The influence of 17 β -oestradiol on lymphopoiesis and immune system ontogenesis in juvenile sea bass, *Dicentrarchus labrax*

Catarina Moreira¹, Matthieu Paiola^{1,2}, Aurélie Duflot¹, Inma Varó³, Ariadna Sitjà-Bobadilla³, Thomas Knigge¹, Patrícia Pinto⁴, Tiphaine Monsinjon^{1,*}

¹ UMR-I 02 Environmental Stress and Aquatic Biomonitoring (SEBIO), University of Le Havre Normandy, F-76600 Le Havre, France;

² Current address: Department of Microbiology and Immunology, University of Rochester Medical Center, 14642 Rochester, NY, United States

³ Instituto de Acuicultura Torre de la Sal, CSIC, 12595 Ribera de Cabanes, Castellón, Spain

⁴ Centro de Ciências do Mar (CCMAR), Universidade do Algarve, 8005-139 Faro, Portugal

*Corresponding author : UMR-I 02 INERIS-URCA-ULH, Unité Stress Environnementaux et Biosurveillance des milieux aquatiques, UFR Sciences et Techniques, University of Le Havre Normandy, 25 rue Philippe Lebon, 76063 Le Havre, France. E-mail address: tiphaine.monsinjon@univ-lehavre.fr (T. Monsinjon)

Abstract

The female sex steroid 17 β -oestradiol (E2) is involved in the regulation of numerous physiological functions, including the immune system development and performance. The role of oestrogens during ontogenesis is, however, not well studied. In rodents and fish, thymus maturation appears to be oestrogen-dependent. Nevertheless, little is known about the function of oestrogen in immune system development. To further the understanding of the role of oestrogens in fish immune system ontogenesis, fingerlings of European sea bass (*Dicentrarchus labrax*) were exposed for 30 days to 20 ng E2 $\cdot$ L⁻¹, at two ages tightly related to thymic maturation, *i.e.*, 60 or 90 days post hatch (dph). The expression of nuclear and membrane oestrogen receptors was measured in the thymus and spleen, and the expression of several T cell-related gene markers was studied in both immune organs, as well as in the liver. Waterborne E2-exposure at 20.2 ± 2.1 (S.E.) ng $\cdot$ L⁻¹ was confirmed by radioimmunoassay, leading to significantly higher E2-contents in the liver of exposed fish. The majority of gene markers presented age-dependent dynamics in at least one of the organs, confirming thymus maturation, but also suggesting a critical ontogenetic window for the implementation of liver resident $\gamma\delta$ and $\alpha\beta$ T cells. The oestrogen receptors, however, remained unchanged over the age and treatment comparisons with the exception of *esr2b*, which was modulated by E2 in the younger cohort and increased its expression with age in the thymus of the older cohort, as did the membrane oestrogen receptor *gpera*. These results confirm that oestrogen-signalling is involved in thymus maturation in European sea bass, as it is in mammals. This suggests that *esr2b* and *gpera* play key roles during thymus ontogenesis, particularly during medulla maturation. In contrasts, the spleen expressed low or non-detectable levels of oestrogen receptors. The E2-exposure decreased the expression of *tcry* in the liver in the cohort exposed from 93 to 122 dph, but not the expression of any other immune-related gene analysed. These results indicate that the proliferation/migration of these innate-like T cell populations is oestrogen-sensitive. In regards to the apparent prominent role of oestrogen-signalling in the late thymus maturation stage, the thymic differentiation of the corresponding subpopulations of T cells might be regulated by oestrogen. To the best of our knowledge, this is the first study investigating the dynamics of both nuclear and membrane oestrogen receptors in specific immune organs in a teleost fish at very early stages of immune system development as well as to examine thymic function in sea bass after an exposure to E2 during ontogenesis.

Key-words: liver; oestrogen receptor; T cell; teleost fish; thymus; spleen.

Abbreviations: *18s*, 18S ribosomal RNA gene; E2, 17 β -oestradiol; ER, oestrogen receptor; ESR, nuclear oestrogen receptor; *fau*, 40S ribosomal protein S30; *foxn1*, transcription factor forkhead-box

34 n1; *foxp3*, forkhead transcriptional regulator P3; GPER, G protein-coupled oestrogen receptor; HSI,
35 Hepato-somatic index; K, Fulton's condition factor; *l13a*, L13a ribosomal protein; *mhc*, major
36 histocompatibility complex; *pcna*, proliferating cell nuclear antigen; qPCR, quantitative reverse
37 transcriptase polymerase chain reaction; *rag*, recombination-activating genes; TEC, thymic epithelial
38 cells; Tregs, regulatory T cells. Protein and gene nomenclature followed that recommended by
39 genenames.org and used for fish at <http://zfin.org/>.

40

41

42 1. Introduction

43 The vertebrate immune system consists of two major components: the adaptive and innate
44 immune responses. The latter generally precedes, activates and determines the nature of the adaptive
45 response, and co-operates in the maintenance of homeostasis (Fearon, 1997; Fearon and Locksley,
46 1996). The adaptive immune system –unique to jawed vertebrates– is highly specialized and based on
47 two lineages of effector immune cells: the B and T lymphocytes (Kasahara et al., 2004), which are
48 characterized by their respective T and B cell receptors (TCR, BCR). Recombination-activating genes
49 (RAG) 1 and 2 encode nuclear proteins that directly mediate the recombination of TCR and BCR gene
50 segments. This recombination is necessary to produce a diversity of receptors that are able to
51 specifically recognize a large number of antigens (Randelli et al., 2009). The TCRs are formed by
52 membrane heterodimers composed either of TCR γ and δ chains, or of TCR α and β chains, thus
53 defining two fundamentally different T cell lineages. The $\gamma\delta$ T cells have innate-like properties,
54 including rapid effector function after activation and the capacity to bind antigens directly. The $\alpha\beta$ T
55 cells, on the other hand, are commonly referred to as ‘conventional T cells’ and recognize antigen
56 peptides presented by the class I or II major histocompatibility complex (MHC), which are expressed
57 by antigen presenting cells such as thymic epithelial cells (TEC) to select self-tolerant T cells (Klein
58 et al., 2009; Mantegazza et al., 2013). Conventional T cells have a highly diverse TCR repertoire
59 allowing a specific adaptive immune response, which follows the innate response.

60 The thymus plays a pivotal role in the ontogenesis of the adaptive immune system. It is the
61 major site for T lymphocyte-education and -maturation, *i.e.*, thymopoiesis. T cell-development,
62 including T cell-commitment and -maturation, depends on the interaction with TECs, the function of
63 which is regulated by the transcription factor forkhead-box n1 (FOXN1) (Bajoghli et al., 2009; Žuklys
64 et al., 2016). Among the various conventional T cell subpopulations, the regulatory T cells (Tregs) are
65 particular in that they develop either in the thymus, or in peripheral immune organs, such as the spleen
66 or the liver, depending on their TCR specificity (Plitas and Rudensky, 2016). Their function is to
67 regulate innate and adaptive immune cell activity and to maintain self- and non-self-tolerance
68 (Quintana et al., 2010; Wing and Sakaguchi, 2010). The forkhead transcriptional regulator P3
69 (FOXP3) is essential for the genetic programming of Treg-differentiation and -function (Sakaguchi et
70 al., 2001; Shevach, 2002). From the thymus, naïve and mature self-tolerant T cells emigrate to
71 peripheral lymphoid organs, including the spleen and the liver (Bowden et al., 2005). Thymopoiesis
72 and the regulation of the abundance of peripheral T cells is dependent on cell migration and on the
73 expansion of proliferating cell nuclear antigen (PCNA)-positive T cells (Goronzy and Weyand, 2019).
74 B and T lymphocytes colonize the spleen after they have been detected in the thymus and head kidney,

proving that the spleen is a secondary lymphoid organ in jawed vertebrates (Boehm et al., 2012; Neely and Flajnik, 2016). The spleen plays an important role in antigen presentation and in the initiation of the adaptive immune response, *i.e.*, T and B cell-activation (Boehm et al., 2012; Mebius and Kraal, 2005). In mammals, the liver is also involved in T cell-education, although its innate immune functions appear to be more important. In fact, the liver promotes their dysfunction and mediates adaptive immune tolerance by acting as a ‘graveyard’ of activated T cells (Racanelli and Rehermann, 2006; Zheng and Tian, 2019). In fish, the role of the liver in immunity is far less documented. Nevertheless, intrahepatic immune cells, which are dominated by T cells, have been described by Möller *et al.* in rainbow trout (2014).

In mammals, early haematopoiesis already begins in the yolk sac and temporarily moves to the liver before definitive haematopoiesis is finally established in the bone marrow and thymus, where the full set of immune and blood cells will be generated (Jagannathan-Bogdan and Zon, 2013). In fish, however, the ontogeny of the haematopoiesis and the immune system is far less studied. It is believed that the protection of larvae and fry depends on maternally transferred components like lectins and antibodies and that a non-specific innate immunity is already present at early egg stages (Ferraresso et al., 2016). In several teleost species, the thymus is the first lymphoid organ in which lymphoid cells appear (Bowden et al., 2005). Nevertheless, the antibody response against T cell-dependent antigens, which highlights a mature adaptive immune response, occurs after the thymus becomes morphologically mature and is delayed in comparison to the maturation of humoral immunity (Lam et al., 2004). Accordingly, fish immunocompetence becomes enhanced as soon as thymic regionalization is completed (Seemann et al., 2017). Nevertheless, before thymus ontogenesis is completed, peripheral T cells are already detected (Huttenhuis et al., 2006; Romano et al., 2011; Scapigliati et al., 2018). Together with the expression of *rag1* in peripheral T cells, this suggests that (i) innate-like T cells arise from both intra- and extrathymic origins in teleost fish, akin to mammals (Boschi et al., 2011; Cheroutre et al., 2011; Huttenhuis et al., 2006) and (ii) during ontogenesis, innate-like T cells probably represent a major component of the immune defence of fish, as has been shown previously for *Xenopus* tadpoles and mammals (Cheroutre et al., 2011; Edholm et al., 2016).

