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

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1 **Abstract**

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

31

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

33 **Abbreviations:** *18s*, 18S ribosomal RNA gene; E2, 17 β -oestradiol; ER, oestrogen receptor; ESR,
34 nuclear oestrogen receptor; *fau*, 40S ribosomal protein S30; *foxn1*, transcription factor forkhead-box
35 n1; *foxp3*, forkhead transcriptional regulator P3; GPER, G protein-coupled oestrogen receptor; HSI,
36 Hepato-somatic index; K, Fulton's condition factor; *l13a*, L13a ribosomal protein; *mhc*, major
37 histocompatibility complex; *pcna*, proliferating cell nuclear antigen; qPCR, quantitative reverse
38 transcriptase polymerase chain reaction; *rag*, recombination-activating genes; TEC, thymic epithelial
39 cells; Tregs, regulatory T cells. Protein and gene nomenclature followed that recommended by
40 [genenames.org](http://zfin.org/) and used for fish at <http://zfin.org/>.

41

42

43 **1. Introduction**

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

61 The thymus plays a pivotal role in the ontogenesis of the adaptive immune system. It is the
62 major site for T lymphocyte-education and -maturation, *i.e.*, thymopoiesis. T cell-development,
63 including T cell-commitment and -maturation, depends on the interaction with TECs, the function of
64 which is regulated by the transcription factor forkhead-box n1 (FOXP1) (Bajoghli et al., 2009;
65 Žuklys et al., 2016). Among the various conventional T cell subpopulations, the regulatory T cells
66 (Tregs) are particular in that they develop either in the thymus, or in peripheral immune organs, such
67 as the spleen or the liver, depending on their TCR specificity (Plitas and Rudensky, 2016). Their
68 function is to regulate innate and adaptive immune cell activity and to maintain self- and non-self-
69 tolerance (Quintana et al., 2010; Wing and Sakaguchi, 2010). The forkhead transcriptional regulator
70 P3 (FOXP3) is essential for the genetic programming of Treg-differentiation and -function
71 (Sakaguchi et al., 2001; Shevach, 2002). From the thymus, naïve and mature self-tolerant T cells
72 emigrate to peripheral lymphoid organs, including the spleen and the liver (Bowden et al., 2005).
73 Thymopoiesis and the regulation of the abundance of peripheral T cells is dependent on cell
74 migration and on the expansion of proliferating cell nuclear antigen (PCNA)-positive T cells
75 (Goronzy and Weyand, 2019). B and T lymphocytes colonize the spleen after they have been

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

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

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

110 while mammals have only the GPER1 form. The functions of GPERs are less well understood, but it
111 is now evident that both types of signalling and receptors may interact with each other (Amenyogbe
112 et al., 2020; Lafont et al., 2016; Pinto et al., 2014; Thomas et al., 2010). The expression pattern of
113 the full set of nuclear and membrane receptors determines the global cellular response to oestrogens.
114 According to the conserved immunomodulatory function of oestrogen, the various ER-isoforms have
115 been detected in immune cells and immune organs, including the thymus, across numerous
116 vertebrates from mammals to fish (Paiola et al., 2018; Segner et al., 2013). In addition to its
117 modulatory role in thymic T cell-differentiation, as evidenced in mammals with a thymus atrophy
118 during pregnancy or after puberty, oestrogen appears to be involved in thymus ontogenesis. In fact,
119 aromatase-, ESR2-, GPER- and especially ESR1-knockout mice display defective thymus
120 ontogenesis (Chunhe et al., 2008; Erlandsson et al., 2001; Li et al., 2002; Yellayi et al., 2000).
121 Interestingly, studies from various jawed vertebrates, including the European sea bass
122 (*Dicentrarchus labrax*), have shown that oestrogen can also increase thymus volume during critical
123 windows of the thymus ontogenesis, during which immunocompetence is established (DeWitt et al.,
124 2012; Kondo et al., 2004; Seemann et al., 2015). Therefore, oestrogen-exposure during thymus
125 ontogenesis may have long-term consequences for the T cell-dependent immune response in
126 mammals (Chapman et al., 2009; Forsberg, 2000) and in fish (Rehberger et al., 2020).

