Normandie Université

THESE

Pour obtenir le diplôme de doctorat

Spécialité
Physiologie et Biologie des organismes – Populations – Interactions
Préparée au sein de « Université Le Havre Normandie »

Immunomodulation of thymic function and T cell differentiation by oestrogens in the European sea bass, *Dicentrarchus labrax.* An evolutionary and ecotoxicological perspective

Présentée et soutenue par
Matthieu PAIOLA

Thèse soutenue publiquement le 19 février 2018 devant le jury composé de		
M. Helmut SEGNER	Professeur à Université de Bern (Suisse)	Rapporteur
M. Giuseppe SCAPIGLIATI	Professeur à Université de Tuscia (Italie)	Rapporteur
Mme Deborah POWER	Professeur à Université de Algarve (Portugal)	Examinateur
M. François BRION	Chargé de recherche INERIS	Examinateur
M. Thomas KNIGGE	Maître de conférence HDR à Université Le Havre Normandie	Directeur de thèse
Mme Tiphaine MONSINJON	Maître de conférence à Université Le Havre Normandie	Examinateur et responsable scientifique

Thèse dirigée par Thomas KNIGGE sous l'encadrement scientifique de Tiphaine MONSINJON

UMR-I 02 Sebio – Stress environnementaux et biosurveillance des milieux aquatiques

UNIVERSITÉ LE HAVRE NORMANDIE

ED 497 NBISE

Sebio UMR-I 02 Stress Environnementaux et BIOsurveillance des milieux aquatiques

Remerciements :

Je voudrais remercier mes encadrants de thèse **Thomas Knigge** et **Tiphaine Monsinjon** de m'avoir donné l'opportunité d'effectuer cette thèse. Un grand merci pour votre confiance et votre précieuse aide tout au long de ce projet et en particulier lors de la rédaction des articles et de ce manuscrit.

Un grand merci à mon jury de thèse **Helmut Segner**, **Giueseppe Scapigliati**, **Deborah Power** et **François Brion** d'avoir accepté d'évaluer mon travail et de faire le déplacement jusqu'au Havre pour ma soutenance. C'est un honneur et un plaisir de vous le présenter.

Je suis également très reconnaissant envers **Patricia Pinto** et ses collègues, sans qui ce travail de thèse aurait été bien différent.

J'aimerai exprimer ma gratitude à **Giueseppe Scapigliati** et **Simona Picchietti** de m'avoir accueilli au sein de leur laboratoire pour m'initier à l'art de l'immunohistologie sur le thymus de bar.

Je tiens à remercier **Emilie Farcy** de m'avoir de nouveau accueilli au sein de son laboratoire lors de cette thèse et surtout de m'avoir permis d'avancer grandement dans mes travaux de thèse. C'est un plaisir de travailler avec toi. Je voudrais également te remercier ainsi que **Catherine Lorin-Nebel**. Le stage de M2 avec vous et au sein de l'équipe AEO m'a donné la fibre du chercheur.

Merci à **Aurélie Duflot** pour ton aide pendant ces 3 années et en particulier lors de ton stage de M2 et lors les échantillonnages.

Je remercie grandement **Matthieu Bonnet** pour l'élevage des bars et ta constante bonne humeur et gentillesse.

J'aimerai remercier mes collègues de thèse et en particulier **Alexandre Erraud** et **Catarina Moreira** pour leur soutien et leur bonne humeur. C'est un plaisir de travailler avec vous. Bon courage pour votre thèse.

Un remerciement tout spécial à mes amis et particulièrement à Julie et ma famille pour leurs immenses soutiens.

Table of Contents

Abstract:

Jawed vertebrates have developed an efficient adaptive immune system to protect them against pathogens or transformed cells. Partly, this immune system relies on T lymphocytes, which are responsible for orchestration of the immune reaction as well as for immune tolerance. These T cells develop in an evolutionary conserved organ, the thymus. In mammals, endogenous oestrogens are powerful regulators of the thymic function and plasticity. The question is, therefore, whether this is also the case in lower vertebrates, such as teleost fish. This thesis aimed (1) at elucidating the mode of oestrogenic regulation of T cell development and (2) at investigating the effects of changes in the internal oestrogen-levels on the thymic T cell differentiation in the European sea bass, *Dicentrarchus labrax.*

To achieve these aims, firstly the distribution of oestrogen receptor subtypes was investigated by immunohistochemistry on the background of a detailed description of the functional anatomy of the thymic microenvironment. Secondly, thymic function- and T cell-related gene expression was analysed in the thymus, and in two other lymphoid organs, the head-kidney and the spleen of sea bass exposed *in vivo* to 17β-oestradiol. Moreover, to assess whether the modification of T cell differentiation results in a modulation of a physiological parameter of immunocompetence, *i.e.*, microbicidal activity, the oxidative burst capacity was evaluated in leucocytes isolated from the head-kidney and spleen following *in vivo* and *in vitro* exposure to oestrogen. Eventually, age- and size-dependent variations in leucocyte number in the thymus were examined in sea bass caught at various time points over three years in the wild or taken from hatchery.

The thesis provides new insights into the evolution of the immunomodulatory function of oestrogen with respect to the thymic and peripheral T cell differentiation in vertebrates. As a matter of fact, in addition to a highly conserved morpho-functional organisation, the distribution of oestrogen receptor subtypes as well as the oestrogenic effects appear to be evolutionary conserved. Our results suggest that, similar to mammals, oestrogen (1) stimulates a thymic alternative pathway of T cell maturation with innate-like properties, and (2) enhances peripheral T cell-mediated immune tolerance by promoting Treg differentiation, and (3) actively regulates thymic plasticity.

The astonishing degree of resemblance between the teleost and the mammalian thymus and the oestrogenic regulation of key events in T cell differentiation leads to the conclusion that xenoestrogens, abundantly released into the aquatic environments could interfere with the fish immune system. Exogenous oestrogens and oestrogen mimics may disturb T cell-mediated immunity in sea bass and, therefore, the capacity to fight pathogenic agents.

From an evolutionary point of view, this study provides new aspects that may facilitate the understanding of the biological significance that oestrogenic regulation of T cell development and thymic function may have in vertebrates. As a matter of fact, in sea bass, high oestrogen-levels are related to wintertime events including the reproduction and the migration. Accordingly, oestrogenic regulation of T cell development and differentiation is likely to be involved in the redirection of energy from immune to reproductive functions, and putatively in the modification of thymopoiesis towards the production of wintertime adapted immune cells, which promote immune tolerance and display innate-like characteristics.

Keywords: thymus, T lymphocyte, teleost fish, comparative immunology, endocrinology, regulatory T lymphocyte, gamma-delta T lymphocyte,

Résumé

Les vertébrés gnathostomes ont développé un système immunitaire adaptatif efficace pour se protéger des agents pathogènes ainsi que des cellules transformées. Ce système immunitaire repose en grande partie sur les lymphocytes T qui sont responsables de la coordination de la réponse immunitaire ainsi que du maintien de la tolérance immunitaire. Ces lymphocytes T se développent dans un organe conservé évolutivement : le thymus. Chez les mammifères, les œstrogènes endogènes constituent de puissants régulateurs de la fonction et de la plasticité thymique. La question soulevée ici est donc de savoir si c'est également le cas chez des vertébrés inférieurs tels que les poissons téléostéens.

Dans ce contexte, cette thèse vise (1) à élucider le mode de régulation œstrogénique du développement des cellules T et (2) à rechercher les effets des changements des niveaux d'œstrogènes endogènes sur la différentiation thymique et périphérique des lymphocytes T chez le bar européen *Dicentrarchus labrax*.

Dans ce but, en tout premier lieu la distribution des différents sous-types de récepteurs aux œstrogènes a été étudiée par immunohistochimie dans le contexte d'une description détaillée de l'anatomie fonctionnelle du microenvironnement thymique. Par la suite, l'expression de gènes relatifs à la fonction thymique et aux différents sous-types de lymphocyte T a été analysée dans le thymus et deux autres organes lymphoïdes, le rein-antérieur et la rate de bars exposés au 17β-œstradiol. De plus, pour évaluer si la modification de la différenciation des cellules T entraine une modulation d'un paramètre d'immunocompétence, c'est-à-dire l'activité microbicide, la capacité de flambée oxydative a été évaluée sur des leucocytes isolés de rein-antérieur et de rate à la suite d'une exposition *in vivo* et *in vitro* à un œstrogène. Finalement, pour étudier la plasticité, les variations du nombre de leucocytes dans le thymus selon différents âges et tailles ont été examinés sur des bars capturés durant trois ans.

La thèse fournit de nouvelles connaissances dans l'évolution des fonctions immunomodulatrices des œstrogènes quant à la différenciation des cellules T périphériques et thymiques chez les vertébrés. En effet, en plus d'une organisation morpho-fonctionnelle fortement conservée, la distribution des sous-types de récepteurs aux œstrogènes ainsi que les effets œstrogéniques apparaissent conservés au cours de l'évolution. Nos résultats suggèrent que, comme chez les mammifères, les œstrogènes (1) stimulent une voie thymique alternative de maturation des lymphocytes T ayant des propriétés similaires aux cellules immunitaires innées, (2) augmentent la tolérance immunitaire en stimulant la différentiation de lymphocytes T régulateurs, et (3) régulent activement la plasticité du thymus.

Le degré étonnant de ressemblance entre le thymus des téléostéens et mammifères et la régulation œstrogénique d'événements clés dans la différenciation des cellules T mènent à la conclusion que les xénœstrogènes abondamment libérés dans les environnements aquatiques pourraient interférer avec le système immunitaire des poissons. Les œstrogènes exogènes et les composés mimant les œstrogènes peuvent perturber l'immunité médiée par les cellules T chez le bar et ainsi altérer la capacité à lutter contre des agents pathogènes.

D'un point de vue évolutif, cette étude ouvre à de nouveaux aspects qui peuvent faciliter la compréhension de la signification biologique que peut exercer la régulation par les œstrogènes du développement des cellules T et des fonctions thymiques chez les vertébrés.

En fait chez le bar, des taux d'œstrogène élevés sont associés à des événements hivernaux incluant la reproduction et la migration. Par conséquent, la régulation œstrogénique du développement et de la différenciation des cellules T est probablement impliquée dans la réallocation énergétique des fonctions immunitaires vers celles de la reproduction et putativement dans la modification de la thymopoïèse vers une production des cellules immunitaires adaptées à la saison hivernale qui promeut une tolérance immunitaire et affiche des caractéristiques de type innées.

Mots clefs : thymus, lymphocyte T, poisson téléostéen, immunologie comparative, endocrinologie, lymphocyte T régulateur, lymphocyte T gamma-delta

List of figures and tables

Abbreviations:

AIRE, autoimmune regulator
APC, antigen-presenting cell
BCR, B cell receptor
BERKO, ESR2-knockout mouse
BPA, bisphenol A
CD, cluster of differentiation
CMJ, cortico-medullary junction
cTEC, cortical thymic epithelial cell
CYP, cytochrome P450 enzyme
CYP19, aromatase
DAMP, damage-associated molecular pattern
DC, dendritic cell
DES, diethylstilbestrol
DLT15, sea bass pan-T cell antibody
DN, double negative
DP, double positive
Dpf, day post-fertilization
Dph, day post-hatch
DPN, diarylpropionitrile (ESR2-agonist)
E1, oestrone
E2, 17β-oestradiol
E3, oestriol
EDC, endocrine disrupting chemical
EE2, 17α-ethinylestradiol
EEDC, oestrogenic endocrine disrupting chemical
ERKO, ESR1-knockout mouse
ESR, nuclear oestrogen receptor
ETP, early thymic progenitor
FOXN1, forkhead-box n1
FOXP3, forkhead box p3
FSC, forward-scatter
G-1, GPER-agonist
GALT, gut-associated lymphoid tissue
GIALT, gill-associated lymphoid tissue
GPER, G protein-coupled oestrogen receptor
H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate
HC, Hassall's corpuscle
HSC, hematopoietic stem cell
HSD, hydroxysteroid dehydrogenase
ICI 182,780, ESR-antagonist
IDO, indoleamine 2,3-dioxygenase
IEL, intraepithelial lymphocyte
IFN-γ, interferon gamma
Ig, Immunoglobulin
IL, interleukin
iNKT, invariant natural killer T cell
KLH, Keyhole limpet hemocyanin
LPS: lipopolysaccharide
lTEC, limiting thymic epithelial cell
MALT, mucosa-associated lymphoid tissues
MAMP, microbe associated molecular pattern
MC, mast cell
MHC, major histocompatibility complex
MISS, membrane-initiated steroid signalisation
MLR, mixed leucocyte reaction
mTEC, medullary thymic epithelial cell
NADPH, nicotinamide adenine dinucleotide phosphate
NALT, nasopharynx-associated lymphoid tissues
NET, Neutrophil Extracellular Trap
NISS, nuclear-initiated steroid signalisation
NLR, nucleotide-binding oligomerization domain (NOD) like receptor
NO, nitric oxide
NOS, nitric oxide synthase
NP, nonyl phenol
PAMP, pathogen associated molecular pattern
PBL, peripheral blood leucocyte
PCB, polychlorinated biphenyl
PCNA, proliferating cell nuclear antigen
PKC, protein kinase c
PMA, phorbol 12-miristate 13-acetate
pMHC; peptide MHC complex
pIEL, progenitor of intraepithelial lymphocyte
PPT, propyl pyrazole triol (ESR1-agonist)
PRR, pathogen recognition receptor
pTreg, peripheral regulatory T cell
RAG, recombination-activating gene
RNS, reactive nitrogen species
ROS, reactive oxygen species
SALT, skin-associated lymphoid tissue
sIg, soluble immunoglobuline
SP, single positive
SSC, side-scatter
Tc, cytotoxic T cell
TCDD, 2,3,7,8- Tetrachlorodibenzo-p-dioxin
TCR, T cell receptor
TEC, thymic epithelial cell
TGF-β, transforming growth factor beta
Th, helper T cell
TLR: toll-like receptors
TNF-α: tumor necrosis factor alpha
Treg, regulatory T cell
TSA: tissue specific antigen
tTreg, thymus-derived regulatory T cell
VLR: variable lymphocytes receptor
WFD, water framework directive
Wpf, week post-fertilization

1) State of the art

1) State of the art

The immune system is a complex and powerful physiological system that evolved to protect metazoans from pathogenic microorganisms and abnormal cells. In vertebrates, the immune system is commonly subdivided in two systems: innate and acquired immune system (Boehm and Swann, 2014). Both systems are fundamentally different but intimately connected so as to develop an efficient immune response (Rauta et al., 2012). The innate or non-specific immune system is the most ancestral and primitive component, which is already present in primitive plants and metazoans (Parra et al., 2013; Rauta et al., 2012). This innate immune system constitutes the first barrier of protection against foreign cells. It is composed of several primordial elements: the physical, humoral and cellular components (Magnadóttir, 2006). The physical barrier, *i.e.*, the epithelia, will prevent the majority of microorganisms from invading the body. It is reinforced by a mucus layer composed of numerous immune-related proteins (Cordero et al., 2015; Parra et al., 2016). Physical injury, however, enables the entry of pathogenic microorganisms and foreign cells. To efficiently counteract a potential infection, the organism needs to respond rapidly. To this end, sentinel cells will recognize the pathogenic/foreign agents and initiate an inflammatory response. This inflammation facilitates the recruitment of other immune cells (neutrophils, macrophages and dendritic cells), which eliminate the opportunistic microorganisms. Vertebrate innate immune cells, however, are more limited in their capacity to recognize invading organisms (Rauta et al., 2012). Evolution has, therefore, developed a second component, the acquired/adaptive immune system, which synergistically and more efficiently eliminates pathogenic agents (Boehm and Swann, 2014; Rauta et al., 2012). In jawed vertebrates, B and T lymphocytes are the effector cells of this adaptive immune system. Their central role in this specific immune response derives from their memory function and the capacity to recognize a wide variety of antigens, thus allowing a powerful and efficient immune response against the pathogenic agents (Boehm and Swann, 2014; Parra et al., 2013). In mammals, T cells have been associated to several major functions such as the (1) coordination of the immune response including the amplification and the suppression of the response with notably the secretion of pro- and anti-inflammatory cytokines, (2) the elimination of infected or tumor cells with the secretion and expression of cytolytic or pro-apoptotic factors and (3) for “unconventional” T cell the initiation of the immune response (innate-like activity). Those different T cell functions are generally associated to specific T cell populations respectively named helper, regulatory and γδ T

lymphocytes (Chien et al., 2014; Tafalla et al., 2016; Ulges et al., 2016). All of them developed in the thymus which provides a complex microenvironment necessary for their development in self-tolerant and immunocompetent cells (Abramson and Anderson, 2017; Klein et al., 2014; Muñoz-Ruiz et al., 2017).

In jawed vertebrates, or gnathostomes, comprising cartilaginous fish (sharks, rays, skates and sawfish as well as ghost sharks), teleost fish and tetrapods, the innate and adaptive immune systems share numerous features at the gene-, cellular- and organ-level (Boehm et al., 2012; Boehm and Swann, 2014; Hayday, 2000). Jawed vertebrates (cartilaginous fish) diverged from cyclostomes (lampreys) around 530 millions years ago (Boehm and Swann, 2014). Teleost fish have a successful evolutionary story, as they represent the majority of the existent vertebrate species (over 28,000 species) and have colonized a large variety of aquatic environments (Cordero et al., 2015; Parra et al., 2016). Even if in fish the innate system is considered predominant compared to the adaptive immunity (Rauta et al., 2012), the conservation of adaptive immunity throughout evolution must be considered a determinative innovation for the immune response in vertebrates.

To date, the vertebrate adaptive immune system has been investigated in mammals for the most part. Teleosts emerge, however, as model organisms and study objects of comparative immunology. Teleost fish represent a model of choice to better understand primary mechanisms, as despite the divergence from mammals hundreds of million years ago, mammals and teleost share many fundamental mechanisms-related to T cell development as well as B and T cell function (Bajoghli, 2013; Langenau and Zon, 2005; Rauta et al., 2012; Tafalla et al., 2016). Furthermore, zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) are important model organisms that provide powerful genetic tools and *in vivo* imaging (Bajoghli et al., 2015; Langenau and Zon, 2005; Rauta et al., 2012). More specifically, teleost fish represent an interesting model for comparative studies of the endocrine-immune crosstalk, since they display a high ecological diversity of life-history traits. For instance, teleost fish display every mode of reproduction that can be found in vertebrates (Cockburn, 1992; Smith and Wootton, 2016). In addition some species can well adapt to a large range of physicochemical conditions in changing environments (Bly and Clem, 1992; Laverty and Skadhauge, 2012). Because reproductive traits and adaptation to environmental stressors relate to hormonal control and largely depend on the energetic resources available and energetic allocation, they must be tightly connected to the immunological defence. Hence, the more “basic” evolutionary position and the enormous diversity of species provide a unique

opportunity to investigate the mechanisms of adaptation and endocrine regulation of the immune system in vertebrates. Besides, the interest in fish immunology has a strong applied aspect as fish consumption has been continuously increasing worldwide resulting in a fall in wild fish stock, which endangers the survival of several economic important teleost species. One of the solutions to these problems is seen in an increase of aquaculture production (Food and Agriculture Organization of the United Nations, 2016). To improve fish survival and, hence, productivity, the control and improvement of cultured fish immune status represent a major impetus in the research of fish immunology. Despite the quotas and fishing restrictions imposed by governments and supra-national organisations, anthropogenic environmental pollution may represent another menace for fish populations. Especially the estuaries, which constitute important nurseries for many marine teleost species, are highly impacted by chemical pollution (Kelley, 1988; Morin et al., 1999). Detoxification and perturbation of endocrine regulation, may affect reproduction but also the immunocompetence of fish and, in all probability, these processes are linked by the endocrine-immune system crosstalk. The estuaries are notably polluted by endocrine disrupting chemicals (EDCs), many of which with a supposed or proven oestrogenic activity (*i.e.*, oestrogenic endocrine disrupting chemicals, EEDCs) (Aris et al., 2014; Tappin and Millward, 2015). In both fish and mammals these chemicals have been reported to also affect the immune system (Burgos-Aceves et al., 2016; Milla et al., 2011; Segner et al., 2013; Szwejser et al., 2016). Assuming reduced immunocompetence as a result of such interference with the fish immune system, a reduced capacity to fight pathogenic agents would represent a supplementary stress, which could be detrimental to wild fish populations (Burgos-Aceves et al., 2016; Segner et al., 2013). Advanced knowledge in the fundamental relations between the endocrine and the immune system of fish are, therefore, relevant for ecological preservation and risk assessment. It will, however, also improve comparative immunotoxicology as well as our understanding of the evolution of the immune system and ecological immunology. Indeed, with regards to the impact of EEDCs, it is important to determine to which extent the immune system and its hormonal regulation are conserved. The higher the degree of conservation, the higher the likelihood that mechanisms can be extrapolated between teleost and mammals.

In mammals, the thymus and associated T cell development have long been identified as a substantial evidence of the endocrine-immune system interaction, indeed the thymus plasticity is strongly modulated by both endogen and exogenous oestrogens. This interaction has been reported for some decades now and has been intensively investigated because it was believed

responsible of female immune system aging and it is notably suspected to be responsible of the high autoimmune prevalence in female (Chapman et al., 2015; Dragin et al., 2016; Hince et al., 2008; Ross and Korenchevsky, 1941). Most investigations have been dedicated to the description of thymus atrophy observed with high-plasmatic level of 17β-oestradiol (E2) (Chapman et al., 2015; Hince et al., 2008; Screpanti et al., 1991; Zoller and Kersh, 2006). However, the mechanisms responsible of this phenomenon remained not completely established and misunderstood. In teleost, early work in Masu salmon suggests that similar effects occur (Sufi et al., 1980) and more recently Seemann et al. (2016) observed that E2-exposure of juvenile sea bass modulates the thymus plasticity.

Based on the knowledge acquired in teleost and mammals, the present work of my PhD thesis investigated possible mechanisms of E2-action on the thymic function of European sea bass. To reach this purpose, we sequentially investigated:

1. The distribution of the different oestrogen receptor subtypes in the thymus of adolescent female.

   Aim: identify the potential thymic cells targeted by oestrogens and depict the possible oestrogen effects and mechanisms of action (**Article 1**).

2. The *in vivo* effect of E2-exposure on the thymus of sea bass. A set of specific genes of the thymic function as well as of different T cell subtypes and cellular homeostasis were chosen. Gene expression-level analysis was conducted on the thymus as well as on two major lymphoid organs, the head-kidney and the spleen. The molecular biology approach was combined with respiratory burst analysis on leucocytes isolated from head-kidney and spleen.

   Aim: (1) unravel the E2-effect on the thymic T cell development including the potential change in thymic T cell output and peripheral T cell differentiation in the head-kidney and spleen (2) determinate if the potential E2-mediated changes in peripheral T cell modulate the innate immune cell activity (**Article 2**).

3. the E2-effect on viability and redox capacity of leucocytes isolated from the thymus, head-kidney and spleen after *in vitro* exposure. In addition, for the leucocytes of the head-kidney and spleen, the oxidative burst capacity was evaluated. We also examined the expression at transcript-level of all the oestrogen receptor subtypes in the head-kidney, the spleen and the associated leucocytes.

Aim: determinate if oestrogen could directly and similarly modulates immune cells of thymus, head-kidney and spleen (**Article 3**).

In parallel of this laboratory research focussing on the thymus and T cells, two supplementary projects were conducted:

1. In a field project entitled ECOTONES (Effect of Contamination on OrgaNisms of the Seine Estuary), we measured on wild juvenile sea bass caught in the Seine estuary from June 2015 to October 2017 different physiological tests on leucocytes isolated from the thymus, head-kidney and spleen by flow cytometry (*i.e.*, viability, phagocytosis and oxidative burst capacity), in order to assess the fish immunocompetence in a contaminated environment.
2. Effect of E2-exposure on the pituitary gland proteome of adolescent female sea bass. The increase of knowledge in that matter would help to understand how E2 regulates non-hormonal proteins which interfere in synthesis, cleavage, post-translational modifications and release of follicle-stimulating and luteinising hormone.

However, those two projects are still ongoing project and only preliminary results are provided in supplementary data at the end of this manuscript.

A) Immune system with an evolutionary point of view from teleosts to mammals

i) Innate immunity

In teleost fish, as in invertebrates and other vertebrates, the first barrier against infection (the physical component) is represented by several mucosae, such as skin, gills and intestine. Mucus hosts commensal non-pathogenic bacteria and protects from pathogenic microorganisms (Parra et al., 2016). The integrity of the mucosal barrier is performed by humoral and cellular factors (Magnadóttir, 2006; Parra et al., 2016). The humoral factors are also interconnected with other body fluids, such as the blood (Magnadóttir, 2006). In fish, several types of humoral factors have been documented: microorganism growth inhibitor (*e.g.,* transferrin), protease inhibitor (*e.g.,* α2-macroglobulin) and lytic enzymes (*e.g.,* lysozyme, chitinase and complement system), agglutinin (*e.g.,* mannose-binding and fucose-binding lectin) and natural antibodies (Cordero et al., 2015; Magnadóttir, 2006; Parra et al., 2016). Antibodies are generally considered as components of adaptive immune system, but natural antibodies can be classified as a component of the innate immune system, since their production occurs without apparent antigen stimulation and gene-rearrangement (Magnadóttir, 2006; Salinas, 2015). Humoral factors, such as antibodies, lytic enzymes and agglutinin will notably facilitate the subsequent cellular response, *i.e.*, phagocytosis by opsonisation (Cordero et al., 2015; Magnadóttir, 2006).