The oestrogenic regulation of the immune system, including the thymus, seems to be conserved across vertebrates (Paiola et al., 2018; Segner et al., 2013). Oestrogenic regulation in different tissues can result from either slow (*via* nuclear-initiated steroid signalization - NISS), or rapid (*via* membrane initiated steroid signalization - MISS) signalling through their binding to multiple oestrogen receptors (ERs). Most fish species have three intracellular nuclear receptors (ESRs), *i.e.*, one Esr1 and two Esr2, while terrestrial vertebrates have only one Esr1 and one Esr2. In addition, fish may have two G Protein-Coupled Estrogen Receptors (GPERs), *i.e.*, Gpera and Gperb, while mammals have only the GPER1

109 form. The functions of GPERs are less well understood, but it is now evident that both types of
 110 signalling and receptors may interact with each other (Amenyogbe et al., 2020; Lafont et al., 2016;
 111 Pinto et al., 2014; Thomas et al., 2010). The expression pattern of the full set of nuclear and membrane
 112 receptors determines the global cellular response to oestrogens. According to the conserved
 113 immunomodulatory function of oestrogen, the various ER-isoforms have been detected in immune
 114 cells and immune organs, including the thymus, across numerous vertebrates from mammals to fish
 115 (Paiola et al., 2018; Segner et al., 2013). In addition to its modulatory role in thymic T cell-
 116 differentiation, as evidenced in mammals with a thymus atrophy during pregnancy or after puberty,
 117 oestrogen appears to be involved in thymus ontogenesis. In fact, aromatase-, ESR2-, GPER- and
 118 especially ESR1-knockout mice display defective thymus ontogenesis (Chunhe et al., 2008;
 119 Erlandsson et al., 2001; Li et al., 2002; Yellayi et al., 2000). Interestingly, studies from various jawed
 120 vertebrates, including the European sea bass (*Dicentrarchus labrax*), have shown that oestrogen can
 121 also increase thymus volume during critical windows of the thymus ontogenesis, during which
 122 immunocompetence is established (DeWitt et al., 2012; Kondo et al., 2004; Seemann et al., 2015).
 123 Therefore, oestrogen-exposure during thymus ontogenesis may have long-term consequences for the
 124 T cell-dependent immune response in mammals (Chapman et al., 2009; Forsberg, 2000) and in fish
 125 (Rehberger et al., 2020).

126 In the European sea bass, Seemann et al. (2015) observed that 17 β -oestradiol (E2)-exposure
 127 triggers a change in thymus size, *i.e.*, hypertrophy, in fish exposed for one month from 90 dph onward,
 128 but not from 60 dph or 120 dph. This E2-sensitive window of thymus ontogenesis seems to begin at
 129 90 dph, which corresponds to the late stages of thymic regionalization. Therefore, in this study, we
 130 tested the hypothesis whether E2 is involved the immune system ontogeny by interfering with the
 131 thymic T cell development. To test this hypothesis, oestrogen responsiveness of the developing
 132 immune system was studied in European sea bass, at two developmental stages from 60 to 90 dph and
 133 from 93 to 122 dph. We investigated (i) the expression of the five fish oestrogen receptor isoforms in
 134 a central and a major secondary immune organ, thymus and spleen, respectively, and (ii) the thymic
 135 function, by studying the expression of thymic specific genes, as well as by measuring T cell-related
 136 genes in the periphery (spleen and liver). Potential effects on extrathymic T cell-differentiation were
 137 assessed by the measurement of *rag2* expression in the spleen and liver.

138
 139
 140
 141

2. Materials and methods

2.1. Animal rearing

European sea bass fingerlings were obtained from the hatchery ‘Poissons du Soleil’ (Sete, France) at 40 dph, and then reared at the facilities of the Institute of Aquaculture Torre de la Sal (CSIC, Castellon, Spain) until they reached the appropriate age for the experimental assay (see Fig. 1 for details on the experimental design). During this time, fish were fed daily *ad libidum* with a diet manufactured by Life Bioencapsulation S. L. (Spin-Off Universidad de Almeria, Spain) and maintained in 20 L aquaria with aerated filtered natural seawater (35‰ salinity), and a photoperiod of 12h light:12h darkness. Physicochemical parameters were assessed weekly and 80% of the water was changed daily (see Table 1 for details).

The experimental protocols involving fish were approved by the Ethics and Animal Welfare Committee of IATS (CSIC) and Generalitat Valenciana. They were carried out in a registered installation facility in accordance with the principles published in the European animal directive (2010/63/EU) and Spanish laws (Royal Decree RD53/2013) for the protection of animals, and all efforts were made to minimize the suffering of animals.

Table 1. Experimental information, physicochemical parameters and biometric indices of fish of the two age cohorts. The physicochemical parameters (Mean $\pm$ SD) were measured weekly in all aquaria. Biometric indices (Mean $\pm$ SD) were measured after each sampling.

Age on sampling	90 dph	122 dph
Water volume	16 L	16 L
Number of tanks	4 replicate tanks per group (CTR or E2)	6 replicate tanks per group (CTR or E2)
Number of fish per tank	25 fish *	13 fish *
Temperature (°C)	17.98 $\pm$ 0.21 °C	18.02 $\pm$ 0.30 °C
pH	7.84 $\pm$ 0.33	7.78 $\pm$ 0.37
Salinity	35.70 $\pm$ 0.87 ‰	35.10 $\pm$ 1.36 ‰
Oxygen	95.20 $\pm$ 1.72 %	96.33 $\pm$ 2.03 %
Fish Length	CTR: 32.75 $\pm$ 4.69 mm E2: 32.40 $\pm$ 4.14 mm	CTR: 45.90 $\pm$ 6.04 mm E2: 46.90 $\pm$ 5.48 mm
Fish Body Weight	CTR: 0.32 $\pm$ 0.15 g E2: 0.30 $\pm$ 0.13 g	CTR: 0.96 $\pm$ 0.39 g E2: 1.02 $\pm$ 0.33 g

K ¹	CTR: $0.87 \pm 0.17 \text{ g} \cdot \text{cm}^{-3}$ E2: $0.81 \pm 0.11 \text{ g} \cdot \text{cm}^{-3}$	CTR: $0.94 \pm 0.15 \text{ g} \cdot \text{cm}^{-3}$ E2: $0.95 \pm 0.06 \text{ g} \cdot \text{cm}^{-3}$
HSI ²	CTR: $2.71 \pm 1.03 \%$ E2: $1.84 \pm 0.67 \%$	CTR: $1.58 \pm 0.58 \%$ E2: $1.49 \pm 0.33 \%$

*The results presented are part of a larger study. Some of the fish were sampled for other analyses, which were not included in this article.

¹Fulton's condition factor (K) = body weight (g) · (total length (cm))⁻³

²Hepato-somatic index (HSI) = liver weight (g) / body weight (g) x 100

2.2. Treatment and sampling

Following the acclimation period, animals were exposed in the same 20 L aquarium under semi-static conditions for 30 days (beginning at either 60 or 93 dph, see Fig.1) to a concentration of 20 ng·L⁻¹ of waterborne E2 (Sigma-Aldrich) diluted in methanol, or to the solvent control only (*i.e.*, filtered sea water with the equivalent concentration of methanol at 0.000001%, v/v). Previous studies have shown this waterborne E2 concentration to be most effective in soliciting immune-related responses in juvenile sea bass at similar ages (Seemann et al., 2016; Seemann et al., 2015; Seemann et al., 2013).

Water quality and E2-concentrations were assured by the daily exchange of 80% of the water body with subsequent E2-spike for the treatment groups. Once a week, a water sample from each aquarium (before and after dosing) was collected and stored at 4 °C in the dark until analysis of E2-concentrations. After 30 days of exposure, *i.e.*, at a final age of 90 dph and 122 dph, respectively, 12 fish were randomly chosen from the different tank replicates of both treatments (control and exposed – see Table 1 for details) to avoid tank effects. Fish were sacrificed with an overdose of MS-222 (Sigma-Aldrich) and body weight and length were recorded. Blood was not collected from the sampled fish but instead, the tissues were carefully dissected on paper to absorb the excess of blood. Whole thymus, spleen, and liver comprising all cell types contained in these organs (including lymphocytes) were dissected from the same 12 fish that were embedded in RNA later (Sigma-Aldrich) and stored at -20 °C until RNA extraction. All fish analysed in the present study were considered as 'sexually immature', as the first signs of histological sex differentiation in sea bass usually appear only after 150 dph in females to 200 dph in males (Blázquez et al., 2008; Carrillo et al., 2015), while at the analysed ages (60 to 122 dph) the undifferentiated gonads are not sufficiently developed to differentially produce sex steroid hormones. Papadaki et al. 2005 (Papadaki et al., 2005) reported a differential peak in circulating E2-levels only after 200 dph, when the first primary oocytes appeared.

