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Oestrogen receptor distribution related to functional thymus anatomy of the European sea bass, *Dicentrarchus labrax*

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Abbreviations: Ab, antibody (mAb, monoclonal Ab, pAb, polyclonal Ab); CD, cluster of differentiation; Ct, connective tissue; Ck, cytokeratin; E2, 17-β-oestradiol; 17 Esr, nuclear oestrogen receptor; Gper, G protein-coupled oestrogen receptor; HCs, Hassall’s Corpuscles; HES, Hematoxylin-Eosin-Saffron; IHC, immunohistochemistry; IS, immune system; MCs, mast cells; MyCs, myoid cells; OZ, outer zone; PAS, Periodic Acid-Schiff; PBS, Phosphate-Buffered Saline; Pcna, Proliferating Cell Nuclear Antigen; PVS, perivascular space (iPVS, inner PVS, oPVS, outer PVS); SDF-1, stromal cell-derived factor; TECs, thymic epithelial cells (cTECs, cortical TECs, mTECs, medullar TECs, LTECs, limiting TECs)

Abstract: In jawed vertebrates, the crosstalk between immune and endocrine system as well as many fundamental mechanisms of T cell development are evolutionary conserved. Oestrogens affect mammalian thymic function and plasticity, but the mechanisms of action and the oestrogen receptors involved remain unclear. To corroborate the oestrogenic regulation of thymic function in teleosts and to identify the implicated oestrogen receptor subtypes, we examined the distribution of nuclear and membrane oestrogen receptors within the thymus of the European Sea bass, *Dicentrarchus labrax*, in relation to its morpho-functional organisation. Immunohistological analysis specified thymus histology and organisation in teleosts and described, for the first time, Hassall's corpuscle like structures in the medulla of sea bass. All oestrogen receptors were expressed at the transcript and protein level, both in T cells and in stromal cells belonging to specific functional areas. These observations suggest complex regulatory actions of oestrogen on thymic function, notably through the stromal microenvironment, comprising both, genomic and non-genomic pathways that are likely to affect T cell maturation and trafficking processes. Comparison with birds, rodents and humans supports the thymic localisation of oestrogen receptors and suggests that oestrogen modulates T cell maturation in all gnathostomes.

1. Introduction

The immune system (IS) forms an integrated network destined to detect and eliminate invading pathogens or transformed cells. The immune response may be mounted by two major entities, the innate and adaptive immunity, the latter being an innovation of the vertebrate lineage (Cooper and Alder, 2006). Innate and adaptive immunity operate synergistically *via* innate immune cells (*e.g.* phagocytes), which initiate a protective response and activate adaptive immune cells (*i.e.* B or T lymphocytes) for an efficient and specific immune reaction (Boehm and Swann, 2014; Esteban et al., 2015). Pathogen recognition by innate immune cells is based on germline gene-coded receptors that confer limited and non-specific capacity. Contrariwise, lymphocytes clonally expresses a monoallelic somatically diversified antigen receptor conferring high antigen specificity (Boehm and Swann, 2014). Basal jawed vertebrates, such as teleosts, also display other elements of higher vertebrate immunity including (1) lymphoid organs, such as thymus, spleen and mucosa-associated lymphoid tissue (Boehm et al., 2012), and (2) fundamental steps of thymus development and thymopoiesis (Boehm et al., 2012; Bajoghli et al., 2015). As for the latter, the thymus provides the appropriate microenvironment for T cell development, comprising proliferation, maturation and the generation of their antigen receptor repertoire (Boehm et al., 2012; Nakanishi et al., 2015). In fish like in mammals, mature and self-tolerant T cells leave the thymus towards the secondary lymphoid organs in order to coordinate the adaptive immune reaction (Langenau and Zon, 2005; Nakanishi et al., 2015).

In mammals, the IS is modulated by the reproductive system *via* sexual hormones, notably oestrogens, as reflected by sexual dimorphisms in the IS performance and female autoimmune disease prevalence, but also by the high oestrogen levels during pregnancy (Hince et al., 2008; Klein and Flanagan, 2016). The thymus and T cell development are particularly targeted by oestrogenic regulation, as evidenced by numerous studies over three decades of research (Bernardi et al., 2015; Glucksmann and Cherry, 1968; Screpanti et al., 1991). Indeed, thymus structure and volume vary throughout lifetime in relation to endogenous oestrogen levels, reproductive status and age (Hince et al., 2008), not only in mammals but also in reptiles and birds (Lutton and Callard, 2006). In fish, the thymus shows a considerable seasonal plasticity of its volume (Tatner, 1996). To what extent these variations of the thymus are linked to the reproductive cycle and, consequently, to changes in hormone titres, is, however, less clear (Tatner, 1996). However, oestradiol exposure has recently been shown to

modify thymic volume and regionalization in juvenile European sea bass, *Dicentrarchus labrax* (Seemann et al., 2015).

In mammals, the structural changes provoked by naturally elevated oestrogen levels or by experimental oestrogen exposure have been ascribed to numerous processes in thymus, including: (1) the induction of thymocyte apoptosis (Do et al., 2002; Okasha et al., 2001; Wang et al., 2008); (2) an inhibition of thymocyte proliferation (Gould et al., 2000; Zoller and Kersh, 2006; Zoller et al., 2007); (3) decreased infiltration of T cell progenitors into the thymus (Zoller and Kersh, 2006) and (4) extensive T cell leakage through the blood vessels into the periphery (Chapman et al., 2015; Martín et al., 1995). Notwithstanding uncertainties and conflicting results obtained in mammals, it is generally accepted that oestrogens (1) modulate T cell maturation by increasing the proportion of single positive CD4+/CD8+ T cell (Bernardi et al., 2015; Erlandsson et al., 2001; Screpanti et al., 1991) and (2) block T cell maturation, as suggested by the increased proportion of immature double negative CD4-/CD8- phenotypes (Bernardi et al., 2015; Rijhsinghani et al., 1996; Wang et al., 2008). The cellular players and the respective oestrogen receptors were, however, only partially identified (Erlandsson et al., 2001; Staples et al., 1999; Wang et al., 2008). Although a growing body of knowledge on oestrogenic regulation of the teleost IS supports that the immunomodulatory role E2 probably exists across vertebrates (reviewed in Burgos-Aceves et al., 2016; Segner et al., 2017; Szwejser et al., 2016), the interplay between stromal and T cells as well as the extent of conservation along vertebrate evolution remains to be detailed (Segner et al., 2017).

Oestrogens mediate their effects on target cells and tissues through oestrogen receptors. These include both genomic pathways (classically associated to nuclear oestrogen receptors, Esrs) and non-genomic pathways (associated to membrane localized Esrs or to recently characterized G-protein-coupled oestrogen receptors, *i.e.* Gpers). In numerous teleost species, the Esrs are represented by three isoforms: Esr1 (also known as Erα), Esr2a (Erβ1) and Esr2b (Erβ1) (Burgos-Aceves et al., 2016). More recently, two GPER isoforms, Gpera and Gperb, have been described in some teleost species (Lafont et al., 2016; Pinto et al., 2016), indicating that both pathways can interact in teleosts (Nelson and Habibi, 2013; Pinto et al., 2014). The presence of oestrogen receptors in the thymus has been demonstrated in mammals (Nancy and Berrih-Aknin, 2005; Seiki and Sakabe, 1997; Wang et al., 2008), chicks (Katayama et al., 2014; Yonezawa et al., 2008) and common carp (Szwejser et al., 2017). It may, therefore, be hypothesized that the different receptors may have a similar distribution in the fish thymus. The morpho-functional organization of the thymus is well documented for the European sea

bass, describing the thymic microenvironment and the expression of genes related to the T cell development (Picchietti et al., 2008, 2009, 2015), with the different steps of T cell maturation taking place in specific regions of the thymus, comparable to the processes described for mammals (Bajoghli et al., 2015).

With respect to the complex and not fully understood oestrogenic regulation of thymopoiesis, in this study we aimed at investigating how and at which point oestrogens influence T cell maturation and selection in the European sea bass (Bajoghli et al., 2015; Langenau and Zon, 2005). The expression of the three nuclear Esr isoforms and the Gper genes in thymic cells was confirmed by RT-PCR. Their localisation within the thymic substructures of *D. labrax* was established in conjunction with a detailed histological analysis, using oestrogen receptor specific antisera previously validated for other teleost species (Cabas et al., 2013; Pinto et al., 2009; Szwejser et al., 2017). The presence of both membrane and nuclear ER-isoforms in most cell types of the thymic microenvironment and their strong occurrence in certain thymic zones, such as the medulla and connective tissue, point to a functionally conserved regulation of thymopoiesis by oestrogens across all vertebrate taxa.

2. Material and Methods

2.1 Animals and sampling

Fingerlings of *D. labrax* were obtained from the hatchery “L’écloserie marine de Gravelines” (Gravelines, France) and raised in the facilities of “Aquacaux” (Octeville, France) in 1,800 l tanks with continuous flow of marine seawater at environmental temperatures. The animals were fed daily *ad libidum* with “Turbot label rouge” fish feed (Le Gouessant, Lamballe, France). All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 2010/63/EU).

Two different fish batches were employed for this study: three-year-old female fish with a total length of 30.6 cm ± 1.42 standard deviation (s.d.) and a weight of 391 g ± 58.02 s.d. for histology and IHC and one-and-a-half-year-old male and female fish with a total length of 21.8 cm ± 2.9 s.d. and a weight of 97.8 g ± 10 s.d. for molecular biology and cytometric analysis. Specimens were sacrificed at the end of November/beginning of December 2014 for the IHC and histological analysis and in June 2015 for molecular biology and immunofluorescence.

2.2 Leucocyte preparation

All solutions for leukocyte preparation were adjusted to 360 mOsm/kg. Dissected thymuses were cut into pieces, immersed in cold Leibovitz 15 (L-15) containing 1 mM Na_2EDTA, and forced through a 100 µm cell strainer. The cell suspension was centrifuged at 1,200 *g* for 5 min at 4 °C prior to erythrocyte lysis in ammonium chloride-Tris solution for 30 min at room temperature under stirring followed by another round of centrifugation at 1,200 *g* for 8 min after which the supernatant was discarded. Pellets were resuspended in L-15 and filtered through a 40 µm mesh before loading on a Ficoll gradient (Pancoll, PAN BIOTECH) at a density of 1.077 g/ml and centrifugation at 400 *g* for 5 min at 4 °C. The leukocyte layer, occurring at the interface of medium and Ficoll, was collected, washed and centrifuged twice with L-15 medium at 1,200 *g* for 5 min and 4 °C. The cell concentration was adjusted to 10^6 cells/ml. Before flow cytometric measurements, cell viability was estimated with trypan blue exclusion and 50 µg/ml propidium iodide (Sigma) over 10 min at room temperature.