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

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143 2. Materials and methods

144 2.1. Animal rearing

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

153 The experimental protocols involving fish were approved by the Ethics and Animal Welfare
154 Committee of IATS (CSIC) and Generalitat Valenciana. They were carried out in a registered
155 installation facility in accordance with the principles published in the European animal directive
156 (2010/63/EU) and Spanish laws (Royal Decree RD53/2013) for the protection of animals, and all
157 efforts were made to minimize the suffering of animals.

158

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

162

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 ± 0.17 g·cm ⁻³ E2: 0.81 ± 0.11 g·cm ⁻³	CTR: 0.94 ± 0.15 g·cm ⁻³ E2: 0.95 ± 0.06 g·cm ⁻³
HSI ²	CTR: 2.71 ± 1.03 % E2: 1.84 ± 0.67 %	CTR: 1.58 ± 0.58 % E2: 1.49 ± 0.33 %

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

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

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

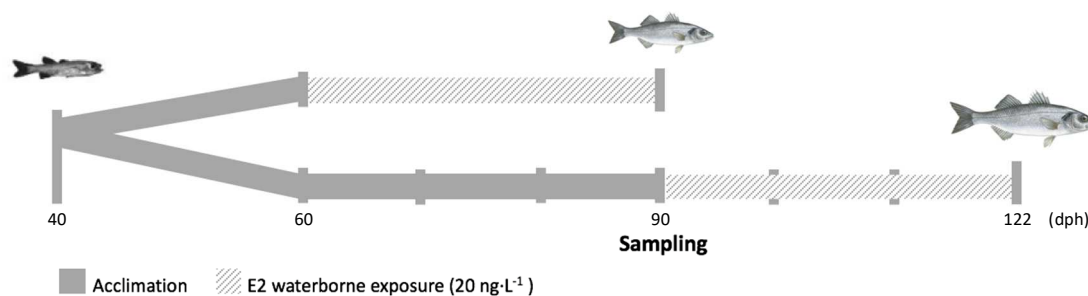
167

168 2.2. Treatment and sampling

169 Following the acclimation period, animals were exposed in the same 20 L aquarium under
170 semi-static conditions for 30 days (beginning at either 60 or 93 dph, see Fig.1) to a concentration of
171 20 ng·L⁻¹ of waterborne E2 (Sigma-Aldrich) diluted in methanol, or to the solvent control only (*i.e.*,
172 filtered sea water with the equivalent concentration of methanol at 0.000001%, v/v). Previous studies
173 have shown this waterborne E2 concentration to be most effective in soliciting immune-related
174 responses in juvenile sea bass at similar ages (Seemann et al., 2016; Seemann et al., 2015; Seemann
175 et al., 2013).

176 Water quality and E2-concentrations were assured by the daily exchange of 80% of the water
177 body with subsequent E2-spike for the treatment groups. Once a week, a water sample from each
178 aquarium (before and after dosing) was collected and stored at 4 °C in the dark until analysis of E2-
179 concentrations. After 30 days of exposure, *i.e.*, at a final age of 90 dph and 122 dph, respectively, 12
180 fish were randomly chosen from the different tank replicates of both treatments (control and exposed
181 – see Table 1 for details) to avoid tank effects. Fish were sacrificed with an overdose of MS-222
182 (Sigma-Aldrich) and body weight and length were recorded. Blood was not collected from the
183 sampled fish but instead, the tissues were carefully dissected on paper to absorb the excess of blood.
184 Whole thymus, spleen, and liver comprising all cell types contained in these organs (including
185 lymphocytes) were dissected from the same 12 fish that were embedded in RNA later (Sigma-
186 Aldrich) and stored at -20 °C until RNA extraction. All fish analysed in the present study were
187 considered as 'sexually immature', as the first signs of histological sex differentiation in sea bass
188 usually appear only after 150 dph in females to 200 dph in males (Blázquez et al., 2008; Carrillo et
189 al., 2015), while at the analysed ages (60 to 122 dph) the undifferentiated gonads are not sufficiently
190 developed to differentially produce sex steroid hormones. Papadaki et al. 2005 (Papadaki et al.,
191 2005) reported a differential peak in circulating E2-levels only after 200 dph, when the first primary
192 oocytes appeared.