The Fulton's condition factor (K) and hepato-somatic index (HSI) were calculated according to Hadidi et al. (2008) and Zha et al. (2007), respectively: $K (g \cdot cm^{-3}) = \text{body weight (g)} \cdot (\text{total length (cm)})^{-3}$; $HSI (\%) = \text{liver weight (g)} / \text{body weight (g)} \times 100$. Details of the experiment, physiochemical parameters and biometric indices of the experiment are specified in Table 1.

Figure 1. Experimental design: juvenile European sea bass, *Dicentrarchus labrax*, from the same batch were acclimated and exposed to $20 \text{ ng} \cdot \text{L}^{-1}$ of waterborne 17β -oestradiol (E2) at two developmental stages, *i.e.*, at 60 and 93 dph, for 30 days. At the end of exposure time, the fish were sampled ($n = 12$ fish per treatment and the same eight fish were used for gene expression analysis in the thymus, spleen and liver) at a final age of 90 dph and 122 dph, respectively.

2.3. Oestradiol radioimmunoassay

The oestrogenic compounds were extracted from water using Sep-Pak cartridges Vac C18 1cc (Waters) from a 1.8 mL sample of each aquarium, resolved in 4 mL ethanol (Sigma-Aldrich), evaporated in a $40 \pm 2 \text{ }^{\circ}\text{C}$ water bath and 0.5 mL of gelatine buffer was added. Fish livers of the 122 dph cohort were macerated, followed by an ether extraction with 200 μL of distilled water and 3 mL of diethyl ether (VWR) and centrifuging at 40 g for 5 min. Samples were frozen in liquid nitrogen for 5 s, the ether phase was decanted and evaporated and 1 mL of gelatine buffer was added. Water and liver E2-levels were determined in duplicate using an optimized radioimmunoassay (RIA) and a specific antiserum against E2 (Guerreiro et al., 2002). Free hormones were separated from total bound hormones using dextran-coated charcoal and a control thin-layer chromatography (TLC) was performed to confirm that E2 has been quantified in both water and liver samples. The quantification of E2 in the liver was only used as a technical validation E2-uptake by the fish. Because the older fish provided enough material for both molecular and E2 measurements, these were used as representatives for the validation of the E2 exposure.

2.4. Gene expression

219 The expression of several genes was assessed in the three different immune organs (thymus,
220 spleen, and liver) unless stated otherwise. Details on the genes and their respective primers are given
221 in Table 2. Total RNA was extracted using PureZOL (BioRad) according to the supplier's instructions.
222 Briefly, whole organs (except for liver, which was sectioned) were grinded in a glass homogenizer
223 with 850 μ L of PureZOL. Then, 170 μ L of chloroform (Sigma-Aldrich) was added and the samples
224 were centrifuged at 12,000 g for 15 min at 4 °C to eliminate the debris. Glycogen (Glycobblue, Ambion)
225 and ammonia acetate (Sigma-Aldrich) were added to have a final concentration of 0.05 mg·mL⁻¹ and
226 0.5 M, respectively, as well as one half of the volume of isopropyl alcohol and then incubated overnight
227 at -80 °C, in order to improve the precipitation and visualization of the pellet. Subsequently, the
228 samples were centrifuged at 12,000 g for 15 min at 4 °C, washed with 75% ethanol and the pellet was
229 resuspended in RNase-free water (MilliQ water, Sigma-Aldrich). After RNA-extraction, possible
230 DNA-contamination was removed by digestion with the TURBO DNA-free Kit (Invitrogen Ambion)
231 according to the supplier's instructions. The yield and purity of RNA were quantified with a Nanodrop
232 One (ThermoFisher) at 260 nm and 280 nm. Absorbance ratios 260/280 between 1.90 and 2.00
233 indicated pure RNA samples. Eight extracts that yielded RNA with good quality and quantity from
234 isolated tissues were selected for gene expression, with a representation across all replicate tanks.
235 Samples were stored at -80 °C until further processing. Reverse transcription was performed using 500
236 ng of RNA for all samples with the exception of thymus and spleen from the younger cohort (250 and
237 350 ng, respectively), 200 ng· μ L⁻¹ of random hexamers, 10nM of dNTP and MMLV-RT (reverse
238 transcriptase, Promega) in a final volume of 20 μ L. RNA was denatured for 5 min at 70 °C and the
239 cDNA-synthesis proceeded for 60 min at 42 °C, after which the cDNA was stored at -20 °C. Real-time
240 quantitative PCR (qPCR) was performed using two technical replicates per individual sample by
241 separately adding cDNA diluted 1:20 in MilliQ water.

242 All genes were analysed with Rotor-Gene Q (Quiagen) using one of two commercial master
243 mixes, QuanTitec (Quiagen) or EvaGreen® (BioRad), according to the manufacturer's
244 recommendations (see Table 2, for details). For each organ and primer pair, the amplification
245 efficiency was calculated by running serial dilutions of cDNA-pools from samples of all conditions.
246 The efficiencies ranged from 1.90 and 2.20. All qPCR reactions had a single peak melt curve and
247 primer specificity was confirmed by sequencing the amplicons. Three reference genes, *i.e.*, the
248 ribosomal protein L13a (*l13a*), the 40S ribosomal protein S30 (*fau*) and the 18S ribosomal RNA sub-
249 unit (*18S*) were used for the quantification of relative gene expression. The primers of the reference
250 genes were previously tested with the same species following oestrogenic exposure (Paiola et al., 2018;
251 Pinto et al., 2016) and they were evaluated as generally stable during ontogenesis (Mitter et al., 2009).
252 Using Pfaffl's formula (2001), the relative expression of each gene of interest was calculated using the

253 efficiency of each primer pair for each organ and the geometric mean of the three reference genes as
 254 recommended by Vandesompele et al. (2002). Gene-expressions were calibrated by normalizing
 255 individual expression to the average of all cDNAs across treatments and age.

256

257 **Table 2.** Analysed genes and their functions, primer sequences or sources, qPCR conditions and
 258 analysed organs.

Genes	Function	Primer source	Amplicon (bp)	Annealing Temperature	Analysed organs
<i>esr1</i>	Oestrogen regulation (<i>via</i> NISS [†])	Seemann et al. (Seemann et al., 2015)	139	60 °C	Thymus, Spleen
<i>esr2a</i>	Oestrogen regulation (<i>via</i> NISS)	Seemann et al. (Seemann et al., 2015)	118	61 °C	Thymus, Spleen
<i>esr2b</i>	Oestrogen regulation (<i>via</i> NISS)	Seemann et al. (Seemann et al., 2015)	295	61.5 °C	Thymus, Spleen
<i>gpera</i>	Oestrogen regulation (<i>via</i> MISS [‡])	Pinto et al. (Pinto et al., 2016)	157	60°C	Thymus, Spleen
<i>gperb</i>	Oestrogen regulation (<i>via</i> MISS)	Pinto et al. (Pinto et al., 2016)	122	60°C	Thymus, Spleen
<i>foxn1</i>	TEC function	Paiola et al. (Paiola et al., 2018)	84	61°C	Thymus, Spleen, Liver
<i>foxp3</i>	Treg differentiation and function	Paiola et al. (Paiola et al., 2018)	80	62°C	Thymus, Spleen, Liver
<i>pcna</i>	Cell proliferation	Paiola et al. (Paiola et al., 2018)	137	60.5°C	Thymus, Spleen, Liver
<i>rag2</i>	Early T cell development	This study ¹	123	62°C	Thymus, Spleen, Liver
<i>tcrα</i>	conventional T cell lineages	Paiola et al. (Paiola et al., 2018)	213	62°C	Thymus, Spleen, Liver
<i>tcrγ</i>	innate-like T cells	Paiola et al. (Paiola et al., 2018)	164	61°C	Thymus, Spleen, Liver
<i>mhcIIa</i>	Conventional T cell selection	Paiola et al. (Paiola et al., 2018)	67	63°C	Thymus, Spleen, Liver
<i>il6</i>	proinflammatory cytokine*	Seemann et al. (Seemann et al., 2013)	221	60°C	Spleen

<i>tnfa</i>	proinflammatory cytokine*	Seemann et al. (Seemann et al., 2013)	154	60°C	Spleen
<i>hepcidin</i>	iron absorption regulation and modulation of innate immunity*	This study ²	97	60°C	Liver

[†]nuclear-initiated steroid signalization, [‡]membrane initiated steroid signalization; * proinflammatory cytokines and *hepcidin* were measured as potential indicators of E2-responsiveness

¹ F: CCGTGGCTGCAATCGTAAAC; R: ACACAAGCTGTCTTCCCTCG

² F: GACACTCGTGCTCGCCTTA; R: TTGAGTGTCATTGCTCCCTG

2.5. Statistical analysis

Statistical analyses were conducted using GraphPad Prism (version 8.0.1, GraphPad Software) and R software (R Core Team, 2013). Prior to the analyses, outliers were eliminated using the ROUT (Q=1%) outlier test. The datasets were checked for normality and equal variances using the Shapiro-Wilk test and the Levene Median test, respectively. The results from the gene expression were LogL transformed, when necessary, to assure normal distribution. Two-Way ANOVA was performed to determine the effect of the two factors, ‘age’ and ‘treatment’, on the expression of the selected genes in each organ, followed by a Tukey HSD post-hoc test. Significant differences were considered at an α -level of 5% ($p \leq 0.05$), with a total of $n=8$ for all genes, organs and age cohorts. Gene-expression data are shown as box-and-whisker plots, indicating the median, the 25 and 75 percentiles, as well as the minimum and maximum values.