Innate immune cells can be considered as sentinel cells. In vertebrates, several innate immune cells have been described, *e.g.*, mast cells and dendritic cells, but mainly professional phagocytes (granulocytes and macrophages) (Esteban et al., 2015; Galindo-Villegasa and Mulero, 2015). These innate immune cells express different subtypes of the pathogen recognition receptor (PRR), such as toll-like receptors (TLR) or nucleotide-binding oligomerization domain like receptor (NLR; Galindo-Villegasa and Mulero, 2015; Rauta et al., 2012). PRRs recognize microorganisms that express conserved structures named pathogen- or microbe-associated molecular pattern (PAMP, MAMP; Galindo-Villegasa and Mulero, 2015). Interestingly, teleost have been described to express a higher diversity of PRR, notably TLR, which initiate the transduction signal and the reaction against the bacterial invasion (Galindo-Villegasa and Mulero, 2015; Rauta et al., 2012). In professional phagocytes of vertebrates, pathogenic bacteria and PAMP (*e.g.,* lipopolysaccharide, LPS) induce the formation of superoxide as well as nitric oxide (NO) respectively catalysed by the

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the NO synthase (NOS). This phenomenon is named oxidative burst or respiratory burst and is used to kill the invading organisms (Esteban et al., 2015; Havixbeck and Barreda, 2015). In mammals, it was also shown that innate immune cells can be activated by damage-associated molecular pattern (DAMP), which is released by cellular injury (necrotic changes and natural apoptosis) (Galindo-Villegasa and Mulero, 2015; Magnadóttir, 2006). Furthermore, numerous inflammatory cytokines (TNF-α, IFN-γ…) can also induce a respiratory burst response of neutrophils in mammals, but this has not yet been investigated in teleosts (Havixbeck and Barreda, 2015).

(1) Macrophages

In all vertebrate classes, macrophages are one of the best known innate immune cells (Galindo-Villegasa and Mulero, 2015). With their capacity to engulf and to kill pathogens, they activate subsequent adaptive and specific immune responses when needed by presenting antigens to lymphocytes as well as by the production of numerous cytokines (Galindo-Villegasa and Mulero, 2015; Lewis et al., 2014). In fact from mammals to teleost, during the immune response against microbial or parasitic infection, macrophages differentiate into type M1 or M2, which are associated to the helper T cell (Th) 1 and Th 2, respectively (Wiegertjes et al., 2016). Type M1 and M2 macrophages are generally referred as classically- and alternatively- activated macrophages with pro-inflammatory and anti-inflammatory (healing) phenotypes. From a comparative immunological point of view, it was hypothesised that macrophages, instead of T cells, could by sensing the microbial/parasitic infection initiate the immune system polarization for type 1 (M1) or type 2 (M2) immunity (Fig. 1; Wiegertjes et al., 2016).

Microbial stimuli activate the macrophages *via* multiple PRR, which drive M1 polarization characterized by increased oxidative burst capacity (NO production) and the production of pro-inflammatory cytokines (*e.g.,* IFN-γ and TNF-α) provoking Th1 differentiation. Differentially, parasite-related stimuli such as chitin from helminths are sensed by macrophages which trigger the differentiation of alternatively-activated M2 macrophages. The M2 polarized macrophages have decreased microbicidal activity but increased arginase activity, as well as increased extracellular matrix and polyamine production for the healing process. Consequently, M2 activity drives Th2 differentiation by secreting IL-4/13. Then,

both M1/M2 and Th1/2 synergistically drive the immune response with a positive feedback loop (Wiegertjes et al., 2016).

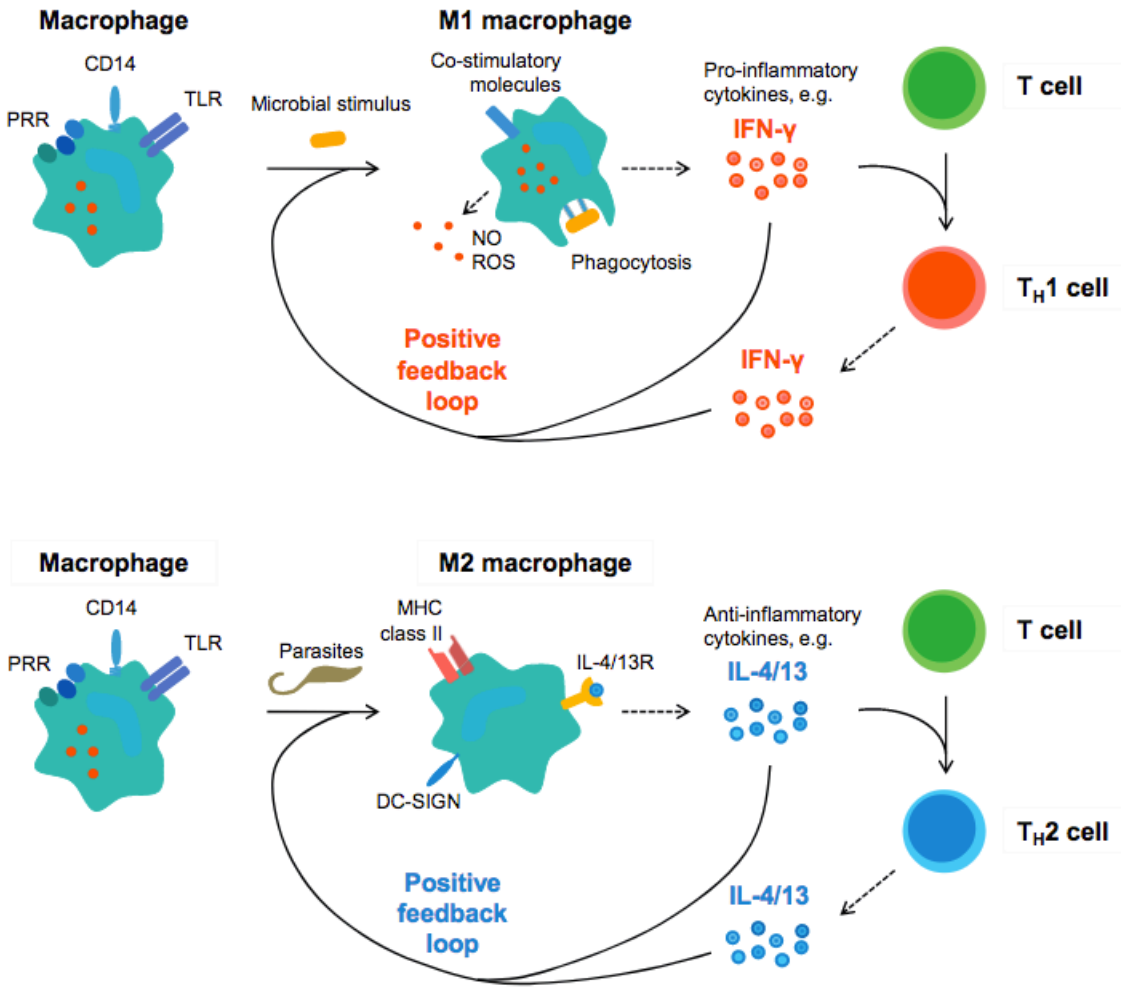


Figure 1: "Macrophage first" point of view: immune response polarization is initiated by macrophage activation and sustained by Th1 (IFN-γ) and Th2 (IL-4/13) cytokines. Microbe stimulus activates macrophage through PRR such as TLR which leads to M1 differentiation (classically-activated macrophage) with increased anti-microbial activity (NO production) and increased secretion of pro-inflammatory cytokines, *e.g.*, IFN-γ. Those cytokines promote Th1 differentiation and IFN-γ secretion which act in a positive feedback loop on M1-polarized macrophage to increase their capacity of antigen presentation. Differentially, parasite-related molecular pattern (PAMP) sensed by macrophage leads to the development of alternatively-activated M2 macrophages. M2 macrophages have increased arginase activity (L-arginine being the substrate of NOS), decrease microbicidal activity and increased production of numerous factors involved in healing process. Furthermore, M2 phenotype drives Th2 differentiation and IL-4/13 secretion which act in a feedback positive loop to further increase M2 polarization and activity. From Wiegertjes *et al.* (2016).

The M2 phenotype was further subdivided on the basis of their activation pathways: (1) M2a is activated by IL-4/13, (2) M2b is activated by protozoan, helminths, apoptotic cells and

immune complex and (3) M2c also called regulatory macrophage is activated by glucocorticoids/TGF-β/IL-10/MCSF-1 (Wiegertjes et al., 2016).

Macrophages have also important role for tissue homeostasis (*i.e.*, apoptotic body clearance) after the immune response or in primary lymphoid organs. In fact, adaptive immune response both implies high clonal proliferation and migration of numerous immune cells (*e.g.*, neutrophil and T cell), which, mainly die by apoptosis during the resolution of the inflammation (Havixbeck and Barreda, 2015). In the thymus as an example, most of the T cell produced do not express a functional TCRαβ and therefore die by apoptosis in the cortex (Klein et al., 2014). Consequently, in the thymus of mammals and teleost, macrophages have been described to be more abundant in the cortex (Halkias et al., 2014; Romano et al., 1999b).

(2) Granulocytes

Teleost and mammal neutrophils share numerous characteristics. They are critical elements of the first line of defence against pathogens. Upon early pro-inflammatory signals, neutrophils will be the first to migrate to the site of inflammation *via* the blood circulation (Fig. 2; Havixbeck and Barreda, 2015). In addition to their high phagocytic capacities, neutrophils display a high anti-microbial activity involving oxidative burst and the release of antimicrobial and cytotoxic substances stored in neutrophilic granules (Havixbeck and Barreda, 2015). As in mammals, teleost neutrophils can confine and kill extracellular pathogen with the degranulation of Neutrophil Extracellular Traps (NET), which are filaments of DNA associated to granular proteins (Esteban et al., 2015; Havixbeck and Barreda, 2015). Recent studies indicate that neutrophils actively take part in the inflammatory resolution process also in teleosts by the production of pro-resolving lipids (Fig. 2; Havixbeck and Barreda, 2015). Indeed, to preserve the host health and tissue homeostasis, the inflammation needs to be controlled and down-regulated rapidly once the pathogenic threat is eliminated. Subsequently, the damaged tissue needs to be cleared and the anti-inflammatory signals are followed by wound healing signals. A critical step of the inflammation resolution process is neutrophil apoptosis, which is tightly regulated by complex signalling pathways involving the intrinsic and extrinsic pathways of apoptosis that are mediated by the mitochondria and death receptors, respectively. Neutrophil apoptosis will stimulate the apoptotic corpse clearance by macrophages during the resolving phase of inflammation (Havixbeck and Barreda, 2015).

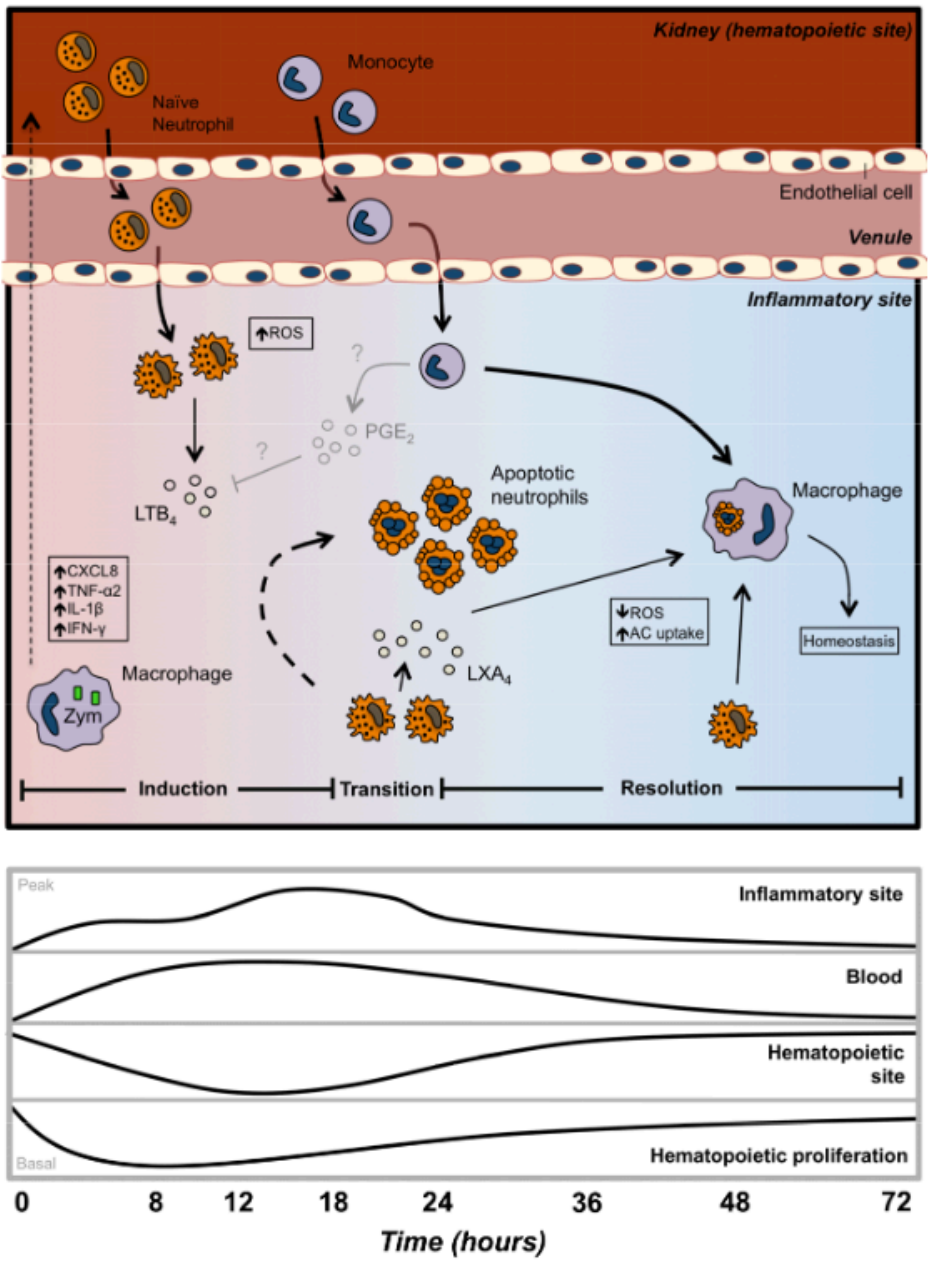


Figure 2: Contribution of neutrophils to the 3 steps of the immune response: induction, regulation and resolution. Peritoneal injection of zymosan provokes a release by sentinel cells (*e.g.*, macrophage) of pro-inflammatory cytokines such as TNF-α, IL-1β and IFN-γ as well as chemoattractants like CXCL8 and LTB_4 (pro-inflammatory lipid mediator), promoting the successive infiltration of neutrophils and other leucocytes. Circulating neutrophils penetrate into the site of inflammation and are subsequently activated by the inflammatory milieu which notably promote ROS production capacity and the release of LTB_4. With the progression of the inflammation, an inflammatory transition begins to occur (18 h-24 h post-infection, hpi). Neutrophils decrease their production of lipid mediators and increase the release LXA_4 which is followed by an entry into apoptotic cascades, peaking at 24 hpi. Havixbeck and Barreda (2015) hypothesised that this switch from pro-inflammatory to anti-inflammatory activity, *i.e.*, LTB_4 to LXA_4 (a pro-resolving lipid) release is mediated in part, by the release of PGE (a major prostaglandin) from neighbouring cells. The inflammatory site slowly becomes pro-resolving as macrophages are stimulated by LXA_4 which decrease their ROS-production capacity and increase their uptake of apoptotic neutrophils. This progression eventually leads to the return to homeostasis by 72 hpi. The relationship between neutrophil production within the kidney hematopoietic tissue, their migration through the blood, and infiltration into the challenge site highlight unique features of the teleost system. From Havixbeck and Barreda (2015).

(3) Mast cells

The teleost mast cells (MC), also called eosinophilic granular cells, remain poorly described. Because mammalian and teleost MCs have been described to share differentiation process, they are believed to be homologous (Prykhozhij and Berman, 2014; Sfacteria et al., 2015). Most likely, MC progenitors emerge from hematopoietic organs, *i.e.*, the bone marrow in mammals and, consequently, the head-kidney in teleost, from where they migrate into multiple organs, in which they undergo differentiation (Prykhozhij and Berman, 2014; Sfacteria et al., 2015). MCs are found in connective tissues, close to blood vessels and nerves in numerous organs. They are particularly present in the digestive tract, but also in the skin, the respiratory and reproductive system of all vertebrate groups (Prykhozhij and Berman, 2014; Sfacteria et al., 2015). As described for mammals, MCs are both resident and motile cells, they can migrate to the site of infection after parasitic or bacterial infection. With respect to their localization close to the tissue-environment interface, they represent an important first line-sensor of invading pathogens. MCs are important for the initiation of inflammation and the immune response. Activated MCs release antimicrobial and vasodilator compounds from cytoplasmic granules (Prykhozhij and Berman, 2014; Sfacteria et al., 2015). In perciform fish, a family considered as the most evolutionary advanced, such as gilthead seabream and sea bass, the granules of MCs contain histamine, a powerful vasodilator (Galindo-Villegas et al., 2016; Mulero et al., 2007). Hence, the MCs of these fish are very similar to those of higher vertebrates. Mammalian MCs are activated through PRR, such as TLR1-9, and produce cytokines, chemokines and eicosanoids such as prostaglandins to attract in the inflammatory site macrophages, neutrophils, dendritic cells and T-cells to initiate the adaptive immune response. Similar observations have been made in fish (Cardamone et al., 2016; Prykhozhij and Berman, 2014). The importance of MCs for the immune response is underscored by their implication in allergic reactions, autoimmune diseases as well as cancer and asthma described for mammals (Cardamone et al., 2016; Sfacteria et al., 2015).

(4) Dendritic cells

In mammals, dendritic cells (DC) are considered as the main antigen-presenting cells (APC). They, therefore, represent the main bridge between innate and adaptive immunity (Audiger et al., 2017; Steinman and Banchereau, 2007). DCs are also important sentinel cells, widely present in different secondary lymphoid organs, such as skin, lung and mucosa (Devi and

Anandasabapathy, 2016). Different DCs with either immunogenic or tolerogenic phenotypes will be produced upon varying stimuli such as DAMP, MAMP and other environmental stimuli. Both types of DCs modulate T cell activity by presenting antigens. Upon infection, upon, for instance, MAMP or DAMP stimuli, immunogenic DCs promote Th differentiation which stimulate T cell dependent adaptive immune response and memory. Differentially, in steady-state, upon cytokine stimulation, for example, tolerogenic DCs regulate immune tolerance by notably promoting regulatory T cell differentiation and function (Audiger et al., 2017; Devi and Anandasabapathy, 2016; Steinman and Banchereau, 2007). After antigen stimulation, DCs modify their chemokine receptor and migrate to lymph nodes to either induce adaptive immune response or immune tolerance *via* cross-presentation of the antigen to T cells (Audiger et al., 2017; Steinman and Banchereau, 2007). In mammals, the activated immunogenic DCs also synthesise cytokines in order to enhance innate and adaptive immune responses as well as chemokines to attract, one after another, phagocytes, memory lymphocytes and naïve T cells (Steinman and Banchereau, 2007). Dendritic-like cells have been isolated from teleost fish and characterized using adhesion protocols developed for mammalian species (Bassity and Clark, 2012; Shao et al., 2015) as well as cell sorting based on their affinity for peanut agglutinin or the expression of CD8α and CD209 (also named DC-SIGN for Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; Granja et al., 2015; Lugo-Villarino et al., 2010; Shao et al., 2015). It was found that these cells have the same tree-like morphology and a similar functionality. Their capacity to stimulate T cell proliferation and differentiation in a Mixed Leucocyte Reaction (MLR) is higher than that of B cells and macrophages and they also express mammalian DC markers, *e.g.,* CD209, CD83, CD80/86 and MHC-II among others which confirm conserved function as professional APC (Bassity and Clark, 2012; Granja et al., 2015; Shao et al., 2015). As in mammals, dendritic-like cells of fish are present in several lymphoid organs such as the head-kidney, the spleen and the skin (Bassity and Clark, 2012; Granja et al., 2015; Shao et al., 2015; Steinman and Banchereau, 2007). Furthermore, they express several PRR and are activated by PAMP such as LPS and zymosan (Granja et al., 2015; Shao et al., 2015). They also have a high capacity of migration and to phagocyte small particles (Bassity and Clark, 2012; Granja et al., 2015), all this being properties they share with mammalian DCs. A recent study in rainbow trout suggest that the tissue resident skin CD8α$^+$ DC-like, which is homologous to the mammalian form, is involved in the promotion of the tolerance in steady-state and CD8α$^+$ T cell immunity after activation by virus infection (Granja et al., 2015).

Dendritic-like cells have not been isolated from sea bass yet, but DC-like cells expressing MHC-II transcript have been observed in the cortico-medullary junction (CMJ) of the thymus (Avilés-Trigueros and Quesada, 1995; Picchietti et al., 2015) where numerous apoptotic cells were also observed (Abelli et al., 1998). Overall those observations suggest that dendritic cells are actively involved in immune tolerance in teleost fish as well.

ii) Adaptive immunity

(1) Definition

In the vertebrate lineage, comprising jawless (shark, teleost, tetrapod) and jawed vertebrates (hagfish and lampreys), an adaptive immune system evolved, which is based on effector cells: the lymphocytes. Lymphocytes clonally express a monoallelic, somatically diversified antigen receptor; in other words, each clone expresses a single receptor and, subsequent to receptor-mediated activation, the lymphocytes undergo intensive proliferation (Boehm and Swann, 2014; Litman et al., 2010). The antigen receptors of jawed and that of jawless vertebrates are, however, fundamentally different in structure. In jawed vertebrates, the receptors comprise the B cell receptor (BCR) and the T cell receptor (TCR), both of which belong to the immunoglobulin (Ig) superfamily. They consist of heterodimer transmembrane proteins with several Ig folds. Other than the germline encoded receptor of the innate immune system, which allows only limited antigen recognition, those of the adaptive immune system are based on somatic diversification of antigen receptor genes. This, in theory, allows for an infinite capacity of antigen recognition and therefore the development of a wide repertoire of antigen receptors (Boehm and Swann, 2014; Litman et al., 2010). The respective somatic diversification takes place during the lymphocyte development when the variable (V), diversity, (D) only for heavy chains, and joining (J) segments are assembled into a complete gene. In jawed vertebrates, the somatic gene rearrangement is enabled by the recombination-activating gene (RAG) family of proteins. Eventually, the lymphocyte repertoire of antigen receptors is further increased by randomly added nucleotides through the terminal deoxyribonucleotidyl transferase (tdt) at the junction of the V, (D), J segments (Litman et al., 2010). In lamprey, a well-studied representative of jawless vertebrates, three types of variable lymphocyte receptors (VLR), have been identified that are formed by the insertion of leucine-rich-repeated modules. The VLRA, VLRB and VLRC, are membrane-bound proteins, and just as TCR and BCR assembled in such a way that no complete sequence of these receptors

can be found in the lamprey genome (Boehm and Swann, 2014; Litman et al., 2010). Their rearrangement is probably accomplished through the cytosine deaminase (Boehm and Swann, 2014; Litman et al., 2010).

Classically, and contrariwise to the innate immune system, the adaptive immune system involves a memory that enables a more efficient immune response in the case of further responses directed against the same antigen. This capacity derives from the differentiation of activated effector lymphocytes into a memory phenotype, which will maximize the clonal expansion during a secondary response. However, in some cases, a variant of immunological memory has been described in invertebrates as well as unicellular organisms such as prokaryotes, thus indicating that the memory is not a faculty restricted exclusively to the adaptive immune system (Boehm and Swann, 2014).

(2) Humoral immunity

(a) Definition

The adaptive humoral immunity is also called B-cell-mediated immunity. It is generally associated with circulating antibodies (soluble Immunoglobulin, sIg), which are an important component of the adaptive immune system destined to eliminate viruses and toxins. Circulating antibodies will bind to and coat the antigen, thus activating the complement system, which neutralizes and aggregates antigens (opsonisation) to form antigen-antibody complex. Such antigen-antibody complexes will facilitate phagocytosis and hence the destruction of foreign particles. Soluble antibodies are secreted by plasma cells, which are activated and differentiated B cells.

(b) B lymphocytes

The B cells were first described in birds (Parra et al., 2013). Together with T cells they represent the two lymphocyte lineages of jawed vertebrates. B cells are characterized by their antigen-binding surface immunoglobulin receptor (BCR, B cell receptor). The high diversity of B cell Igs is obtained, just as in T cells, by somatic gene rearrangement associated to the TdT. However, B cells display supplementary diversification process mediated by cytosine deaminase comprising class switch recombination, gene conversion and somatic hypermutation involving a point mutation and sequence change between V gene segments.

The former arise only in tetrapod (Parra et al., 2013). Gene conversion and somatic hypermutation allow to modify and improve the clonally expressed antigen-receptor by randomly changing the V, (D), J exon sequences. From this highly variable repertoire, clones with higher antigen affinity can be selected (Litman et al., 2010). Somatic hypermutation as a mechanism of somatic gene diversification and affinity maturation has been described in all jawed vertebrates, including cartilaginous fish (Litman et al., 2010).