193 The Fulton's condition factor (K) and hepato-somatic index (HSI) were calculated according
194 to Hadidi et al. (2008) and Zha et al. (2007), respectively: $K \text{ (g}\cdot\text{cm}^{-3}\text{)} = \text{body weight (g)} \cdot (\text{total}$
195 $\text{length (cm)})^{-3}$; $\text{HSI (\%)} = \text{liver weight (g)} / \text{body weight (g)} \times 100$. Details of the experiment,
196 physiochemical parameters and biometric indices of the experiment are specified in Table 1.
197



198
199 **Figure 1.** Experimental design: juvenile European sea bass, *Dicentrarchus labrax*, from the same batch were
200 acclimated and exposed to $20 \text{ ng}\cdot\text{L}^{-1}$ of waterborne 17β -oestradiol (E2) at two developmental stages, *i.e.*, at 60
201 and 93 dph, for 30 days. At the end of exposure time, the fish were sampled ($n = 12$ fish per treatment and the
202 same eight fish were used for gene expression analysis in the thymus, spleen and liver) at a final age of 90 dph
203 and 122 dph, respectively.
204

205 2.3. Oestradiol radioimmunoassay

206 The oestrogenic compounds were extracted from water using Sep-Pak cartridges Vac C18
207 1cc (Waters) from a 1.8 mL sample of each aquarium, resolved in 4 mL ethanol (Sigma-Aldrich),
208 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
209 dph cohort were macerated, followed by an ether extraction with 200 μL of distilled water and 3 mL
210 of diethyl ether (VWR) and centrifuging at 40 g for 5 min. Samples were frozen in liquid nitrogen
211 for 5 s, the ether phase was decanted and evaporated and 1 mL of gelatine buffer was added. Water
212 and liver E2-levels were determined in duplicate using an optimized radioimmunoassay (RIA) and a
213 specific antiserum against E2 (Guerreiro et al., 2002). Free hormones were separated from total
214 bound hormones using dextran-coated charcoal and a control thin-layer chromatography (TLC) was
215 performed to confirm that E2 has been quantified in both water and liver samples. The quantification
216 of E2 in the liver was only used as a technical validation E2-uptake by the fish. Because the older
217 fish provided enough material for both molecular and E2 measurements, these were used as
218 representatives for the validation of the E2 exposure.
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220 2.4. Gene expression

221 The expression of several genes was assessed in the three different immune organs (thymus,
222 spleen, and liver) unless stated otherwise. Details on the genes and their respective primers are given
223 in Table 2. Total RNA was extracted using PureZOL (BioRad) according to the supplier's
224 instructions. Briefly, whole organs (except for liver, which was sectioned) were grinded in a glass
225 homogenizer with 850 μL of PureZOL. Then, 170 μL of chloroform (Sigma-Aldrich) was added and
226 the samples were centrifuged at 12,000 g for 15 min at 4 $^{\circ}\text{C}$ to eliminate the debris. Glycogen
227 (Glycoblu, Ambion) and ammonia acetate (Sigma-Aldrich) were added to have a final
228 concentration of 0.05 $\text{mg}\cdot\text{mL}^{-1}$ and 0.5 M, respectively, as well as one half of the volume of
229 isopropyl alcohol and then incubated overnight at -80 $^{\circ}\text{C}$, in order to improve the precipitation and
230 visualization of the pellet. Subsequently, the samples were centrifuged at 12,000 g for 15 min at 4
231 $^{\circ}\text{C}$, washed with 75% ethanol and the pellet was resuspended in RNase-free water (MilliQ water,
232 Sigma-Aldrich). After RNA-extraction, possible DNA-contamination was removed by digestion with
233 the TURBO DNA-free Kit (Invitrogen Ambion) according to the supplier's instructions. The yield
234 and purity of RNA were quantified with a Nanodrop One (ThermoFisher) at 260 nm and 280 nm.
235 Absorbance ratios 260/280 between 1.90 and 2.00 indicated pure RNA samples. Eight extracts that
236 yielded RNA with good quality and quantity from isolated tissues were selected for gene expression,
237 with a representation across all replicate tanks. Samples were stored at -80 $^{\circ}\text{C}$ until further
238 processing. Reverse transcription was performed using 500 ng of RNA for all samples with the
239 exception of thymus and spleen from the younger cohort (250 and 350 ng, respectively), 200 $\text{ng}\cdot\mu\text{L}^{-1}$
240 of random hexamers, 10nM of dNTP and MMLV-RT (reverse transcriptase, Promega) in a final
241 volume of 20 μL . RNA was denatured for 5 min at 70 $^{\circ}\text{C}$ and the cDNA-synthesis proceeded for 60
242 min at 42 $^{\circ}\text{C}$, after which the cDNA was stored at -20 $^{\circ}\text{C}$. Real-time quantitative PCR (qPCR) was
243 performed using two technical replicates per individual sample by separately adding cDNA diluted
244 1:20 in MilliQ water.