3. Results

3.1. Oestrogen concentrations and fish biometric measurements

The mean effective concentration of E2 in the treatment aquaria water over the 30 days of the exposure was of 20.22 ± 2.12 (S.E.) ng E2·L⁻¹ and did not vary substantially, confirming the treatment nominal concentration in the two groups. The E2-concentration for the control aquaria was below the limit of detection (< 1.39 ng·L⁻¹) in all measurements. The liver samples from the E2-exposed fish presented a significantly higher concentration of E2 than the control group at 122 dph: 2038.6 ± 361.6 ng·g⁻¹ vs. 538.9 ± 186.4 ng·g⁻¹, respectively ($p = 0.007$). No significant differences in fish size or weight were observed between treated and control groups and, consequently, there were no differences in the K condition factor or the hepatosomatic index. However, the K condition factor was significantly different between the two age-cohorts (Two-Way ANOVA, $p = 0.0001$), and there was a trend towards a decrease in the hepatosomatic index (Two-Way ANOVA, $p = 0.052$, see Table 1). The survival rate

was about 95% and did not differ between treated and control groups, which indicates that 30 days exposure to 20 ng E2·L⁻¹ did not negatively affect survival of fish of either of the age cohorts.

3.2. Oestrogen receptors gene expression

The expression of the three ESRs and the two GPERs was analysed in the two immune organs, thymus and spleen. In general, the expression of all receptors was well detectable in the thymus, whereas only *gperb* could be consistently quantified across the age cohorts and treatment groups in the spleen (Fig. 2). Among ESRs, only the *esr2b* seemed to be modulated by age as well as by E2-treatment. A significant increase of its expression in the thymus was detected in the 90 dph cohort when treated with E2 (Fig. 2, Tukey's test, $p = 0.013$). In addition, the 122 dph cohort also had a higher *esr2b*-expression compared to that of the 90 dph cohort (Fig. 2, Tukey's test, $p = 0.001$). Concerning the membrane ERs, only *gpera* was modulated by the age of the fish, with a significantly higher expression level in the thymus of the 122 dph cohort (Fig.2, Tukey's test, $p = 0.008$), but not by E2-exposure. *Gperb* had a relatively constant expression in both organs, irrespective of age or E2-exposure, whereas no expression of *gpera* expression was detected in the spleen.

Fig. 2. Relative expression of the oestrogen receptors in the thymus and spleen of European sea bass, *Dicentrarchus labrax*, sampled at two developmental stages, *i.e.*, 90 and 122 dph, after exposure to E2 (20 ng·L⁻¹) for 30 days, or in non-exposed control fish (CTR). Expression-levels are related to the geometric mean of the expression of three reference genes (*fau*, *113a* and *18S*). The graphs are represented as box-and-whisker plots with the median, 25 % and 75 % quartiles as well as extreme values depicted by the bars. * indicates significant differences between the two age cohorts or between E2-exposed and CTR fish within the same age, at $p \leq 0.05$; nd, non-detectable; n = 8 per treatment for all the organs and ages.

3.3. Immune-related gene expression

The dynamics of *foxp3*-expression in the thymus, spleen or liver were neither modulated by age, nor by E2-treatment (Fig. 3). In the liver, a significantly lower expression of *pcna* was observed

319 at 122 dph compared to the younger cohort (Fig.3, Tukey's test, $p = 0.016$) and an increase was
320 observed in the thymus at 122 dph (Fig.3, Tukey's test, $p = 0.050$). However, there was no significant
321 modulation by age, or by E2 in the spleen for the same gene. The *tcra*-expression was also modulated
322 by age in all the tissues analysed, with a significant increase in expression at 122 dph in the thymus
323 and liver, and a significant decrease in the spleen when compared to the 90 dph cohort (Fig. 3, Tukey's
324 test, $p < 0.0001$, in all tissues). A trend towards an effect of the E2-treatment was detected in the liver
325 of the older cohort, which had higher levels of *tcra*-expression, and in which *tcra* tends to be reduced
326 in the E2-treated group, as compared to the controls of the same age (Fig. 3, Tukey's test, $p = 0.052$).
327 The *tcry* increased significantly between 90 dph and 122 dph in the liver (Fig. 3, Tukey's test, $p <$
328 0.0001), the tissue where generally higher *tcry*-levels were found. The expression of *tcry* in the thymus
329 did not show any alteration with age or treatment, whereas it decreased in the spleen with age to
330 expression-levels below the detection limit at 122 dph; furthermore, there was a down-regulation of
331 the liver *tcry*-expression following E2-exposure in the 122 dph cohort (Fig. 3, Tukey's test, $p = 0.018$).
332 No *foxn1*-expression was detected in the liver and similarly low levels were found in the spleen, but
333 *foxn1* was detected at quantifiable levels in the thymus, although not being significantly modulated by
334 the treatment or by age. The expression of *rag2* was detected in all organs and significantly modulated
335 in the thymus, where it increased at 122 dph compared to 90 dph (Fig. 3, Tukey's test, $p = 0.006$). For
336 liver or spleen, no significant modulation by treatment or age was found. For all the organs, the
337 expression of *mhcIIa* significantly increased at 122 dph compared to 90 dph, whereas no effect of E2-
338 exposure was detected (Fig. 3, Tukey's test, $p < 0.0001$, < 0.0001 and 0.001 , respectively).

Fig. 3. Relative expression of selected genes related to T cells in the thymus, spleen and liver of European sea bass, *Dicentrarchus labrax*, sampled at two developmental stages, *i.e.*, 90 and 122 days post-hatch (dph), either exposed to 20 ng E2·L⁻¹ over 30 days (E2), or in non-exposed controls (CTR). Gene expression-levels are represented relative to the geometric mean of the expression of three reference genes, *fau*, *l13a* and *18S*. Box-and-whisker plots show median, 25 % and 75 % quartiles as well as extreme values depicted by the bars. *, significant differences between the two age cohorts or between E2-exposed and control fish within the same age, at $p \leq 0.05$; nd, non-detectable; n = 8 per treatment for all organs and ages.

348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382

The expression of the two proinflammatory cytokine genes *il6* and *tnfa* in the spleen was neither significantly affected by age, nor by E2-exposure (Fig. 4). In the liver, a significantly higher expression of *hepcidin*, a protein involved in iron-absorption that can indirectly modulate innate immune responses, was observed at 122 dph compared to the 90 dph cohort (Fig. 4, Two-way ANOVA, $p < 0.001$), but no effect of the E2-exposure was found.

Fig. 4. Relative expression of genes for two cytokines (*il6* and *tnfa*) in the spleen and *hepcidin* in the liver of 90 and 122 dph European sea bass, *Dicentrarchus labrax*, either exposed to 20 ng E2·L⁻¹ (E2), or non-exposed controls (CTR). Gene expression levels are related to the geometric mean of the expression of three reference genes (*fau*, *il13a* and *l8s*). Box-and-whisker plots show median, 25 % and 75 % quartiles as well as extreme values depicted by the bars. *Hepcidin* expression was log-transformed for representation. *, significant differences between the two age cohorts at $p \leq 0.05$; $n = 8$ per treatment, organs and ages.

4. Discussion

In this study, we tested the hypothesis that oestrogens are directly involved in thymus maturation and associated T cell development in the European sea bass at two distinct developmental stages, previously identified as oestrogen sensitive and non-sensitive windows of thymus ontogenesis, respectively (Seemann et al., 2015): a) between 60 to 90 dph, comprising the beginning of thymus regionalisation (Romano et al., 2011), and b) between 93 to 122 dph, corresponding to the final phase

383 of thymus maturation, particularly of the medulla (dos Santos et al., 2000; Romano et al., 2011). First
384 of all, we validated the oestrogen uptake, and then investigated oestrogen responsiveness (ERs) and
385 gene expression related to T cell-development in the thymus and in two peripheral immune organs,
386 the spleen and the liver.

387 The E2-concentrations in the water and in the liver of exposed fish confirmed that the correct
388 nominal E2-concentration of the water was obtained and that E2 was indeed taken up by the fish.
389 Nevertheless, no significant effects of E2 were observed in any of the general biometric parameters of
390 the two cohorts. In addition, neither the inflammatory cytokines, nor the *hepcidin*-expression
391 responded to the exposure to 20 ng E2·L⁻¹ at the analysed time points. Together with interleukin 1β
392 (IL-1β), TNFα is one of the first cytokines present in the proinflammatory process of fish and mammals
393 (Chadzinska et al., 2008). Previous studies on juvenile fish have shown a reduction of proinflammatory
394 cytokine *tnfa*- and *il6*-expression, while others found an increase of the same cytokines following
395 exposure to oestrogens (Cabas et al., 2012; Chaves-Pozo et al., 2005; Seemann et al., 2013; Wenger
396 et al., 2014). This suggests that depending on the concentration of oestrogens, the age or the species,
397 E2 can either be a pro- or an anti-inflammatory regulator in fish, as has been reported for mammals
398 (Straub, 2007). Furthermore, *hepcidin*, a protein linked to iron metabolism that may influence innate
399 immunity (Cuesta et al., 2008; Zhou et al., 2016) was previously shown to be down-regulated by E2
400 in the liver of six-month old largemouth bass, four days after E2-injection (Robertson et al., 2009). In
401 summary, *tnfa*, *il6* and *hepcidin* are components or modulators of the immune system that have been
402 previously shown to respond to E2 in particular conditions, but in the present study, the concentration
403 used and the timing chosen did not reveal any significant effect of E2 on their gene expression.
404 However, the increase in *hepcidin*-expression with age was pronounced and similar to that previously
405 detected in whole larvae from 1 to 33 dph in sole (Ferraresso et al., 2016), which can be related to a
406 change in iron metabolism.