After antigen stimulation, the Ig expression changes from the membrane-bound to the secreted form. In tetrapods, this process is made possible by the class switch recombination, which involves a gene change in the constant fragment of the heavy chain (Litman et al., 2010; Parra et al., 2013). In jawed vertebrates, the membrane-associated isoform is the IgM. In teleosts, however, IgM is also the most prevalent form in body fluids and is also present in the mucosal response (Parra et al., 2016; Zwollo, 2016). Indeed, IgM is the best studied Ig subtype in teleosts, and, as such, considered the main teleost isotype (Parra et al., 2016; Zwollo, 2016). Teleost IgM is generally present as a tetramer with a variety of inter-heavy chain disulphide polymerisations of the monomeric and/or dimeric subunits. The various oxidation states have been shown to correspond to its functionality with an increase of disulphides (oxidation) leading to higher affinity and longer half-life (Parra et al., 2016). In addition, teleost B cells also produce IgT (named IgZ in cyprinids) and IgD (Parra et al., 2016; Zwollo, 2016). IgM and IgD have been identified in almost all jawed vertebrates excepted for rabbit and birds which lack IgD (Parra et al., 2013). IgT/Z appear to be the functional equivalent to the mammalian IgA and the amphibian IgX involved in the mucosal defence (Parra et al., 2013; Zwollo, 2016). The IgT is the main immunoglobulin coating microbes for immune exclusion (Parra et al., 2016; Salinas, 2015). IgM+ cells are the most abundant B cell subtype in the spleen, the kidney, the blood and the peritoneal cavity, whereas IgT+ B cells outnumber IgM+ cells in the skin and the gut (Parra et al., 2013). IgM+ and IgD+ B cells do not express IgT/Z; a distinct lineage appears to express uniquely IgT in teleosts (Parra et al., 2013; Picchietti et al., 2017). Both IgM and IgT are important components of the immune response, as the titers of both increase in the serum and mucus after immunization or following infection (Parra et al., 2016). Considering IgT, it is present in the serum as a monomer and in the mucus as a pentamer or tetramer non-covalently bound (Parra et al., 2016). On the contrary, IgD, of which two different secreted monomeric forms have been observed in the serum, is less characterised. Due to its rather weak induction during

infection, however, its role appears to be minor in comparison to the two other isoforms (Parra et al., 2016).

Compared to mammalian B cells, the teleost IgM are less diverse, show little increase of IgM-affinity and a longer response time to those of mammals. Also, the response latency appears to be temperature dependent in fish (Parra et al., 2013). As teleosts are poikilotherm, their metabolic rate is lower than that of mammals, which limits lymphocyte proliferation and, therefore, impedes an early adaptive immune response. These particularities probably also result in an lower capacity of antibody production, repertoire, affinity maturation and memory, as compared to mammals (Galindo-Villegasa and Mulero, 2015; Magnadóttir, 2006; Segner et al., 2017).

Although the main function of B cells is antibody production, they also have innate-immunity-like functions in teleosts. Indeed, it was discovered that B cells of fish also display phagocytic activity (Basten and Silveira, 2010; Li et al., 2006). Presumably, the recently described capacity of oxidative burst of teleost B cells is related to their phagocytic activity (Zhang et al., 2017) and their antimicrobial activity described in mammals and teleost (Kovats, 2015; Li et al., 2006). These innate-like properties of teleost B cells correspond to that of the mammalian B-1 cells. Furthermore, across all vertebrate classes, B cells also act as APCs (Basten and Silveira, 2010; Zhu et al., 2014). Eventually, mammalian B cells can also participate to the regulation of the immune response by the secretion of pro-inflammatory and anti-inflammatory cytokines (Basten and Silveira, 2010).

(3) Cellular immunity

(a) Definition

In jawed vertebrates, the second component of adaptive immunity is referred as “cellular immunity” or “T lymphocyte-mediated immunity”. Accordingly, it is based on T lymphopoiesis (thymopoiesis), which is accomplished in the thymus, from where naïve and mature self-tolerant T cells migrate to the peripheral lymphoid organs. T lymphocytes are characterized by their antigen-binding receptor: TCR (T cell receptor), a membrane heterodimer protein associated to the CD3-complex, which is involved in the TCR-signal transduction. The CD3-signalling complex is composed of a CD3-γ and a CD3-δ chain and two CD3-ε and CD3-ξ chains. In teleost only three CD3 chains have been found, *i.e.*, CD3-γδ,

-ε and -ξ (Tafalla et al., 2016). T cells can be further identified by the expression of lymphocyte-specific protein tyrosine kinase (Lck) or by the well conserved ZAP70, both of which are involved in the TCR-signalling (Dee et al., 2016; Nakanishi et al., 2015).

Based on the TCR heterodimers presented on their membrane, two fundamentally different T cell populations can be distinguished in jawed vertebrates: the TCRγδ or the TCRαβ. These confer different antigen-recognition capacities: for TCRαβ+ cells (αβ cells), the antigen must be presented by another cell on the major histocompatibility complex (MHC) of type 2 or 1; for TCRγδ the antigen-recognition is not related to MHC so that TCRγδ+ T cells (γδ T cells) can directly bind the antigen in order to become activated. The four TCR gene-families are strongly conserved across jawed vertebrate (Hayday, 2000). However, the major and best-described T cell population is the αβ T cells. Based on the expression of CD4 and CD8 membrane glycoproteins, γδ T cells are generally referred as double negative (DN). From these, two populations of mature αβ T cell with specific function emerge: the single positive CD4 or CD8 αβ T cells. Single positive (SP) CD4 coordinates and regulates other immune cells during the adaptive immune response (T helper and T regulatory, Th and Treg). SP CD8 αβ T cells have cytotoxic activity (Tc): they are involved in cellular surveillance of transformed cells (tumor or infected cells) (Nakanishi et al., 2015; Tafalla et al., 2016). Those αβ T cell subsets have been firstly described in human and rodents but phenotypic and functional homologous populations have been described in teleost (Nakanishi et al., 2015; Tafalla et al., 2016).

(b) TCRαβ+ lymphocytes

(i) CD4+ αβ lymphocytes

Different to tetrapods, which have a single CD4 co-receptor with four Ig domains, teleost species have two CD4 isoforms, designated *CD4-1* and *-2* with four and two Ig domains, respectively (Dee et al., 2016; Tafalla et al., 2016; Takizawa et al., 2016). The functional structure of the two isoforms was assessed in pufferfish by investigating their affinity to IL-16 and MHC-II. CD4-2 appears to be the functional equivalent to the mammalian CD4 in this species (Wen et al., 2011). In rainbow trout, the two CD4 discriminate two populations of CD4 positive lymphocytes, the main population displaying both DP CD4-1 and CD4-2, whereas the minor population expresses only CD4-2 (Takizawa et al., 2016). The latter SP

CD4-2 αβ T cell appears to be a distinct population with a more restricted TCRβ repertoire and minor proliferation capacity (Takizawa et al., 2016). Teleost CD4 αβ T cells appear to have similar properties as their mammalian homologue, *i.e.*, they are non-phagocytic cells and non-cytotoxic cells (Tafalla et al., 2016; Takizawa et al., 2016). In fish, as in mammals, CD4-1 expression is not restricted to lymphocytes; it is also expressed in trout and zebrafish by a population of macrophages/monocytes with high phagocytotic capacity (Dee et al., 2016; Takizawa et al., 2016).

Based on the expression of specific cytokines and transcription factors, several subtypes of CD4+ αβ T cells were described for mammals. In teleost, the main cytokines- and genes-related to the transcription factors have also been characterised (Fig. 3, Boschi et al., 2011; Tafalla et al., 2016). Moreover, the teleost Th subtypes are believed to have a fundamental role in the immune response coordination as well (Nakanishi et al., 2015; Tafalla et al., 2016). In mammals, after antigen stimulation, CD4+ αβ T cell subsets mainly differentiate in the peripheral immune organs. As shown in Figure 3, the cytokine microenvironment directs the naïve CD4+ T cells to differentiate into one of the different Th subsets. In turn the cytokines inhibit the differentiation into other Th subsets (Straub, 2007; Tafalla et al., 2016).

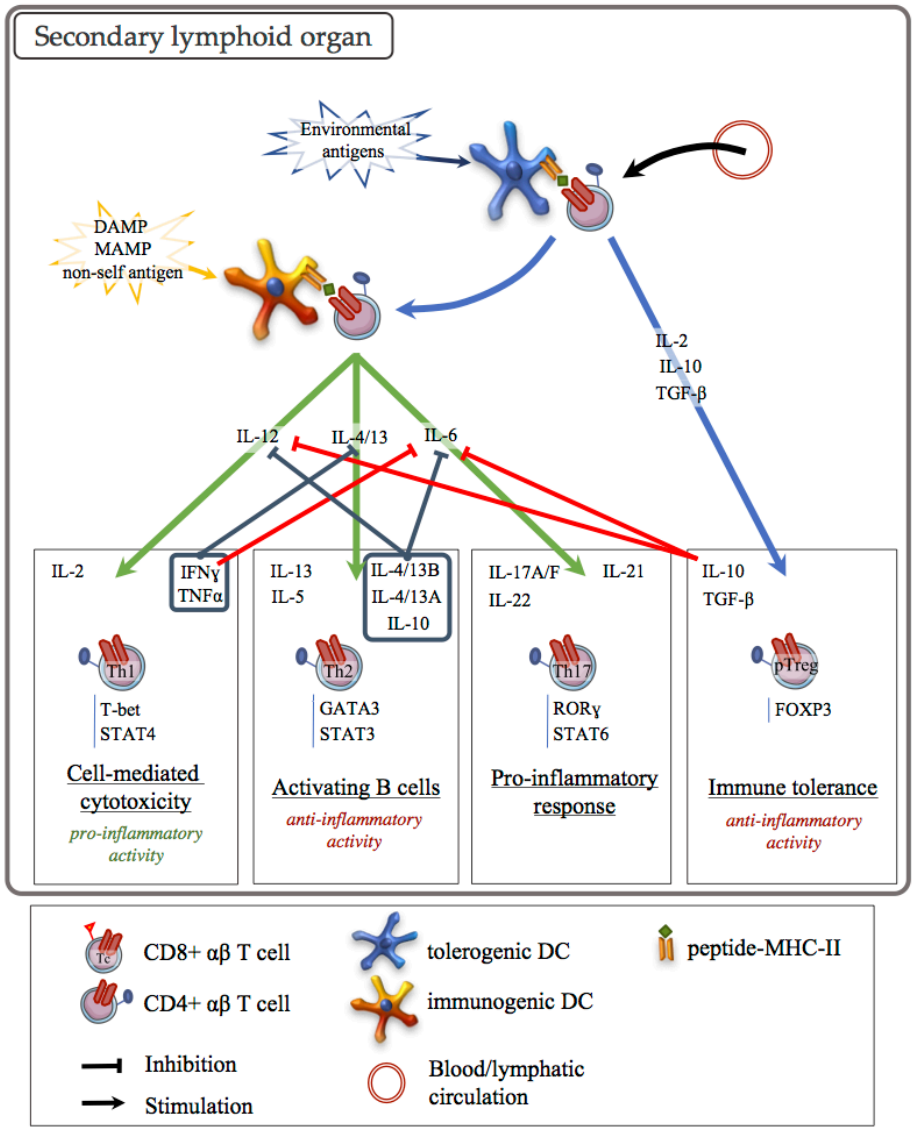


Figure 3: Model for peripheral CD4+ αβ T cell differentiation in teleost fish as adapted from mammals. Naïve cells migrate from the thymus to the secondary lymphoid organs. In steady-state (in non-infection), tolerogenic DCs promote the differentiation of specific peripheral Treg (pTreg) toward environmental and not harmful antigens (*e.g.*, commensal organism and food antigens). Upon infection, damage-associated molecular pattern (DAMP) and microbe-associated molecular pattern (MAMP) promote Th1, Th2 or Th17 depending on cytokine environment. Cytokines associated to differentiated CD4 αβ T cell subset inhibit the differentiation of other subtypes.

1. Treg

In mammals, the regulatory T cell (Treg) function and differentiation is controlled by the master transcription factor forkhead box winged helix p3 (FOXP3; Fig. 3) (Tafalla et al., 2016). Tregs are also characterized by the membrane expression of CD4 and CD25 (IL-2Rα). Their action is notably mediated by the production of interleukin 10 (IL-10) and transforming

growth factor β (TGF-β). IL-10 has an anti-inflammatory activity and inhibits the Th1/Th17 polarization, but stimulates the Th2 immunity *via* B cell proliferation (Tafalla et al., 2016). Different to the above-described other CD4+ αβ T cell subtypes, two populations of Tregs have been distinguished: natural or thymus derived Treg (tTreg) and induced or peripheral Treg (pTreg) which respectively differentiate in the thymus and peripheral lymphoid organs. After first antigen encounter of naïve CD4+ αβ T cells, Treg differentiation is notably promoted by IL-2, IL-10 and TGF-β secretion in its close environment (Fig. 3) (Audiger et al., 2017; Li and Rudensky, 2016). Tregs are involved in peripheral tolerance (*e.g.*, self-tolerance and mucosal tolerance), but also in the regulation of the immune response in order to avoid an excessive immune response for instance (Nakanishi et al., 2015; Tafalla et al., 2016). A homolog of the mammalian FOXP3 gene was identified in several teleost species (Jia et al., 2015; Quintana et al., 2010; Yang et al., 2012). Although teleost Foxp3 displays gene promotor region, protein-structure and functional properties homologous to the mammalian counterpart (Quintana et al., 2010; Yang et al., 2012), the functionally important N-terminal domain, which is important for the interaction with the protein partner, as well as key amino acid residues in the DNA-binding element are missing in teleost (Andersen et al., 2012). It is, therefore, discussed controversy whether FOXP3 function is indeed conserved across vertebrate classes. In Nile tilapia, however, FOXP3 is only expressed in lymphoid cells in the thymus, spleen, and head-kidney (Jia et al., 2015). Furthermore, CD4+ T cells with a Treg phenotype, and hence Treg response and function have been identified in fish *in vitro* and *in vivo* (Dee et al., 2016; Quintana et al., 2010; Wen et al., 2011; Yang et al., 2012). Treg-like cells expressing CD4-2, CD25-like (IL-2Rα/IL-15Rα-like) and Foxp3 have been described in the pufferfish. These Tregs appear to have a similar inhibitory effect on mixed leucocyte culture and non-specific cytotoxic cells *in vitro* (Wen et al., 2011). And the depletion of Tregs by injection of an anti-CD25-like antibody induced intestinal inflammatory lesions, lymphocyte infiltration and induction of pro-inflammatory (Th1) IFNγ and TNFα transcript which corroborate their anti-inflammatory roles. Moreover, in the zebrafish similarly to what was found in mammals, CD4-1 T cells isolated from the gut show a Treg-like phenotype with a high expression of *foxp3a* and *il-10* in comparison to the cells isolated from the spleen and head-kidney (Dee et al., 2016). Eventually, the anti-inflammatory role of Tregs in fish was confirmed by a foxp3a zebrafish mutant, which shows an autoimmune-like phenotype, *i.e.*, an increased inflammatory mononuclear cell infiltration (mostly T cells) in

mucosa-associated lymphoid tissues (MALTs), *e.g.*, gills and intestine as well as the overexpression of gene relative to Th1 and Th2 immune response (Sugimoto et al., 2017).

Overall, these studies demonstrate that teleost Foxp3 is also implicated in Treg function and the peripheral tolerance, albeit the significant difference in the structure with the mammalian FOXP3. This suggests a similar mechanism of immune tolerance between mammals and teleosts.

2. Th1

In mammals, following antigen stimulation, the differentiation of naïve CD4+ αβ T cell into pro-inflammatory Th1 is promoted by the IL-12 (Fig. 3). IL-12 induces the expression of the lineage specific transcription factor T-bet and STAT4 (Fig. 3) (Boschi et al., 2011; Dee et al., 2016; Tafalla et al., 2016). Th1 are characterized by the production of several cytokines such as interferon γ (IFN-γ), tumor necrosis factor α (TNF-α) and interleukin 2 (IL-2) (Boschi et al., 2011; Straub, 2007; Tafalla et al., 2016). Th1 cytokines induce cellular immunity, IFN-γ and TNF-α are involved in the defence against intracellular pathogens and IL-2 induces lymphocyte proliferation. In turn IFN-γ and TNF-α inhibit Th2 and Th17 differentiation (Fig. 3) (Straub, 2007; Tafalla et al., 2016). In teleost fish, induction of IFN-γ, and TNF-α gene transcription have been measured in the intestine following infection with pathogenic bacteria and protozoa (Tafalla et al., 2016).

3. Th2

The differentiation of SP CD4 T cells into Th2 is regulated by the transcription factors GATA3 and STAT6 (Fig. 3) (Dee et al., 2016; Tafalla et al., 2016). GATA-3 expression itself is induced by IL-4 (Dee et al., 2016). Upon APC stimulation Th2 differentiation is promoted by IL-4 secretion (Boschi et al., 2011; Straub, 2007). Th2 are characterized by the production of IL-4, IL-5, IL-10 and IL-13, all of which induce humoral immunity by stimulating antibody secretion of B cells (Straub, 2007; Tafalla et al., 2016). It was hypothesised that IL-13 arose from a gene duplication of IL-4. Hence, the two related genes have been nominated *il-4/il-13a* and *il-4/il-13b* in teleosts (Tafalla et al., 2016). In steady-state, CD4-1 T cells isolated from the gills of zebrafish show a Th2-like phenotype with a high expression of *gata3a* and *il-4/13b* (Dee et al., 2016). And infection with carp reovirus resulted in an induction of IFN-γ and T-bet gene expression and a decrease of GATA3 gene expression

(Tafalla et al., 2016). This suggests the existence of the Th1 and Th2 paradigm like in mammals (Tafalla et al., 2016).

4. Th17

Th17 differentiation is mediated by the transcription factor RORγ and STAT3 (Fig. 2) (Boschi et al., 2011; Tafalla et al., 2016). Th17 are characterized by the production of the pro-inflammatory cytokines IL-17, IL-21 and IL-22 (Ouyang et al., 2008; Rathore and Wang, 2016; Tafalla et al., 2016). In addition to APC stimulation, Th17 differentiation in mice require IL-6 and TGF-β and, IL-6 and IL-1 in human (Rathore and Wang, 2016). Th17 are implicated in the induction of the immune response against extracellular bacteria and fungi (Rathore and Wang, 2016; Tafalla et al., 2016). They are particularly important for mucosa defence (Ouyang et al., 2008; Rathore and Wang, 2016). Th17 offer a rapid amplification and termination of the immune response due to a rapid cell division and apoptosis (Rathore and Wang, 2016). IL-17 is involved in the maturation and recruitment of macrophages and neutrophils (Chien et al., 2014; Rathore and Wang, 2016). In both mammals and teleosts, the IL-17 family includes several members; the mammalian Th17 mainly produce IL-17A and IL-17F, both of which have teleost orthologues (Tafalla et al., 2016).

(ii)CD8αβ+ TCRαβ+ lymphocytes

Teleost CD8+ αβ T cells appear to express the heterodimer CD8 formed by α and β chains and to share similar function with their mammalian counterparts (Tafalla et al., 2016). Indeed, CD8α is expressed in a teleost T cell subtype distinct from CD4+ αβ T cells (Nakanishi et al., 2015; Takizawa et al., 2016). Furthermore, in teleost, CD8α+ αβ T cells have a high diversity in the TCR repertory (Tafalla et al., 2016). As in mammals, CD8α+ αβ T lymphocytes are implicated in the cell-mediated cytotoxicity against allogeneic cells and tissues (cells from the same species) and against virus- and bacteria-infected cells (syngenic/isogenic infected cells, *i.e.*, genetically identical; Nakanishi et al., 2015). The cytotoxic activity of teleost CD8α+ αβ T cells is mediated by the recognition of the MHC-I, followed by the induction of programmed cell death (apoptosis) of the target cells (Nakanishi et al., 2015; Tafalla et al., 2016). As in mammals, apoptosis appears to be mediated by two distinct pathways: (1) the degranulation of perforin and granzymes (Nakanishi et al., 2015; Takizawa et al., 2011) and

(2) the induction of apoptosis *via* death receptor such as Fas. The cytotoxic cells express the Fas ligand (FasL), which will activate the external pathway of the caspase-dependent apoptosis (Nakanishi et al., 2015). In mammals, perforin forms pores, which allow the entry of granzyme (serine protease) into the cytoplasm to induce apoptosis (Nakanishi et al., 2015; Takizawa et al., 2011).

In the gill and the intestine of rainbow trout, CD8α+ positive cells represent the majority of lymphocytes (25 and 54.6% respectively), whereas in head-kidney and spleen they are scant (*i.e.*, 4.1% and 2.3%, respectively, Takizawa et al., 2011). Numerous CD8α+ cells have also been found in the epithelium and the *lamina propria* of the intestine in sea bass (Picchietti et al., 2011).

(c) TCRγδ+ lymphocytes

In human and mouse, γδ T cells comprise around 4% of the total of T cells in all lymphoid organs (Chien et al., 2014; Tafalla et al., 2016). In the mouse, but not in human, γδ T cells represent an elevated proportion of total T cell in the MALTs (*e.g.,* 50% to up to 70% in the skin; Chien et al., 2014). In higher vertebrates, *i.e.*, mouse, cattle, sheep, or chicken, γδ T cells represent around 25% of the lymphocyte in the blood and from 50 to 70% in the skin (Chien et al., 2014). Regardless of their tissue localization, γδ T cells express a highly diverse TCR repertoire in these tetrapods (Chien et al., 2014). The anatomical repartition is based on the expression of Vγ, which is determined by the V, (D), J recombination (Hayday, 2000; Muñoz-Ruiz et al., 2017; Turchinovich and Pennington, 2011). γδ T cells appear to have a distinct role in the immune response, as they respond earlier than αβ T cells (Chien et al., 2014). They are usually described as innate immune cell-like: (1) during the infection their number in the blood increases drastically, followed by their early infiltration in the lesion (Chien et al., 2014; Turchinovich and Pennington, 2011) in which they (2) act as a first line of defence by initiating the inflammation, notably by the early production of IL-17 and IFNγ (Chien et al., 2014; Turchinovich and Pennington, 2011). γδ T cells are (3) not restricted to MHC for antigen stimulation. Instead, they express PRR offering the capacity to initiate the immune response (Tafalla et al., 2016). Moreover, γδ T cells have also been described to have (4) anti-bacterial activity with the production of antimicrobial peptides (Chien et al., 2014).

Human and mouse γδ T cells have been described to develop a Th1/Th2 response with pro-inflammatory, anti-inflammatory and cytotoxic activity in infections and tumours similar to αβ T cells, albeit at a lower extent (Chien et al., 2014; Hayday, 2000). γδ T cells are also implicated in the balance between the immune response and tolerance towards commensal microbiota and other environment-related antigens on the skin and intestine (Chien et al., 2014; Tafalla et al., 2016).

In teleost fish, the γδ T cells and TCRγδ repertoire have been poorly investigated (Wan et al., 2017). In common carp and sea bass TCRγ gene in teleost is mainly expressed in the thymus, and, to a lesser extent, in MALT, such as gut and gill (Boschi et al., 2011; Shang et al., 2008).

In the gut of sea bass, using sea bass pan-T cell antibody named DLT15, purified DLT15+ leucocytes have been shown to highly express TCRγ gene in comparison of the TCRβ gene (Boschi et al., 2011). And during sea bass ontogenesis, Romano et al., (2011) quantified the density of *tcrβ+ and* DLT15+ cells in the thymus, head-kidney, spleen and gut. And they observed lower density of tcrβ+ than DLT15+ cells in the gut and the head-kidney. They, therefore, hypothesis that the majority of DLT15+*tcrβ-* cells corresponds to γδ T cells. Based on that hypothesis, in the oldest juvenile investigated (180 days post fertilization) γδ T cells represent respectively 92%, 54%, 45% of total T cells in the head-kidney, spleen and gut. Because in teleost and mammals γδ and αβ T cells express CD8α, both cell populations are likely to correspond to the numerous *cd8α+* cells observed in the gut of sea bass by Picchietti et al. (2011). Those results indicate that γδ T cells are likely to play a key role in mucosal immunity of sea bass. As a confirmation, Nuñez Ortiz et al. (2014), observed that phytohemagglutinin stimulation of leucocytes isolated from the gills strongly increased TCRβ and much more TCRγ gene expression-level.

Very recently, and for the first time in teleost, γδ T cells have been isolated from zebrafish (Wan et al., 2017). As in mammals, they have the typical lymphoid cell phenotype with a TCRγδ+ CD4- CD8α+ surface that is also found in a minor population of mammalian γδ T cells (Hayday, 2000; Wan et al., 2017). Their relative proportion amongst lymphoid cells of spleen, head-kidney, skin, intestine and peripheral blood lymphocyte (PBL) of 7.7%, 9.3%, 16.9%, 18.3% and 20.5%, respectively, confirm their importance in mucosal immunity of zebrafish. As described for mammals, zebrafish γδ T cells appear to have phagocytic and APC capacity (Chien et al., 2014; Wan et al., 2017). By immunisation against the T cell

dependent antigen keyhole limpet hemocyanin (KLH), *in vivo* depletion and transfer, the authors have shown that teleost γδ T cells are implicated in the CD4+ αβ T cell activation, followed by the production of systemic anti-KLH IgM and mucosal IgZ. Consequently, in all jawed vertebrates, γδ T cells seem to initiate the adaptive immune response (Wan et al., 2017).

iii) Immune tolerance *vs*. immune reaction

Immune tolerance has been almost exclusively investigated in mammals. It can be divided into two subtypes intimately connected: the thymic (central) and the peripheral tolerance (Audiger et al., 2017; Devi and Anandasabapathy, 2016). In mammals, APCs, and especially DCs, have a key role in central and peripheral immune tolerance (Devi and Anandasabapathy, 2016). In addition to DCs, thymic specific APCs with non-redundant function in immune tolerance have been identified: the medullary thymic epithelial cells (mTEC) and cortical TEC (cTEC) (Audiger et al., 2017; Klein et al., 2014).