245 All genes were analysed with Rotor-Gene Q (Quiagen) using one of two commercial master
246 mixes, QuanTitec (Quiagen) or EvaGreen® (BioRad), according to the manufacturer's
247 recommendations (see Table 2, for details). For each organ and primer pair, the amplification
248 efficiency was calculated by running serial dilutions of cDNA-pools from samples of all conditions.
249 The efficiencies ranged from 1.90 and 2.20. All qPCR reactions had a single peak melt curve and
250 primer specificity was confirmed by sequencing the amplicons. Three reference genes, *i.e.*, the
251 ribosomal protein L13a (*l13a*), the 40S ribosomal protein S30 (*fau*) and the 18S ribosomal RNA sub-
252 unit (*18S*) were used for the quantification of relative gene expression. The primers of the reference
253 genes were previously tested with the same species following oestrogenic exposure (Paiola et al.,
254 2018; Pinto et al., 2016) and they were evaluated as generally stable during ontogenesis (Mitter et

255 al., 2009). Using Pfaffl's formula (2001), the relative expression of each gene of interest was
 256 calculated using the efficiency of each primer pair for each organ and the geometric mean of the
 257 three reference genes as recommended by Vandesompele et al. (2002). Gene-expressions were
 258 calibrated by normalizing individual expression to the average of all cDNAs across treatments and
 259 age.

260

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

Genes	Function	Primer source	Amplicon (bp)	Annealing Temperature	Analysed organs
<i>esr1</i>	Oestrogen regulation (via NISS [†])	Seemann et al. (Seemann et al., 2015)	139	60 °C	Thymus, Spleen
<i>esr2a</i>	Oestrogen regulation (via NISS)	Seemann et al. (Seemann et al., 2015)	118	61 °C	Thymus, Spleen
<i>esr2b</i>	Oestrogen regulation (via NISS)	Seemann et al. (Seemann et al., 2015)	295	61.5 °C	Thymus, Spleen
<i>gpera</i>	Oestrogen regulation (via MISS [‡])	Pinto et al. (Pinto et al., 2016)	157	60°C	Thymus, Spleen
<i>gperb</i>	Oestrogen regulation (via 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>pna</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>mhclla</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	221	60°C	Spleen

		al. (Seemann et al., 2013)			
<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