2.3. Gene expression

Gene expression studies were performed using five replicates (n = 5) per measurement. For each animal one of the thymuses was subjected to direct RNA extraction whereas the second thymus was used for RNA extraction from isolated thymocytes obtained by Ficoll gradient separation as described above. For whole organ RNA extraction, the thymus and liver were snap frozen in liquid nitrogen and stored at -80 °C. The frozen organs were transferred to Tri Reagent® (Sigma), ground twice in Precellys® tubes (CK14; Bertin technologies) for 10 s at 5,000 rpm and centrifuged at 12,000 *g* for 15 min at 4 °C to eliminate debris. Supernatants were mixed with 190 µl of chloroform (Sigma) and incubated for 15 min at room temperature with slow stirring. Following centrifugation at 12,000 *g* for 15 min at 4 °C the aqueous phase was gently mixed with 500 µl of isopropanol for 10 min at room temperature and centrifuged at 12,000 *g* for 10 min at 4° C. Pellets were washed twice with 1 ml of ethanol. Each wash was followed by centrifugation at 10,000 *g* for 5 min at 4° C. Subsequently, the pellets were air-dried and dissolved in DNase/RNase free water. RNA extraction from isolated thymocytes was carried out with the RNeasy mini kit (Qiagen) according to the supplier instructions. DNA removal was performed with Turbo DNA-free (Ambion). RNA quality was assessed on 1% agarose gels and the yield was quantified with a Nanodrop spectrometer (ND1000). Samples were stored at -80 °C until further processing.

Reverse transcription was performed on 1 µg of total RNA using M-MLV Reverse Transcriptase H^- (Promega) and oligo(dT) 15 primer for 10 min of incubation at 40 °C, 60

min at 45 °C and 15 min at 70 °C. cDNA was stored at -20 °C until performing PCR using oestrogen receptor subtype specific primers (Table S1) and 1µl of cDNA (or 2 µl for *gpera*) as template. In the case of *esr1*, *esr2a* and *gpera*, Purple Taq (Ozyme) was used and PCR conditions were 95 ºC for 2 min followed by 35 cycles at 95 °C for 30 s, 30 s at the primer specific annealing temperature (table S1) and 72 °C for 45 s, followed by a final extension of 72 °C for 5 min. For *esr2b* and *gperb*, Platinum® Taq DNA Polymerase (Thermo Fisher Scientific) was used and PCR condition were 95° for 5 min followed by 35 cycles at 94 °C for 30 s, 30 s at the specific annealing temperature (table S1) and 68 °C for 1 min, followed by a final extension of 68 °C for 5 min. Negative controls were performed with DNA free water. The size of the various amplicons was verified on 2 % agarose gels. For *gperb* the PCR identify was confirmed by sequencing.

2.4 Antibodies

Antisera specific to fish Esr subtypes were raised in rabbit against synthetic peptides as described in detail by Pinto et al. (2009) - see Table 1 for antibodies characteristics. To minimise the likelihood of interspecies cross-reactivity, sequence alignments of the respective predicted amino acid sequences were carried out (Fig. S1-3). In addition, *in silico* predictions based on these alignments and on protein structure prediction, carried out by the Proteogenix (Schiltigheim, France) antibody design team, gave a high likelihood that the previously antibodies validated for sea bream and Mozambique tilapia Esrs would detect well the corresponding sea bass Esrs (data not shown).

For sea bass Esr2b, a new specific polyclonal antibody (pAb) was produced by Proteogenix against a synthetic peptide targeting the C-terminal region of *D. labrax* Esr2b (dlEsr2b). This peptide (PQPPSHLQPGSHQC) was designed to target the C-terminus of the dlEsr2b protein in a region with high predicted antigenic index and hydrophilic profile; this position was also selected based on the alignment between the Esrs of sea bass and sea bream (Fig. S4), to increase the cross-reactivity with sea bream Esr2b (and permit cross-species use) and to avoid cross-reactivity with Esr1/Esr2a from both species (Fig.S4). The peptide was synthesized, conjugated to keyhole limpet hemocyanin and used for immunization of two New Zealand White rabbits, after sampling of pre-immune serum. The final antiserum was tested by direct ELISA titration using the synthetic peptide. Lyophilized antisera were reconstituted in 0.2 M Phosphate-Buffered Saline (PBS), pH 7.2, with 40 % of glycerol. Specificity of the antibody was validated by Western Blot as previously described (Pinto et al., 2009). Briefly, *E. coli*

BL21 lysates containing the recombinant sea bream Esr2b protein were incubated with the new pAb anti-dlEsr2b (1:60000 and 1:100000) or with the respective pre-immune serum at the same dilutions, for 1h, followed by 1h incubation with an anti-rabbit secondary antibody (1:35000) and detection by the ECL Plus Western Blotting Chemiluminescent Detection System (GE Healthcare, UK) until the best signal was achieved. The expected band size of approx. 85 kDa was detected with the new anti-dlEsr2b while the pre-immune control gave none or negligible detection (Fig. S5).

For sea bass Gper immunostaining, two distinct pAb rabbit anti-human GPER were used (see Table 1). To assess potential specificity for sea bass Gper isoforms, sequence alignments of the predicted amino acid sequences were carried out (Fig. S6).

The epithelial cells were immunostained using a mouse monoclonal antibody (mAb) against human Cytokeratin (Table 1). For proliferating cells a mouse mAb anti-rat PCNA was used (Table 1). For the S100-immunostaining a rabbit pAb against cow S100 was used (Table 1), S100 protein family is express in dendritic cells, neurons, fibroblasts, lymphocytes, smooth and skeletal muscle cells (Picchietti et al., 2015; Zimmer et al., 1995). Those antibodies were previously validated for the thymus of sea bass (Picchietti et al., 2015).

Mouse mAb and rabbit pAb were diluted in PBS supplemented with 5 % of horse or goat serum according to the species of origin of the secondary antibody in order to decrease unspecific binding of the secondary antibody.

Unspecific binding was assessed for each antibody by various negative controls: (1) omitting the primary antibody, (2) isotype-matched controls (1/100 rabbit IgG anti-BSA; Sordalab) and (3) a commercial blocking peptide (sc-48525 P; Santa Cruz Biotechnology) preincubated with the corresponding GPER N15 (sc-48525-R; Santa Cruz Biotechnology) overnight at 4°C for competitive binding studies.

2.4 Immunohistochemistry

Thymuses from four different female fish were fixed in Histochoice® Tissue Fixative (Sigma) for 3 h at 4 °C and dehydrated in successive baths of cold graded ethanol. After clearing with Histochoice® Clearing Agent (Sigma) and embedding in paraffin, blocs were serially sectioned at 7 µm. Some sections were stained with Hematoxylin-Eosin-Saffron or Pappenheim or Periodic Acid-Schiff solutions whilst others were used for IHC.

IHC was performed by ABC-peroxidase with nickel enhancement as previously described (Scapigliati et al., 1995). Briefly, serial sections were deparaffinised with Histochoice® Clearing Agent, hydrated in PBS and endogenous peroxidase activity was inactivated with 0.5 % H_2O_2 in PBS for 20 min. The slides were incubated overnight at room temperature with the different primary antibodies (Table 1). Subsequently, the slides were washed twice with PBS and incubated during 1 h at room temperature with a biotinylated horse or goat secondary antibody (Vector laboratories) with a dilution of 1/1000 in PBS/1 % BSA. Slides were rinsed twice in 0.05 M TRIS (pH 7.6) and incubated for 1 h with an avidin-biotinylated peroxydase complex (ABC KIT, Vector laboratories) diluted 1/1000 in TRIS. Peroxydase activity was revealed for 5 min with a solution of 0.05 M TRIS (pH 7.6) with 0.41 % $(NH_4)_2Ni(SO_4)_2$, 0.034 % Diaminobenzidine (Sigma) and H_2O_2. The slides were washed in TRIS (pH 7.6) solution and then dehydrated, cleaned in toluene before mounting. The slides were observed with Eclipse TE2000-U (Nikon) and EVOS FLAuto (life technology) microscopes.

2.5 Flow cytometry

Leucocytes from four fish were adjusted to $1x10^6$ cells/mL with L-15. For immunofluorescence staining, cells were fixed in 1.6 % paraformaldehyde at 16°C for 20 min adding ice cold 4% PFA immediately after defrosting, followed by centrifugation at 1,200 *g* for 3 min at 4 °C, washing and resuspension in PBS. Cells were incubated with the different primary antibodies (Table 1) diluted in PBS/5 % goat serum/0.1 % Tween20 and incubated for 1 h at room temperature. The cells were centrifuged at 1,200 *g* 3 min at 4 °C, washed twice with PBS and stained with a secondary anti-rabbit IgG, coupled to Fluorescein (FITC), diluted in PBS with 1 % of bovine serum albumin for 30 min at room temperature. Before flow cytometry using a NovoCyteTM (ACEA, Ozyme), washing with PBS was repeated two more times. For the fluorescence analysis, doubled and aggregated cells were gated out in the forward-scatter area (FSC-A) and height (FSC-H) signal. In the FSC-H/SSC-H (side-scatter) a gate "thymocytes" was created with the software NovoExpress. Immunofluorescent measurements were carried out for two independent experiments.

3. Results

3.1 Oestrogen receptor expression in sea bass thymus

The expression of the five oestrogen receptor genes (nuclear receptors *esr1, esr2a, esr2b* and membrane receptors *gpera* and *gperb*) in adult sea bass thymus is shown in Fig. 1a. The liver, a classical oestrogenic target, was utilized as a control tissue and confirmed the correct

amplification of the oestrogen receptors using the same primers and reaction conditions (Fig. 1a). The expression of the *er*-subtypes in the thymus was generally well detected.

3.2 Presence of oestrogen receptors in isolated thymocytes

The isolated thymocytes also produced well-defined *esr1* and *gperb* amplicons (Fig. 1a). Amplification of *esr2b* and *esr2a* resulted in slightly weaker bands, but was still clearly detectable, whereas no *gpera* expression was detected in isolated thymocytes. Examination of oestrogen receptors by immunofluorescence and flow cytometry (Fig. 1b-f), revealed that thymocytes displayed oestrogen receptor isoforms at both transcript and protein level. The signals obtained for the two different antibodies against Esr2b and Gper, respectively, also provided positive results. All negative controls, including the isotype-matched control and the blocking peptide, showed a weaker immunostaining, confirming the binding specificity (Fig. 1 and S7).

3.3 Histochemistry of the thymus

3.3.1 Thymus organisation

Our study confirmed the structure previously described for adult sea bass (Avilés-Trigueros, 1993; O'Neill, 1989). Thymuses were mainly composed of tightly packed lymphoid cells with deeply stained nuclei and scant cytoplasm (Fig.2). The thymic parenchyma was clearly subdivided into several lobules by invaginations of connective tissue (Ct) named trabeculae deriving from the capsule. The trabeculae commonly contain migrating lymphocytes but also nerves and blood vessels that extend into the perivascular space (PVS) composed of Ct, limiting thymic epithelial cells (LTEC) and a basal membrane of endothelial cells forming the blood-thymus barrier (Avilés-Trigueros, 1993; Chilmonczyk, 1983). The trabeculae dwindle gradually and the vessels terminate as thin capillaries of very flattened endothelial cells in the medulla of the thymus, constituting a continuous blood vascular system with the gill (Avilés-Trigueros, 1993; Sailendri and Muthukkaruppan, 1975). The lobules were further differentiated into cortex and medulla with the cortex containing much more lymphoid cells (Fig. 2a, b). Moreover, the cortex was characterized by higher density of proliferating Pcna positive cells, which were localised mainly within the subcapsular zone (Fig. 2c, d), as observed in one-year-old sea bass and rat (Forsberg, 1996; Picchietti et al., 2015). Medially, a continuous capsule of Ct separated the thymus from the muscles of the head (Fig. 2b). As typical for teleost species (Castillo et al., 1991; Gorgollon, 1983), the thymus was limited laterally from the gill cavity by an outer zone (OZ) formed by pharyngeal epithelia and a

more or less developed subepithelial zone (Fig. 3), having no homolog structure in higher vertebrates.