407 Initially, we investigated the expression-levels of the full set of five fish oestrogen receptors,
408 which allow for potential direct oestrogenic regulation (Segner et al., 2013). The presence of ESRs in
409 the immune organs spleen and thymus clearly establishes the existence of their paracrine or autocrine
410 regulation by oestrogens (Szwejsner et al., 2017). However, few studies have yet revealed the dynamics
411 of ER-expression at early stages of fish development and they have focused on the nuclear isoforms
412 only. Chandrasekar et al. (Chandrasekar et al., 2010) detected ESR gene-expression (especially for
413 *esr2a* and *2b*) from less than three hours post fertilization in zebrafish embryos, while for sea bass
414 aromatase-related expression and activity was detected in 50 dph animals and onwards (Blázquez et
415 al., 2008) in a study describing the interplay between ESRs during sexual maturation in the brain and
416 in the gonads. Seemann et al. (Seemann et al., 2016) have shown that all three nuclear receptors are

417 expressed in sea bass head kidney at about 70 dph and the expression of *esr1* increased steadily after
 418 120 dph. Correspondingly, we found *esr1*, *esr2a* and *esr2b* to be expressed in the thymus from 90 dph.
 419 We also observed a significant increase in *esr2b*- and *gpera*-expression with thymus maturation at 122
 420 dph, which more specifically corresponds to the late stage of medulla maturation (dos Santos et al.,
 421 2000; Romano et al., 2011). This up-regulation of one nuclear and one membrane oestrogen receptor
 422 in the developing thymus at this stage suggests putative key functions during medulla differentiation,
 423 and points to a possible role of oestrogen in the sea bass immune system development. According to
 424 the stereological study from Seemann et al. (2015), oestrogen-exposure during this specific
 425 developmental stage triggers significant morphological changes, *i.e.*, thymus hypertrophy. Similarly,
 426 in mice, oestrogen exposure during neonatal stages has been shown to trigger thymus hypertrophy
 427 (Forsberg, 2000; Leceta et al., 1988). This developmental stage in mice also corresponds to a critical
 428 window of medulla maturation (Klein et al., 2009; Li and Rudensky, 2016; Watanabe et al., 2005).
 429 The neonatal period is associated with the generation and release of $\alpha\beta$ Tregs that have a specific
 430 function in maintaining T cell self-tolerance in order to prevent autoimmunity (Fujikado et al., 2016;
 431 Li and Rudensky, 2016). As a matter of fact, thymic-derived $\alpha\beta$ Treg-differentiation takes place in the
 432 medulla. Functional and developmental homology between fish and mammals would suggest that in
 433 sea bass, Tregs probably expand during the oestrogen-sensitive developmental window. Nevertheless,
 434 the expression of *foxp3* (involved in Treg-differentiation and -function) was not significantly increased
 435 at 122 dph in the thymus as well as in the analysed peripheral lymphoid organs, which does not support
 436 Treg-expansion during this period of medulla maturation in European sea bass. Nevertheless, in the
 437 spleen, *foxp3*-expression was not down-regulated, despite the decrease of *tcra*-expression. Since the
 438 density and proportion of $\alpha\beta$ T cells in the spleen of sea bass is expected to be reduced after 90-101
 439 dph (dos Santos et al., 2000; Romano et al., 2011), this suggests that $\alpha\beta$ Treg may represent a higher
 440 proportion of splenic $\alpha\beta$ T cells and, therefore, expand at this developmental period. Accordingly, in
 441 zebrafish, the increase of *foxp3a* corresponded to the period of thymus ontogenesis when the
 442 regionalization and the maturation of the medulla are acquired, confirming that Treg-development in
 443 teleosts takes place in the medulla, as in the case of mammalian Tregs (Lam et al., 2004; Sugimoto et
 444 al., 2017). Our results, therefore, suggest that *esr2b*- and *gpera*-signalling is important for the
 445 maturation of the thymic medulla. In adult sea bass, *Esr2b* and *Gpers* have been detected in the thymic
 446 parenchyma including endothelial cells, Hassall's corpuscles and the myeloid cells, which reside in
 447 the medulla (Paiola et al., 2017). The increase of *esr2b* and *gpera* expression corroborate, therefore,
 448 the maturation of the medulla as well as the development of the vascular system. Yet, the first
 449 appearance of the myoid cells and Hassall's corpuscles during sea bass ontogenesis remains unknown.
 450 Interestingly, despite their function being unclear, Hassall's corpuscles are apparently involved in Treg

451 production (Watanabe et al., 2005). In fact, ER-subtypes may accomplish different regulatory
452 functions, depending on the timing when and the cells and organs in which they are expressed (Morani,
453 2008; Zhang and Trudeau, 2006). Our results contrast with the observation made in mice, in which
454 ESR1-signalling is apparently crucial for medulla maturation at the late stage. In fact, only ESR1-
455 deficient neonatal mice, but not foetal mice, showed an altered formation of the thymic medulla
456 (Yellayi et al., 2000). Nevertheless, in mice ESR1 is not restricted to the medulla. It is localized in
457 TEC of the medulla and the cortex and its expression is detected during early during gestation at the
458 beginning of the medulla formation (Seiki and Sakabe, 1997). Its dynamics during the neonatal stage
459 as well as the localization of the other oestrogen receptors remain unknown to date. In mice, the effects
460 of neonatal exposure to oestrogen have been poorly described, but include (1) long lasting and time-
461 delayed modulation of ‘delayed-type hypersensitivity’, which is a T cell-dependent immune response
462 (Forsberg, 2000) and (2) induced polycystic ovarian syndrome, which may involve thymic-derived
463 Tregs (Chapman et al., 2009). In summary, these data and our results indicate that E2 is probably
464 involved in the long-lasting establishment of immune tolerance during teleost development through
465 both *gper* and *esr*-signalling. This further supports previous findings in adult sea bass, which also
466 suggested that E2 unbalances immune tolerance by acting on Treg and $\gamma\delta$ T cells (Paiola et al., 2018).

467 Concomitantly, with the thymus maturation, *tcra*- and *tcrg*-expression drastically increased in
468 the liver indicating that this developing stage may correspond to the thymic development of liver-
469 specific $\alpha\beta$ and $\gamma\delta$ T cells, as observed for $\gamma\delta$ T cells of the mammalian skin and intestine, which persist
470 throughout life (Cheroutre et al., 2011; Chien et al., 2014; Muñoz-Ruiz et al., 2017). In fact, *rag2*- and
471 *pcna*-expression, which are indicators of extra-thymic regulation (Boschi et al., 2011; Cheroutre et al.,
472 2011; Goronzy and Weyand, 2019; Huttenhuis et al., 2006), were not significantly altered. In
473 mammals, as probably in teleosts, the involvement of the liver in adaptive immunity allows the
474 clearance of activated T cells and signalling molecules following inflammatory reactions and promotes
475 immunologic tolerance towards potentially antigenic proteins that are absorbed from the intestinal tract
476 (Racanelli and Rehmann, 2006; Zheng and Tian, 2019). In mammals, the thymic medulla is also
477 involved in $\gamma\delta$ T cell-development. Nevertheless, its possible modulation by oestrogen remains to be
478 demonstrated. In fact, in mammals E2 has been suggested to stimulate adult intrathymic development
479 of $\gamma\delta$ T cells, which results in the increase of the number of $\gamma\delta$ T cells in the peripheral organs (Abo,
480 2001; Chapman et al., 2015). The results of the present study also contrast with the observation made
481 in adult sea bass, in which E2-treatment increased *tcrg*-expression in the spleen of males only (Paiola
482 et al., 2018). Overall, the results reinforce our hypothesis that (1) the investigated developmental stages
483 correspond to implementation of the adaptive immune system, including immune tolerance, and that
484 (2) oestrogen plays a key role in its establishment by potentially acting on the thymus.

485 Apart from markers characterising T cell subpopulations, we investigated genes that reflect the
486 selection and differentiation of T cells. In parallel to the increased expression of *mhcIIa* and *rag2*, the
487 $\alpha\beta$ T cells were increased in the thymus, which suggests an intensive T cell-differentiation followed
488 by proliferation activity in the thymus. Notably, RAG2 is expressed in the cortex of the thymus by
489 early immature T cells until positive selection occurs (Ruscher et al., 2017). The up-regulation of *rag2*
490 with age in the thymus indicated that the thymus became functional over the developmental periods
491 investigated in this study. Correspondingly, Øvergård et al. (2011) found an increase of *rag1* from
492 hatching to 159 dph in the whole body of halibut. Furthermore, the same observation was made in
493 whole-body extracts from Senegalese sole larvae from 1 to 33 dph (Ferraresso et al., 2016).
494 Additionally, Picchietti et al. (2015) described an increase of *mhcIIa* in the thymus of the European
495 sea bass with age (from 16 dph to 1 year old), especially at the beginning of the thymus formation and
496 after the regionalization is finalised, which is coherent with our findings.