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2.5. Statistical analysis

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

¹ F: CCGTGGCTGCAATCGTAAAC; R: ACACAAGCTGTCTCCCTCG

² F: GACACTCGTGCTCGCCTTFA; R: TTGAGTGTGTCATTGCTCCCTG

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

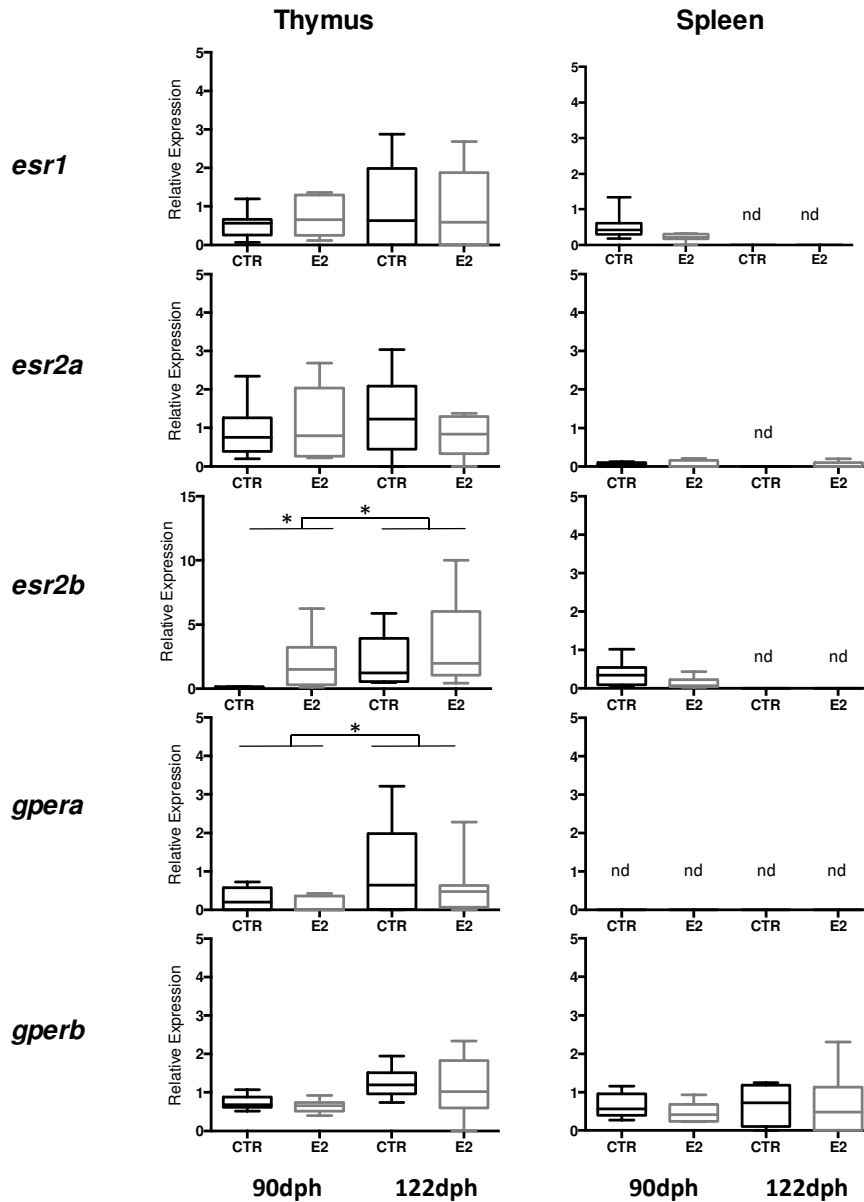
294 1). The survival rate was about 95% and did not differ between treated and control groups, which
295 indicates that 30 days exposure to 20 ng E2·L⁻¹ did not negatively affect survival of fish of either of
296 the age cohorts.

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298 **3.2. Oestrogen receptors gene expression**

