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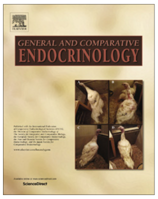

Research paper

Effects of the soya isoflavone genistein in early life stages of the Senegalese sole, *Solea senegalensis*: Thyroid, estrogenic and metabolic biomarkers

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ABSTRACT

This study examines the effects induced by environmentally relevant concentrations of the isoflavone genistein (3 mg/L and 10 mg/L) during early life stages of the Senegalese sole. Throughout the hypothalamus-pituitary-thyroid (HPT) axis, several neurohormonal regulatory thyroid signalling patterns (thyroglobulin/*Tg*, thyroid peroxidase/*TPO*, transthyretin/*TTR*, thyroid receptors/*TRβ*, and iodothrynonine deiodinases, *Dio2* and *Dio3*) were analysed. Furthermore, the expression patterns of estrogen receptor *ERβ* and haemoprotein *Cyp1a* were also evaluated. In the control larvae, progressive increases of constitutive hormonal signalling pathways have been evidenced from the pre-metamorphosis phase onwards, reaching the highest expression basal levels at the metamorphosis (*Tg*, *TPO*, *Dio2*) and/or during post-metamorphosis (*TTR*, *TRβ*, *ERβ*). When the early larvae were exposed to both genistein concentrations (3 mg/L and 10 mg/L), a statistically significant down-regulation of *TPO*, *TTR* and *Tg* mRNA levels was clearly detected at the metamorphic stages. In addition, the *Dio2* and *Dio3* transcript expression levels were also down and up-regulated when exposed to both genistein concentrations. In the larvae exposed to genistein, no statistically significant responses were recorded for the *TRβ* expression patterns. Nevertheless, the *ERβ* and *Cyp1a* transcript levels were up-regulated at the middle metamorphic stage (S2, at 16 dph) in the larvae exposed to high genistein concentrations and, only the *ERβ* was down-regulated (S1, at 12 dph) at the lower doses. Finally, all these pointed out imbalances were only temporarily disrupted by exposure to genistein, since most of the modulated transcriptional signals (i.e. up or down-regulation) were quickly restored to the baseline levels. Additionally, the control and genistein-exposed Senegalese sole specimens showed characteristic ontogenetic patterns and completely suitable for an optimal development, metamorphosis, and growth.

1. Introduction

In teleostean farming species, but most notably in Pleuronectiformes, such as the flatfish Senegalese sole, *Solea senegalensis*, in which the complex metamorphosis phase is also highly sensitive to internal and external signals, endo-exotrophic phase and weaning onto artificial diets have been considered as the most critical periods in terms of duration, vulnerability and mortality (Dinis et al., 1999; Imsland et al., 2003; Morais et al., 2014). Furthermore, many experimental designs focusing on inadequate feeding practices, nutrient deficiencies, and dependent-alterations and disorders (i.e. skeletal, thyroid, pigmentation, etc.) have been carried out in early life stages of the Senegalese sole (Boglino et al., 2014; Boglione et al., 2013; Darias et al., 2013; Fernández et al., 2009; Gavaia et al., 2009; Gisbert et al., 2008, among others). On the other hand, interesting findings have been published about functional properties and effects of different phytochemical flavonoids (i.e. antioxidant, immunostimulant, anti-inflammatory, hormonal alterations, feminisation, apoptosis, skeletal disorders, growth imbalances, etc.) in several different fish species (Benneteau-Pelissero et al., 2001; Chen et al., 2016; Francis et al., 2001; Gatlin et al., 2007; Ingham et al., 2004; Pelissero et al., 1991).