3.3.2 Cellular localization of oestrogen receptors within the thymic parenchyma

The three nuclear Esr subtypes and the Gper could be localised in the thymus of adult female sea bass with varying signal positions and intensities according to the oestrogen receptor subtypes. In agreement with the results of immunofluorescence obtained in one-and-a-half-year-old male and female fish, in the thymic parenchyma and the connective tissue, lymphoid cells were immunopositive to all the nuclear and membrane oestrogen receptors (Fig. 4d-g). Moreover, especially in the subcapsular zone, the antibodies anti-Esr1 (Fig. 5b) and both anti-Gper (Fig. 5a) strongly labelled cells with a morphology and a localisation similar to LTECs adjacent to the connective capsule (Fig. 5c). IHC without the primary antibodies and with the isotype-matched did not result in any staining (Fig. 4c). IHC against GPER (N15) with the blocking peptide showed a significant decrease of the immunostaining confirming the binding specificity as observed for IF (Fig. S8).

In the medulla, and to a lesser extent in the cortex, large pyknic cells (Fig. 6a, b) of a size of *ca*. 17.5 x 15.5 µm (± 3.2 x 3.4 s.d.), similar to myoid cells (MyCs), stained positive for the various receptor isoforms. The visual impression of staining intensities suggested an order as follows: anti-Gper Abs (Fig. 6g, h) > Esr2a (Fig. 6k) > Esr1 (Fig. 6i, j) > Esr2b (Fig. 6l, m). These cells also stained immunopositive for S100 (Fig. 6d-f), but negative for Ck (Fig. 6c).

In the medulla, but rarely in the cortex, onion-like structures with a size of ca. 26.3 x 19.7 µm (± 9.1 x 4.2 s.d.) reminding of Hassall's Corpuscles (HCs) were observed with variable frequency between individuals (Fig. 7). Basically, three structure types could be distinguished: firstly, structures of a generally smaller size with a solid centre and eosinophilic staining (Fig. 7a, b), which were immunopositive for Ck (Fig. 7k, l) and S100 (Fig. 7n). Secondly, larger HC-like structures with either eosinophilic or saffron staining, PAS staining and a degenerative centre (Fig. 7c-g) that were frequently surrounded by a ring of Ck immunopositive cells (Fig. 7j) and occasionally associated with S100 immunoreactive cells (Fig. 7o). Thirdly, large degenerative structures, slightly frayed on the edges, which were strongly PAS positive (Fig. 7h) and colourless in HES (Fig. 2a). The HC-like structures, generally those of the smaller type, were immunopositive, to a certain extent, for Esr2a (Fig. 7u, v), Gper (Fig. 7w-x), Esr1 (Fig. 7q-s) and Esr2b (Fig. 7t).

3.3.3 Cellular localization of oestrogen receptors within thymic capsule and vascular system

Mast cells (MC), previously described in the thymic Ct of perciform fish (Mulero et al., 2007), were identified by eosinophilic staining and a size of 9.4 x 6.7 μm (± 1.3 x 0.85 s.d.) (Fig. 4a, b). These cells were strongly immunopositive for Esr1 (Fig. 4d), Esr2a (Fig. 4e) and, to lesser extent, Esr2b (Fig. 4f) and Gper (Fig. 4g). The Ct is also composed of S100 immunopositive cells with fusiform shapes that may be characterized as fibroblasts (Fig. 8a) and S100 immunopositive vessels in the trabeculae (Fig. 8a, c). The vessels in the trabeculae were also strongly Gper immunopositive (Fig. 8b). In the Ct of the capsule the vessels were strongly Esr2a (Fig. 8f) and Gper immunopositive (Fig. 8b), but stained to a lesser extent for Esr1 (Fig. 8d) and Esr2b (Fig. 8e).

The outer zone (OZ) was composed of epithelial, mucous, chloride cells, lymphocytes and macrophages (Fig. 3a) (Castillo et al., 1991; Gorgollon, 1983). As in the entire thymic parenchyma, numerous Ck immunopositive cells were present within the OZ (Fig. 3b). The OZ strongly expressed all of the oestrogen receptors (Fig. 3d-g). Outer perivascular spaces (oPVS) were observed between the Oz and the cortex (Fig. 3a), within which vessels of different sizes were strongly S100 immunopositive (Fig. 3c). The fibroblasts forming the oPVS around the vessels also showed some S100-positive immunostaining (Fig. 3c). The oPVS vessels were also immunopositive for Esr2a, Esr2b, Gper and, to a lesser extent Esr1 (Fig. 3d-g).

Within the thymic parenchyma we could observe inner perivascular spaces (iPVS), also containing vessels and Ck immopositive LTECs (Fig. 3i), which encompassed Gper immopositive cells (Fig. 3m). Vessels of the iPVS expressed the entire set of the oestrogen receptors (Fig. 3j-m).

Table 2 summarizes the observations on the Esr and Gper localization in the thymus of adult sea bass with the oestrogen receptors distribution retrieved in other vertebrates.

4. Discussion

4.1 Whole organ level

To the best of our knowledge this study for the first time presents a concurrent expression of membrane and nuclear oestrogen receptor isoforms in the thymus at both the transcriptional and the protein level.

The expression of *esr1*, *esr2a, esr2b, gpera* and *gperb* in the whole thymus corroborates earlier findings for the common carp (Szwejser et al., 2017) and is in line with studies that

report the expression of multiple oestrogen receptor isoforms in higher vertebrates, such as birds or rodents. The expression of *esr1* was reported for the thymus of human, rat, mouse, chicken and common carp (Seiki and Sakabe, 1997; Yonezawa et al., 2008; Szwejser et al., 2017) and the expression of *esr2* can also be retrieved in the thymus of human, rat, mouse and common carp (Mor et al., 2001; Mosselman et al., 1996; Szwejser et al., 2017). Similarly, the membrane receptor *gper* is expressed in the thymus of human, mouse and common carp (Wang et al., 2008; Olde and Leeb-Lundberg, 2009; Szwejser et al., 2017). However, for teleost *gper* isoforms no expression data for a primary lymphoid organ is available, but expression in the spleen of the European eel has been reported (Lafont et al., 2016). In teleosts and mammals the presence and expression of multiple oestrogen receptor isoforms (including GPER) in lymphoid organs have been associated with an immunoregulation by oestrogen (Cabas et al., 2013; Massart et al., 2014; Seemann et al., 2016; Szwejser et al., 2017). Together these data are suggestive of a role of oestrogens in regulating thymus and T cell development.

4.2 Cellular level

4.2.1 Isolated leucocytes

Isolated leucocytes from the thymus expressed membrane and nuclear oestrogen receptors, both at the transcript and protein level. This is in agreement with previous reports for fish, in which oestrogen receptors transcripts and protein were detected in peripheral blood and head-kidney leucocytes of several teleost species (Iwanowicz et al., 2014; Liarte et al., 2011b; Szwejser et al., 2017). Thymic leucocytes constitute a heterogeneous cellular fraction, which in sea bass is composed of at least 80 % of DLT15 cross-reactive cells, *i.e.* thymocytes that are immunopositive for sea bass pan-T cell antibody (Romano et al., 1997; Scapigliati et al., 1995). We, therefore, conclude that T cells of sea bass appear to express all the nuclear Esr and Gper isoforms. In mammals, mouse and rat thymocytes were reported to express *esr1* and *esr2* (Kawashima et al., 1995; Mor et al., 2001) and human thymic T cells display ESR1 and ESR2 (Nancy and Berrih-Aknin, 2005). Binding assays allowed for the detection of oestrogen receptors in rat, fetal guinean pig and human thymocytes (Danel et al., 1983; Gulino et al., 1985). More recently, Katayama and co-worker have shown that ESR1 to be present in thymocytes of chicken (Katayama et al., 2014).

Regarding Gper isoform expression, the absence of *gpera* transcript in isolated thymocytes suggests that teleost Gpera and Gperb have a specific repartition and thus non-redundant

biological functions. GPERs have been reported to be present and functional on various immune cells, such as human and teleost granulocytes (Cabas et al., 2013; Rodenas et al., 2017; Szwejser et al., 2017). Murine T lymphocytes and human eosinophils also display GPER (Schneider et al., 2014; Tamaki et al., 2014).

4.2.2 Thymic epithelial cells

Thymic epithelial cells (TEC), located in the cortex (cTECs) and medulla (mTECs), respectively, form a three-dimensional network necessary to develop the thymic T cell repertoire by positive and negative selection, though other antigen-presenting cells in the thymus are also involved. A third type of TECs can be distinguished in the subcapsular, perivascular and peritrabecular zones, between the thymic parenchyma and the connective tissue as single layer of LTECs (Romano et al., 1999). Fish LTECs have similar features as those of mammals with apparent secretory capacity related to the formation of a basal membrane (Avilés-Trigueros, 1993; Castillo et al., 1991; van de Wijngaert et al., 1984). LTECs of the subcapsular zone of the sea bass thymus strongly displayed Gper and Esr1, the latter also being reported for LTECs of the human and rat thymus (Seiki and Sakabe, 1997). In rats, humans and chicken, mTECs highly express ESR1, but this specific expression could not be confirmed in our study for female adult sea bass. Nevertheless, the strong immunolabelling of Gper and Esr1 in subcapsular LTECs suggests that their function in sea bass can potentially be modulated by oestrogens, as reported for mammals (Glucksmann and Cherry, 1968; Martín et al., 1995; Moreno and Zapata, 1991). *In vitro* and *in vivo* studies have shown that oestrogen inhibits thymosin α-1 and the SDF-1α/CXCL12 production in cortical TEC, but stimulates thymulin secretion resulting in increased thymulin serum levels (He et al., 1996; Jin et al., 2003; Laan et al., 2016; Savino et al., 1988). CXCL12 is implicated in the homing of immature T cells in the outer zone, where their proliferation is accompanied by somatic gene rearrangement and selection (Bajoghli et al., 2009; Plotkin et al., 2003).

4.2.3 Myoid cells

MyCs are cells with an ultrastructure and antigen characteristics similar to muscle cells. The cells observed in the thymus of sea bass displayed several similarities with MyCs, described as oval/round eosinophilic cells with a cytoplasm containing myofilaments organised in sarcomere-like structures around the nucleus. In fish, MyCs seem to be rare medullary cells (Avilés-Trigueros, 1993; Gorgollon, 1983; O'Neill, 1989), the increased frequency of which appears to be associated with thymic involution (Franchini and Ottaviani, 1999; O'Neill,

1989). The latter can be a seasonal phenomenon in fish and occur towards wintertime (Attia et al., 2010; Tatner, 1996). The high number of myoid-like cells in the specimens analysed may, therefore, be explained by the sampling dates at end of November and beginning of December. Notwithstanding possible thymus involution, the number of proliferating cells indicates a high organ activity in T cell formation. Although, the functional significance of MyCs is still uncertain (reviewed by Zapata, 1996), some authors suggest a role in T cell maturation, proliferation and apoptosis (Kamo et al., 1985; Le Panse and Berrih-Aknin, 2005). In view of the predominant localisation in the medulla, one may assume that oestrogenic regulation of myoid cells would mostly impact differentiation, negative selection and apoptosis. In the sea bass thymus we observed that myoid-like cells displayed all oestrogen receptors, but at quite variable signal intensities, which could be due to varying maturation states of these cells (Avilés-Trigueros, 1993; Bornemann and Kirchner, 1998).