497 Concerning the modulation of oestrogen receptor-expression by oestrogens, it may vary
498 between species and tissues. The presence of multiple ERs on immune cells and the fact that E2-
499 signalling pathways may be simultaneously induced can result in the highly diverse, cell type-
500 dependent effects in response to oestrogens (Thilagam et al., 2014). In several studies, a differential
501 regulation by E2 was shown for the different ESR-isoforms and between tissues, in both developing
502 and in adult fish (e.g. Casanova-Nakayama et al., 2018; Pinto et al., 2019; Pinto et al., 2016; Seemann
503 et al., 2016). In the present study, among the various ERs analysed in the thymus, only *esr2b* was
504 significantly modulated by E2-treatment for the younger cohort (90 dph). Apparently, E2 triggered the
505 precocious increase of *esr2b* in the thymus, which normally increases with thymus maturation. Similar
506 to our study, the *esr2b* of sea bass head kidney responded with increased expression to E2-exposure at
507 $20 \text{ ng} \cdot \text{L}^{-1}$ in the study of Seemann et al. (Seemann et al., 2016) at the same age. These E2-dependent
508 dynamics in *esr2b* in both, the thymus and the head kidney, suggest a key function during thymopoiesis
509 and hematopoiesis and point to important roles of oestrogen and *esr2b* in the primary lymphoid organ
510 development. Interestingly, the increase of ER-expression in the thymus corresponds to the gonad
511 development and the early production of gonadal E2 (Blázquez et al., 2008). This period could,
512 therefore, correspond to the establishment of interactions along the hypothalamic–pituitary–gonadal-
513 thymus axis. Moreover, in the spleen, only *esr1* and *esr2b* are expressed at 90 dph, but at much lower
514 levels than in the thymus, and appear to decrease in older fish, thus suggesting a lower importance of
515 the classical oestrogenic modulation of this peripheral immune organ during immune system ontogeny.
516 Notwithstanding, the transcripts for the membrane receptor *gperb* are consistently expressed in the
517 spleen of sea bass in both age cohorts, *i.e.*, 90 or 122 dph.

518

5. Conclusion

To the best of our knowledge, this study is the first to investigate the regulatory role of E2, both in thymus and in secondary lymphoid organs, and the first to examine the dynamics of ESRs and GPERs gene expression in immune organs of a teleost fish during thymic development. We have confirmed the importance of oestrogen-signalling in the thymus development across different vertebrates. In fact, although the ER-isoforms have low or undetected levels of expression in the spleen of juvenile European sea bass, all ERs were detected in the maturing thymus and nuclear and membrane ERs, *i.e.*, *esr2b* and *gpera* were upregulated with age along with the maturation of the thymic medulla. In addition, *esr2b* was further modulated by supplementary oestrogen-treatment. These results are indicative of critical roles for these two ER-isoforms in the maturation of the thymic medulla of European sea bass, which contrasts with the establishment of the immunocompetence in mice, in which only ESR1 has been identified as a critical element in the medulla formation.

The expression of different nuclear and membrane receptors suggests that E2 may have complex roles in the immune system ontogenesis. Further research in both, mammals and fish, is needed to identify the extent of conservation of the signalling pathways and the function of E2 throughout evolution. Our results show that in sea bass, as in mammals, the examined oestrogen-sensitive stage of thymus maturation corresponds to the implementation of the adaptive immune system, including immune tolerance, *via* the maturation of the thymic medulla and Treg-development. Finally, our results suggest that the liver plays an important role in extrathymic regulation during the development of the immune system, as it does for mammals.

Acknowledgments

This research was supported by ANR financed project ETaT (ANR-15-CE32-0014), by FCT (project UID/Multi/04326/2019, fellowship SFRH/BPD/84033/2012 and contract with the University of Algarve with reference DL57/2016/CP6321/CT0015 for P.I.S.P.) and by the EU H2020 Research and Innovation Programme AQUAEXEL2020 (grant agreement 652831; TNA project AE050029) and ASSEMBLE PLUS (contract SR02022018976f).

References

- Abo, T., 2001. Extrathymic pathways of T-cell differentiation and immunomodulation. *Int. Immunopharmacol.* 1, 1261-1273.
- Amenyogbe, E., Chen, G., Wang, Z., Lu, X., Lin, M., Lin, A.Y., 2020. A review on sex steroid hormone estrogen receptors in mammals and fish. *Int. J. Endocrinol.* 2020, 5386193.

552 Bajoghli, B., Aghaallaei, N., Hess, I., Rode, I., Netuschil, N., Tay, B.-H., Venkatesh, B., Yu, J.-K.,
 553 Kaltenbach, S.L., Holland, N.D., 2009. Evolution of genetic networks underlying the emergence
 554 of thymopoiesis in vertebrates. *Cell* 138, 186-197.
 555 Blázquez, M., González, A., Papadaki, M., Mylonas, C., Piferrer, F., 2008. Sex-related changes in
 556 estrogen receptors and aromatase gene expression and enzymatic activity during early
 557 development and sex differentiation in the European sea bass (*Dicentrarchus labrax*). *Gen.*
 558 *Comp. Endocrinol.* 158, 95-101.
 559 Boehm, T., Hess, I., Swann, J.B., 2012. Evolution of lymphoid tissues. *Trends Immunol.* 33, 315-
 560 321.
 561 Boschi, I., Randelli, E., Buonocore, F., Casani, D., Bernini, C., Fausto, A.M., Scapigliati, G., 2011.
 562 Transcription of T cell-related genes in teleost fish, and the European sea bass (*Dicentrarchus*
 563 *labrax*) as a model. *Fish Shellfish Immunol.* 31, 655-662.
 564 Bowden, T., Cook, P., Rombout, J., 2005. Development and function of the thymus in teleosts.
 565 *Fish Shellfish Immunol.* 19, 413-427.
 566 Cabas, I., Liarte, S., García-Alcázar, A., Meseguer, J., Mulero, V., García-Ayala, A., 2012. 17 α -
 567 Ethynylestradiol alters the immune response of the teleost gilthead seabream (*Sparus aurata*
 568 *L.*) both *in vivo* and *in vitro*. *Dev. Comp. Immunol.* 36, 547-556.
 569 Carrillo, M., Espigares, F., Felip, A., Escobar, S., Moles, G., Rodriguez, R., Alvarado, M.V., Gomez,
 570 A., Zanuy, S., 2015. Updating control of puberty in male European sea bass: a holistic approach.
 571 *Gen. Comp. Endocrinol.* 221, 42-53.
 572 Casanova-Nakayama, A., Wernicke von Siebenthal, E., Kropf, C., Oldenberg, E., Segner, H., 2018.
 573 Immune-Specific Expression and Estrogenic Regulation of the Four Estrogen Receptor
 574 Isoforms in Female Rainbow Trout (*Oncorhynchus mykiss*). *Int. J. Mol. Sci.* 19 (4), 932.
 575 Chadzinska, M., Leon-Kloosterziel, K.M., Plytycz, B., Verburg-van Kemenade, B.L., 2008. *In vivo*
 576 kinetics of cytokine expression during peritonitis in carp: evidence for innate and alternative
 577 macrophage polarization. *Dev. Comp. Immunol.* 32, 509-518.
 578 Chandrasekar, G., Archer, A., Gustafsson, J.-Å., Lendahl, M.A., 2010. Levels of 17 β -estradiol
 579 receptors expressed in embryonic and adult zebrafish following *in vivo* treatment of natural or
 580 synthetic ligands. *PLOS ONE* 5, e9678.
 581 Chapman, J.C., Chapman, F.M., Michael, S.D., 2015. The production of alpha/beta and
 582 gamma/delta double negative (DN) T-cells and their role in the maintenance of pregnancy.
 583 *Reprod. Biol. Endocrinol.* 13, 1-14.
 584 Chapman, J.C., Min, S.H., Freeh, S.M., Michael, S.D., 2009. The estrogen-injected female mouse:
 585 new insight into the etiology of PCOS. *Reprod. Biol. Endocrinol.* 7, 47.
 586 Chaves-Pozo, E., Muñoz, P., López-Muñoz, A., Pelegrín, P., Ayala, A.G., Mulero, V., Meseguer, J.,
 587 2005. Early innate immune response and redistribution of inflammatory cells in the bony fish
 588 gilthead seabream experimentally infected with *Vibrio anguillarum*. *Cell Tissue Res.* 320, 61-68.
 589 Cheroutre, H., Lambolez, F., Mucida, D., 2011. The light and dark sides of intestinal
 590 intraepithelial lymphocytes. *Nat. Rev. Immunol.* 11, 445-456.
 591 Chien, Y.-h., Meyer, C., Bonneville, M., 2014. $\gamma\delta$ T cells: first line of defense and beyond. *Annu.*
 592 *Rev. Immunol.* 32, 121-155.
 593 Chunhe, C., Dehghani, B., Magrisso, I.J., Rick, E.A., Bonhomme, E., Cody, D.B., Elenich, L.A.,
 594 Subramanian, S., Murphy, S.J., Kelly, M.J., 2008. GPR30 contributes to estrogen-induced thymic
 595 atrophy. *Mol. Endocrinol.* 22, 636-648.
 596 Cuesta, A., Meseguer, J., Esteban, M.A., 2008. The antimicrobial peptide hepcidin exerts an
 597 important role in the innate immunity against bacteria in the bony fish gilthead seabream. *Mol.*
 598 *Immunol.* 45, 2333-2342.
 599 DeWitt, J.C., Peden-Adams, M.M., Keil, D.E., Dietert, R.R., 2012. Current status of developmental
 600 immunotoxicity: early-life patterns and testing. *Toxicol. Pathol.* 40, 230-236.