In the past two decades, several reports demonstrated that a large number of pollutants, even present at trace concentrations in the environment, can have negative impacts on exposed organisms. There are numerous references about environmentally relevant concentrations of many xenobiotics acting as endocrine disrupting compounds (EDCs)-or endocrine modulators-, including several phytoestrogen compounds (isoflavones, phytosterols,

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coumestans, lignans) and, especially, a higher number of reports about chemical xenobiotics which have been most widely analysed in the environment and largely studied on exposed fish species (Brown et al., 2004; Kiparissis et al., 2003; Ng et al., 2006; Patisaul and Adewale, 2009; Rocha et al., 2013; Sassi-Messai et al., 2009; Schiller et al., 2013a,b, among others). Indeed, many natural and synthetic phytochemicals (i.e. flavonoids, non-flavonoids) possessing phytoestrogen properties can be present in agricultural, domestic and industrial wastewater effluents as a result of anthropogenic activities, and they can be discharged in the environment. In several different worldwide ecosystems have been reported findings from trace concentrations, in the nanomolar range, until elevated concentrations (e.g., from 10 μg/L to 0.25 mg/L) of several phytoestrogen isoflavones (genistein, daidzein, equol, etc.), among other phytochemicals (Kiparissis et al., 2001; Rearick et al., 2014; Ribeiro et al., 2016; Rocha et al., 2013; Spengler et al., 2001). In addition, in previous unpublished studies on several freshwaters and marine fish species, some toxicity data as for instance the no observed effect concentration (NOEC) for the genistein were determined at high nominal concentrations (i.e., at around 5–10 mg/L). On the other hand, the increasing sustainable expansion and utilisation of different vegetal compounds in aquafeeds may be advantageous, but it could also provoke undesirable or harmful effects for consumers (Patisaul and Jefferson, 2010; Setchell and Cassidy, 1999; Wang et al., 2013). Soya bean meal is frequently used to partially substitute fishmeal because of its protein content, good amino acid profile, palatability to fishes, availability and low price. In fact, most of the utilised leguminous derived-products (soya, corn, wheat, etc.) also contain different estrogenic and anti-nutritional factors (e.g. flavones, trypsin inhibitors, lectins, saponins, antigens, etc.), contributing to several disorders in different fish species (Francis et al., 2001; Gatlin et al., 2007). Nevertheless, as far as we know, there are no studies on the effects of flavonoids (e.g. genistein, daidzein) in the larval development and, particularly, focusing on the complex thyroid-driven metamorphosis process of flatfish species, like Senegalese sole.

In vertebrates, but most notably in anuran amphibians, in flatfish and in other teleosteans, the thyrotrophic negative feedback control is regulated by the hypothalamus-pituitary-thyroid (HPT) axis. The complex neuroendocrine-thyroid system is the main key for the metamorphosis, and serves to transduce environmental information into a developmental response (Brown and Cai, 2007; Darras et al., 2015; Power et al., 2001, 2008; Schreiber, 2013). Briefly, under the control of pituitary thyrotropin (TSH), the sodium/iodide symporter (NIS) transports iodide from the blood into thyrocytes, where the enzyme thyroid peroxidase (TPO) catalyses iodide oxidation, iodination of tyrosine residues in thyroglobulin (Tg) and iodothyronine coupling in the presence of H_2O_2; iodinated Tg is stored in the colloid and subsequently hydrolysed by proteases (i.e. cathepsins), releasing T4 into the circulation. The conversion of prohormone-thyroxine to active T3 depends on the expression and catalytic activity of selenocysteine deiodinases (DIOs 1, 2, 3), which also participate in the hormonal homeostasis. Thyroid hormones (THs) are transported by transthyretin (TTR) and other binding proteins, and thyroid hormones activate a downstream signalling pathway when they bind specifically to thyroid hormone receptors (TRs). The thyroid receptors function as hormone-activated transcription factors; and thereby act by modulating gene expression (Darras et al., 2015; Itoh et al., 2010; Orozco and Valverde, 2005; Power et al., 2000a,b, 2001; Santos et al., 2002; Yamano, 2005, among others). In particular, THs are known to play a crucial role in many ontogenetic (i.e. metamorphosis, eye migration, skeletogenesis, pigmentation, etc.) and metabolic processes. Therefore, the thyroid system is essential for normal growth, differentiation and development, since THs and signalling pathways directly or indirectly stimulate processes of proliferation and apoptosis patterns, as well as resorption and remodelling of larval tissues (Alves et al., 2016; Power et al., 2001, 2008; Schreiber, 2013, among others). In this context, some of the main steps for thyroid gland development, TH biosynthesis, metabolism and accumulation in the thyroid follicles through a negative feedback regulation of the HPT axis during development of the Senegalese sole are fairly well established (Campinho et al., 2015; Infante et al., 2007, 2008; Isorna et al., 2009; Izigar et al., 2010; Klaren et al., 2008; Manchado et al., 2008, 2009; Ortiz-Delgado et al., 2006; Ponce et al., 2010). For example, among other neuroendocrine signalling patterns, the constitutive *TSH* transcript levels decrease significantly at the onset of metamorphosis. On the contrary, *THs*, *Tg*, *TRs*, and iodothyronine deiodinases (e.g. *Dio2*) increase significantly at the metamorphic climax, showing variable temporal pattern changes during the larval development of this flatfish species, as recently reviewed by Darras et al. (2015).