4.2.4 Hassall's corpuscles

Although HCs were considered to be generally lacking in sea bass (Avilés-Trigueros, 1993; O'Neill, 1989; Zapata et al., 1996), we observed the presence of HC-like structures of variable size and number in the medulla of adult sea bass. The sea bass HC-like observed here correspond to the morphology and immunostaining of HC described in human foetuses and new-borns as well as in amphibian, avian and reptile thymuses (Bodey and Kaiser, 1996; Raica et al., 2006; Sakabe et al., 1993; Yurchinskij, 2016), which are generally formed by TEC arranged concentrically around a degenerative centre in the medulla of chicken and humans (Bodey and Kaiser, 1996; Kannan et al., 2015). Similar structures have, however, been reported occasionally in teleost fish (Bowden et al., 2005; Cao et al., 2017; Romano et al., 1999). The cellular origin of HCs in human is not fully elucidated and remains controversial. In sea bass, as in other vertebrates, HC-like immunoreactivity (CK and S100) indicates that TEC and MyC are likely to be responsible for HC-like formation (Bodey and Kaiser, 1996). Our study demonstrated immunostaining of all ER isoforms in sea bass HC-like, however with variable staining intensity. This finding is consistent with findings from mammals and birds, where HCs were also immunopositive for ESR1 (Seiki and Sakabe, 1997; Yonezawa et al., 2008) and modulated by oestrogen treatment increasing both in size and number (Ebbesen and Christensen, 1972; Selvaraj and Pitchappan, 1985). HC-function remains unclear, but they are believed to be implicated in regulatory T cell differentiation (Watanabe et al., 2005) induced during pregnancy in mouse (Laan et al., 2016; Zoller et al., 2007).

4.2.5 Vascular system

The vessels dispersed throughout the thymus show particularly intense staining for Gper and to a lesser extent for the other oestrogen receptors, thus suggesting rapid oestrogenic action on the vascular system of the thymus of sea bass to represent an important mechanism. In mammals, oestrogen treatment induces, in the thymus, the degeneration of endothelial cells and vasodilatation (Martín et al., 1995; Öner and Ozan, 2002). As a matter of fact, oestrogens are known to affect the cardiovascular system and inflammation through the genomic and non-genomic action *via* ESR1, ESR2 and GPER expressed in human endothelial cells and vascular smooth muscle cells (Prossnitz and Barton, 2014; Usselman et al., 2016). In gilthead seabream, Liarte et al., (Liarte et al., 2011a) suggested that oestrogen also modulates the inflammatory response and vasodilatation through *esr1* and *er2a* expressed in endothelial cells.

4.2.6 Mast cells

In the thymic connective tissue of sea bass, MCs displayed the different oestrogen receptor isoforms, but it appears that they were especially immunopositive for Esr1 and Esr2a. In mammals and birds, MCs are present in the medulla and the connective tissue of the foetal and adult thymus (Ribatti and Crivellato, 2016). The exact role of MCs in the thymus is not clear, but MCs appear to have a role in thymus blood vessel homeostasis and organogenesis (Crivellato et al., 2005; Raica et al., 2010). MCs are highly conserved cells that are key to the crosstalk between the innate and acquired immune system, for instance in the induction of inflammation (Galindo-Villegas et al., 2016; Sfacteria et al., 2015). This study is the first to report the localization of oestrogen receptors in thymic MCs and, therefore, suggests that oestrogens could modulate sea bass MC activity in the thymus. Human MCs have been shown to express ESR1 and its rapid activation by oestrogen induced a progressive influx of extracellular Ca^{2+} into MCs and subsequent release of histamine (Narita et al., 2006; Zaitsu et al., 2007). Oestradiol has been described to increase the number of MC in the testis of lizards, the proliferation and the differentiation of MC in the gonad of toads and frogs (Lutton and Callard, 2006) and the number of MC in the thymus of rat (Öner and Ozan, 2002; Ross and Korenchevsky, 1941). More recently, Chapman et al. (2015) hypothesized that the oestrogen-mediated activation of MCs plays a fundamental role in thymic involution during post-puberty and pregnancy. They hypothesized that oestrogen mediated secretion of histamine and serotonin by MCs could mediate thymic vasodilation leading to a T cell release and thymic atrophy. Considering the immunolabelling of the different oestrogen receptors in MCs

and in the vascular system of sea bass thymus, oestrogens could regulate T cell progenitor recruitment and T cell output as it has been proposed for mammals.

5. Conclusion

Oestrogens typically induce volume changes of the thymus in practically all classes of vertebrates from fish to mammals, which can be manifested as either atrophy (Lutton and Callard, 2006; Sufi et al., 1980; Zoller and Kersh, 2006), or hypertrophy (Forsberg, 1996; Kondo et al., 2004; Seemann et al., 2015) depending on the period, *i.e.* the "critical window" of exposure (DeWitt et al., 2012). Histological examination of the distribution of multiple oestrogen receptors isoforms, including Gper, in *D. labrax* suggests that thymocytes as well as the thymic microenvironment would be responsive to oestrogens. Thus, oestrogenic regulation is likely to be evolutionary conserved amongst gnathostomes. The latter is inferred from (*i*) similarities obtained for oestrogen receptor distribution (*e.g.* ESR1 and ESR2) in thymic cells of the sea bass and other vertebrates, notably mammals. Furthermore, (*ii*) identified mammalian target cells for oestrogenic action (*e.g.* LTEC, T cells and mast cells) having key roles in thymic function (*e.g.* T cell migration, maturation and apoptosis) express the different oestrogen receptor subtypes also in sea bass. To the best of our knowledge, this study represents the first description of the Esr2 and Gper localization within the thymus and hence suggests a number of different potential target cells with their oestrogen receptor subtypes, representing new target cells likely to be common with higher vertebrates (for instance mast cells or myoid cells) in teleosts that call for further investigation of their role in thymus function.

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Author contribution statement

Compliance with ethical standards

All applicable national guidelines for the care and use of animals were followed.

Conflict of interest: The authors declare that they have no conflict of interest.

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Figures and tables

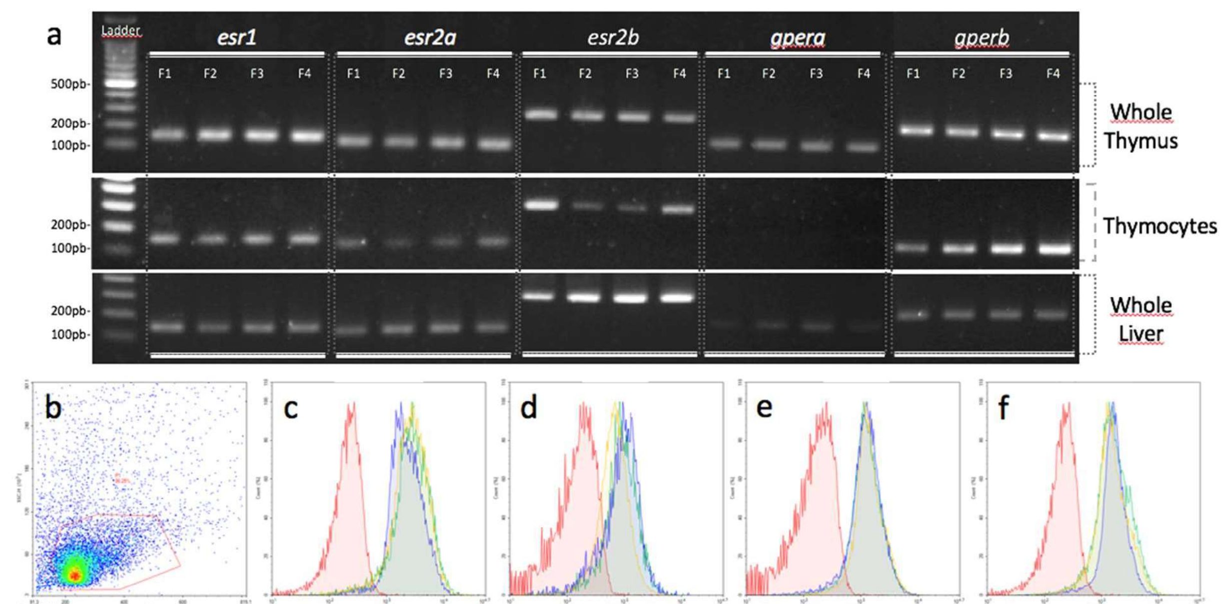

Fig. 1: a, PCR gel for the detection of Esr (*esr1, esr2a, esr2b*) and Gper (*gpera* and *gperb*) transcripts in the whole thymus, isolated thymocytes and liver of one-year-old European sea bass, *Dicentrarchus labrax*, for four different fish (F1–F4); b, Flow cytometric analysis of isolated thymocytes in side-scatter (SSC-H)/forward-scatter (FSC-H) with the gate "thymocytes" used for the green fluorescence (FITC-H) analysis (c-f). Histogram overlays

from thymocytes incubated (c-f) without primary antibody (i.e. negative control, autofluorescence) in red, with anti-Esr1 (c), Esr2a (d), Esr2b (e) and Gper (f). The histograms in blue, green and yellow correspond to three different fish. Experimental details in the text.