Immune tolerance is essential to prevent autoimmune diseases in mammals (Audiger et al., 2017; Devi and Anandasabapathy, 2016). Tolerogenic DCs have a key role because they maintain and induce immune tolerance through different processes: (1) clonal deletion (*i.e.*, elimination of autoreactive T cells by apoptosis) through the up-regulation of death receptors, such as FasL (or CD95L), (2) the induction of anergy and (3) the induction of Treg differentiation and function by antigen-presentation, and the production of IL-10, indoleamine 2,3-dioxygenase (IDO) and NO (Audiger et al., 2017; Devi and Anandasabapathy, 2016). IDO is the rate-limiting enzyme of tryptophan catabolism, which contributes to immune regulation and tolerance by depleting tryptophan, causing effector T cell apoptosis and an increase of Treg frequency and activity (Audiger et al., 2017; Devi and Anandasabapathy, 2016).

To promote immune tolerance, APCs express a wide variety of self and non-self-peptides loaded on the MHC, which are presented to immature or mature T cells that escape the thymic clonal deletion (Audiger et al., 2017; Devi and Anandasabapathy, 2016; Klein et al., 2014). And as stated, potentially harmful autoreactive T cells are eliminated by apoptosis (clonal selection). In mammals, this apoptosis depends on the two caspase-dependent pathways, *i.e.*, the intrinsic and extrinsic pathway of apoptosis (Audiger et al., 2017; Bouillet and O'Reilly,

2009). The extrinsic pathway and the intrinsic pathway are respectively initiated by the binding of death receptor on the target cell and various external stresses (Bouillet and O'Reilly, 2009). They are notably mediated by the activation of CASPASE 8 and 9, respectively (Bouillet and O'Reilly, 2009).

In steady-state, tolerogenic DCs with an immature phenotype are present in MALT associated to the gut and skin and promote peripheral immune tolerance towards commensal organisms and food antigens (Audiger et al., 2017; Devi and Anandasabapathy, 2016). Differentially, and after activation, immunogenic DCs express high levels of co-activator membrane proteins, such as CD40, which interact with CD40-ligand on T cells. Immunogenic DC highly express also B7-1 (CD80) and B7-2 (CD86) which bind to CD28 on T cells to stimulate the production of cytokines (*e.g.*, IL-2, IL-1β) and effector CD4+ T cell differentiation (Audiger et al., 2017; Devi and Anandasabapathy, 2016).

iv) Lymphoid organs

The adaptive immune system emerged in jawed vertebrates with the lymphoid organs that can be classified primary and secondary lymphoid organs. By definition, the primary lymphoid organs will provide the microenvironment necessary for the development of the B and T cell (lymphocyte) repertoire. The secondary lymphoid organs are specialized in the initiation of the adaptive immune response. These specialized organs support the interactions between APC and lymphocytes (Drayton et al., 2006). Lymphocytes, as all hematopoietic cells, differentiate from hematopoietic stem cells (HSC). The HSCs can differentiate into the myeloid cell progenitor or lymphoid cell progenitor which respectively differentiate in various innate immune cells (granulocytes, macrophages, mast cells and dendritic cells) and lymphocytes including B and T cells (Boehm and Swann, 2014; Prykhozhij and Berman, 2014). The HSCs are present in hematopoietic tissues, *i.e.*, the bone marrow in tetrapods, or its equivalent, the kidney in teleosts (Fig. 4) (Boehm and Swann, 2014). B and T cells are produced in specialized and distinct microenvironments. Contrariwise to the development of B cells, which can occur in anatomical diverse sites across the vertebrate lineage, the development of T cells, *i.e.*, thymopoïesis, occurs exclusively in an ancient primary lymphoid organ located near the pharynx, called thymoid in lamprey larvae and thymus in all jawed vertebrates (Boehm and Swann, 2014).

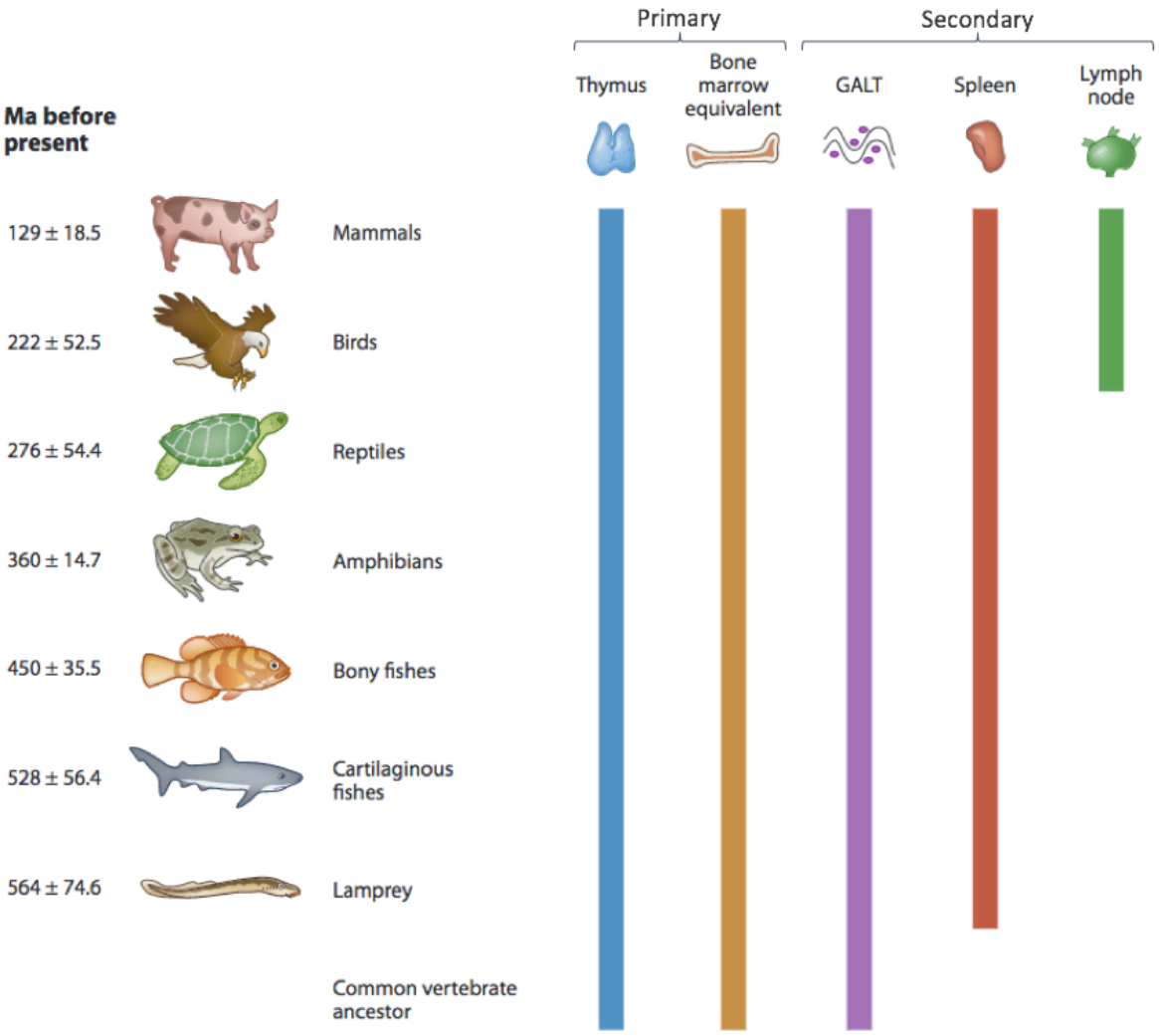


Figure 4: Evolutionary emergence of primary and secondary lymphoid organs/tissues in the different vertebrate lineages. The vertebrate immune system is characterized by the presence of several distinct primary and secondary lymphoid organs/tissues of various evolutionary origins. The time points when major groups of vertebrate emerged are indicated on the left; vertical bars indicate the time of emergence of different tissue types. GALT, gut associated lymphoid tissue; Ma, million years. Adapted from Boehm and Swann (2014).

(1) Primary lymphoid organs

(a) Bone Marrow equivalent

Hematopoiesis, such as myelopoiesis and B lymphopoiesis, occurs in the head-kidney of teleosts, the bone marrow of mammals and in the bursa of Fabricius of birds (Boehm and Bleul, 2007; Parra et al., 2013). The main developmental steps of B lymphopoiesis, which are defined by the expression of B cell specific gene and transcription factors, appears to be well conserved amongst jawed vertebrates (Zwollo, 2016). In mammals, multiple mechanisms of self-tolerance, *i.e.,* elimination of autoreactive B cells have been identified in the bone marrow and the spleen (Basten and Silveira, 2010). In the spleen, B cell clones with a high

self-affinity that (1) escaped to the first line of selection (in the bone marrow) and which were (2) formed in the germinal centers after somatic hypermutation are eliminated by apoptosis (Basten and Silveira, 2010).

(b) Thymus

(i) General presentation

The anatomic location and the number of lobes of the thymus, however, varies between species. In fish, the thymus is a bilobed organ of which either lobe is on each side of the fish near the gill cavities. In birds and in mammals, the lobes are gathered in the neck and in the thorax, respectively (Boehm et al., 2012; Rezzani et al., 2013). In almost all jawed vertebrates, the lobes are divided into lobules that present two distinct regions: the cortex and the medulla. The cortex is characterised by densely packed immature T cells. In the various teleost species so far investigated, the thymus is the first lymphoid organ in which lymphoid cells appear during ontogenesis, however the cortico-medullary regionalization was not always observed (Bowden et al., 2005).

In mammals, and most likely in teleosts, the different states of T cell maturation can be characterized by the differential expression of CD4 and/or CD8. Using antibodies against CD4-1 and CD8α, the distribution of the different T cell populations was described in the thymus and in the peripheral lymphoid organs of Crucian carp, *Carassius carassius* (Fig. 5). In the thymus, the main population that was identified were SP CD4-1 cells (37.3%), followed by the DN cells (31,3%), the SP CD8α cells (1,9%) and the DP cells (17,5%) (Nakanishi et al., 2015; Toda et al., 2011). More recently, the expression CD4-2 was investigated in rainbow trout (Takizawa et al., 2016). Based on the expression of CD8 and CD4-2 (being expressed on all rainbow trout CD4+ T cells), the main population were the DP cells with 66.3%, followed by SP CD4-2 cells with 16.1%, the DN cells with 10,7% and SP CD8α cells with 6,9%. These relative proportions are close to those found in mice (Fig. 3) where the DPs also constitute the main population (80-85%), followed by the SP CD4 (10%), SP CD8 (5%) and DNs (5%) (Nakanishi et al., 2015). As in mammals, the DP population was observed mainly in the thymus, which corresponds to an immature state of maturation (Nakanishi et al., 2015). These results (1) may confirm that CD4-2 is the teleost functional equivalent to the mammalian CD4 as observed by Wen et al. (2011) in pufferfish and (2) suggest evolutionary conserved feature in T cell maturation from teleosts to mammals.

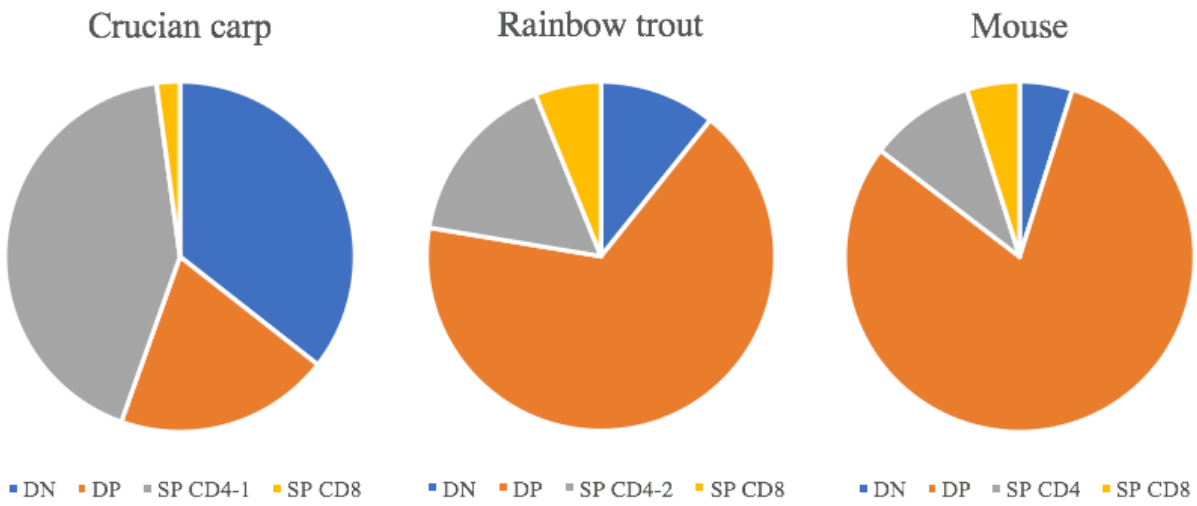


Figure 5: Thymic distribution of the different T cell populations based on the expression of CD4 and CD8 in teleosts and rodents.

(ii) Thymopoiesis and morpho-functional specialization of the thymus

Thymopoïesis is believed to share the same basic fundamental mechanisms across the vertebrate lineage (Boehm and Swann, 2014). In mammals and teleosts, early thymic progenitors (ETPs) reach the thymic lobe, enter the thymus, migrate in the subcapsular zone of the cortex and later relocate to the medulla (Fig. 6) (Bajoghli et al., 2015; Gameiro et al., 2010; Klein et al., 2014). The journey of the immature T cells passing through the different zones of the thymus relates to distinct and sequential steps of maturation where maturing T cells will interact with different stromal cells (Gameiro et al., 2010; Klein et al., 2014). This morpho-functional specialisation has been well described for mammals. And, in fish, a number of studies strongly suggest that teleost thymopoïesis and thymus organisation are similar to that of mammals. Notably, immature T cell migration through the different subregions of the thymic microenvironment as well as interaction with other thymic cells has been recently confirmed with non-invasive *in toto* imaging on a medaka transgenic reporter line (Bajoghli et al., 2015).

A major component of the thymic stroma is composed of the thymic epithelial cells (TEC) (Gameiro et al., 2010). Across all vertebrate classes, at least three sub-types are commonly distinguished according to their morphology and thymic localisation: limiting TEC (lTEC) adjacent to the connective tissue, medullary (mTEC) and cortical TEC (cTEC) (Fig. 6, Klein et al., 2014; Romano et al., 1999a). The TECs provide growth factors for the immature T cell

proliferation as well as extracellular matrix components and chemokines for ETP homing and T cell migration through the different thymic areas (Abramson and Anderson, 2017; Gameiro et al., 2010; Halkias et al., 2014).

Chemokines are a major family of secreted proteins that induce chemotaxis. Usually, chemokines are divided into four subfamilies (CC, CXC, C, or CX3C), identified according to the pattern of their N-terminal cysteine residues (Gameiro et al., 2010; Halkias et al., 2014). In mammals, several chemokines and associated receptors are implicated in T cell maturation, such as the chemokine CCL25 associated to CCR9, CXCL12 (also known as Stromal cell-derived factor 1α, SDF-1α) which binds to CXCR4, and CCL19/21 promoting chemotaxis of cells expressing CCR7 (Gameiro et al., 2010; Halkias et al., 2014). Chemokines do not only provide directional cues, but also modulate the activity of integrins for the modulation of cell motility that are involved in the adhesion of immature T cells to the extracellular matrix, such as fibronectin, laminin and collagen (Gameiro et al., 2010; Halkias et al., 2014). TCR-signalisation and numerous other molecules are believed to direct the distinct migration pattern of immature T cells, such as cytokines, galectins, neurophiline-1/semaphoring-3A, for instance (Gameiro et al., 2010; Halkias et al., 2014).

1. Cortical and subcapsular zone

a. Homing and T cell lineage commitment

In jawed vertebrates during the thymus ontogenesis, thymic lobules are shaped by connective tissue and the vascular system, which participates to the colonisation of the thymic cortex with ETPs, coming from the bone marrow equivalent, *i.e.*, the head-kidney in teleosts (Fig. 6 A; Gameiro et al., 2010; Langenau and Zon, 2005). Across all vertebrate classes, the homing of ETPs is mediated by CXCL12 and CCL25 (*e.g.*, *ccl25a and cxcl12a* in medaka and zebrafish; Bajoghli, 2013; Halkias et al., 2014). In mammals, CCR7/CCL21 are also involved in this process (Bajoghli, 2013; Halkias et al., 2014). Following ETP homing, the progenitors are committed to the T cell lineage (Fig. 6 B), a process which is mediated by the Notch ligation to Delta-like 2 (Dll2a in medaka) express on TEC (Bajoghli et al., 2009). In all vertebrates, ETP homing and differentiation into T cells are regulated by the transcription factor FOXN1 expressed in TECs, which induces *ccl25* and *Dll2a* expression (Bajoghli,

2013; Halkias et al., 2014). The committed immature T cells represent the common T cell progenitors which can differentiate in either γδ and αβ T cell lineages.

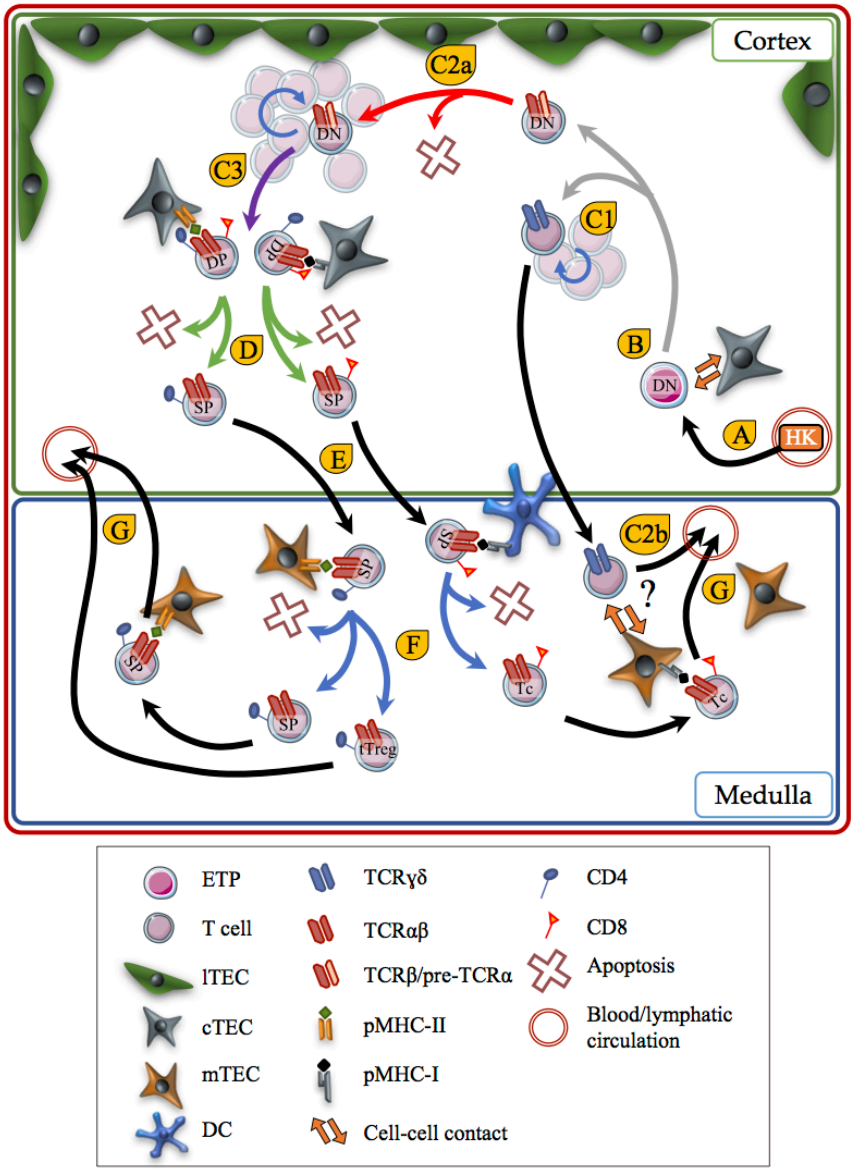


Figure 6: Model for teleost T cell development in the thymic microenvironment, *i.e.*, morpho-functional specialization as adapted from mammals. A, early thymic progenitor (ETP) homing in the thymus, ETP migrate from the head-kidney (HK) in the cortex likely *via* the vascular system in non-larval fish as in mammals. B, T cell lineage commitment after Notch and Delta-like 2a interaction. C1, γδ selection, *i.e.*, γδ T cell commitment; C2a, β selection, *i.e.,* αβ T cell commitment and C2b, maturation signalling for γδ T cells; C3, DP differentiation. D, positive selection or death by neglect; E, migration to the medulla; F, central tolerance which either eliminate by apoptosis autoreactive αβ T cells (negative selection or clonal deletion) and promote tTreg differentiation (thymus derived Treg); G, migration toward peripheral lymphoid organs of mature and naive γδ and αβ T cells. The question mark indicates that the γδ T cell development as well as thymic localisation has not been investigated yet in teleost. lTEC, limiting epithelial cells; cTEC, cortical epithelial cells; mTEC, medullary epithelial cells; DC, dendritic epithelial cells; pMHC, complex self-peptide major histocompatibility complex.

In mice, the early immature DN T cells are usually subdivided into four subsets on the basis of the expression CD117 (c-kit), CD44 and CD25 (Halkias et al., 2014). In humans, analogous stages have been characterized by the expression of CD34, CD38 and CD1a (Halkias et al., 2014). To date, no similar subset has been described in teleost fish. The early thymic progenitors of teleost fish express the transcription factor Ikaros, the expression of which is down-regulated when ETP begin to express *rag1* (Hess and Boehm, 2012; Lam et al., 2004).

b. γδ T cell lineage commitment

This process as well as the development of γδ T cells have been exclusively investigated in mammals. In mammals, and possibly in teleost as well, DN RAG1/2+ T cells rearrange the TCRβ, δ and γ gene segments differentiate in the two T cell lineages (Muñoz-Ruiz et al., 2017; Turchinovich and Pennington, 2011). In mammals, the common T cell progenitors first experience γδ selection which trigger the γδ T cell lineage commitment (Fig. 6 C1) or further maturation toward the αβ T cell lineages. The γδ T cell commitment is a complex process that, to date, is not understood in all details. Indeed, different mechanisms have been described that determinate the γδ T cell lineage choice: (1) on the one hand it is proposed that the T cell destiny would be independent from the TCRγδ-ligand signalling, and that it depends on specific transcription factors or efficiently paired TCRγ/TCRδ chains, for instance. On the other hand, (2) the DN T cell differentiation has also been described to be determined by the strength of TCR-signalling, *i.e.,* T cells with a strong TCRγδ-signalling will differentiate into the γδ T cell lineage, whereas the others will differentiate into αβ T cells (Muñoz-Ruiz et al., 2017; Turchinovich and Pennington, 2011). Consequently, the development of γδ T cell can be initiated without TCR-ligand mediated signals. However such stimulation is mandatory to mature and leave the thymus (Chien et al., 2014; Turchinovich and Pennington, 2011). In jawed vertebrates, γδ T cells are the first cells to appear during the ontogenesis of the thymus (Chien et al., 2014; Hayday, 2000). In contrast to the majority of αβ T cells, the γδ T cell effector function is acquired in the thymus. The γδ T cells are "developmentally programmed". The different effector γδ T cell subsets, *e.g.*, IFNγ or IL-17 producing cells, are defined by (1) the Vγ (which also determines the anatomical localisation), (2) γδ T cell commitment initiation (TCR-ligand dependent or independent) and

the (3) TCR signal strength (Chien et al., 2014; Muñoz-Ruiz et al., 2017; Turchinovich and Pennington, 2011).

c. αβ T cell lineage commitment

Immature DN T cells which did not differentiate into the γδ T cell lineage undergo β selection (Fig. 6 C2a; Trampont et al., 2010; Turchinovich and Pennington, 2011). At this step, immature DN T cells express on the membrane recombined TCRβ chain coupled to an invariant TCRα chain (TCRβ/pre-TCRα complex), consequently clones with a functional TCRβ receive pro-survival signal which triggers the TCRα gene-rearrangement as well as the differentiation into DP CD4 and CD8 (Fig. 6 C3; Gameiro et al., 2010; Klein et al., 2014; Turchinovich and Pennington, 2011). In the following, DP T cells undergo positive selection step in order to eliminate non-MHC restricted clone and to determine the αβ T cell differentiation into SP CD4 or CD8. DP CD4 and CD8 expressing a TCRαβ with low affinity for the complex self-peptide MHC-I or II differentiate into SP CD8 or CD4 (Klein et al., 2014). The αβ T cell and γδ T cell lineage commitment are respectively highlighted by the expression of both CD4 and CD8 (DP cells) and TCRγδ (Fig. 6 C1, C2a and C3; Turchinovich and Pennington, 2011). However, if the different steps of αβ T cell maturation have been well described in mammals, the selection step during γδ T cell maturation remained poorly known (Abramson and Anderson, 2017; Cowan et al., 2015). Notwithstanding, The β-selection and apparently γδ selection at a lesser extent are highlighted by intensive steps of proliferation (Fig. 6; Taghon et al., 2006).