dos Santos, N.M., Romano, N., de Sousa, M., Ellis, A.E., Rombout, J.H., 2000. Ontogeny of B and T cells in sea bass (*Dicentrarchus labrax*, L.). *Fish Shellfish Immunol.* 10, 583-596.
 Edholm, E.-S., Banach, M., Robert, J., 2016. Evolution of innate-like T cells and their selection by MHC class I-like molecules. *Immunogenetics* 68, 525-536.
 Erlandsson, M.C., Ohlsson, C., Gustafsson, J.Å., Carlsten, H., 2001. Role of oestrogen receptors α and β in immune organ development and in oestrogen - mediated effects on thymus. *Immunology* 103, 17-25.
 Fearon, D.T., 1997. Seeking wisdom in innate immunity. *Nature* 388, 323-324.
 Fearon, D.T., Locksley, R.M., 1996. The instructive role of innate immunity in the acquired immune response. *Science* 272, 50-53.
 Ferrarresso, S., Bonaldo, A., Parma, L., Buonocore, F., Scapigliati, G., Gatta, P.P., Bargelloni, L., 2016. Ontogenetic onset of immune-relevant genes in the common sole (*Solea solea*). *Fish Shellfish Immunol.* 57, 278-292.
 Forsberg, J.-G., 2000. Neonatal estrogen treatment and its consequences for thymus development, serum level of autoantibodies to cardiolipin, and the delayed-type hypersensitivity response. *J. Toxicol. Environ. Health Part A* 60, 185-213.
 Fujikado, N., Mann, A.O., Bansal, K., Romito, K.R., Ferre, E.M., Rosenzweig, S.D., Lionakis, M.S., Benoist, C., Mathis, D., 2016. Aire inhibits the generation of a perinatal population of interleukin-17A-producing $\gamma\delta$ T cells to promote immunologic tolerance. *Immunity* 45, 999-1012.
 Goronzy, J.J., Weyand, C.M., 2019. Mechanisms underlying T cell ageing. *Nat. Rev. Immunol.* 19, 573-583.
 Guerreiro, P.M., Fuentes, J., Canario, A.V., Power, D.M., 2002. Calcium balance in sea bream (*Sparus aurata*): the effect of oestradiol-17 β . *J. Endocrinol.* 173, 377-385.
 Hadidi, S., Glenney, G.W., Welch, T.J., Silverstein, J.T., Wiens, G.D., 2008. Spleen size predicts resistance of rainbow trout to *Flavobacterium psychrophilum* challenge. *J. Immunol.* 180, 4156-4165.
 Huttenhuis, H.B., Romano, N., Van Oosterhoud, C.N., Taverne-Thiele, A.J., Mastrolia, L., Van Muiswinkel, W.B., Rombout, J.H., 2006. The ontogeny of mucosal immune cells in common carp (*Cyprinus carpio* L.). *Anat. Embryol.* 211, 19-29.
 Jagannathan-Bogdan, M., Zon, L.I., 2013. Hematopoiesis. *Development* 140, 2463-2467.
 Kasahara, M., Suzuki, T., Pasquier, L.D., 2004. On the origins of the adaptive immune system: novel insights from invertebrates and cold-blooded vertebrates. *Trends Immunol.* 25, 105-111.
 Klein, L., Hinterberger, M., Wirnsberger, G., Kyewski, B., 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat. Rev. Immunol.* 9, 833-844.
 Kondo, Y., Goto, C., Abe, A., 2004. Effects of estrogen treatment during the embryogenic period on chick antibody production. *J. Poult. Sci.* 41, 85-93.
 Lafont, A.-G., Rousseau, K., Tomkiewicz, J., Dufour, S., 2016. Three nuclear and two membrane estrogen receptors in basal teleosts, *Anguilla* sp.: Identification, evolutionary history and differential expression regulation. *General and comparative endocrinology* 235, 177-191.
 Lam, S., Chua, H., Gong, Z., Lam, T., Sin, Y., 2004. Development and maturation of the immune system in zebrafish, *Danio rerio*: a gene expression profiling, in situ hybridization and immunological study. *Dev. Comp. Immunol.* 28, 9-28.
 Leceta, J., Fernandez-Galaz, C., Sanchez, C., Navarro, R., Gomariz, R., Zapata, A., Tejero, A., 1988. Effects of neonatal treatment with estrogens on the development of the thymus in rats. *Dev. Comp. Immunol.* 12, 375-383.
 Li, C.-L., Toda, K., Saibara, T., Zhang, T., Ono, M., Iwasaki, S., Maeda, T., Okada, T., Hayashi, Y., Enzan, H., 2002. Estrogen deficiency results in enhanced expression of Smoothed of the

649 Hedgehog signaling in the thymus and affects thymocyte development. *Int. Immunopharmacol.*
 650 2, 823-833.
 651 Li, M.O., Rudensky, A.Y., 2016. T cell receptor signalling in the control of regulatory T cell
 652 differentiation and function. *Nat. Rev. Immunol.* 16, 220-233.
 653 Mantegazza, A.R., Magalhaes, J.G., Amigorena, S., Marks, M.S., 2013. Presentation of
 654 phagocytosed antigens by MHC class I and II. *Traffic* 14, 135-152.
 655 Mebius, R.E., Kraal, G., 2005. Structure and function of the spleen. *Nat. Rev. Immunol.* 5, 606-
 656 616.
 657 Mitter, K., Kotoulas, G., Magoulas, A., Mulero, V., Sepulcre, P., Figueras, A., Novoa, B.,
 658 Sarropoulou, E., 2009. Evaluation of candidate reference genes for QPCR during ontogenesis
 659 and of immune-relevant tissues of European seabass (*Dicentrarchus labrax*). *Comp. Biochem.*
 660 *Physiol., Part B: Biochem. Mol. Biol.* 153, 340-347.
 661 Möller, A.-M., Korytář, T., Köllner, B., Schmidt-Posthaus, H., Segner, H., 2014. The teleostean
 662 liver as an immunological organ: Intrahepatic immune cells (IHICs) in healthy and benzo [a]
 663 pyrene challenged rainbow trout (*Oncorhynchus mykiss*). *Dev. Comp. Immunol.* 46, 518-529.
 664 Morani, A., 2008. Role of estrogen receptor beta in epithelia lacking estrogen receptor alpha
 665 expression. *Biovetenskaper och näringslära/Biosciences and Nutrition*.
 666 Muñoz-Ruiz, M., Sumaria, N., Pennington, D.J., Silva-Santos, B., 2017. Thymic determinants of $\gamma\delta$
 667 T cell differentiation. *Trends Immunol.* 38, 336-344.
 668 Neely, H.R., Flajnik, M.F., 2016. Emergence and evolution of secondary lymphoid organs. *Annu.*
 669 *Rev. Cell Dev. Biol.* 32, 693-711.
 670 Øvergård, A.-C., Fiksdal, I.U., Nerland, A.H., Patel, S., 2011. Expression of T-cell markers during
 671 Atlantic halibut (*Hippoglossus hippoglossus* L.) ontogenesis. *Dev. Comp. Immunol.* 35, 203-213.
 672 Paiola, M., Knigge, T., Duflot, A., Pinto, P.I.S., Farcy, E., Monsinjon, T., 2018. Oestrogen, an
 673 evolutionary conserved regulator of T cell differentiation and immune tolerance in jawed
 674 vertebrates? *Dev. Comp. Immunol.* 84, 48-61.
 675 Paiola, M., Knigge, T., Picchietti, S., Duflot, A., Guerra, L., Pinto, P.I.S., Scapigliati, G., Monsinjon,
 676 T., 2017. Oestrogen receptor distribution related to functional thymus anatomy of the
 677 European sea bass, *Dicentrarchus labrax*. *Dev. Comp. Immunol.* 77, 106-120.
 678 Papadaki, M., Piferrer, F., Zanuy, S., Maingot, E., Divanach, P., Mylonas, C.C., 2005. Growth, sex
 679 differentiation and gonad and plasma levels of sex steroids in male- and female-dominant
 680 populations of *Dicentrarchus labrax* obtained through repeated size grading. *J. Fish Biol.* 66,
 681 938-956.
 682 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR.
 683 *Nucleic acids research* 29, e45-e45.
 684 Picchietti, S., Abelli, L., Guerra, L., Randelli, E., Serafini, F.P., Belardinelli, M., Buonocore, F.,
 685 Bernini, C., Fausto, A., Scapigliati, G., 2015. MHC II- β chain gene expression studies define the
 686 regional organization of the thymus in the developing bony fish *Dicentrarchus labrax* (L.). *Fish*
 687 *Shellfish Immunol.* 42, 483-493.
 688 Pinto, P.I., Estevao, M.D., Power, D.M., 2014. Effects of estrogens and estrogenic disrupting
 689 compounds on fish mineralized tissues. *Mar. Drugs* 12, 4474-4494.
 690 Pinto, P.I.S., Andrade, A.R., Moreira, C., Zapater, C., Thorne, M.A.S., Santos, S., Estevao, M.D.,
 691 Gomez, A., Canario, A.V.M., Power, D.M., 2019. Genistein and estradiol have common and
 692 specific impacts on the sea bass (*Dicentrarchus labrax*) skin-scale barrier. *J. Steroid Biochem.*
 693 *Mol. Biol.* 195, 105448.
 694 Pinto, P.I.S., Estevao, M.D., Andrade, A., Santos, S., Power, D.M., 2016. Tissue responsiveness to
 695 estradiol and genistein in the sea bass liver and scale. *J. Steroid Biochem. Mol. Biol.* 158, 127-
 696 137.