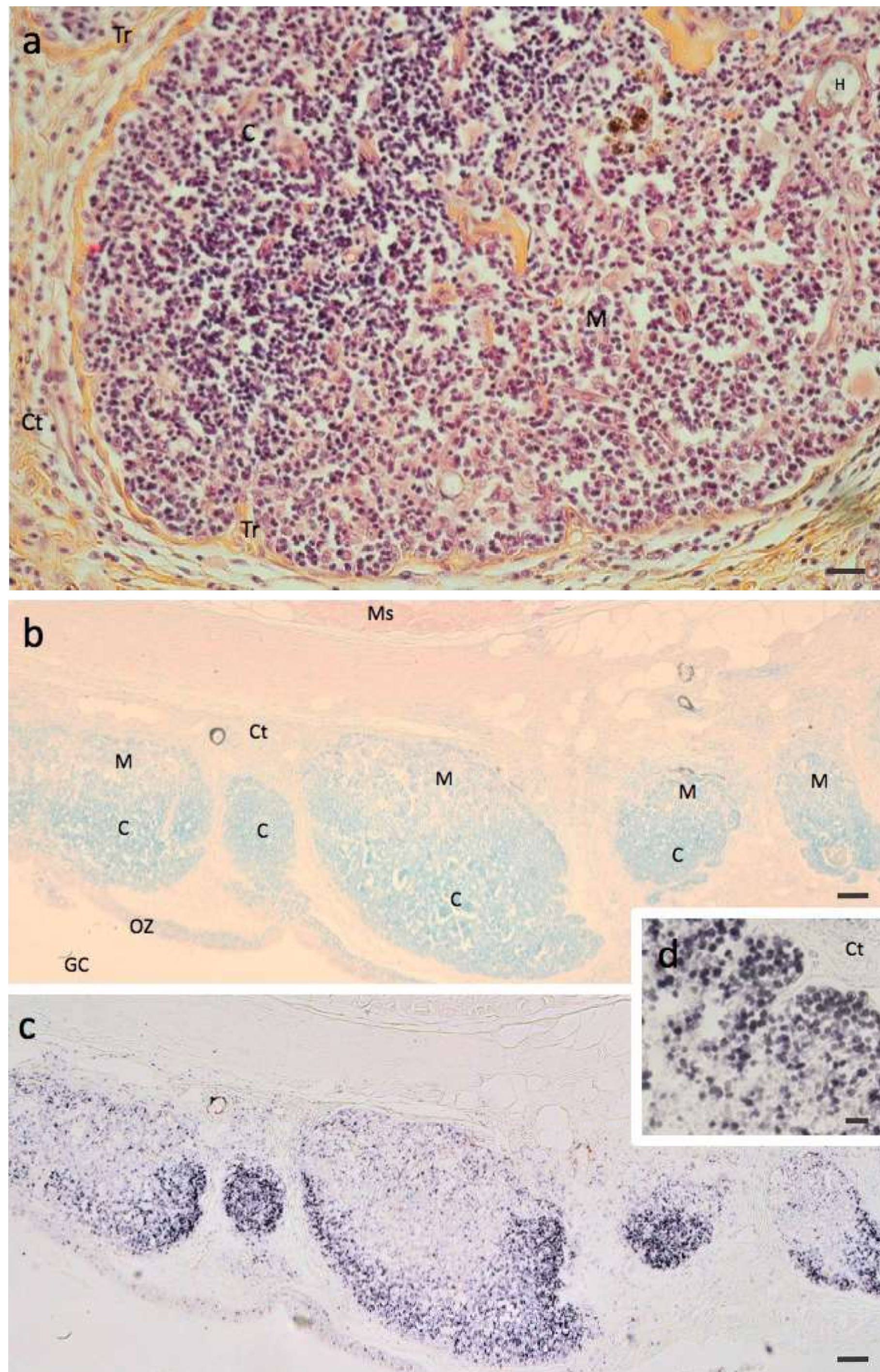

Fig. 2: Representative histological section of a thymus from adult *Dicentrarchus labrax* showing thymic lobules and their regionalisation in the cortex (C) and medulla (M) with HES (a) and Pappenheim staining (b). The thymic parenchyma is partially encapsulated in a connective tissue (Ct) forming the lobules with the trabeculae (Tr). b, c show histology and IHC of successive sections with anti-Pcna staining (for details see text). Pcna immunopositive

cells occur mainly within the subcapsular and cortical zone (c, d). OZ: Outer zone, GC: gill cavity, Ct: connective tissue, Ms: muscle, H: degenerated HC-like. Scale bar: 50 µm in a, b, c and 10 µm in d.

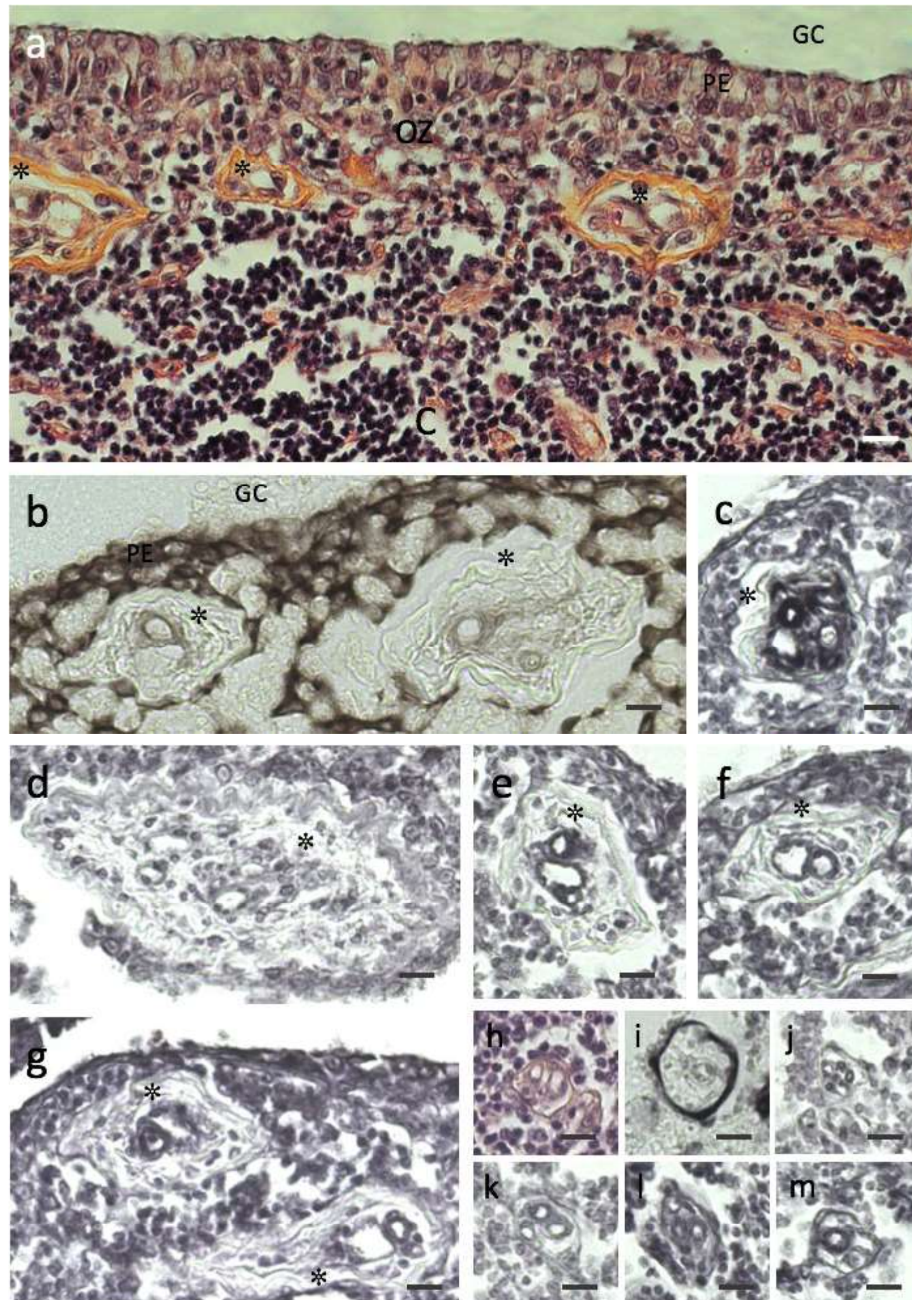

Fig. 3: Histological sections of the thymus of *Dicentrarchus labrax* representing the thymic perivascular system with HES staining (a, h) and IHC using anti-Ck (b, i), anti-S100 (c) and the five different antibodies against oestrogen receptors (d-g, j-m); for details see text. a, histological organisation of the thymus adjacent to the gill cavity (GC) with the cortex (C) being separated from the cavity by the pharyngial epithelium (PE) and the outer zone (OZ). Various outer perivascular spaces (oPVS) can be recognised between the outer zone and the cortex (*). Numerous Ck-positive cells are present in the outer zone (b) as well as S100-

positive vessels in the outer perivascular spaces (c). Vessels of the outer perivascular spaces and numerous cells of the outer zone are immunopositive for Esr1 (d), Esr2a (e), Esr2b (g) and Gper (f). h, inner perivascular spaces (iPVS) of the thymic parenchyma with Ck immunopositive staining of limiting epithelial cells surrounding inner perivascular space (i). Vessels of the inner perivascular space are immunopositive for Esr1 (j), Esr2a (k), Esr2b (l) and Gper (m). Limiting epithelial cells of the inner perivascular space also stain positive for Gper (m). Scale bar: 10 µm.

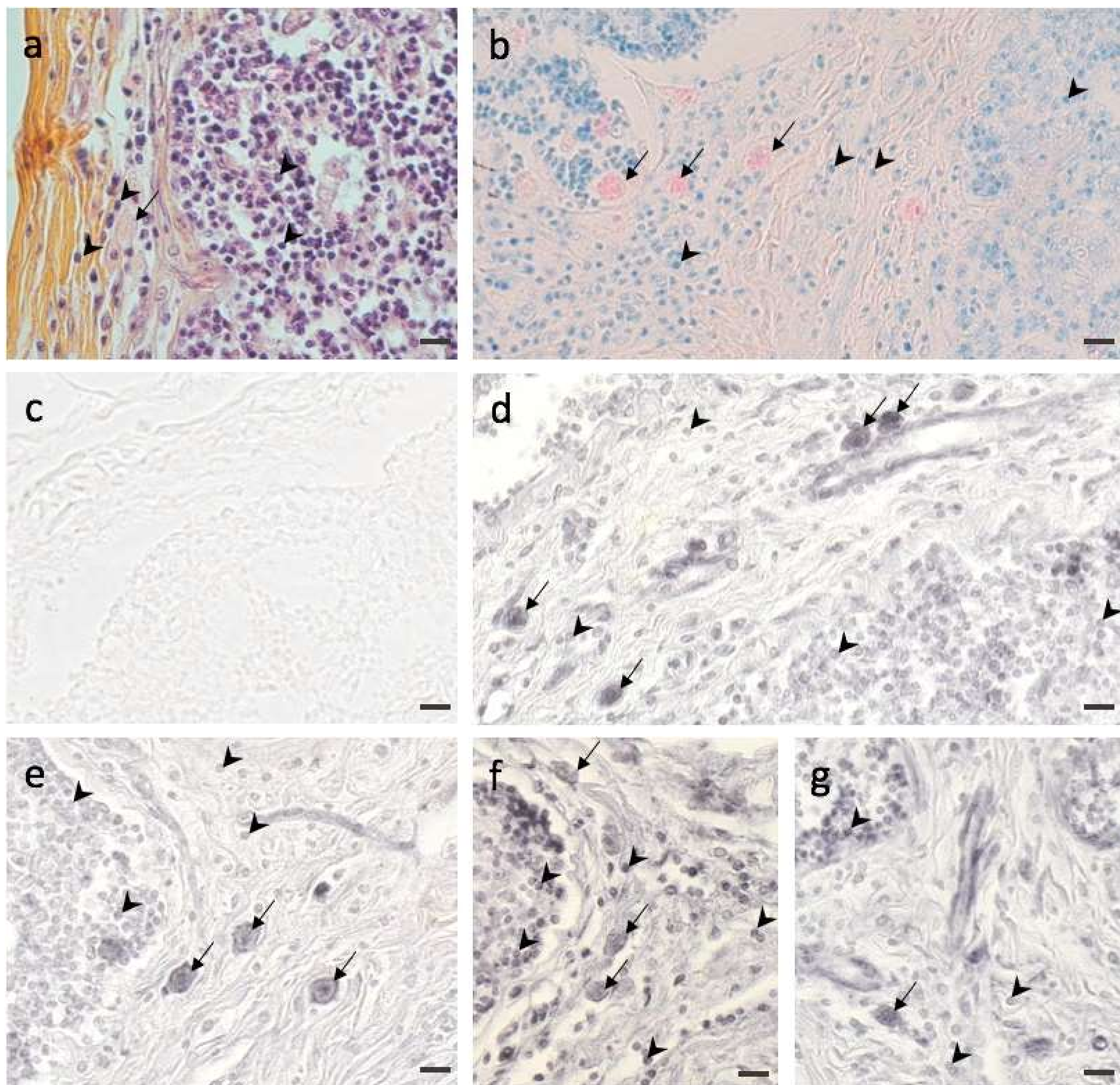

Fig. 4: Histological sections of the thymus of *Dicentrarchus labrax* stained with either HES (a) or Pappenheim (b), respectively showing mast cells with an eosinophilic staining (arrow) in the connective tissue and lymphoid cells (head-arrows) in the connective tissue (left) and in the thymic parenchyma (right). Cells with morphology similar to mast cells and lymphocytes

are immunopositive for Esr1 (d), Esr2a (e), Esr2b (f) and Gper (g). c, negative control without primary antibodies. Scale bar: 10 µm.