In medaka, the investigation of RAG2 gene expression revealed a morpho-functional specialisation of the cortex for somatic gene recombination (Bajoghli et al., 2015). In mammals as well, RAG2 gene is expressed in the cortex by early immature T cell until positive selection (Ruscher et al., 2017). In mammals, subsequent to homing and T cell lineage commitment, DN3 T cells migrate to the subcapsular zone for β selection allowing for DN3-DN4 transition and the commencement of a high proliferation step, which is highlighted by the predominance of proliferating cell nuclear antigen (PCNA) positive lymphocytes in this area (Fig. 6; Forsberg, 1996; Klein et al., 2014; Trampont et al., 2010; Zoller and Kersh, 2006). Similar processes probably occur in sea bass, where DN *cd4-/cd8α-* and PCNA+ lymphocytes are localised in the subcapsular zone (Picchietti et al., 2015, 2009).

In the cortex of mice, 90% of DP T cells are not positively selected and therefore die by apoptosis (Klein et al., 2014). Accordingly, in the cortex of carp and sea bass, apoptotic cells are mainly found in the cortex and the CMJ (Abelli et al., 1998; Romano et al., 1999b). Moreover, in sea bass, DP *cd4-1*+ *cd8α*+ *tcrβ*+ are abundantly localized in the cortex whereas *mhc2β*+ were detected in subcapsular zone (Picchietti et al., 2009).

In mammals, positive selection is also highlighted by the high level expression of TCRαβ (Halkias et al., 2014; Ruscher et al., 2017), similar process is likely to occur in medaka because *tcrβ* appeared to be mainly expressed in the medulla in the larvae (Bajoghli et al., 2015). However, the results of Bajoghli et al. (2015) contrast with the results obtained in post-larvae and one-year-old sea bass, where *tcrβ*+ lymphocytes were mainly detected in the medulla (Picchietti et al., 2008). Nevertheless, altogether those results indicate that similar steps of T cell maturation occur in the cortex of teleosts and mammals which would confirm the functional homology.

In mammals, after β and positive selection, the transition from DN4 to DP and then to SP is associated with the migration from the cortex to the medulla (Fig. 6E) directed by the downregulation of CXCR4 and the upregulation of CCR7 in mammals (Halkias et al., 2014; Klein et al., 2014; Trampont et al., 2010). In medaka, because T cells express *ccr9b* in the medulla only, *ccr9b* expression is, therefore, activated in late stage of somatic recombination (*rag2* being expressed only in the cortex) when the cell move to the medulla (Bajoghli et al., 2015). As CCR7 in mammals, this chemokine receptor may be involved in the migration from the cortex to the medulla during T cell development in the medaka.

2. Medulla

In the CMJ and medulla, SP αβ T cells are negatively selected by professional APCs, such as mTEC and DC, in order to eliminate clone expressing a TCRαβ with a too high affinity for self-pMHC, *i.e.*, autoreactive T cells (Fig. 6 F; Halkias et al., 2014; Klein et al., 2014). Moreover, in the medulla, CD4+ αβ T cells with an intermediate or a high affinity for p-MHC-II differentiate in Treg (Fig 6 F; Klein et al., 2014; Li and Rudensky, 2016). In agreement, Picchietti et al. (2009, 2015) observed in the medulla of sea bass, SP *cd4*+ and *cd8α*+lymphocytes as well as *mhc-2β*+ cells corresponding to TEC and DC-like cells which were also observed in the CMJ (Picchietti et al., 2015). In medaka, Bajoghli et al. (2015)

observed that immature T cells interact with phagocytes at the CMJ during their migration or *via* extending long protrusions with phagocytic cups at their tips (Macrophage/DC). Moreover, as a confirmation that in teleost as well medullary T cells undergo intensive selection, *nur77b* which is upregulated after TCR-signalling in mammals, is only expressed in the inner zone of the thymus of medaka (Bajoghli et al., 2015).

Anomalies in the selection may induce the release of self-reactive T cells into the periphery, which could cause deleterious autoimmune diseases (Klein et al., 2014). For central tolerance, *i.e.*, negative selection and tTreg differentiation, mammalian mTECs express a wide variety of tissue specific antigen (TSA) also called tissue-restricted autoantigens (Anderson and Su, 2016; Romano et al., 2013). The transcriptional factor AIRE regulates a large panel of TSAs and AIRE-deficiency results in an altered AIRE-dependent TSA expression leading to autoimmune diseases in mouse and human (Anderson and Su, 2016). In teleost, *aire* is mainly expressed in the thymus as in mammals (Saltis et al., 2008) and, more specifically, could be detected in the medulla of medaka (Bajoghli et al., 2015). The comparative analysis of *aire* sequences in jawed vertebrates revealed a potential conservation of its function in central tolerance (Saltis et al., 2008). Surprisingly, a high correlation between the mutation in human *aire* entailing autoimmunity and the evolutionary conserved residues was found.

These data indicate that in the medulla of teleost also take place important steps of central tolerance including negative selection and likely tTreg differentiation because *foxp3* is well expressed in the thymus of teleost (Wang et al., 2010; Yang et al., 2012). The teleost medulla is, therefore, apparently the functional homologue of the mammalian counterpart.

Afterwards, the selected self-tolerant and mature SP αβ T cells that constitute the repertoire of naïve and mature T cells, reach the secondary lymphoid organs (Halkias et al., 2014; Turchinovich and Pennington, 2011). T cell egress occurs *via* the blood vessels of the CMJ (Fig. 6 G), which has been clearly demonstrated in mice, but is probably also the case in humans (Halkias et al., 2014). In teleost fish, αβ T cell egress has been poorly investigated.

Considering the γδ T cells, the γδ-selected T cells apparently migrate to the medulla to complete their maturation before they leave the thymus (Fig. 6 C2b) (Abramson and Anderson, 2017; Cowan et al., 2015).

(2) Secondary lymphoid organs

(a) Head-kidney

The head-kidney can also be considered as a secondary lymphoid organ, as it is actively involved in mounting the immune response. Furthermore, numerous T cells, which differentiate in the thymus, have been reported in the head-kidney of various teleost species (Table 1). In the head-kidney, like in the spleen, T cells have been described to be randomly distributed (Nakanishi et al., 2015; Romano et al., 2007) and grouped close to melanomacrophages (Romano et al., 2007). In response to microbial stimulation, IgM+ and IgT+ proliferate and secrete the respective Igs in the head-kidney (Parra et al., 2013). The IgM are secreted by plasma-like cells, which are mainly localized in the head-kidney where they will bear memory function (Parra et al., 2013).

Table 1: Distribution of the different lymphocyte subtypes (%) isolated from the head-kidney and spleen in various teleost species. By flow cytometry the population distributions in the lymphoid gate were determined using specific antibodies (see references). *Indicates population distribution calculated on the total leukocytes.

Head-kidney

	B cells		αβ T cells			γδ Tcell	trombocyte	references
species \ antibodies	IgM	IgT	CD4.1	CD4.2	CD8a	Tcrγ		
rainbow trout	37.94				2,7		0.56	Takizawa 2011
			22.4	24.9	3.4			Takizawa 2016
	14.7*	5.1*						Zhang et al. 2010
ginbuna crucian carp	18.0		10.3		23.6			Toda et al. 2011
zebrafish						9,3		Wan 2017
			DLT15					
sea bass	33*		7*					Romano et al. 1997
		22*						Picchietti et al. 2017

Spleen

	B cells		αβ T cells			γδ Tcell	trombocyte	references
species \ antibodies	IgM	IgT	CD4.1	CD4.2	CD8a	Tcrγ		
rainbow trout	57.71				2,15		15,85	Takizawa 2011
			8.3	9.4	2.0			Takizawa 2016
	42.9*	11.9*						Zhang et al. 2010
ginbuna crucian carp			5.2		11.5			Toda et al. 2011
zebrafish						7,7		Wan 2017
			DLT15					
sea bass	30*		6*					Romano et al. 1997
		16*						Picchietti et al. 2017

(b) Spleen

The spleen is present in all jawed vertebrates and is considered as the most ancient secondary lymphoid organ (Fig. 4) (Boehm et al., 2012). As in mammals, the teleost spleen is believed to act primarily as a secondary lymphoid organ, where mature B cells are activated by antigens (Zwollo, 2016). The spleen has also been reported to be an important organ for T cell activation, because CD4 T cells from the spleen, but not from the head-kidney, have been observed to respond to T cell-dependent-antigen (Takizawa et al., 2016). The spleen has also a key role in the filtration and clearance of blood, *i.e.*, antigen trapping and removal of damaged blood cells, and in hematopoiesis (Boehm et al., 2012; Rauta et al., 2012). The spleen is subdivided in different areas and this subdivision is largely conserved across vertebrates. The white pulp, for instance, which is essentially formed by leucocytes and involved in the coordination of the immune response between the different leucocyte populations can be found in teleosts as well as in mammals (Boehm et al., 2012). The teleost spleen, however, lacks germinal centres. It was, therefore, hypothesised that the melanomacrophages may present the antigen to B cells like follicular dendritic cell in mammals (Zwollo, 2016). Teleost leucocytes from the spleen have been shown to be abundantly composed of mature B cells, but also T cells (Table 1). Using a sea bass pan-T cell antibody named DLT15, isolated immunopositive cells or gathered in small groups have been described in the lymphoid area close to the melanomacrophages in sea bass (Romano et al., 2007).

(c) Mucosa-associated lymphoid tissues

The MALTs comprise the main surfaces in contact with the environment and therefore represent the main routes of entry for pathogens (Parra et al., 2016). Considering their localization, four subtypes of MALTs can be distinguished in teleosts: SALT (skin-associated lymphoid tissue), GIALT (gill-associated lymphoid tissue), NALT (nasopharynx-associated lymphoid tissues) and GALT (gut-associated lymphoid tissue; Parra et al., 2016; Salinas, 2015). MALTs and specially GIALT and GALT combine two different fundamental functions: (1) absorption of nutrients and regulation of the gas/ionic exchanges (respiration and osmoregulation), which require a high surface and thin epithelia, (2) immune defence to avoid the invasion of potential pathogen across the epithelia and (3) immune tolerance (Parra et al., 2016; Salinas, 2015). Indeed, MALTs present symbiotic bacterial microbiota and are

constantly exposed to a microbial-rich environment. Therefore to avoid constant immune response against non-harmful antigens comprising food-related and other environmental antigens, the balance between immune tolerance and immune response must be tightly regulated to maintain mucosal homeostasis (Salinas, 2015). In teleost, it is well known that mucosal present an innate and adaptive immune cells and potentially an adaptive immune response (Fig. 7 and Table 2; Salinas, 2015). However, in contrast to mammals, fish do not have an organised lymphoid structure that resembles the classical lymph nodes, such as Peyer's patches, mesenteric lymph nodes, isolated lymphoid follicles and the mucosa-draining lymph nodes (Parra et al., 2016; Tafalla et al., 2016). In teleosts, diffused B and T lymphocytes seem to be associated with the mucosa (Fig. 6 and Table 2). As mammals, the teleost leucocytes of the intestine are present in two distinct immunological compartments: the *lamina propria* formed by macrophages, granulocytes, plasma cells as well as T and B lymphocytes; and in the intraepithelial compartment comprising mainly T cells and very few B cells (Fig. 7; Parra et al., 2016; Tafalla et al., 2016). The activation of B cells and production of pathogen-specific Igs in teleosts rather occur at the mucosal surface (Fig. 7; Parra et al., 2016). However, it remained to be determined if teleost MALTs represent true secondary lymphoid organs because the area where APC, T and B cells interact to mount mucosal immune response or immune tolerance have not been clearly established even for the most investigated GALT (Fig. 7; Parra et al., 2016; Salinas, 2015; Tafalla et al., 2016).

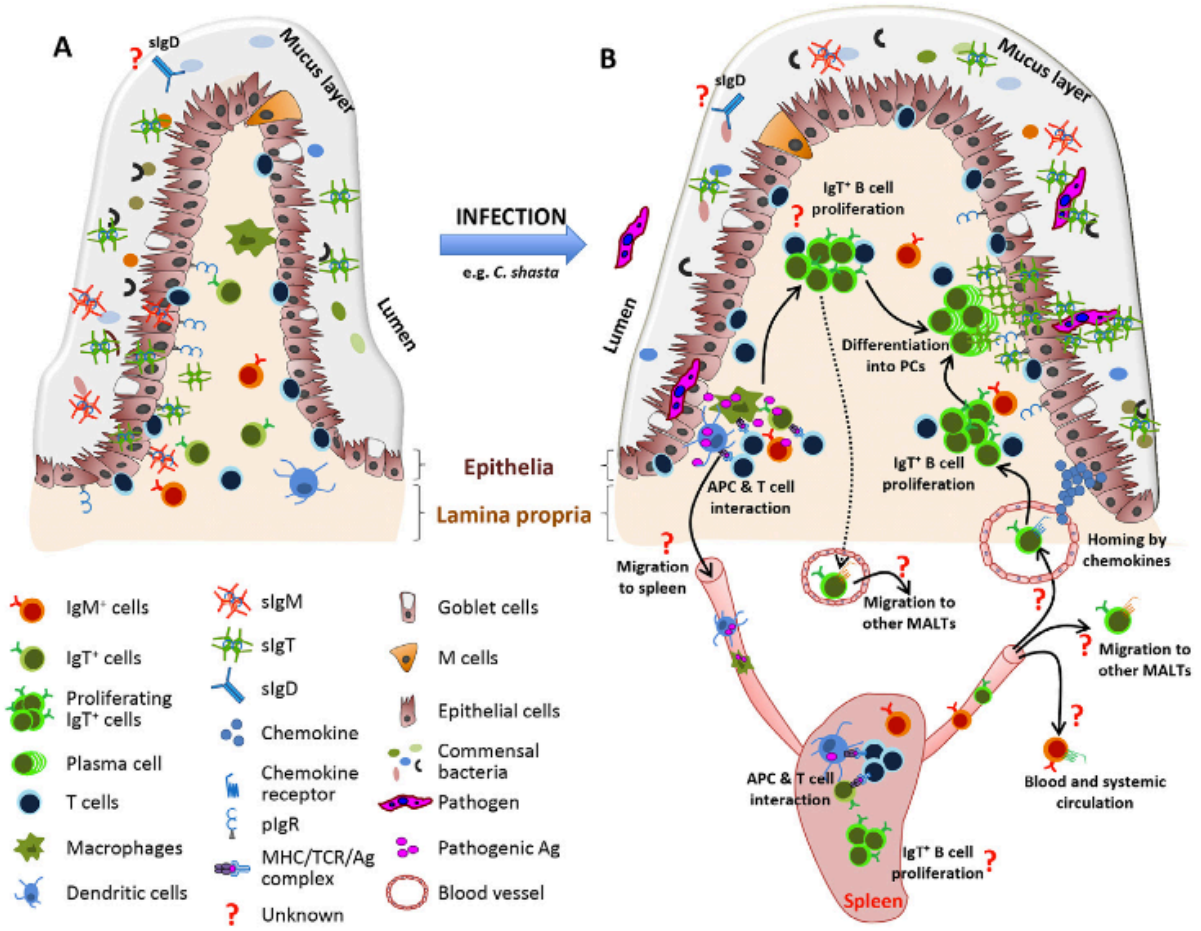


Figure 7: Induction of mucosa IgT immune response in the teleost GALT. Scheme of typical intestinal villus structure in healthy teleost (A) and suggested model for the immune response upon pathogen infection in rainbow trout (B). In healthy fish (A) the number of IgT+ and IgM+ B cells is low and they are generally located in the *lamina propria.* Natural IgM and IgT are respectively produced by IgM and IgT secreting B cells and transported from the epithelium into the mucus layer *via* polymeric Ig receptor (pIgR). IgM and specially IgT coat the microbiota, however, whether IgD contributes to the microbiota recognition remained to be evaluated. In infected rainbow trout (B), in the *lamina propria*, antigen from the pathogen is apparently taken up by antigen-presenting cells which trigger CD4+ αβ T cell activation. In response, CD4+ αβ T cells proliferate (not represented) and activate IgT+ B cells which also proliferate locally and start to differentiate into plasmablast or plasma cell-like cells. The possibility exists that upon antigen uptake, loaded gut APCs, *i.e.*, B cells, dendritic-like cells, macrophages and γδ T cells may migrate into the spleen or head-kidney where they will present antigen to naïve and memory CD4+ αβ T cell. Consequently, activated IgT+ cells may home into the gut. Alternatively, antigens from the parasite may travel from the gut into the lymphoid tissues through the vascular system to initiate the immune response. In the case that immune response develops in the spleen or the head-kidney, Parra et al. (2016) hypothesises that IgT+ B cell may proliferation in those organs. Alternatively, activated IgT+ B cell may migrate into the gut where they may proliferate. From Parra et al. (2016).

Table 2: Summary table of the main characteristics of teleost MALT. Question points highlight unknown aspects, not studied. Total % of B cells represent the B cell proportion in total leucocytes. ILT, interbranchial lymphoid tissue. T cells appear to be very abundant in MALTS, representing, for instance, 50%-70% of lymphoid cells in the GALT, SALT and GIALT of common carp. From Salinas (2015).

Characteristic	GALT	SALT	GIALT	NALT
Anatomical localization	Intestine	Skin	Gills	Olfactory organ
Organization	Diffuse only	Diffuse	Diffuse with one organized tissue in salmon (ILT)	Diffuse
Presence of goblet cells	Yes	Yes	Yes	Yes
Total % of B cells	4-5%	4-5%	?	35%–40%
Approximate IgT/IgM B cell ratio	1:1	1:1	?	1:1
Expression of pIgR (protein level)	Yes	Yes	?	Yes
Compartmentalized specific IgT responses against pathogens (protein level)	Yes	Yes	Not demonstrated	Not demonstrated
Abundant T cells	Yes	Yes	Yes	?
Presence of bacterial microbiota	Yes	Yes	Yes	Yes
Microbiota coated by secretory immunoglobulins	Yes	Yes	?	Yes

In mammals and likely in teleost, in the *lamina propria* compartment, T cells are mainly represented by conventional SP CD4 monomer and CD8αβ heterodimer (Picchietti et al., 2011; Salinas, 2015; Tafalla et al., 2016). Differentially in mammals, intraepithelial lymphocyte (IEL) of the intestine are represented by γδ and αβ T cells which express CD8αα homodimer or do not express CD4 and CD8β (Cheroutre et al., 2011; Tafalla et al., 2016). IELs are extremely heterogeneous populations commonly referred as unconventional T cell populations. They are believed to play an important role for mucosal immunity including defence and tolerance (Cheroutre et al., 2011; Tafalla et al., 2016). In teleost fish, CD8α, CD8β, TCRβ and TCRα transcripts have been detected in MALT and in purified CD8α+ leucocytes isolated from the GALT and GIALT (Tafalla et al., 2016; Takizawa et al., 2011). Furthermore, for instance, numerous *cd8α*+ and *tcrβ*+ cells have been observed in the epithelia of the sea bass GALT (Picchietti et al., 2011), as well as numerous CD8α+ cells were observed in the epithelial of GIALT and GALT in rainbow trout (Takizawa et al., 2011). Altogether these observations suggest the existence of IELs similar to the mammalian counterpart. However, differentially from mammals and birds, an investigation on adult rainbow trout has shown that the TCRβ repertoire of gut IEL seems to be diverse and polyclonal as in the spleen and head-kidney (Salinas, 2015; Tafalla et al., 2016).

B) Endocrine System: oestrogenic regulation, evolution and disruption

Three major endogenous oestrogens are implied in the hormonal regulation: oestrone (E1), 17β-oestradiol (E2) and oestriol (E3). E2 is the most potent and abundant endogen oestrogen. It represents the major female hormone, and increases notably during reproductive period (Bondesson et al., 2015; Samavat and Kurzer, 2015). At lower serum-level, oestrogens are also present in males. Functional oestrogen-signalling and synthesis are well conserved in the vertebrate lineage and can be detected across all vertebrate classes as well in cephalochordates (Baker et al., 2015; Bondesson et al., 2015; Eick and Thornton, 2011; Tokarz et al., 2015). Endogenous oestrogens are generally associated with the hypothalamus-pituitary-gonad axis and regulate sexual development and differentiation as well as reproduction (Segner et al., 2017). However, in mammals, oestrogens are also well known to modulate numerous other physiological functions, which may be, indirectly, related to reproduction. Notably the immune system as well as the cardiovascular, the central nervous, metabolism, behaviour and musculoskeletal system are subject to oestrogenic regulation and, therefore, display sexual dimorphisms (Cooke et al., 2017; Jochmanova et al., 2015; Prossnitz and Barton, 2014). In view of the evolution of oestrogen metabolism and oestrogenic signalling in cephalochordates/vertebrates, it is not surprising that in teleost fish oestrogens have been described to also interact with numerous physiological processes, such as the sensory system, the behaviour, the osmoregulation and mineral homeostasis as well as, importantly, the immune system (P. Pinto et al., 2014; Segner et al., 2013). Of course, this regulation is not unidirectional and, in turn, the immune and central nervous system modulate the reproductive system (Segner et al., 2017). Such tight crosstalk is notably necessary to allocate limited energetic resources to the function where they are most needed and to preserve internal homeostasis between energetically costly physiological systems, such as reproduction and the immunity.

i) Oestrogen metabolism and function

Oestrogens are small steroid hormones, the synthesis of which, as for all steroid hormones, begin with cholesterol (Fig 8). Oestrogen synthesis is a complex biochemical pathway involving several enzymes, such as the aromatase cytochrome P450 (CYP19) and

hydroxysteroid dehydrogenases (HSD, Fig. 8). CYP19 converts testosterone into E2 and androstenedione in E1. E2 is also produced by 17-β-hydroxysteroid dehydrogenase 1 conversion of E1 (Baker, 2011; Tokarz et al., 2015). Aromatase (CYP19) is the rate-limiting enzyme in the conversion of androgens to oestrogens. It is, therefore, an indicator of oestrogen production (Baker et al., 2015; Bondesson et al., 2015).

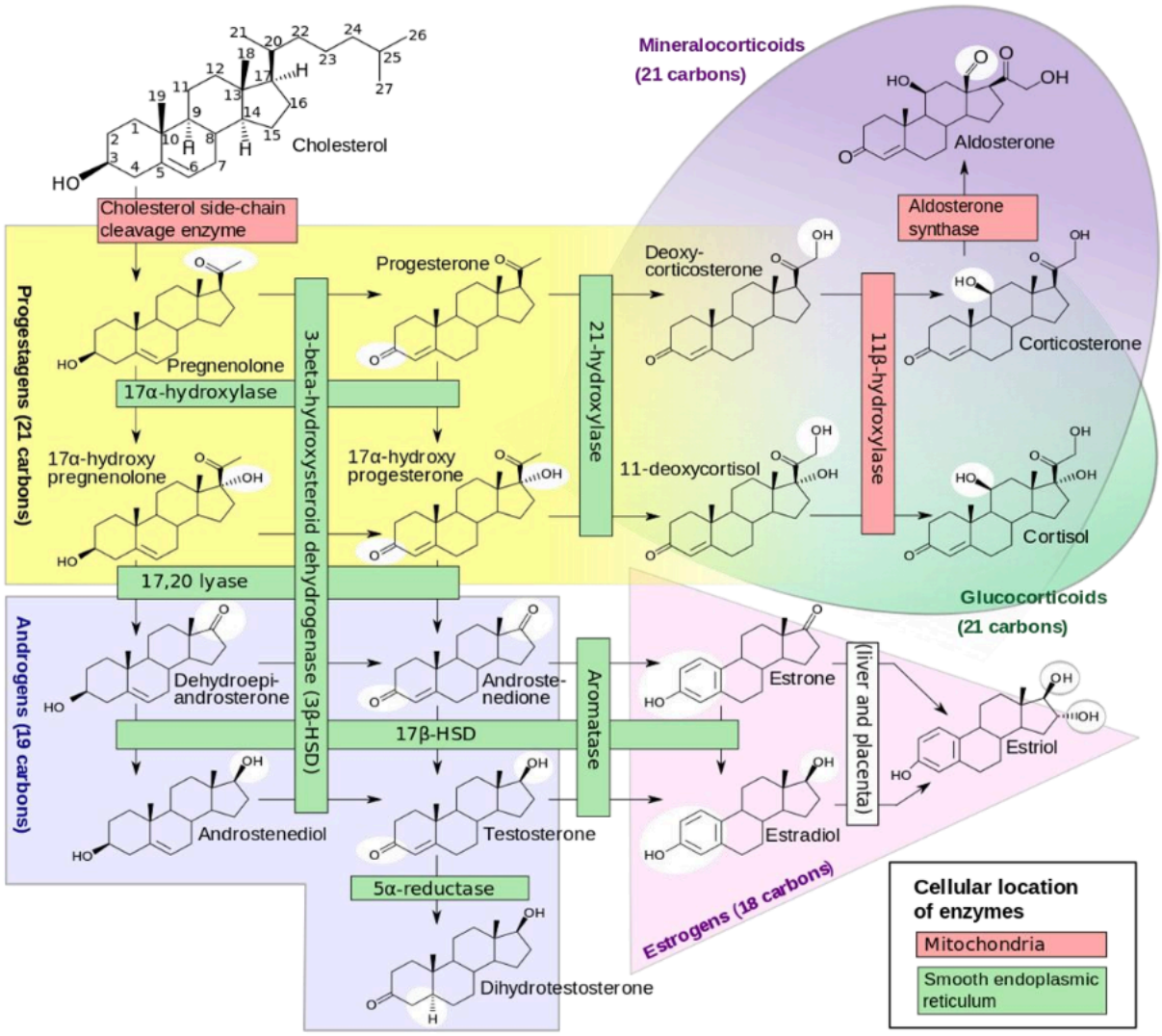


Figure 8: Human steroidogenesis with the enzymes involved as well as the major classes of steroid hormones and molecular structure changes. Steroid hormones are formed from the common precursor the cholesterol. The synthesis pathway begins with the cleavage of the cholesterol side chain to form the pregnenolone (Cyp11a, not shown). Downstream, to form androgens and then oestrogens, several enzymes add and modify functional groups by hydroxylation, reduction and aromatization. Aromatase: CYP19, cytochrome P450 and HSD: hydroxysteroid dehydrogenase. From Häggström and Richfield (2014).