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

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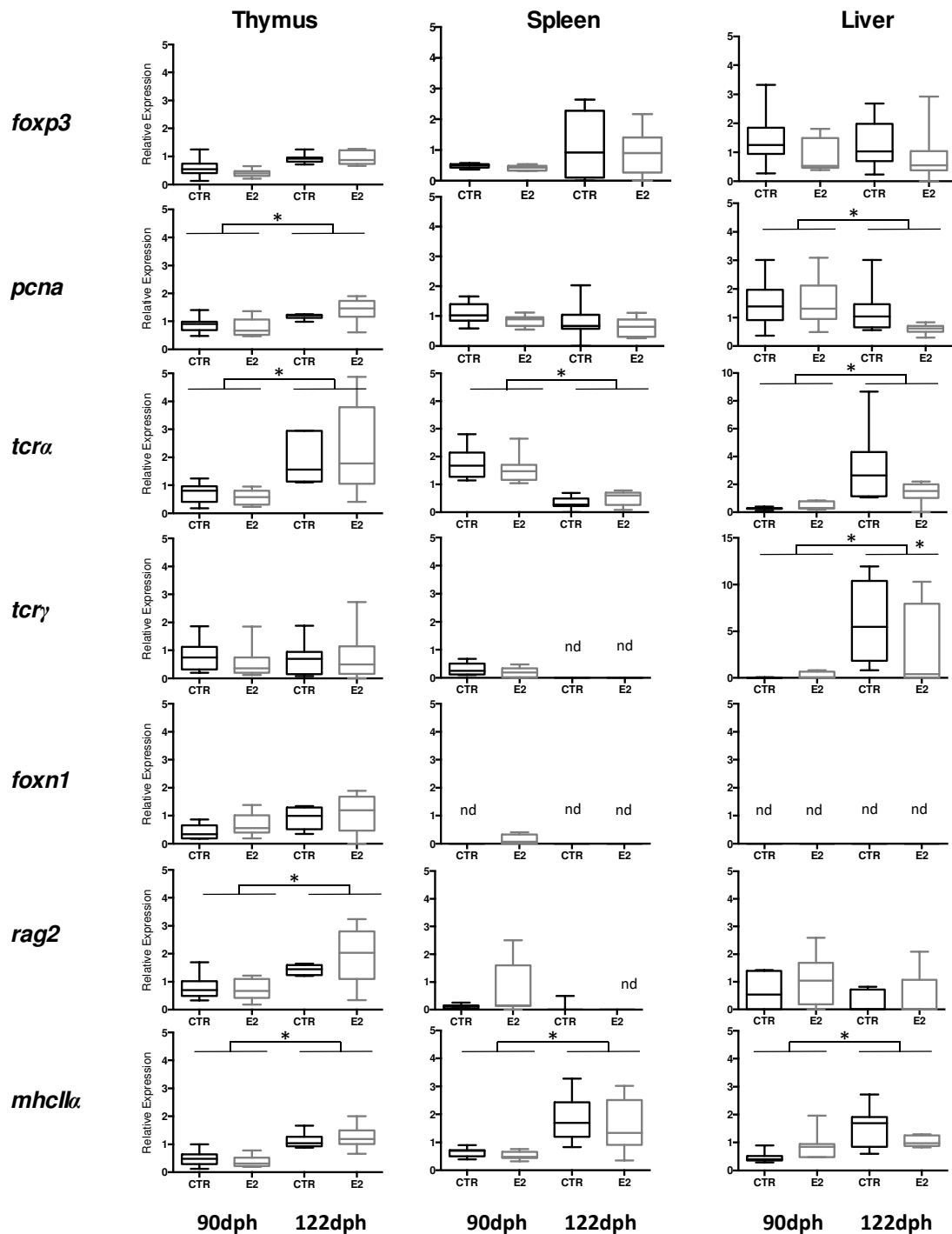
313 **Fig. 2.** Relative expression of the oestrogen receptors in the thymus and spleen of European sea bass,
 314 *Dicentrarchus labrax*, sampled at two developmental stages, *i.e.*, 90 and 122 dph, after exposure to E2 (20
 315 ng·L⁻¹) for 30 days, or in non-exposed control fish (CTR). Expression-levels are related to the geometric mean
 316 of the expression of three reference genes (*fau*, *l13a* and *18S*). The graphs are represented as box-and-whisker
 317 plots with the median, 25 % and 75 % quartiles as well as extreme values depicted by the bars. * indicates
 318 significant differences between the two age cohorts or between E2-exposed and CTR fish within the same
 319 age, at $p \leq 0.05$; nd, non-detectable; n = 8 per treatment for all the organs and ages.

320

321 3.3. Immune-related gene expression

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

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



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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*, *il3a* 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.