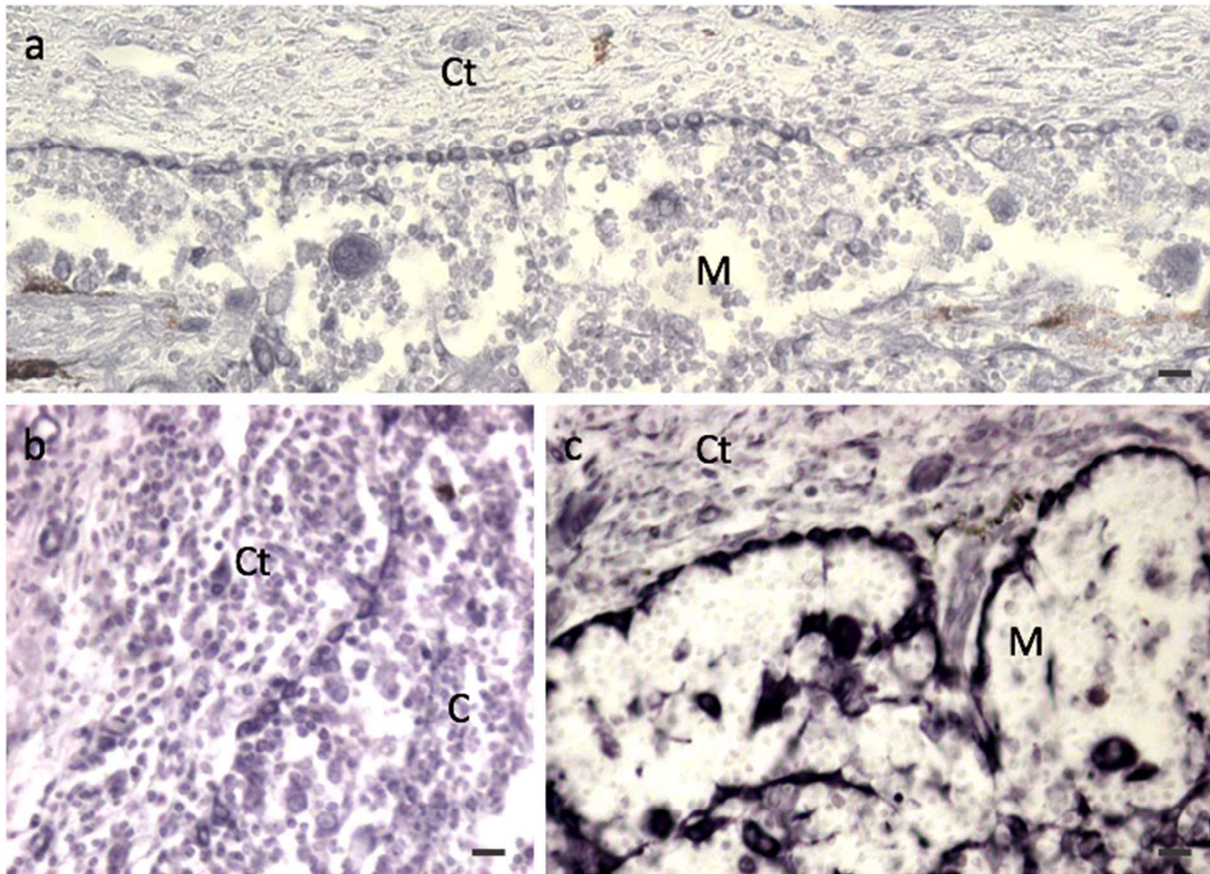

Fig. 5: IHC of histological sections of the thymus of *Dicentrarchus labrax* stained with anti-Gper (a), anti-Esr1 (b) and anti-Ck (c). Cells situated in the subcapsular zone between the connective tissue and the thymic parenchyma with the same morphology as the Ck-positive limiting epithelial cells in (c) are also immunopositive for Gper and Esr1. Ct: connective tissue, C: cortex, Ct: connective tissue, M: medulla. Scale bar: 10 µm.

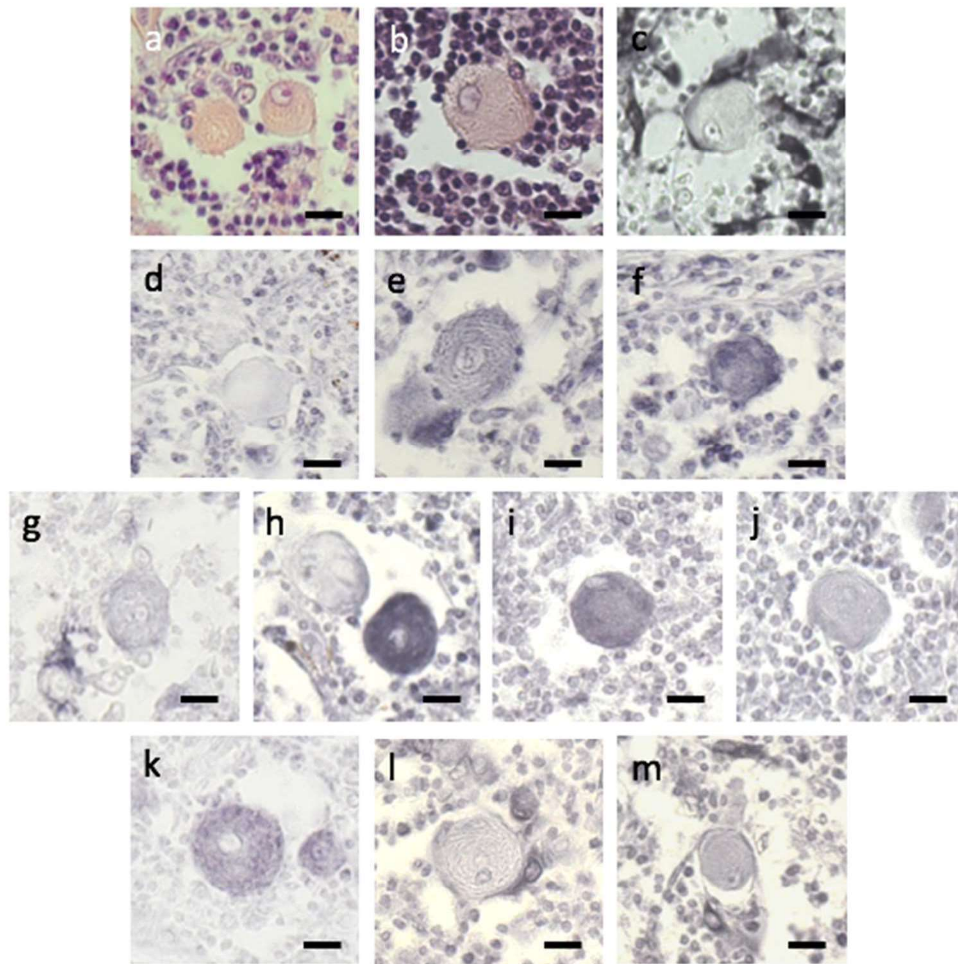

Fig. 6: Histological sections showing myoid cell-like structures in the thymus of *Dicentrarchus labrax*, as rounded cells with an eosinophilic staining with HES both in the medulla (a) and the cortex (b). IHC against Ck (c), S100 (d-f) and the five different oestrogen receptors (g-m) shows that Ck-negative myoid cell-like are associated with Ck immunopositive cells in their periphery (c). IHC against S100 show negative (d), intermediate (e) and positive (f) myoid like cells. IHC against Gper (g, h), Esr1 (I, j), Esr2a (k), Esr2b (l, m) results in variable immunostaining of myoid cell-like structure. Scale bar: 10 µm.

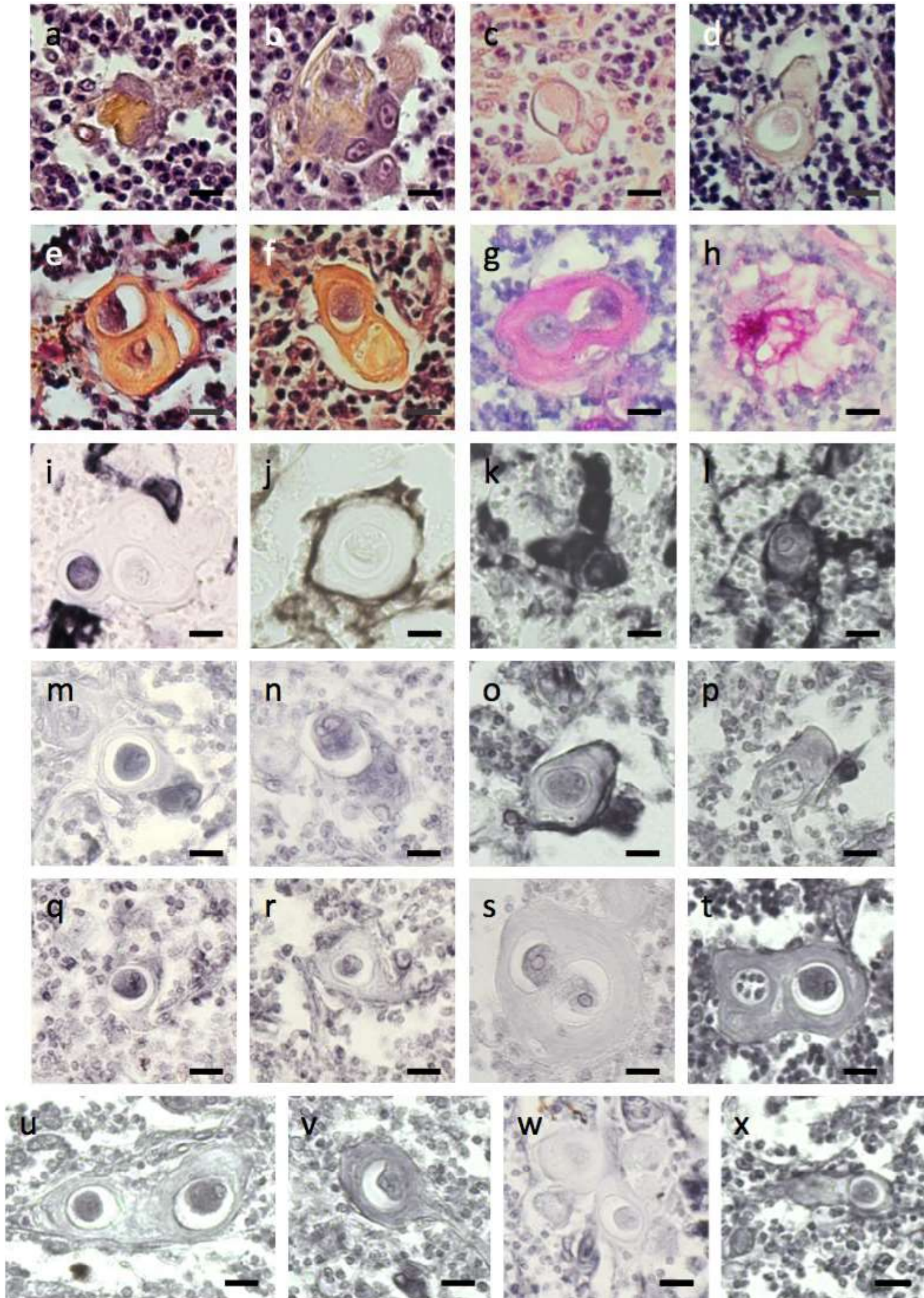

Fig. 7: Histological sections showing Hassall's corpuscle-like structures (HC-like) in the thymus of *Dicentrarchus labrax*, with HES (a-f) and PAS (g, h) staining, IHC using anti-Ck (g-j), anti-S100 (k-n) and five different anti-oestrogen receptors (o-y). a, b, immature HC-like with a solid centre; d-g, mature HC-like with fibrillary structure and/or a degenerative centre; h, degraded HC-like; i, j, mature HC-like with Ck positive cells in the periphery; k, l, immature HC-like composed of concentrically associated Ck positive cells; m-p, polymorphic

HC-like with varying S100-immunoreactivity; o-x, polymorphic HC-like immunopositive for Esr1 (q-s), Esr2a (u, v), Esr2b (t) and Gper (w, x). Scale bar: 10 µm.