Oestrogen-biosynthesis and -signalling appear to be well conserved throughout evolution. The evolution of steroidogenic enzymes involved in the vertebrate type biosynthesis of oestrogen is not known but already evolved in jawless vertebrates (Baker et al., 2015; Bondesson et al., 2015; Eick and Thornton, 2011; Tokarz et al., 2015). In mammals, E2 is abundantly synthesised by the ovaries (granulosa cells) during the reproductive cycle and the placenta during pregnancy (Barakat et al., 2016; Nelson and Bulun, 2001). Although in most vertebrates, aromatase(s) are mainly expressed in gonads and brain (Lange et al., 2002; Piferrer and Blázquez, 2005), it is also expressed in numerous other tissues, such as testis, adipose tissue, liver, blood tissue, intestine and lymphoid tissue which are referred as extra-gonadal or peripheral sites of oestrogen synthesis (Barakat et al., 2016; Bondesson et al., 2015; Piferrer and Blázquez, 2005; Szwejser et al., 2017). In fish, two aromatase genes have been well described: *cyp19a1a* and *cyp19a1b* also named respectively *cyp19a* and *cyp19b* (Guiguen et al. 2010). They encode two structurally different proteins with similar catalytic activity that are predominantly expressed in the ovaries and in brain, respectively (Guigen et al. 2010; Piferrer and Blázquez, 2005; Tokarz et al., 2015). In all vertebrates, oestrogens are essential for sexual differentiation, the regulation of reproduction of both gender as well as control of metabolism investment (Bondesson et al., 2015; Cooke et al., 2017; Lange et al., 2002; Piferrer and Blázquez, 2005). This notably implies the production of oestrogens by the gonads and the regulation of gonad development *via* signals from the brain involving a feedback-loop along the hypothalamus-pituitary-gonad axis (HPG; Fig. 9). In oviparous vertebrates, gonadal secretion of oestrogen controls the metabolic investment for oogenesis (egg yolk production with induction of vitellogenin synthesis; Lange et al., 2002; Piferrer and Blázquez, 2005). In viviparous eutherians, oestrogens control endometrium proliferation and cervical mucus, in addition oestrogen is involved in pregnancy maintenance and preparation of lactation period (Lange et al., 2002).

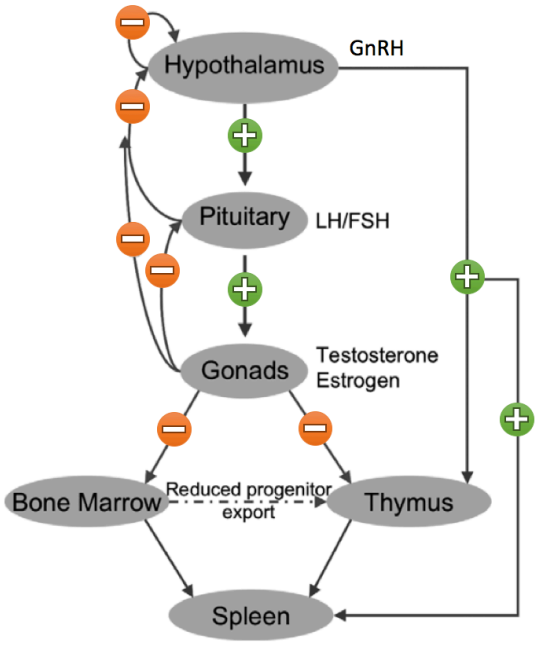


Figure 9: Simplified diagram of the mammalian hypothalamus-pituitary-gonad axis and the immunomodulatory effect on major lymphoid organs. The hypothalamus produces gonadotropin releasing hormone (GnRH) which is transported to the anterior pituitary where it controls the reproduction and stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). FSH and LH stimulate the production of gonadal steroids (testosterone and oestrogen) which negatively affect the thymus (atrophy) and bone marrow (suppression of B lineage precursor). Differentially, GnRH has a positive immunomodulatory effect. From Hince et al. (2008).

In mammals, two distinct pathways of oestrogen action have been distinguished: the gonadal steroidogenesis (ovaries) that releases sex steroid hormones into the bloodstream so as to deliver them to all tissues; this is the classical endocrine action. The endocrine action of oestrogen is evidenced by high aromatase activity and triggers notably the brain and the liver. On the contrary, peripheral steroidogenesis exert their action more locally with paracrine and autocrine action (Barakat et al., 2016; Nelson and Bulun, 2001).

Extra-gonadal oestrogen synthesis has been also highlighted by their incapacity to synthetize the testosterone precursor, *i.e.*, androstenedione (commonly abbreviated A4 or Δ4-dione) and dehydroepiandrosterone (commonly abbreviated DHEA; Barakat et al., 2016; Nelson and Bulun, 2001). In teleost fish as well numerous extra-gonadal sites of oestrogen synthesis have been identified (Piferrer and Blázquez, 2005). Considering the immune system, leucocytes have been described to express CYP19(s), both in mammals and teleosts. Common carp monocytes/macrophages and human macrophages express CYP19(s), have aromatase activity and likely 17β-HSD and 3β-HSD activity (Schmidt et al., 2000; Szwejser et al., 2017). Similarly, murine immune cells and specially splenic T cell express at transcript-level CYP19, 17β-HSD, 3β-HSD, α-reductase and bears the corresponding enzymatic activities (Samy et al., 2001). Interestingly in all of those studies, the activity of testosterone and oestrogen synthesis is apparently modulated by the immune cells activity, as hypothesised by Szwejser and co-worker (2016). Those data suggest that hormone synthesis by immune cells is actively regulated for a fine tuning of their function or neighbouring cells (*e.g.*, endothelial cells).

Oestrogen-level is determined by the activity of steroidogenic enzymes but also by the enzyme involved in biotransformation. In mammals and likely in teleost, E2 and E1 are transformed by three competitive pathways involving irreversible hydroxylations catalysed by the NADPH-dependent cytochrome P450 enzymes comprising CYP1A, CYP1B1, and CYPA2 (Fig. 10). Depending on the carbon position which is hydroxylated, oestrone and oestradiol are converted by CYPs in different catechol oestrogens: 4 or 2-hydroxyoestrone, 2 or 4-hydroxyoestradiol, and 16a-hydroxyoestrone (Phase I of the biotransformation). The hydroxylation of oestradiol or 16-hydroxyoestrone produce oestriol. Afterwards, catechol oestrogens can be methylated to methoxyoestrogens by the cathechol-O-methyltransferase. Then, methoxyoestrogens, catechol oestrogens and oestrogens are conjugated with glucuronic acid or sulfate by hepatic phase II enzymes including UDP-glucuronosyltransferases and sulfotransferases, respectively (Lange et al., 2002; Liu et al., 2009; Samavat and Kurzer, 2015). The step of conjugation increases the polarity, *i.e.*, water solubility of the compounds which is considered as detoxification reaction important for the excretion. Natural oestrogens, are excreted *via* the urine and at a lesser extent in the faeces as free hormones but mainly as sulfate or glucoronide conjugated oestrogens in human but not in all species of mammals (Lange et al., 2002; Liu et al., 2009; Samavat and Kurzer, 2015).

Interestingly, from mammals to teleost, lymphocytes express at the transcript-level various CYPs such as CYP1A which is upregulated by an exposure to various xenobiotic (Bellamri et al., 2016; Phalen et al., 2017). Human T cells have also been shown to have CYP1A1 and CYP1B activity which is stimulated upon xenobiotic-exposure but also more intriguingly upon T cell activation (Bellamri et al., 2016). Therefore, because catechol oestrogens have low or no oestrogen activity (Liu et al., 2009), we can hypothesis that, in addition to locally produce oestrogen, lymphocyte may also metabolize oestrogens and locally deplete the availability of biologically active oestrogen.

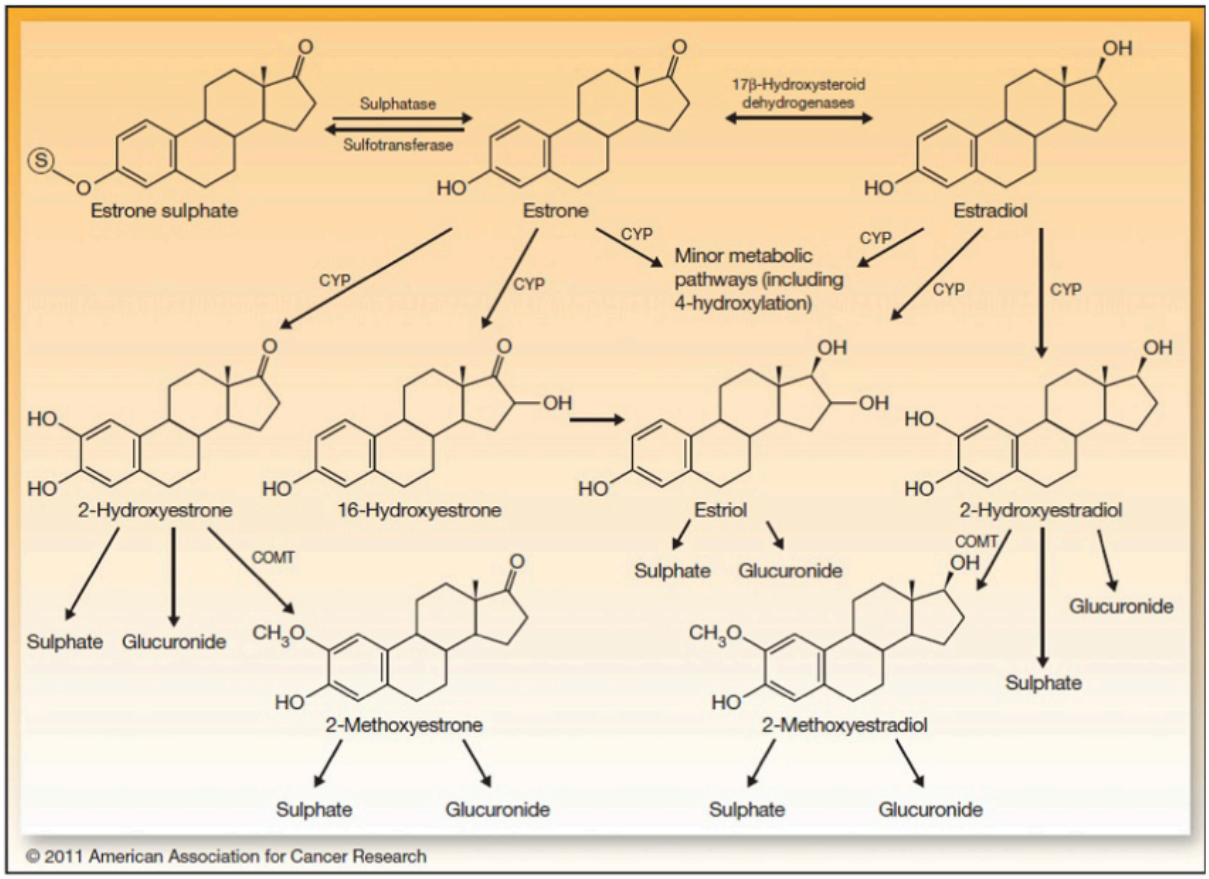


Figure 10: Endogen oestrogen metabolism in human. Several cytochrome P450 enzymes such as CYP1A, CYP1B1, and CYPA2 add hydroxyl group (-OH) to form different catechol oestrogens. Afterwards before excretion, catechol oestrogens are conjugated with sulfate or glucuronide acid or methylated to methoxyoestrogens by the catechol-O-methyltransferase (COMT). From Samavat and Kurzer (2015).

ii) Oestrogen-signalling pathways

Signal transduction of oestrogen can be realised *via* two fundamentally different, but connected pathways: the nuclear-initiated steroid signalisation (NISS) and the membrane-initiated steroid signalisation (MISS) also characterized as genomic and non-genomic signalling (Fig. 11), which will be described in more details in the following:

(1) Nuclear-initiated steroid signalisation

NISS is mediated in mammals *via* two receptors ESR1 (ERα) and ESR2 (ERβ), encoded by duplicate genes belonging to the family of ligand-dependent transcription factors. In numerous teleost species two receptors related to ESR2 have been identified: Esr2a (Erβ1 or Erβ) and Esr2b (Erβ2 or Erγ) (Nelson and Habibi, 2013; P. Pinto et al., 2014; Segner et al.,

2013). These nuclear ESRs have been detected in the cytoplasm and the nucleus across all vertebrates from teleosts to mammals (P. Pinto et al., 2014). In absence of ligand, the cytoplasmatic ESRs are associated to inhibitory heat shock proteins (Nelson and Habibi, 2013). The lipophilic property of oestrogen is assumed to facilitate the diffusion across the cell membrane, followed by binding to ESRs in the cytoplasm and subsequent nuclear translocation to act as a transcription factor (Fig. 11; Bondesson et al., 2015). Three types of ESR-mediated transcription regulation have been described: (1) the classical pathway involving ESRs dimerization, which activates gene transcription by binding to oestrogen-response elements (EREs) in the promotor region of oestrogen-responsive genes (Fig. 11 A); (2) the alternative pathway, involving the dimerization of ESRs with other transcription factors, such as activated protein 1 (AP-1), specific protein 1 (SP-1) or NFκB. The resulting transcription factor complexes bind to non-ERE elements to activate gene transcription (Fig. 11 B; (Klein and Flanagan, 2016; Nelson and Habibi, 2013; P. Pinto et al., 2014; Straub, 2007). (3) In mammals, it was also shown that ESRs can also activate gene transcription independently from ligand fixation (Fig. 11 E; Khan et al., 2012; Nelson and Habibi, 2013).

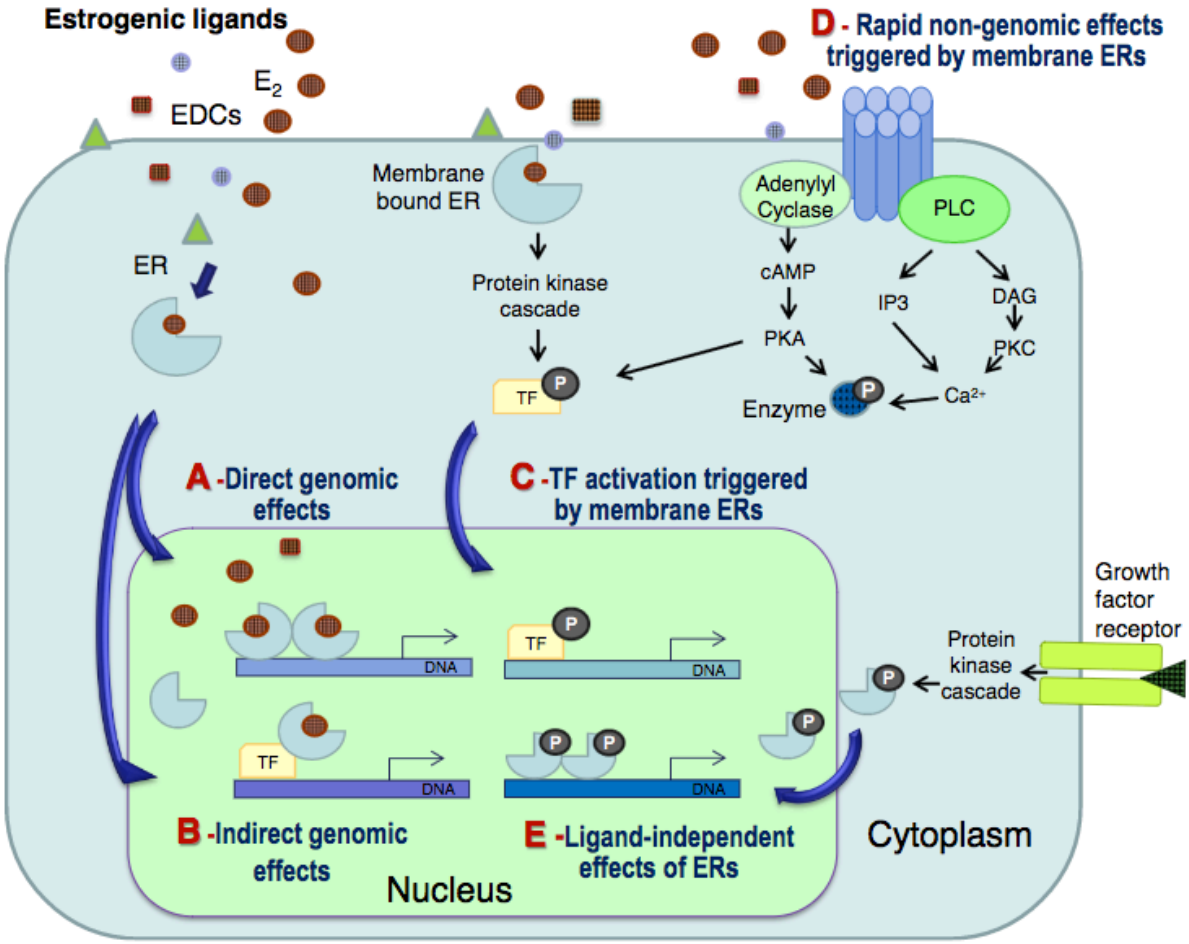


Figure 11: Scheme of the different signalling pathways of endogen oestrogen and EEDC (with an E2-mimicking action). From P. Pinto *et al.,* (2014).

(2) Membrane-initiated steroid signalisation

Recent studies in fish provide evidence for membrane-associated oestrogen receptors (*e.g.,* the G protein-coupled oestrogen receptor, Gper, previously named Gpr30) mediating a rapid non-genomic pathway as in mammals (Fig. 11 D; Cabas et al., 2013; Rodenas et al., 2017b; Seto et al., 2016; Szwejser et al., 2017). This signalling pathway was notably characterized in the fish gonad and in granulocytes (Cabas et al., 2013; P. Pinto et al., 2014). However, differentially from mammals, several teleost species, such as European sea bass or European eel, appear to have different genes corresponding to two GPER-isoforms, *i.e.*, *gpera* and *gperb*, or, alternatively, named *gper* and *gper-like* (Lafont et al., 2016; P. Pinto et al., 2014). Mammalian MISS is also initiated by membrane bound ESR1 (Fig. 11 D). The cellular localisation of ESR1 is influenced by post-transcriptional modification and alternative splicing. Full-length ESR1 can act a ligand-dependent transcription factor or activate a cytoplasmic signalling cascade after post-translation modification, *i.e.*, palmitoylation that

allows the anchoring to plasma membrane (Romano and Gorelick, 2017). In human, the alternative spliced proteins that lack one or both transactivation domains were also associated to the membrane after post-translation modifications. Because truncated human ESR1s have been shown to be less efficient transcription factors, they are, therefore, believed to be predominantly involved in MISS (Romano and Gorelick, 2017). In teleosts numerous alternative splice variants are expressed but their functions have not been identified yet. In contrast, no splice variant of post-translation modification has been described for GPER which, however, can be localized on the plasma membrane or endoplasmic reticulum (Romano and Gorelick, 2017).

MISS activates cytosolic signalling cascades involve calcium/cyclic adenosine monophosphate/protein kinase A (Ca^{2+}/cAMP/PKA), Diacylglycerol/phospholipase C/protein kinase C (DAG/PLC/PKC) and kinase signalling cascades (Fig. 11 D). Consequently, MISS modulates specific enzyme activity and also gene expression by activating transcription factor (Fig. 11 C; P. Pinto et al., 2014; Romano and Gorelick, 2017).

(3) Integration of oestrogen-signalling pathways – Biological significance

Both, in teleost fish and in mammals the different Esrs and Gper(-s) isoforms are expressed in a large variety of tissues and cells with its specific subtype-ratio (Iwanowicz et al., 2014; Kuiper et al., 1997; Lafont et al., 2016; Massart et al., 2014; Nagler et al., 2007; Olde and Leeb-Lundberg, 2009; Segner et al., 2013). This wide oestrogen receptor distribution illustrates the pleiotropic and complex activities of oestrogens in the modulation of numerous physiological functions (Iwanowicz and Blazer, 2011; P. Pinto et al., 2014; Segner et al., 2013). However, even in mammals, the biological significance of the most investigated ESR1 and ESR2 is not fully understood due to a complex interplay (Leitman et al., 2010; Matthews and Gustafsson, 2003; Tetel and Pfaff, 2010). In teleosts, knowledge on the importance of ESR1 and ESR2, is scarce. Much work has been conducted on the liver, the primary source of vitellogenin (vtg). *Via* both ESR1 and ESR2 activation, E2 is well known to induce Vtg expression in the liver of both teleost male and female (Nelson and Habibi, 2013). Therefore, Vtg-level is commonly used as a biomarker and, the liver as a reference organ to validate and evaluate oestrogen-exposure as well as oestrogen receptor probes (Nelson and Habibi, 2013; Segner et al., 2013; Szwejser et al., 2017).

In mammals, the contribution of MISS is far less understood, but has been given attention more recently in order to elucidate if it provides possibilities for therapeutic interventions in cancer, cardiovascular and autoimmune diseases. In fact, MISS is involved in the modulation of immune and vascular system but requires further validation (Barton and Prossnitz, 2015; Prossnitz and Barton, 2014; Seto et al., 2016). In teleost, GPER has been involved in the modulation of the reproduction and, more recently, the immune system (Cabas et al., 2013; Rodenas et al., 2016; Szwejser et al., 2017; Thomas, 2012).

As in mammals, MISS and NISS have non-redundant and complementary functions also for fish (Menuet et al., 2004; Nelson and Habibi, 2013; Rodenas et al., 2016, 2016; Szwejser et al., 2017; Thomas, 2012). In mammals and likely in teleost, their interplay depends on the cell type, differentiation and pathological status (Maggioli et al., 2016; Prossnitz and Barton, 2014; Romano and Gorelick, 2017; Seto et al., 2016). Notwithstanding, in teleost fish, the presence of two Esr2-subtypes and two Gper-isoforms in addition to Esr1 suggests amplified complexity of oestrogenic regulation, although its physiological and evolutionary significance remains to be elucidated.

In summary, this diversity of signalling pathways involved in the cellular transduction of E2 as well as the wide oestrogen receptor expression in various organs and cell types investigated are likely to explain the complexity of oestrogen-action, which is specific for each tissue, cell type and physiological state (Khan et al., 2012; Segner et al., 2013).

iii) Oestrogenic Endocrine Disrupting Chemicals

The endocrine system which represents the body's communication system mediating with hormones the coordination of major physiological functions such as immune system, reproduction, metabolism, development or behaviour. Chemicals notably released by anthropogenic activity that interfere with this system are named endocrine disrupting chemicals (EDC). Following the WHO's definition, "*an endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations"* (Bergman et al., 2012). Recently, the WHO evaluated that EDCs are represented by 800 chemicals including the suspected and well known ones (Bergman et al., 2012). EDCs have been widely retrieved in the environment. They, therefore represent a special concern for human and wild

life because of their wide environmental distribution, which, eventually, ends up in aquatic environment (Bergman et al., 2012; Ting and Praveena, 2017; Wee and Aris, 2017). Consequently, international governments and organisations (European Union, the USA, Canada and Japan; OECD) are establishing testing approaches and regulatory frameworks to assess the risk for human and aquatic organisms (Ting and Praveena, 2017). As an example, in Europe, the water framework directive (WFD, 2000/60/EC) establishes standards for a good chemical and ecological status for surface and ground waters and intends to prevent their degradation (Ting and Praveena, 2017).

EDC-risk assessment is complex, because EDCs are plentiful and they are likely (1) to act at very low doses with a non-monotonic dose-response, (2) to have long term effects and (3) an additive effect in mixture. This is complicated by different actions depending on the developmental stage and on the species (MacLatchy et al., 2009; Segner et al., 2013; Wee and Aris, 2017; Xu et al., 2017). Moreover, EDCs interfere with hormonal signalisation by multiple mechanisms of action by acting as (1) an agonist to the hormone receptor and thus mimicking endogenous hormone, as (2) an antagonist, which blocks the transduction of the endogenous hormone, or by modulating (3) the availability of the natural hormone or hormone receptor (metabolism and synthesis). In addition, EDCs can act in a complex manner on various physiological functions *via* multiple receptors, (Fig. 12; Jochmanova et al., 2015; P. Pinto et al., 2014; Wee and Aris, 2017). For example, bisphenol A, generally attributed oestrogenic activity, has also the capacity to act as an androgen receptor antagonist (Jochmanova et al., 2015).