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

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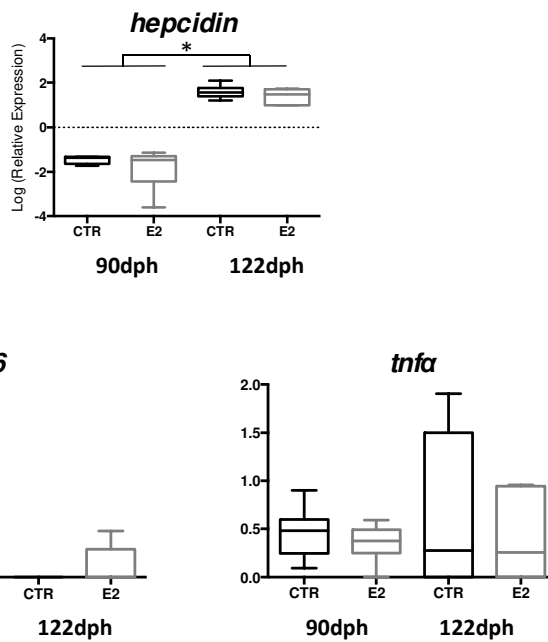
375

376 **Fig. 4.** Relative expression of genes for two cytokines (*il6* and *tnfa*) in the spleen and *hepcidin* in the liver of
377 90 and 122 dph European sea bass, *Dicentrarchus labrax*, either exposed to 20 ng E2·L⁻¹ (E2), or non-
378 exposed controls (CTR). Gene expression levels are related to the geometric mean of the expression of three
379 reference genes (*fau*, *il13a* and *l8s*). Box-and-whisker plots show median, 25 % and 75 % quartiles as well as
380 extreme values depicted by the bars. *Hepcidin* expression was log-transformed for representation. *,
381 significant differences between the two age cohorts at $p \leq 0.05$; n = 8 per treatment, organs and ages.

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383 4. Discussion

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



389 final phase of thymus maturation, particularly of the medulla (dos Santos et al., 2000; Romano et al.,
390 2011). First of all, we validated the oestrogen uptake, and then investigated oestrogen responsiveness
391 (ERs) and gene expression related to T cell-development in the thymus and in two peripheral
392 immune organs, the spleen and the liver.

393 The E2-concentrations in the water and in the liver of exposed fish confirmed that the correct
394 nominal E2-concentration of the water was obtained and that E2 was indeed taken up by the fish.
395 Nevertheless, no significant effects of E2 were observed in any of the general biometric parameters
396 of the two cohorts. In addition, neither the inflammatory cytokines, nor the *hepcidin*-expression
397 responded to the exposure to 20 ng E2·L⁻¹ at the analysed time points. Together with interleukin 1β
398 (IL-1β), TNFα is one of the first cytokines present in the proinflammatory process of fish and
399 mammals (Chadzinska et al., 2008). Previous studies on juvenile fish have shown a reduction of
400 proinflammatory cytokine *tnfa*- and *il6*-expression, while others found an increase of the same
401 cytokines following exposure to oestrogens (Cabas et al., 2012; Chaves-Pozo et al., 2005; Seemann
402 et al., 2013; Wenger et al., 2014). This suggests that depending on the concentration of oestrogens,
403 the age or the species, E2 can either be a pro- or an anti-inflammatory regulator in fish, as has been
404 reported for mammals (Straub, 2007). Furthermore, *hepcidin*, a protein linked to iron metabolism
405 that may influence innate immunity (Cuesta et al., 2008; Zhou et al., 2016) was previously shown to
406 be down-regulated by E2 in the liver of six-month old largemouth bass, four days after E2-injection
407 (Robertson et al., 2009). In summary, *tnfa*, *il6* and *hepcidin* are components or modulators of the
408 immune system that have been previously shown to respond to E2 in particular conditions, but in the
409 present study, the concentration used and the timing chosen did not reveal any significant effect of
410 E2 on their gene expression. However, the increase in *hepcidin*-expression with age was pronounced
411 and similar to that previously detected in whole larvae from 1 to 33 dph in sole (Ferrareso et al.,
412 2016), which can be related to a change in iron metabolism.

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

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

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

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

491 hypothesis that (1) the investigated developmental stages correspond to implementation of the
492 adaptive immune system, including immune tolerance, and that (2) oestrogen plays a key role in its
493 establishment by potentially acting on the thymus.

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

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

525 immune organ during immune system ontogeny. Notwithstanding, the transcripts for the membrane
526 receptor *gperb* are consistently expressed in the spleen of sea bass in both age cohorts, *i.e.*, 90 or 122
527 dph.

528

529 **5. Conclusion**

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

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

550

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557

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