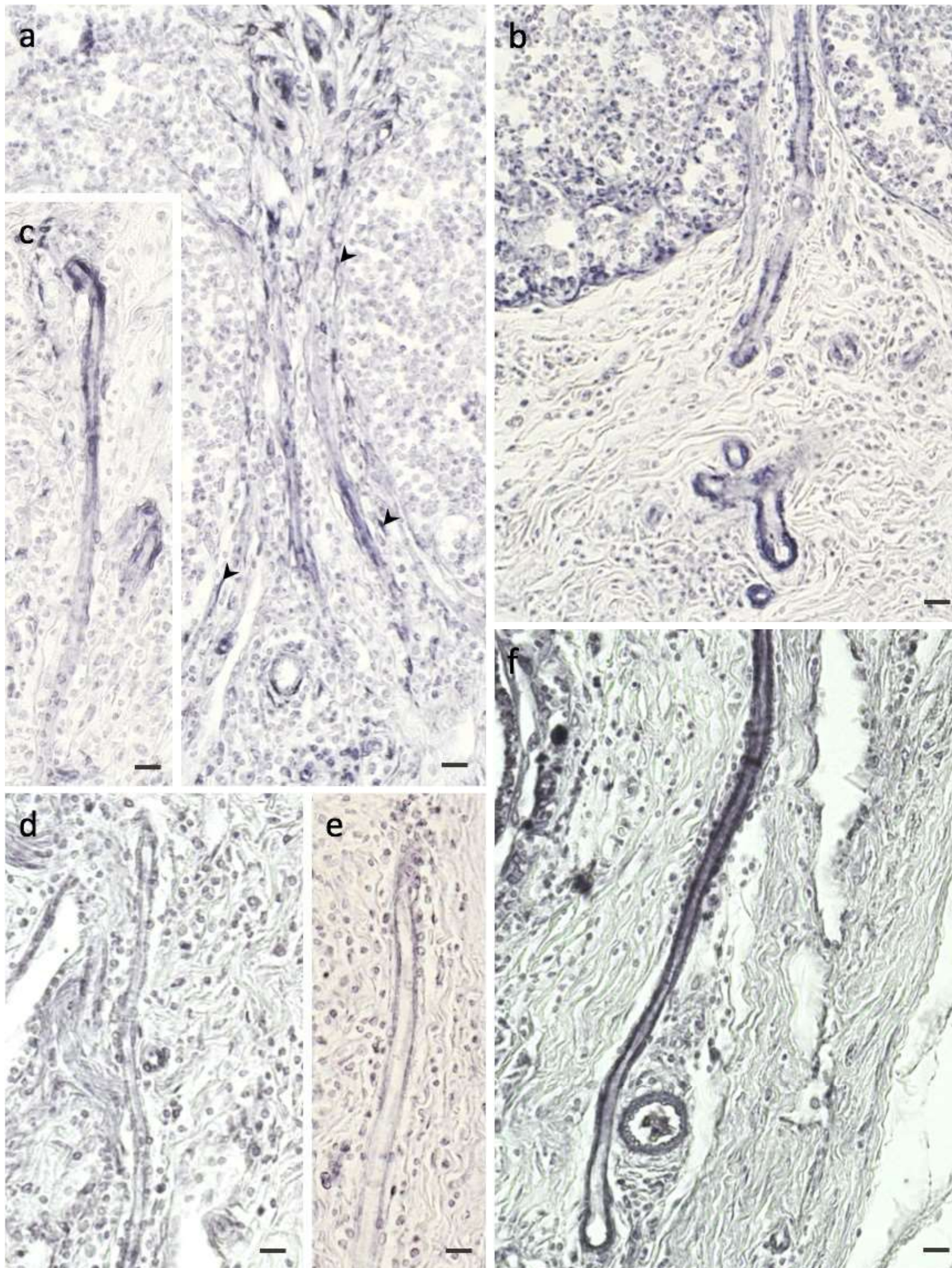

Fig. 8: Histological sections showing the vascular system of the thymus of *Dicentrarchus labrax*; IHC against S100 (a, c) and the five oestrogen receptors (d-f). S100 immunostaining shows S100-positive vessels inside the trabeculae (a, c) formed by S100 positive fibroblasts (arrowheads). IHC against Esr1 (d), Esr2a (f), Esr2b (e) and Gper (b) show immunopositive vessels within the capsula of connective tissue (b, e, f) and trabeculae (b). Scale bar: 10 µm.

Table 1: Characteristics of the primary antibodies used in immunohistochemistry (IHC) and immunofluorescence (IF); abbreviations as defined in the text.

Antibodies	Antigen recognized	Clone	Antibodies types	Working dilution		Source	Bibliographic references
				IHC	IF		
pan-Ck	-Human Cytokeratin	AE1/AE3	Mouse mAb	1/500	-	SCB	Sea bass (Picchietti et al. 2015)
Pcna	-Rat Proliferating cell nuclear antigen	PC10	Mouse mAb	1/500	-	SCB	
S100	-Cow S100		Rabbit pAb	1/400	-	Dako	
Esr1	- saEsr1 N-term and C-term		Rabbit pAb	1/750	1/250		Sea bream and Mozambique tilapia (Pinto et al. 2009)
Esr2a	-saEsr2a N-term and C-term		Rabbit pAb	1/500	1/500		
Esr2b	-saEsr2b N-term		Rabbit pAb	1/100	1/100		
	-saEsr2b C-term		Rabbit pAb	-	1/600		-
Gper	-Human GPER conserved N-term	N-15	Rabbit pAb	1/100		SCB	Sea bream and Common carp (Cabas et al. 2013, Szweijser et al. 2016)
	-Human GPER conserved 2s extracellular domain		Rabbit pAb	6µg/ml	7,5µg/ml	Thermo Fisher	-

-mAb: monoclonal antibody

-SCB: Santa Cruz Biotechonology, Inc

-saEsr1 N-term and C-term: peptide corresponding to N- and C- terminal region of sea bream *sparus auratus* Esr1

-2s extracellular domain: 15 amino acid peptide from the second extracellular domain

Table 2: Thymic cells and structures of female adult *Dicentrarchus labrax* with their respective nuclear (Esr) and membrane (Gper) oestrogen receptors as well as cellular protein markers (immunohistochemistry as described in the text). Oestrogen receptor-immunolocalization retrieved in other vertebrates is cited for comparison.

Cell subset	Esr1	Esr2a	Esr2b	Gper	Ck	S100	ESR and GPER expression in other vertebrates	References
Limiting epithelial cells	+ +	+	+	+ +	+ + +	+	Human and Rat (ESR1)	Seiki and Sakabe 1996
Mast cells	+ + +	+ + +	+	+	-	-	Human and Rat (ESR1)	Zaitsu et al. 2007
Thymocytes	+	+	+	+	-	+	Human thymic T cell (ESR1, ESR2)	Nancy and Berrih-Aknin, 2005
Myoid cells	+ +	++	+	+ + +	-	+ +	n.a.	
Hassall's corpuscles	+ / -	+ / -	+ / -	+ / -	++ / -	+ / -	Human, Rat and Chicken (ESR1)	Seiki and Sakabe 1996, Yonezawa et al. 2008
Vessels (smooth muscle cells and endotheliale cells)	+	+ +	+	+ + +	+ / -	+ +	Human (ESR1, ESR2 and GPER)	Prossnitz and Barton, 2014; Usselman et al., 2016.

n.a.: not available +/-: relative immunostaining intensity as determined by visual inspection

Table S1: Oligonucleotide sequences, annealing temperatures, length of the targeted fragments and references used for the detection of different oestrogen receptor isoforms

Gene	Primers 5'-3'	Amplicon (pb)	Hybridation Temperature	Reference
esr1	F: TGCACTACTCCGGTTCGTTT R: TGCCACAATATGACCTAACACC	139	60 °C	Seemann et al. 2015
esr2a	F: TGCAGACAGACCAAACTTGC R: TGCAGACAGACCAAACTTGC	118	60 °C	Seemann et al. 2015
esr2b	F: GATGATGTCCCTCACCAACC R: ACTTCAGCAGGTGGATCTGG	116	69 °C TD	Seemann et al. 2015
gpera	F: GCCACCCTTCTCCCTTTCACC R: TTCGCCCAATCAGAGAGTAGCAT	157	69 °C TD	Pinto et al., 2016
gperb	F: GCAGGACTACCCCTTGACAG R: AGACGGATCTTCCTGAGCCT	176	70 °C TD	This study
l13a	F: TCTGGAGGACTGTCAGGGGCATGC R: AGACGCACAATCTTGAGAGCAG	145	60 °C	This study

NB: with TD (touch down), *i.e.* decrease of 1°C at each cycle over 10 consecutive cycles followed by constant annealing temperature.

```
                1                                                  50
     saEsr2b    MAASPELDSR SLLQLQEVDS SKPSERPSSP RQLPAAYSPP LGMDSHTVCI
     dlEsr2b    MASSPGLDAH PLLQLQEVDS SKASERPNSP GPLPAVYSPP LGMDGHTVCI
   Consensus    MAaSPeLDar pLLQLQEVDS SKaSERPnSP rqLPAaYSPP LGMDgHTVCI
```