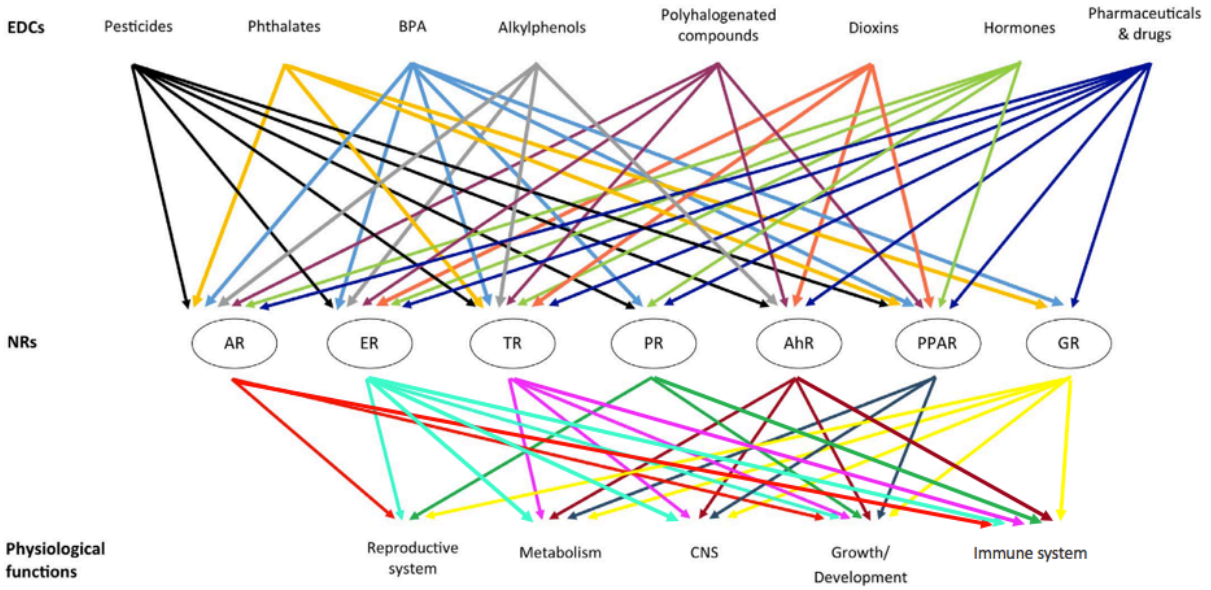


Figure 12: Interaction of endocrine disrupting chemicals (EDCs) with multiple pathways of the endocrine system through several nuclear receptors (NRs) affecting different physiological functions. NR, Nuclear receptor; AR, androgen receptor; ER, oestrogen receptor; TR, thyroid hormone receptor; PR, progesterone receptor; AhR, aryl hydrocarbon receptor; PPAR, peroxisome proliferator-activated receptor; GR, glucocorticoid receptor; CNS, central nervous system. Adapted from Wee and Aris (2017).

(1) Xenoestrogens

Xenoestrogens designate any compound with possible action on the oestrogen-signalisation that is liberated into the environment by organisms (animals and plants) and anthropogenic activities. Oestrogenic EDCs (EEDC) represent one of the best investigated group of EDCs (Segner et al., 2013). They modulate cellular transduction and signalisation pathways of endogenous oestrogens (Oestrone, E1; 17β-oestradiol, E2; Oestriol, E3). EEDCs comprise compounds of various nature and structure (Fig. 13), such as:

- Naturally produced compounds:
  - Endogenous oestrogens
  - Phytoestrogens (isoflavones)
  - Mycoestrogens (zearalenone)
- Synthetic therapeutic drugs:
  - Selective oestrogen receptor modulators (SERM) (*e.g.,* raloxifen and tamoxifen)
  - Synthetic oestrogens (*e.g.,* 17α-Ethinylestradiol, EE2 and Diethylstilbestrol, DES)
- Synthetic compounds found in numerous products:

- Industrial chemicals (*e.g.,* polychlorinated biphenyls, PCBs; dioxins such as 2,3,7,8- Tetrachlorodibenzo-p-dioxin, TCDD; polycyclic aromatic hydrocarbons, PAHs)
- Surfactant and detergent (*e.g.,* alkylphenols; nonylphenol, NP; octylphenol)
- Pesticides (methoxychlor, dichlorodiphenl-trichloroethane, hexachlorobenzene, dieldrin)
- Plastic compounds (phthalates; bisphenol A, BPA; bisphenol F; Aris et al., 2014; Jochmanova et al., 2015; P. Pinto et al., 2014; Wee and Aris, 2017).

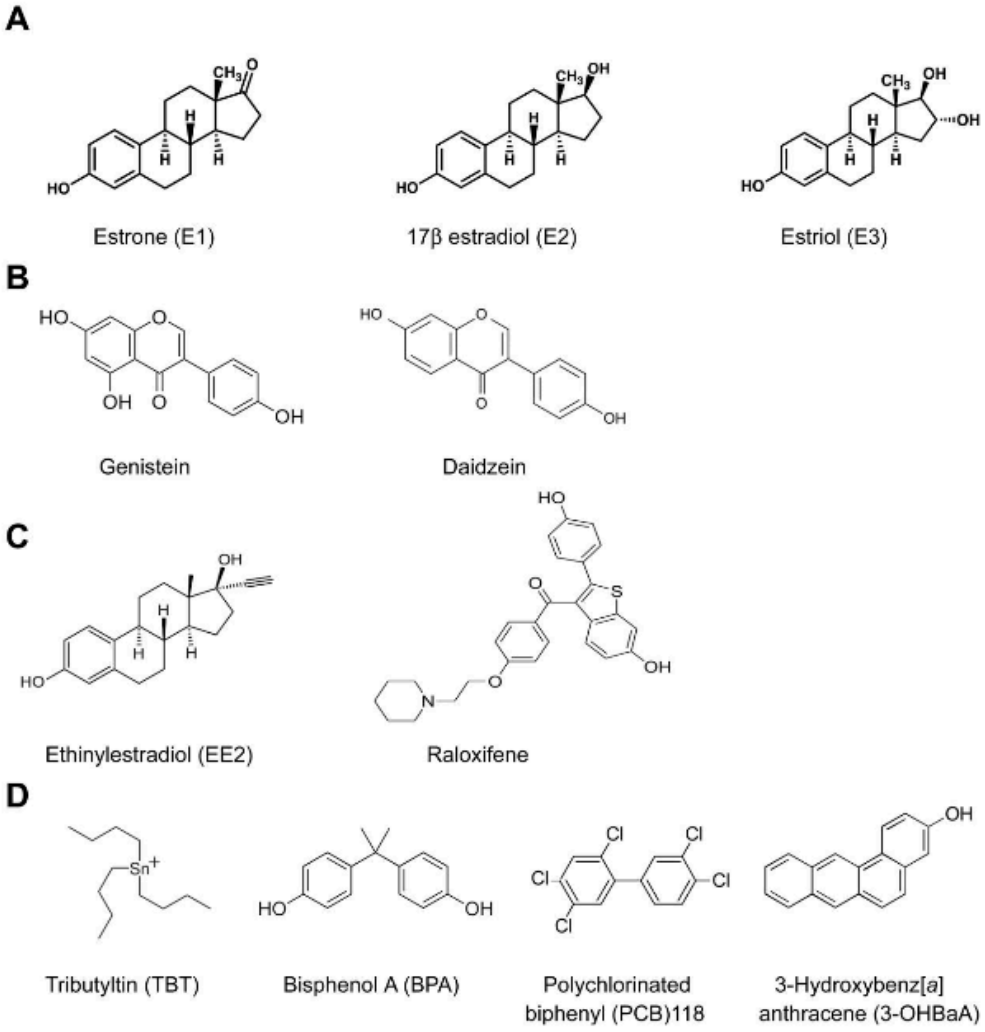


Figure 13: Chemical structure of common oestrogenic disrupting chemicals: endogenous oestrogens (A) and, example of phytoestrogen (B), synthetic EEDC used in endocrine therapy (C) or in industry (D). From P. Pinto *et al.* (2014).

EEDC are detected in the environment across the world, but E2 and EE2 are of particular concern, because they are among the most powerful oestrogenic compounds representing the main environmental oestrogenic activity (Adeel et al., 2017; Aris et al., 2014; Wee and Aris, 2017). Consequently, E2 and EE2 belong to the European WFD watch list of substances for Union-wide monitoring (2015/495/EC). The WFD (2011/0429/EC) proposed thresholds of 0.4 ng/L for E2 and 0.035 ng/L for EE2 as annual average value for environmental quality standards in the inland surface water (rivers and lakes). As the natural ligand for ERs, E2 is the most powerful endogenous oestrogen. However, EE2 has the highest oestrogenic activity in teleosts and mammals with respect to (1) their higher ER binding affinity (Aris et al., 2014; Segner et al., 2013) and (2) *in vitro* and *in vivo* oestrogenic response (Aris et al., 2014; C. Pinto et al., 2014). EE2 has received special interest because of its (1) high capacity to resist the degradation (persistent nature), its (2) tendency to absorb to organic matter and to accumulate in the sediment, (3) its bioaccumulation capacity in the biota and (4) its high potent oestrogenic activity eliciting oestrogenic responses at extremely low concentrations (ng/L) (Aris et al., 2014; P. Pinto et al., 2014).

E1, E2 and E3 are naturally excreted by animal faeces and urine, especially during pregnancy (Adeel et al., 2017; Liu et al., 2009). E2 and EE2 are used in endocrine therapy (*e.g.,* contraceptive pill), in cattle breeding and aquaculture for growth promotion (banned in European Union in 1988 and 1996; Ting and Praveena, 2017). Both, natural and synthetic oestrogens are not completely removed by wastewater treatment plants. Consequently, natural and synthetic oestrogens are mainly released by wastewater effluents of municipal treatment plants into the environment, as well as from livestock activities (Fig. 14, Adeel et al., 2017; Aris et al., 2014; Elnwishy and Sedky, 2016). Aquaculture is another likely source of natural oestrogens' discharge into the environment (Ting and Praveena, 2017). Part of the oestrogens is also present in the sewage sludge and animal manure, which, in turn, are used as fertilizer. With the runoff, these oestrogens may likewise end up in surface water, groundwater and drinking water (Fig. 14, Adeel et al., 2017; Aris et al., 2014; Ting and Praveena, 2017; Wee and Aris, 2017). Indeed, animal manure represents a major environmental source of natural oestrogens (Adeel et al., 2017; Ting and Praveena, 2017).

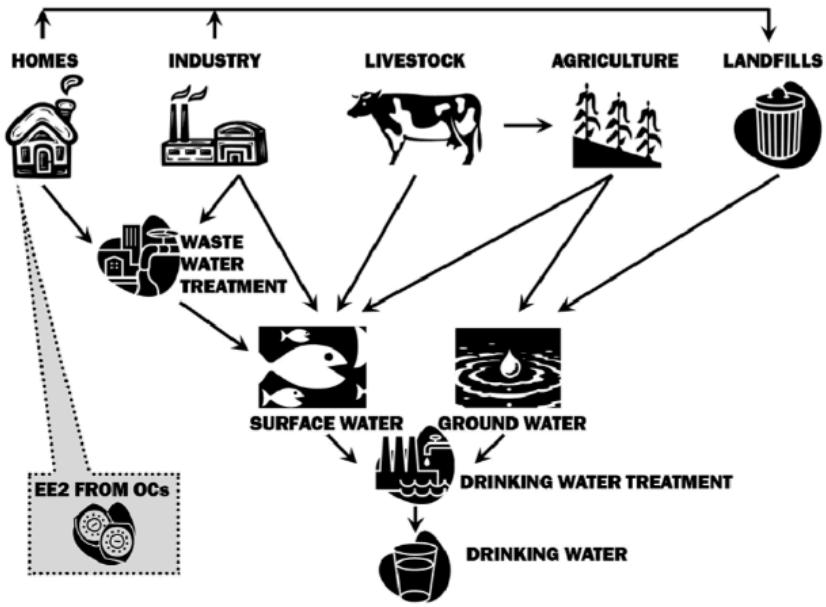


Figure 14: Diagram of the main oestrogenic endocrine disrupting chemicals sources and the link with the aquatic organisms and human health. EE2, ethinylestradiol; OCs, oral contraceptives. From Wise *et al.* (2011).

Natural oestrogens can be easily degraded by several microorganisms such as bacteria. Synthetic EE2, on the contrary, is not readily biodegradable and is not removed from the environment (Adeel et al., 2017; Ting and Praveena, 2017). In the aquatic environment, E2 and to a far lesser extent EE2 are rapidly eliminated by photolysis and photocatalysis (Adeel et al., 2017).

Steroid oestrogens including EE2 and E2 are excreted as sulfate or glucuronide conjugated oestrogens in addition to the free form (Aris et al., 2014; Liu et al., 2009; Ting and Praveena, 2017). These conjugated oestrogens are more polar than free oestrogen and have a far lower affinity for oestrogen receptors. Subsequently, excreted conjugated oestrogens are metabolised by microorganisms to free oestrogens before their final degradation (Aris et al., 2014; Ben et al., 2017; Ting and Praveena, 2017). However, if glucuronide form are readily deconjugated into free form in sewage transport, the sulfate conjugates seem to represent a large part of the oestrogens retrieved in the effluent of wastewater treatment plant (Ben et al., 2017). Consequently, the slow deconjugation of oestrogens is likely to participate in the uncomplete removal of natural oestrogens in the wastewater effluents (Ben et al., 2017; Liu et al., 2015).

Moreover, a recent work has shown that E1 is likely to be underestimated as environmental oestrogen. As a matter of fact, Ankley et al. (2017) demonstrated that E1-exposure of adult male fathead minnow (*Pimephales promelas*) at environmentally relevant concentrations induces high-plasmatic level of E2 of 5 ng/mL, which is similar to the female-level during reproduction. This increase probably results from 17β-HSD conversion (Ankley et al., 2017). In the environment, E1 makes up from a large proportion of oestrogens. It is commonly found in wastewater effluents because it appears to be the main product of E2 and EE2 of microorganismal degradation under aerobic conditions (Adeel et al., 2017). Due to its low oestrogenic activity, however, E1 was rarely considered as an important environmental oestrogen (Adeel et al., 2017; Ankley et al., 2017).

(2) EEDC-effects

The effects of EEDCs on various organisms, in particular vertebrates, are complex. Firstly, their effects differ depending on the nature of a specific EEDC. Secondly, receptor distribution which determinate EEDC-effect is cell and tissue-specific notably dependent on the physiological state and the species (P. Pinto et al., 2014; Segner et al., 2013). Eventually, these compounds, cell and tissue specificities are multiplied by the combined action of EEDCs in mixtures, defining the overall EEDC-concentration assuming additivity. Compound specificity is determined by molecule structure, which confers differential affinity for the ESR-isoforms. This results in a different capacity to modulate oestrogen receptor conformation, which, in turn, initiates the cellular signalling transduction (P. Pinto et al., 2014; Segner et al., 2013). Such characteristics, associated with a pleiotropic expression of the oestrogen receptor and highly complex oestrogen-signalling pathways, increase the challenge for EEDC-risk assessment.

Numerous reports confirm adverse effects on human and teleost fish reproduction (Giusti et al., 1995; Szwejser et al., 2016). However, EEDCs can also be carcinogenic and immunotoxic, both for humans and wild-life (Adeel et al., 2017; Aris et al., 2014; Giusti et al., 1995). An exceptionally well-documented example in humans is diethylstilbestrol (DES), which was prescribed to women with risk pregnancies between 1940 and 1972 in USA and until 1978 in Europe. In USA, an estimated 5 to 10 millions of people were exposed including the mother and the foetus (Giusti et al., 1995). This prenatal exposure produced some of the worst deleterious effects reported for any EEDC, *i.e.*, long-term reprotoxic effects, but also

immune-deficiencies entailing increases in autoimmune diseases or cancer in the offspring as well as into the second generation (Giusti et al., 1995). In teleost fish, both laboratory and field studies have shown that EEDCs increase the plasmatic level of vitellogenin in males and females, increase intersex, reduce testes size, decreased egg and sperm production, which consequently reduces the fertility (Adeel et al., 2017; Aris et al., 2014; Segner et al., 2013). Alike endogenous oestrogens, EEDCs have been described to modulate numerous physiological functions, such as the nervous, the cardiovascular and the immunological system (Jochmanova et al., 2015; Segner et al., 2013; Wee and Aris, 2017). It was notably suggested that the oestrogen-containing oral contraceptives or hormone replacement therapeutics are responsible for disease exacerbation (Jochmanova et al., 2015).

C) Interaction immune system – endocrine system: evolution and oestrogenic regulation

In mammals, the immune system and endocrine system crosstalk is notably characterized by a sexual dimorphism in immune system performance: adult females generally mount a stronger adaptive and innate immune response, but, at the same time, are more susceptible to inflammatory and autoimmune diseases (Klein and Flanagan, 2016). Indeed, women comprise 80% of autoimmune disease cases in the USA and respond stronger to vaccines. Men, on the contrary, are more susceptible to infectious disease and malignant cancers (Klein and Flanagan, 2016; Straub, 2007). Although some of this disease prevalence between men and women derives from multiple factors, such as behavioural and genetic differences (the X chromosome, for instance, has more genes-related to the immune system than its Y counterpart, *e.g.*, PRRs, cytokine receptors and transcription factors), differences in sex steroid-levels are well known to be responsible for a great deal of the sexual dimorphism in immune system performance (Klein and Flanagan, 2016). Sex steroids, and specially E2, profoundly modulate the immune response, which is particularly underscored by female autoimmune disease severity, changes in the immune response throughout the female menstrual cycle, and, accordingly similar effects following experimentally increased levels of E2 (Khan and Ansar Ahmed, 2016; Klein and Flanagan, 2016; Straub, 2007). In female mammals, oestrogens have been reported to have both immunosuppressive and immunoenhancing effects with a context dependent action. These fundamentally different responses depend on numerous factors, such as oestrogen concentration, organ and cellular

target, as well as reproductive and immune status (Khan et al., 2012; Khan and Ansar Ahmed, 2016; Straub, 2007). The dichotomous and specific reaction to oestrogens can be explained by the large number of signalling pathways and cell types which are modulated by E2 including immune cells and other cell types in close interaction, *e.g.*, endothelial cells. And the E2-effect is dependent on the expression oestrogen receptor subtypes which has a spatial and temporal variation (Khan and Ansar Ahmed, 2016; Segner et al., 2013; Straub, 2007). Depending on the targeted immune cell, oestrogens have been reported to modulate various functions such as cytokine secretion, migration, proliferation, differentiation and apoptosis (Straub, 2007). The oestrogen effects on the innate and adaptive immune system has been extensively reviewed mostly for mammals (Khan et al., 2012; Kovats, 2015; Laffont et al., 2017; Straub, 2007). Nevertheless, similar regulations occur in many non-mammalian species such as reptiles, birds and teleosts (Burgos-Aceves et al., 2016; Milla et al., 2011; Segner et al., 2017; Szwejser et al., 2016). Notwithstanding, sexual dimorphisms in the immune system have been also described in various non-mammalian vertebrates, such birds, reptiles and teleost fish (Dong et al., 2017; Klein and Flanagan, 2016; Segner et al., 2017; Szwejser et al., 2016).

i) Immune system and endogenous oestrogens in teleost

Female teleost fish, such as Common carp or European sea bass among other species display a pronounced seasonal reproduction. This discontinuous reproductive cycle entails a seasonal variation of plasmatic E2-levels with higher levels encountered generally at the end of winter (February) to spring (April). In sea bass as in several teleost species two patterns of E2-variation have been described: plasmatic E2-level show an increase during the months of vitellogenesis reaching maximal value during or before the spawning period (Fig. 15; Cerdá et al., 1995; Mañanós et al., 1997; Navas et al., 1998; Szwejser et al., 2016).

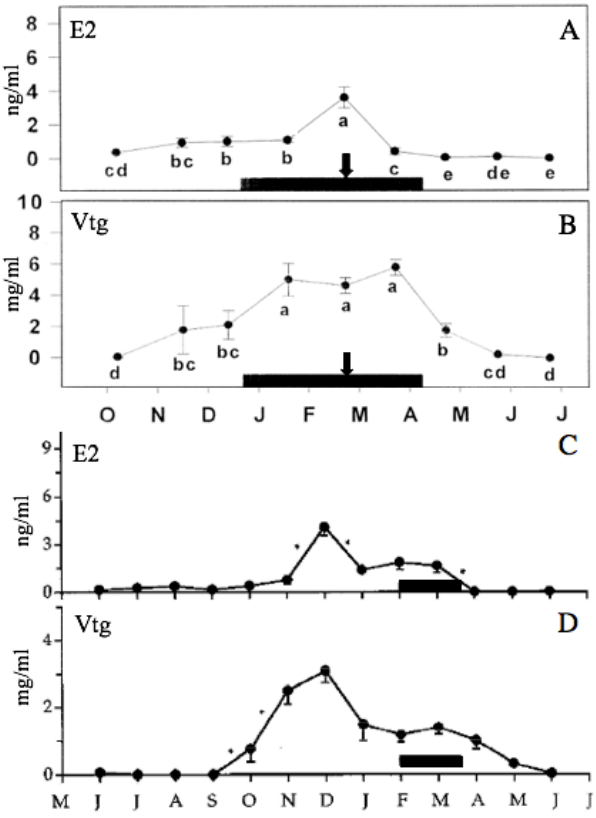


Figure 15: Two distinct patterns of plasmatic level of E2 and Vtg during the reproductive cycle of mature female sea bass kept in captivity under natural photoperiod and temperature in the East coast of Spain (40°N 0°E). The horizontal bars at the bottom of each graph indicate the spawning period (arrows indicate mid spawning time). Different letters indicate significant differences ($p<0.05$). A, B from Navas *et al.* (1998) and C, D from Mañanós *et al.* (1997).

In these studies, maximum E2-levels were associated with up-regulated *cyp19* and ESRs in the ovaries, increased oocyte growth, high gonadosomatic indices (GSIs), high plasmatic vitellogenin and gonadotropin II hormone-levels. E2-levels in fish have been widely studied in conjunction with their reproductive status. The role of E2 in the regulation of the immune system function in fish, however, has received far less attention (Navas et al., 1998; Szwejser et al., 2016). Together with the above-mentioned seasonal variations in E2-levels and the associated gametogenesis processes, variations in numerous immune parameters have been reported, such as innate immune parameters (*e.g.,* number of neutrophils, oxidative burst capacity, phagocytosis, complement system and total IgM plasmatic levels), and adaptive immune parameters (*e.g.,* specific Ig titer and lymphocyte proliferation). In agreement with those immune parameter changes, seasonal variation in the immune response and disease susceptibility have been described in teleost fish (Bowden, 2008; Szwejser et al., 2016).

In mammals, one of the most prominent examples of immune parameters being modulate with high plasmatic E2-level, might well be the changes of the thymus, *i.e.*, thymic volume and thymopoiesis, attenuating immune system-performance during pregnancy. Similar effects, *i.e.*, thymus atrophy accompanied by a drastic diminution of immature T cells, can be obtained when E2-levels are artificially elevated (Hince et al., 2008; Straub, 2007).

ii) Thymus plasticity

In teleost, the size of immune organs has also been reported to vary with the season (Nakanishi, 1986; Szwejser et al., 2016). Accordingly, the thymus displays important involution which can precede and surpass the involution of the head-kidney and spleen (Álvarez et al., 1998; Honma and Tamura, 1984). Seasonal thymus plasticity has been described in several species of teleosts, reptiles and birds but not in rodents and humans, however, from teleosts to mammals the thymus increases in size until the puberty and decrease with ageing in various species (Aw and Palmer, 2012; Cockburn, 1992; Finger and Gogal, 2013; Honma and Tamura, 1984; Peel and Belov, 2017; Rezzani et al., 2013; Tatner, 1996; Torroba and Zapata, 2003). In mammals, the age-related involution has been mostly attributed to the influence of sex hormones. It is, however, initiated before puberty, and accelerated with puberty and the rising level of sexual hormones, a phenomenon that can be observed in humans, rodent, horse and marsupial species (Aw and Palmer, 2012; Peel and Belov, 2017; Rezzani et al., 2013). Similarly, in teleost, correlation between sexual maturation and breeding season was observed in several species (Cockburn, 1992; Honma and Tamura, 1984). However, the timing, *i.e.*, before or after puberty and extent of the observed thymus involution can vary greatly in mammals and among teleost species. Interestingly, it appears to correlate with the different strategies in life-history across the different vertebrate classes (Cockburn, 1992). Cockburn observed that vertebrates which reproduce once (semelparous) such as Salmon, eel and a small marsupial species of the genera *Anthechinus* undergo a complete thymus involution, which begins before puberty. However, in *Anthechinus*, steroid hormones (sex steroids and corticosteroid) were not correlated with the involution. The author hypothesized that thymus involution results from an immune-reproduction trade-off with the high energetic costs allocated to reproduction reducing the amount of energy invested in the immune system.

Interestingly, in teleost, examples of sexual dimorphisms in the immune system are notably provided by the ovoviviparous fish *Sebasticus marmoratus*, in which females exhibit (1) a lower humoral response against a T cell-dependent-antigen with sexual maturity, (2) a higher seasonal thymus atrophy (in winter) than males and (3) higher atrophy during pregnancy following the spawning season (Nakanishi, 1986). Interestingly, winter and pregnancy/spawning correspond to relatively high level of E2 in the serum of this species (Takano et al., 1991). Seasonal thymus size dimorphism has also been reported in the ovoviviparous surfperch, in which females had larger thymuses, especially during gestation,

than males or females of other reproductive conditions (Tamura et al., 1981). The sexual dimorphism in thymus plasticity is probably mediated by an action of both male and female sexual hormones because jawed vertebrate androgens have an immunosuppressive function and both oestrogens and androgens are likely to trigger thymus atrophy in teleost as in birds, reptiles and mammals (Hince et al., 2008; Lutton and Callard, 2006; Sufi et al., 1980).