In the light of current evidence, several environmental chemicals that are known as typical endocrine disruptors appear to interfere with the thyroid system of vertebrates by two principal molecular mechanisms: a) the disruption the thyroid homeostasis, what includes effects on enzymes responsible for iodine uptake and thyroid hormone synthesis, on transport in the blood, and on cellular uptake degradation and excretion: thyroid gland function disruptors; and b) ligand binding to the thyroid receptors in the peripheral target tissues and subsequent activation of thyroid signalling (Boas et al., 2012; Thienpont et al., 2011). This last mode of action does not necessarily involve changes in endogenous hormone levels but in thyroid signalling, that is, in thyroid hormonal action disruptors (Baumann et al., 2016; Brown et al., 2004; Jarque and Piña, 2014). In this context, the majority of phytoestrogen flavonoids (e.g. isoflavones) can induce both agonist and antagonist effects, at estrogenic, androgenic and thyroid levels, among other harmful or healthy effects (Harper et al., 2006; Patisaul and Jefferson, 2010; Setchell and Cassidy, 1999; Wang et al., 2013). As a typical phytoestrogen, the genistein (4′,5,7-trihydroxyisoflavone) can bind to estrogen receptors (ERs) and activate ER-dependent pathways. Besides, genistein gives evidence to also activate estrogen-independent pathways, including pro-apoptotic effects (Akiyama et al., 1987; Amakura et al., 2008; Patisaul and Adewale, 2009; Santos et al., 2014; Sassi-Messai et al., 2009; Shim et al., 2007). Furthermore, phytoestrogen isoflavones (e.g. genistein, daidzein) can affect the thyroid hormone biosynthesis (Tg/T4 and T3) and metabolism, by inhibition of thyroid peroxidase (TPO) and deiodinases/DIOs; and many flavonoids are also potent competitors for pro-hormone T4 binding to the thyroid distributor protein, as the transthyretin (TTR), and as a consequence, these phytochemicals may alter the hormonal thyroid homeostasis (Chang and Doerge, 2000; Li et al., 2011; Schiller et al., 2013a,b; Schmutzler et al., 2007).

On the other hand, cytochromes P-450 (CYPs) catalyse the oxidative biotransformation of a broad spectrum of endogenous substrates (i.e. steroids, fatty acids, vitamins and retinoids, etc.) and participate in the metabolism and detoxification of different xenobiotics, e.g. polyaromatic hydrocarbons, polychlorinated biphenyls, dibenzodioxins, pharmaceutical drugs, flavonoids, etc. (Amakura et al., 2008; Cajaraville et al., 2000; Denison and Nagy, 2003; Nebert and Gonzalez, 1987; Whitlock, 1999). CYP1a is induced by a variety of xenobiotics through ligand binding and activation of the cytoplasmic aryl hydrocarbon receptor (AhR). Typical AhR ligands are hydrophobic, polycyclic, aromatic compounds having a planar structure (Denison and Whitlock, 1995; Nebert and Gonzalez, 1987; Whitlock, 1999). However, the currently defined model of AhR/CYP1a induction may be too simplistic, as indicated from the identification of numerous atypical