Fig. S1: Partial alignments of the predicted Esr2b amino acid sequences of sea bream (*Sparus auratus*) and sea bass (*Dicentrarchus labrax*) transcripts retrieved respectively from Genbank and the sea bass genome at http://seabass.mpipz.de/. Accession numbers are as follows: sea bream (*Sparus auratus*) saEsr2b: Q6H9M4, sea bass (*Dicentrarchus labrax*) dlEsr2b: DLAgn_00027190. Amino acid sequence fragments to which the rabbit pAb raised against the peptide of the N-terminal sea bream Esr2b are directed are shaded.

```
                1                                                    50
     saEsr2a   MAVACSPEKD QSLLQLQKVD SSRV...ILS PVLSSPMETN QPICIPSPYT
     dlEsr2a   MAVASSPEKD QPLLQLQKVD SSRVGGRVLS PILSSSMESS QPICIPSPYT
   Consensus   MAVAcSPEKD QpLLQLQKVD SSRV...!LS P!LSSpMEsn QPICIPSPYT
               501                                                  550
     saEsr2a   PLYDLLLEML DAHIMHSSRL PRRSPQQETV EQCDAPARPH SPGTSGPTNT
     dlEsr2a   PLYDLLLEML DAHIMHGSRL PHRPPQQESR DQREAPAQPQ S.SDNGPSNT
   Consensus   PLYDLLLEML DAHIMHgSRL PrRpPQQEsr #Qr#APArPq S.gdnGPsNT
```

Fig. S2: Partial alignments of the predicted Esr2a amino acid sequences from sea bream (*Sparus auratus*) and sea bass (*Dicentrarchus labrax*) transcripts retrieved respectively from Genbank and the sea bass genome at http://seabass.mpipz.de/. Accession numbers are as follows: sea bream (*Sparus auratus*) saEsr2a: Q9W6M2, sea bass (*Dicentrarchus labrax*) dlEsr2a: DLAgn_00070020. Amino acid sequence fragments to which the rabbit pAb raised against the peptide of the N and C-terminal sea bream Esr2a are directed are shaded.

```
               101                                                  150
      saEsr1   PSDGSLQSLG SGPNSPLVFV PSSPHLSPFM HPPTHHYLET TSTPIYRSSV
      dlEsr1   PSDGSLQSLG SGPTSPLVFV PSSPRLSPFM HPPTHHYLET TSTPVYRSSV
   Consensus   PSDGSLQSLG SGPnSPLVFV PSSPrLSPFM HPPTHHYLET TSTP!YRSSV

               151                                                  200
      saEsr1   PSSQHSVSRE DQCGTSDDSY SVGESGAGAG AAGFEMAKEM RFCAVCSDYA
      dlEsr1   PSSQQPVSRE DPCGTSDDSY SVGESGAGAR AGGFEMAKDM RFCAVCSDYA
   Consensus   PSSQqpVSRE DqCGTSDDSY SVGESGAGAr AaGFEMAK#M RFCAVCSDYA

               601                                   639
      pmEsr1   SSAGSTSGPR VSHESP.... TSPGVLQYGG SRSECTHIL
      dlEsr1   SSAGSSSGPR VSHESPSRGP TCPGVLQYGG SRSDCTHIL
   Consensus   SSAGSsSGPR VSHESP.... TcPGVLQYGG SRS#CTHIL
```

Fig. S3: Partial alignments of the predicted Esr1 amino acid sequence of sea bream (*Sparus auratus*), sea bass (*Dicentrarchus labrax*) and red sea bream (*Pagrus major*) transcripts retrieved from Genbank. Accession numbers are as follows: Gilthead sea bream (*Sparus auratus*) saEsr1: Q9PVZ9, sea bass (*Dicentrarchus labrax*) dlEsr1: CAD43599.1, red sea bream (*Pagrus major*) pmEsr1: O42132. Amino acid sequence fragments to which the rabbit pAb raised against the peptide of the N-terminal sea bream Esr1 and C-terminal red sea bream Esr1 are directed are shaded.

```
               551                                                  600
     dlEsr2b   NVVLVYDLLL EMLDANTSSS GSQPSSSPSS DTYSDQQQQP QPPSHLQPGS
     saEsr2b   NVVLVYDLLL EMLDANTTTS GSQASSSPTS ETFPDQHQYP QAPSHLQPGS
     dlEsr2a   NMVPLYDLLL EMLDAHIMHG SRLPHRPPQQ ESRDQREAPA QPQS.SDNGP
     saEsr2a   NMVPLYDLLL EMLDAHIMHS SRLPRRSPQQ ETVEQCDAPA RPHSPGTSGP
      dlEsr1   NKVPLYDLLL EMLDAHRIQR PDRPAQSWSQ ADGEPPFTIT TNNNNNNISG
      saEsr1   NKVPLYDLLL EMLDAHRVHR PDRPAETWSQ ADREPLFT.S RNSSSSSGGG
   Consensus   N.VplYDLLL EMLDAh..h. ...p..sp.q e..e...... .p.s....g.

               601                                                  650
     dlEsr2b   HQCNTDHGTV PPHGPGVDQI LDGHLQALPL QSSPPFQSLE VPHMDSNDYI
     saEsr2b   DQAAADHTAV PPRGPAEAPI LDGHLQALTL QSSPHFQSLE MTHMDSNQYI
     dlEsr2a   SNTWAPSSST GGGGEPQ... .......... .......... ..........
     saEsr2a   TNTWTPSC.T GGRGEPQ... .......... .......... ..........
      dlEsr1   GGSTSSAGSS SGPRVSHESP SRGPTCPGVL QYGGSRSDCT HIL.......
      saEsr1   GGGSSSAGST SGPQVNLESP ....TGPGVL QLRVHPHPMK PTE.......
   Consensus   ........st .g.g...... .........l q......... ..........
```

Fig. S4: Partial alignments of the predicted ESR amino acid sequences of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*) transcripts retrieved from Genbank and the sea bass genome at http://seabass.mpipz.de/. Accession numbers are as follows: sea bass (*Dicentrarchus labrax*) dlEsr1: CAD43599.1, dlEsr2a: DLAgn_00070020, dlEsr2b: DLAgn_00027190, sea bream (*sparus auratus*) saEsr1: Q9PVZ9, saEsr2a: Q9W6M2,

saEsr2b: Q6H9M4. Amino acid sequence fragments to which the rabbit pAb raised against the peptide of the new C-terminal sea bass Esr2b are directed are shaded.

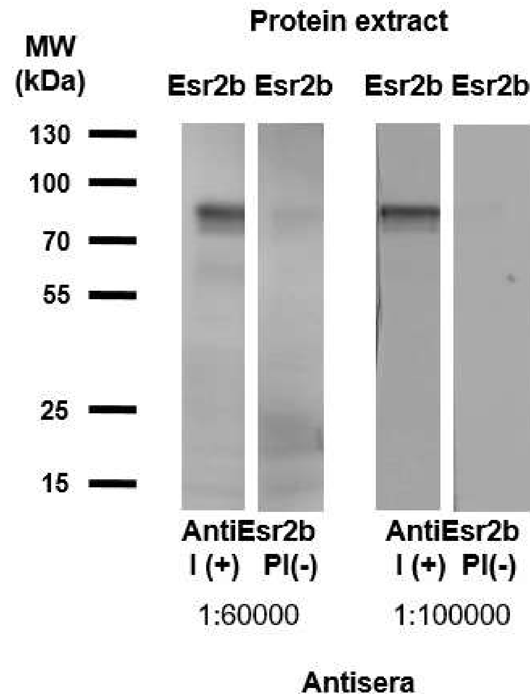

Fig. S5. Western blot of the recombinant sea bream Esr2b protein incubated with the produced anti-dlEsr2b (I, immune, (+) reaction) or with the respective pre-immune serum (PI or (-) control) at two different dilutions, detected by the ECL Plus Western Blotting Chemiluminescent Detection System. Molecular weight marker bands are indicated in kDa.

```
           101                                                150
    hsGPER LAVADLILVA DSLIEVFNLH ERYYDIAVLC TFMSLFLQVN MYSSVFFLTW
   dlGpera LAVADLILVA DSLIEVFNLN EKYYDYAVLC TFMSLFLQVN MYSSIFFLTW
   dlGperb LAVADLALVA DSLIEVFNLK QGYYDMASLC TFMNLFQQLN MYSSVFFLTW
 Consensus LAVADLiLVA DSLIEVFNL. #.YYD.AvLC TFMsLFlQvN MYSS!FFLTW
```

Fig. S6: Partial alignments of the GPER amino acid sequence of human (*homo sapiens*) and sea bass (*Dicentrarchus labrax*) retrieved from Genbank and the sea bass genome at http://seabass.mpipz.de/ (transcript prediction). Accession numbers are as follows: human (homo sapiens) hsGPER: Q99527, sea bass (*Dicentrarchus labrax*) dlGpera: DLAgn_00191960, dlGperb: E6ZGW6. Amino acid sequence fragments to which the rabbit pAb raised against human GPER 2s extracellular domain (ThermoFisher) are directed are shaded.

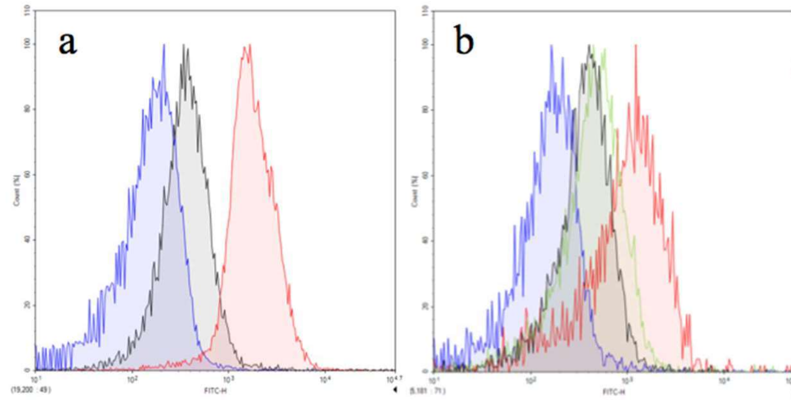

Fig. S7: Flow cytometric analysis of isolated thymocytes in green fluorescence (FITC-H) from two independent experiments (a-b). Histogram overlays from thymocytes incubated without primary antibody in blue, with the isotype-matched control in black, with the blocking peptide in green and with the respective anti-GPER (N15) staining in red. Experimental details in the text.

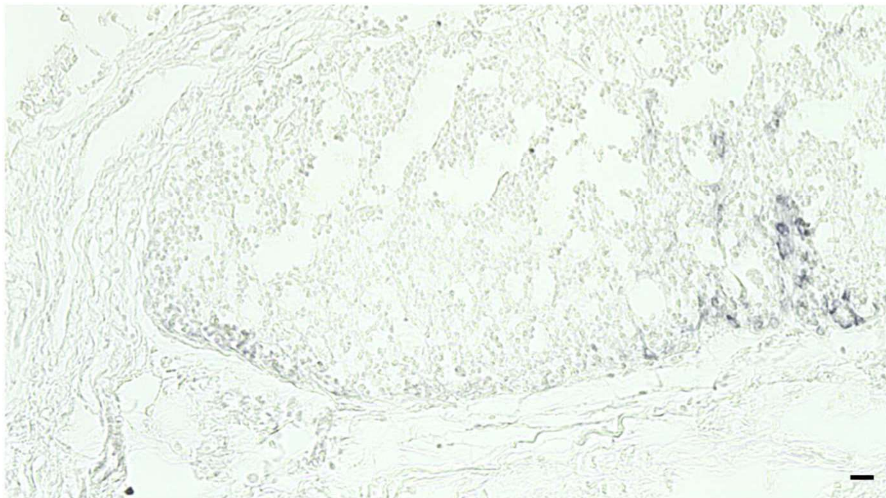

Fig. S8: IHC of histological sections of the thymus of *Dicentrarchus labrax* incubated, for negative control, with the antibody against GPER (N15) preincubated with the specific blocking peptide. Scale bar: 10 µm