697 Plitas, G., Rudensky, A.Y., 2016. Regulatory T cells: differentiation and function. *Cancer*
698 *Immunol. Res.* 4, 721-725.

699 Quintana, F.J., Iglesias, A.H., Farez, M.F., Caccamo, M., Burns, E.J., Kassam, N., Oukka, M., Weiner,
700 H.L., 2010. Adaptive autoimmunity and Foxp3-based immunoregulation in zebrafish. *PLoS One*
701 5, e9478.

702 Racanelli, V., Rehermann, B., 2006. The liver as an immunological organ. *Hepatology* 43, S54-
703 S62.

704 Randelli, E., Buonocore, F., Casani, D., Fausto, A.M., Scapigliati, G., 2009. An “immunome” gene
705 panel for transcriptomic analysis of immune defence activities in the teleost sea bass
706 (*Dicentrarchus labrax* L.): a review. *Ital. J. Zool.* 76, 146-157.

707 Rehberger, K., Wernicke von Siebenthal, E., Bailey, C., Bregy, P., Fasel, M., Herzog, E.L.,
708 Neumann, S., Schmidt-Posthaus, H., Segner, H., 2020. Long-term exposure to low 17alpha-
709 ethinylestradiol (EE2) concentrations disrupts both the reproductive and the immune system
710 of juvenile rainbow trout, *Oncorhynchus mykiss*. *Environ. Int.* 142, 105836.

711 Robertson, L.S., Iwanowicz, L.R., Marranca, J.M., 2009. Identification of centrarchid hepcidins
712 and evidence that 17 β -estradiol disrupts constitutive expression of hepcidin-1 and inducible
713 expression of hepcidin-2 in largemouth bass (*Micropterus salmoides*). *Fish Shellfish Immunol.*
714 26, 898-907.

715 Romano, N., Caccia, E., Piergentili, R., Rossi, F., Ficca, A.G., Ceccariglia, S., Mastrolia, L., 2011.
716 Antigen-dependent T lymphocytes (TcR β +) are primarily differentiated in the thymus rather
717 than in other lymphoid tissues in sea bass (*Dicentrarchus labrax*, L.). *Fish Shellfish Immunol.*
718 30, 773-782.

719 Ruscher, R., Kummer, R.L., Lee, Y.J., Jameson, S.C., Hogquist, K.A., 2017. CD8 $\alpha\alpha$ intraepithelial
720 lymphocytes arise from two main thymic precursors. *Nat. Immunol.* 18, 771.

721 Sakaguchi, S., Sakaguchi, N., Shimizu, J., Yamazaki, S., Sakihama, T., Itoh, M., Kuniyasu, Y.,
722 Nomura, T., Toda, M., Takahashi, T., 2001. Immunologic tolerance maintained by CD25+ CD4+
723 regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and
724 transplantation tolerance. *Immunol. Rev.* 182, 18-32.

725 Scapigliati, G., Fausto, A.M., Picchiatti, S., 2018. Fish lymphocytes: an evolutionary equivalent of
726 mammalian innate-like lymphocytes? *Front. Immunol.* 9, 971.

727 Seemann, F., Knigge, T., Duflot, A., Marie, S., Olivier, S., Minier, C., Monsinjon, T., 2016. Sensitive
728 periods for 17 β -estradiol exposure during immune system development in sea bass head
729 kidney. *J. Appl. Toxicol.* 36, 815-826.

730 Seemann, F., Knigge, T., Olivier, S., Monsinjon, T., 2015. Exogenous 17beta-oestradiol (E2)
731 modifies thymus growth and regionalization in European sea bass *Dicentrarchus labrax*. *J. Fish*
732 *Biol.* 86, 1186-1198.

733 Seemann, F., Knigge, T., Rocher, B., Minier, C., Monsinjon, T., 2013. 17beta-Estradiol induces
734 changes in cytokine levels in head kidney and blood of juvenile sea bass (*Dicentrarchus labrax*,
735 L., 1758). *Mar. Environ. Res.* 87-88, 44-51.

736 Seemann, F., Peterson, D.R., Chiang, M.W.L., Au, D.W.T., 2017. The development of cellular
737 immune defence in marine medaka *Oryzias melastigma*. *Comp. Biochem. Physiol. C Toxicol.*
738 *Pharmacol.* 199, 81-89.

739 Segner, H., Casanova-Nakayama, A., Kase, R., Tyler, C.R., 2013. Impact of environmental
740 estrogens on fish considering the diversity of estrogen signaling. *Gen. Comp. Endocrinol.* 191,
741 190-201.

742 Seiki, K., Sakabe, K., 1997. Sex hormones and the thymus in relation to thymocyte proliferation
743 and maturation. *Arch. Histol. Cytol.* 60, 29-38.

744 Shevach, E.M., 2002. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat. Rev.*
745 *Immunol.* 2, 389-400.

746 Straub, R.H., 2007. The complex role of estrogens in inflammation. *Endocr. Rev.* 28, 521-574.
 747 Sugimoto, K., Hui, S.P., Sheng, D.Z., Nakayama, M., Kikuchi, K., 2017. Zebrafish FOXP3 is
 748 required for the maintenance of immune tolerance. *Dev. Comp. Immunol.* 73, 156-162.
 749 Szejser, E., Pijanowski, L., Maciuszek, M., Ptak, A., Wartalski, K., Duda, M., Segner, H., Verburg-
 750 van Kemenade, B.L., Chadzinska, M., 2017. Stress differentially affects the systemic and
 751 leukocyte estrogen network in common carp. *Fish Shellfish Immunol.* 68, 190-201.
 752 Thilagam, H., Gopalakrishnan, S., Bo, J., Wang, K.-J., 2014. Comparative study of 17 β -estradiol
 753 on endocrine disruption and biotransformation in fingerlings and juveniles of Japanese sea
 754 bass *Lateolabrax japonicus*. *Mar. Pollut. Bull.* 85, 332-337.
 755 Thomas, P., Alyea, R., Pang, Y., Peyton, C., Dong, J., Berg, A., 2010. Conserved estrogen binding
 756 and signaling functions of the G protein-coupled estrogen receptor 1 (GPER) in mammals and
 757 fish. *Steroids* 75, 595-602.
 758 Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F.,
 759 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of
 760 multiple internal control genes. *Genome Biol.* 3, research0034. 0031.
 761 Watanabe, N., Wang, Y.-H., Lee, H.K., Ito, T., Wang, Y.-H., Cao, W., Liu, Y.-J., 2005. Hassall's
 762 corpuscles instruct dendritic cells to induce CD4⁺ CD25⁺ regulatory T cells in human thymus.
 763 *Nature* 436, 1181-1185.
 764 Wenger, M., Shved, N., Akgul, G., Caelers, A., Casanova, A., Segner, H., Eppler, E., 2014.
 765 Developmental oestrogen exposure differentially modulates IGF-I and TNF-alpha expression
 766 levels in immune organs of Yersinia ruckeri-challenged young adult rainbow trout
 767 (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 205, 168-175.
 768 Wing, K., Sakaguchi, S., 2010. Regulatory T cells exert checks and balances on self tolerance and
 769 autoimmunity. *Nat. Immunol.* 11, 7-13.
 770 Yellayi, S., Teuscher, C., Woods, J.A., Welsh, T.H., Tung, K.S., Nakai, M., Rosenfeld, C.S., Lubann,
 771 D.B., Cooke, P.S., 2000. Normal development of thymus in male and female mice requires
 772 estrogen/estrogen receptor- α signaling pathway. *Endocrine* 12, 207-213.
 773 Zha, J., Wang, Z., Wang, N., Ingersoll, C., 2007. Histological alternation and vitellogenin
 774 induction in adult rare minnow (*Gobiocypris rarus*) after exposure to ethynylestradiol and
 775 nonylphenol. *Chemosphere* 66, 488-495.
 776 Zhang, D., Trudeau, V.L., 2006. Integration of membrane and nuclear estrogen receptor
 777 signaling. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol* 144, 306-315.
 778 Zheng, M., Tian, Z., 2019. Liver-mediated adaptive immune tolerance. *Front. Immunol.* 10,
 779 2525.
 780 Zhou, Z., Xu, M.-J., Gao, B., 2016. Hepatocytes: a key cell type for innate immunity. *Cell. Mol.*
 781 *Immunol.* 13, 301-315.
 782 Žuklys, S., Handel, A., Zhanybekova, S., Govani, F., Keller, M., Maio, S., Mayer, C.E., Teh, H.Y.,
 783 Hafen, K., Gallone, G., 2016. Foxn1 regulates key target genes essential for T cell development
 784 in postnatal thymic epithelial cells. *Nat. Immunol.* 17, 1206-1215.