Presumably, among teleosts and also likely in mammals, interspecies differences in thymus atrophy reflect the multiplicity of reproductive strategies and ecological conditions associated to a complex interaction between the thymus and the endocrine system. Indeed, in addition to the sexual hormones, the thymus plasticity is modulated by stress- and circadian rhythm-related hormones in teleosts (Chilmonczyk, 1992; Honma and Tamura, 1984; Torroba and Zapata, 2003). Interestingly, as observed for the seasonal involution by Álvarez et al., (1998), Honma and Tamura (1984), accordingly to Torroba and Zapata, (2003) and Tatner (1996) the most obvious evidence of lymphoid organ aging is the involution of the thymus in all vertebrate investigated. Taken into consideration these observations, in mammals and teleost, thymus regression occurs early and therefore may not only result from ageing but rather from regulated processes (Aw and Palmer, 2012). From teleosts to mammals, the data emphasise the importance of the thymus and confirm the existence of complex mechanism to allocate the expenditure to this energy demanding organ (Aw and Palmer, 2012). Therefore, in all jawed vertebrate, the immune system and particularly the thymus must be tightly regulated by the neuro-endocrine system as in mammals (Savino et al., 2015; Savino and Dardenne, 2000).

iii) Oestrogenic effects on the immune system with special focus on T cell biology

In rodents and humans of both gender, the thymus plasticity and thus T cell development is strongly regulated by E2 (Hince et al., 2008; Straub, 2007). In non-mammalian species, this could be experimentally confirmed in several studies that observed E2-modulated thymic plasticity in birds, lizard and teleosts (Hareramadas and Rai, 2006; Kondo et al., 2004; Seemann et al., 2015; Selvaraj and Pitchappan, 1985). Evidence, therefore, suggests that oestrogenic effects on T cell maturation described below are conserved in jawed vertebrates.

(1) Oestrogenic effects on thymus ontogenesis

In several species of jawed vertebrates, such as lizard, pigeon, salmon and mammals, oestrogen-exposure of adult has shown to induce thymic atrophy (Forsberg, 1996; Hareramadas and Rai, 2006; Selvaraj and Pitchappan, 1985; Sufi et al., 1980). A similar effect has been observed after foetal exposure of Guinea pig and mouse (Holladay et al., 1993; Screpanti et al., 1982). In certain cases, however, thymic hypertrophy was reported (Forsberg, 1996; Kondo et al., 2004; Leceta et al., 1988; Seemann et al., 2015). This was associated with oestrogen-exposure during specific periods of thymus ontogenesis in perinatal rodents, chick embryos and sea bass juveniles, suggesting that in all jawed vertebrates, oestrogen can increase thymus volume during critical windows of the thymus ontogenesis, during which immunocompetence is established (DeWitt et al., 2012; Kondo et al., 2004; Seemann et al., 2017, 2015). In birds and mammals, the importance of oestrogen and ESR1 during the immune system development is highlighted by the modulation of ESR1 distribution during the thymus ontogenesis (Katayama et al., 2012; Seiki and Sakabe, 1997). Hence, these studies point to the implications of E2 in thymus ontogenesis being critical during specific phases of immune system development of all jawed vertebrates.

Studies using aromatase-, ESR2-, GPER- and especially ESR1-knockout mice confirmed that oestrogen plays an important role in thymus ontogenesis of both genders (Erlandsson et al., 2001; Li et al., 2002; Wang et al., 2008; Yellayi et al., 2000). It could be further demonstrated that ESR1, but neither GPER nor ESR2, was necessary for a full size development of the thymus and spleen (Erlandsson et al., 2001; Staples et al., 1999; Yellayi et al., 2000). More specifically, the expression of ESR1 in radio-resistant stromal cells, but not radiosensitive hematopoietic cells, is necessary for the development of a full size thymus (Staples et al., 1999). Interestingly, Yellayi et al. (2000) have shown that, at birth, ESR1-knockout (ERKO) mice have a thymus size similar to the wild-type but neonatal ERKO mice of five days of age exhibited a significant reduction in thymus weight and medullary area, which persisted into adulthood. Overall, these studies emphasise the primordial role of oestrogen and ESR1 during critical windows of the thymic ontogenesis (perinatal period in mammals) in jawed vertebrates.

(2) Oestrogenic effects on thymus function

Although the changes in thymus volume resulting from increased levels of natural (E2) or synthetic (DES) oestrogens and EEDC (TCDD, NP and genistein) are well described (Brown et al., 2006b; Erlandsson et al., 2001; Gould et al., 2000; Staples et al., 1998; Yao and Hou, 2004; Yellayi et al., 2002; Zoller and Kersh, 2006), the mechanisms leading to the oestrogen-induced thymus atrophy are not well understood (Bernardi et al., 2015; Forsberg, 1984; Glucksmann and Cherry, 1968; Screpanti et al., 1991). Thymus atrophy is characterized as drastic and reversible decrease of lymphocytes in the entire organ, but especially in the cortex (Gould et al., 2000; Martin et al., 1994b; Moreno and Zapata, 1991; Öner and Ozan, 2002; Selvaraj and Pitchappan, 1985). The opposite, *i.e.*, thymic hypertrophy is obtained when E2-levels decrease following ovariectomy in lizard and rodents (Hareramadas and Rai, 2006; Hince et al., 2008).

In detail, several steps of T cell maturation were identified to be modulated by oestrogen, such as (1) decrease of T cell progenitor homing in the thymus (Zoller and Kersh, 2006); (2) inhibition of thymocyte proliferation (Gould et al., 2000; Zoller and Kersh, 2006; Zoller et al., 2007); (3) induction of thymocyte apoptosis (Do et al., 2002; Okasha et al., 2001; Wang et al., 2008); and (4) extensive T cell leakage through the blood vessels into the periphery (Chapman et al., 2015; Martín et al., 1995a).

(a) Apoptosis

As discussed above, T cell apoptosis is a major process for negative and positive selection to build a peripheral T cell repertoire with self-tolerant and immunocompetent T cells. Numerous studies have described *in vivo* exposure to oestrogens to induce immature T cell apoptosis (Do et al., 2002; Martín et al., 1994b; Okasha et al., 2001; Wang et al., 2008), and specially DP T cells (Wang et al., 2008). Similar effects were described after DES- and NP-treatment of adult male and female rodents (Brown et al., 2006a; Calemine et al., 2002; Yao and Hou, 2004). Consequently, it was hypothesised that immature T cell apoptosis is responsible of the E2-mediated thymic atrophy.

This oestrogen-induced T cell apoptosis appeared to be mediated by the extrinsic pathway of apoptosis *via* death receptor as indicated by (1) the E2-mediated increase of expression of genes-related to the extrinsic pathway (*e.g.,* FasL) in thymocytes and whole thymus (Do et al.,

2002; MOR et al., 2001); (2) the absence of bcl-2 overexpression effect on the E2-induced atrophy in mice (an anti-apoptotic oncogene that belong to the intrinsic pathway; Staples et al., 1998); and (3) the inhibition of E2-mediated apoptosis when using a caspase 8 inhibitor (Do et al., 2002). In addition, *in vivo* E2-exposure of juvenile mice was also reported to increase *caspase7* and *caspase9* transcript-level in whole thymus extracts (Selvaraj, 2005). This would mean that oestrogen also activates the intrinsic pathway. In fact, FasL/Fas-deficiency mice increased the resistance to the E2-induced apoptosis, but did not prevent the induction of apoptosis (Do et al., 2002). Although the extrinsic pathway does not appear to be essential for thymocyte selection (Daley et al., 2017), nevertheless this pathway is apparently also involved in positive and negative selection (Paulsen and Janssen, 2011).

The E2-induced thymocyte apoptosis could result from a mechanism conserved in all jawed vertebrates, as (1) in lizard, *in vitro* exposure to E2 has also been reported to induce a caspase 9-dependent apoptosis in thymocytes (Hareramadas and Rai, 2006), and (2) oestrogens were apparently able to induce T cell apoptosis in teleosts as well, because degenerated lymphocytes have been observed after oestrogen-treatment in salmon (Sufi et al., 1980).

Although an important decrease of DP T cells was frequently reported, other studies could not associated apoptosis with increased E2-levels (e.g., Staples et al., 1998; Zoller and Kersh, 2006) or EEDCs, such as DES (Gould et al., 2000).

Conflicting results with respect to E2-mediated T cell apoptosis may originate from methodological differences or from the difficulties to detect apoptosis *in vivo* because of rapid clearance of apoptotic cells by phagocytic cells. In several studies, which reported E2-induced apoptosis, the *in vivo* exposure was followed by 24 h of thymocyte culture before apoptosis was measured (Brown et al., 2006a; Do et al., 2002; Okasha et al., 2001). Other studies observed apoptosis directly by electron (Kawashima et al., 1995) and light microscopy (Martin et al., 1994b; Moreno and Zapata, 1991).

(b) Proliferation

Another source of thymus atrophy could be the inhibition of the thymocyte proliferation, which was observed following oestrogen-treatment in atrophied thymus rodents and Guinean pig (Gould et al., 2000; Gulino et al., 1985; Martin et al., 1994b; Moreno and Zapata, 1991; Screpanti et al., 1982). Pregnancy in mice also inhibited the proliferation of all thymocyte

subsets (Zoller et al., 2007). *In vitro* spontaneous proliferation of thymocytes has been reported to precede the decrease in thymocyte number in mice after administration of a single dose of oestradiol (Okuyama et al., 1992). And, as a confirmation that E2-signalling is involved in the regulation of the proliferation, aromatase-deficiency also decreases the proliferation of immature T cells (Li et al., 2002). Contrariwise, oestrogen has also been reported to inhibit thymocyte proliferation in rats with thymus hypertrophy (Forsberg, 1996), which would mean that inhibition of immature T cell proliferation may not be the main cause of thymic atrophy.

Chances are that the inhibition of thymocyte proliferation is evolutionary conserved. Oestrogen-treatment of salmon has been reported to decrease mitotic events in the thymus (Sufi et al., 1980). Moreover, E2-exposure of isolated thymocytes from lizards inhibited ConA-induced proliferation (Hareramadas and Rai, 2006).

(c) Homing

A number of studies discussed E2-modulated cellular migration into the thymus as a possible cause of changes in thymus volume. Zoller and Kersh (2006) observed E2-depleted T cell precursors in the bone marrow and ETP. Correspondingly, decreased E2-levels following ovariectomy increased the number of T cell precursors in the bone marrow and ETP (Ryan et al., 2005). At physiologically high levels of E2, during mouse pregnancy, only the decrease number of ETP was confirmed (Zoller et al., 2007).

In addition, oestrogen-treatments appear to increase the number of CD5+ B cells in the thymus, particularly around the blood vessels in the medulla and CMJ (Martín et al., 1995b), plasma cells (Martín et al., 1994a; Ross and Korenchevsky, 1941), macrophages (Martín et al., 1994; Moreno and Zapata, 1991; Öner and Ozan, 2002), mast cells (Öner and Ozan, 2002) and granulocytes (Martín et al., 1994a). These findings may be explained by an E2-triggered increase in peripheral immune cell migration.

(d) T cell output

Thymus volume might not only change due to a reduced entry of early T cell progenitors, but also by an increased release of T cells. Indeed, E2 has also been reported to increase the

proportion of CD4, CD8 SP and DP (which are generally not present in the spleen and lymph node) in the spleen and lymph nodes two days after oestradiol benzoate injection (Martín et al., 1995a). It was, therefore, hypothesised that E2-induces T cell output. A high level of E2 during pregnancy was also described to decrease total thymic output in the spleen of mice (Zoller et al., 2007). However, ovariectomy increased the number of naive CD4 SP migrating from the thymus to the spleen (Ryan et al., 2005).

(e) Maturation

As almost all studies have reported that E2, but also NP and TCDD, modulate T cell phenotype distribution and markedly decreased the proportion of DP, it was also suggested that E2 modulates T cell maturation (Erlandsson et al., 2001; Okasha et al., 2001; Staples et al., 1998; Wang et al., 2008; Zoller and Kersh, 2006). In addition, oestrogen-treatment of rat and mouse have been shown to increase the proportion of T cells with a mature phenotype CD4 and CD8 SP (Bernardi et al., 2015; Erlandsson et al., 2001; Okasha et al., 2001). Similarly, during mouse pregnancy, (1) the proportion of immature DP was drastically reduced, (2) the proportion of mature CD4, CD8 and CD3 SP T cell phenotypes increased, and (3) the fraction of immature CD3 CD4 CD8 triple negative (TN) T cell was almost entirely composed of DN1 CD44+ CD25- (Rijhsinghani et al., 1996a). The same observations could be made after E2-treatment (Rijhsinghani et al., 1996b; Screpanti et al., 1991; Wang et al., 2008). Thus, it was assumed that oestrogen stimulated an alternative pathway of T cell maturation and blocked early T cell maturation at the DN1 stage. As a confirmation that E2 is involved in those maturation steps, aromatase-deficiency decreased the proportion of mature CD4, CD8 SP, immature DN1 CD44+ CD25- and CD4 CD8 DP. And as proof these effects were mainly reversed using an E2-treatment (Li et al., 2002).

Initially, Zoller et al. (2006) observed similar results, but using CD117 immunolabelling (c-kit) which is more restrictive for early T cell progenitor, they observed that oestrogen-treatment preferentially decreased ETP (also named DN1 CD25- CD117+) and DN2 CD25+ CD117+ (Yui and Rothenberg, 2014; Zoller and Kersh, 2006). In addition, they observed that E2 inhibited the proliferation of TN3-TN4 (β selected T cells) responding to the TCRβ/pre-α signal. It is, therefore, quite possible that E2 inhibits beta selection. The importance of E2-signalling for β selection or maturation-step associated was confirmed with aromatase-

deficient mouse which has a higher proportion of DN4 CD44- CD25- and a lower proportion of DP (Li et al., 2002).

To explain the E2-mediated (1) decrease of immature DP, (2) increase proportion of mature T cells, (3) the potential decrease of β-selected T cells, (4) the drastic and rapid drop of T cell content and (5) the concomitant change in splenic T cells, it was hypothesised that E2 inhibits the main pathway of thymic T cell maturation (CD4 and CD8 SP) and stimulates a thymic alternative pathway of T cell development which are CD3+ SP TCR αβ+ or TCR γδ+ and either NK1.1+ or NK1.1- (Fig. 16; Abo, 2001; Chapman et al., 2015; Narita et al., 1998; Okuyama et al., 1992; Zoller and Kersh, 2006).

This hypothesis might notably be confirmed by the fact that γδ-selected cells apparently proliferate less and differentiate faster than the β-selected cells (Taghon et al., 2006). The E2-mediated inhibition of T cell proliferation may therefore illustrate the modulation of lineage commitment. This idea is substantiated by the extrathymic differentiation of αβ and γδ T cells with intermediate levels of CD3 and either NK1.1+ or NK1.1- that were observed in the liver and in the uterus at the same time as thymic atrophy occurring during pregnancy and E2-treatment (Abo, 2001; Kimura et al., 1995; Narita et al., 1998; Okuyama et al., 1992).

As a matter of fact, the CD3 SP TCRαβ+ T cells are likely to correspond to the precursor of CD8αα+ intraepithelial αβ T lymphocyte (pIELs) which are an abundant population of thymus-derived T cells localized within the intestinal epithelia and protect the gut barrier surface by regulating gut inflammations and host defense against pathogens (Cheroutre et al., 2011). Moreover, CD3 SP TCRαβ+ T cells can represent the precursors of invariant natural killer T cell (iNKT). pIELs can differentiate from early immature T cell at the DN state or from DP T cells (Cheroutre et al., 2011; Mondoon et al., 2017; Ruscher et al., 2017). iNKT differentiates from DP T cells (Hogquist and Jameson, 2014). After their thymic development, the pIELs migrate into the epithelium of the intestine to complete their differentiation by expressing CD8αα and intermediate level of TCR (Cheroutre et al., 2011; Mondoon et al., 2017; Ruscher et al., 2017). Abo (2001) hypothesized that T cells with intermediate TCR/CD3 mainly arise from extrathymic differentiation. Nevertheless, this maturation pathway was a long-standing debate. Indeed, the generation of IELs and their absolute number are highly dependent of the thymus (Abo, 2001; Cheroutre et al., 2011; Mondoon et al., 2017; Ruscher et al., 2017). Therefore, the E2-induced extrathymic T cell

differentiation is likely to correspond to the intrathymic stimulation of alternative T cell maturation followed by the rapid exportation in the periphery. However, the hypothesis of the E2-mediated stimulation of extracellular T cell differentiation cannot be completely rejected because fully committed T cell precursor DN2-DN3 have been described to migrate in the intestine and rearrange *in situ* their TCRs (Cheroutre et al., 2011; Lambolez et al., 2006). And in the liver, E2-treatment increased the number of lymphopoietic precursors which express RAG1 and RAG2 transcripts (Narita et al., 1998). This result may explain the E2-mediated thymic decrease of DN2 proportion as well as the rapid thymic atrophy systematically observed (Fig. 16). Such hypothesis could be confirmed by Okuyama and co-workers (1992) who observed that E2-treatment rapidly inhibits and stimulates spontaneous leucocyte proliferation in the thymus and the liver, respectively, because committed T cell precursors that initiate TCRs rearrangement proliferate extensively to persist in the periphery (Lambolez et al., 2006).

As a confirmation that E2 stimulates pIEL and iNKT differentiation, Screpanti et al. (1991) observed that E2 increases the proportion of TCRαβ+ CD5+ cells which discriminate the precursor of IEL as well as iNKT cells (Hogquist and Jameson, 2014; Mondoon et al., 2017; Ruscher et al., 2017). Furthermore, Narita et al. (1998) observed that the proportion of SP $CD3^{inter}$ $TCR\alpha\beta^{inter}$ both NK1.1+ and NK1.1- increases in the thymus after E2-treatment. And since pIEL and iNKT differentiate from TCRαβ+ DP T cells, the E2-mediated increase of TCRαβ+ SP CD5+ T cell differentiation coupled to the E2-mediated promotion of γδ selection instead of β selection provokes the high decrease of DP proportion observed after E2-treatment (Fig. 16).

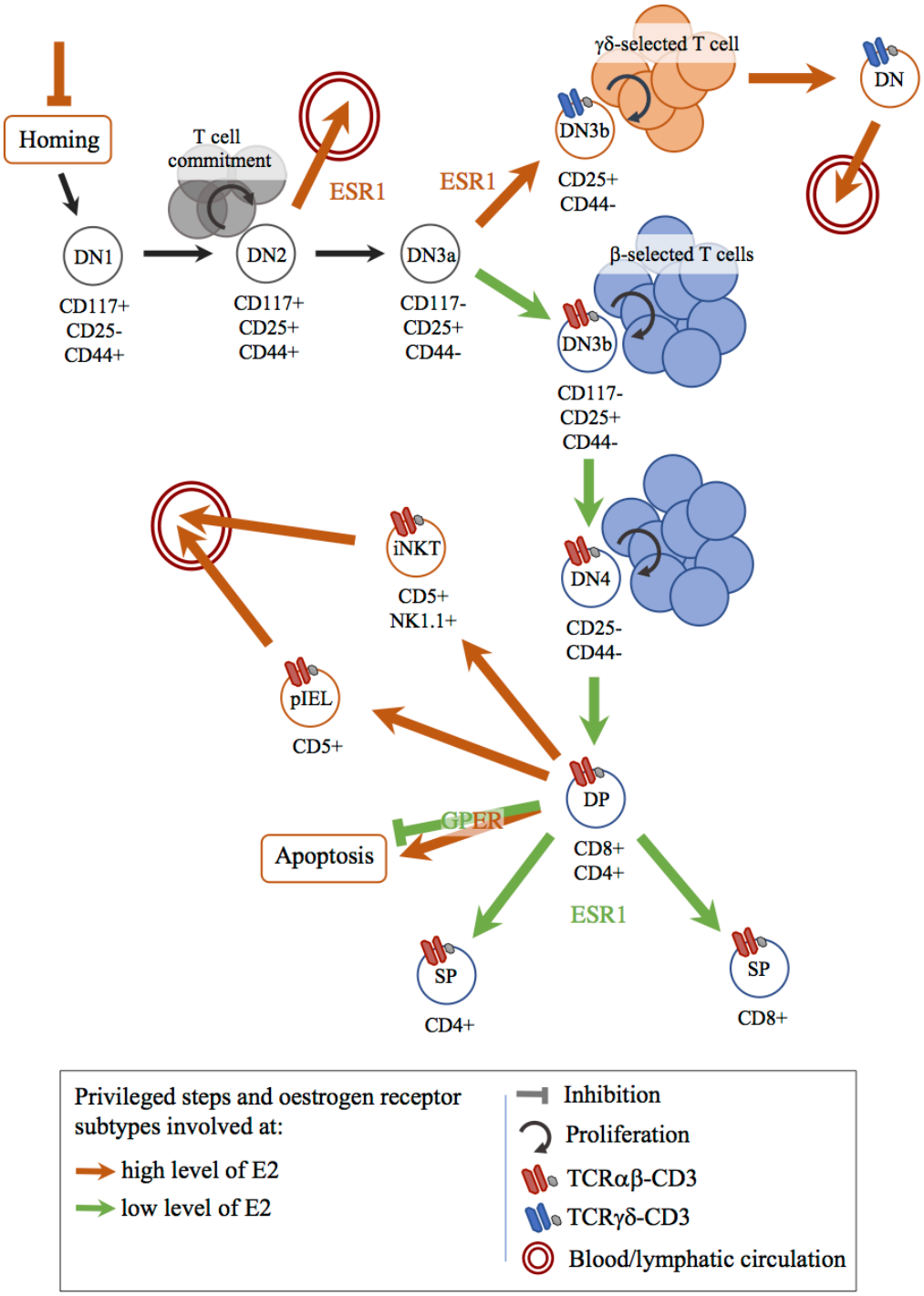


Figure 16: Role of E2-level in thymic T cell development and commitment into the different T cell lineages in mammals. At low level, E2 is crucial for development of conventional T cell (SP CD4 and CD8), more specifically E2-signalling is likely important for β selection because E2-deficiency inhibit DP differentiation and DN proliferation. At high physiological level (pregnancy level) E2 apparently inhibits the homing of early T cell progenitor and likely stimulates the early exportation of T cell precursor. Furthermore, high level of E2 inhibits β selection, promotes γδ T cell differentiation and stimulates the differentiation of unconventional T cell, *i.e.*, intraepithelial lymphocyte progenitors (pIEL) and invariant natural killer T cell (iNKT). E2 at low and high levels are also believed to respectively promote anti- and pro-apoptotic activity.

(f) MISS and NISS

Altogether these data indicate that the E2-mediated regulation of thymus volume can be very complex and may occur on different levels, perhaps even through different signalling pathways. In order to elucidate the latter, the implication of the different oestrogen receptor subtypes was investigated with ESR1- (ERKO), ESR2- (BERKO) and GPER-knockout (KO) mice (Staples et al., 1999; Wang et al., 2008; Yellayi et al., 2000) but also with specific oestrogen receptor agonists and antagonist: the propyl pyrazole triol (PPT, ESR1-agonist), the diarylpropionitrile (DPN, ESR2-agonist), G-1 (GPER-agonist) and ICI 182,780 (ESR-antagonist; McMurray et al., 2001; Wang et al., 2008; Yellayi et al., 2002).

(i) Atrophy

E2-mediated atrophy is almost completely suspended in ERKO and GPER KO mice, but is not attenuated in BERKO mice (Staples et al., 1999; Wang et al., 2008). In BERKO mice, the oestrogen mediated cortical atrophy notably changed the ratio between cortex and medulla (Erlandsson et al., 2001). The ESR1 and GPER implications in the E2-mediated thymus atrophy could be demonstrated by preventing atrophy with ICI 182,780 (Yellayi et al., 2002) and inducing of thymic atrophy with G-1 and PPT but not DPN (McMurray et al., 2001; Wang et al., 2008).

(ii) Apoptosis

The above mentioned studies ESRs/GPER KO mice suggested that GPER is implicated in the E2-induced apoptosis, as G-1-treatment indeed induced DP-apoptosis (Wang et al., 2008). Furthermore, it was observed that GPER KO mice increase the number of apoptotic DP T cells. Hence, Wang et al. (2008) hypothesised that E2 would have an anti-apoptotic effect at low concentrations and a pro-apoptotic at high concentration and that this effect would be conveyed *via* the GPER signalling pathway (Fig. 16). Accordingly to the anti-apoptotic effect of GPER, in mice, the GPER-inactivation reduced the release of all T cell subtypes, but especially SP CD4 and CD8 (Isensee et al., 2009). Overall, GPER expression in the thymus of mice and humans and its direct implication in the modulation of thymic function remains controversial (Isensee et al., 2009; Olde and Leeb-Lundberg, 2009; Wang et al., 2008).

(iii) T cell maturation

Considering the E2-mediated alteration of T cell phenotypes, currently available data are inconsistent: using ERKO and BERKO mice, Erlandsson et al. (2001) observed that E2-mediated phenotype alteration is likely to be mediated by ESR2 but not ESR1. However, Staples et al. (1999) observed that ESR1 KO did not manifest this CD8/CD4 shift after E2-treatment, pointing to an implication of ESR1. The results of Staples et al. (1999) were further confirmed by McMurray et al. (2001) using the specific agonists (DPP and DPN). These results indicate that, at high level of E2, ESR1 is involved in the E2-mediated promotion of the alternative pathway of T cell differentiation.

On the other hand, several studies have shown that both young and aged ERKO but not BERKO mice have higher proportion of DP and less SP CD4 and CD8 in the thymus mice, indicating that rather ESR1 would be involved in the transition from DP to SP (Erlandsson et al., 2001; Islander et al., 2003; Yellayi et al., 2000). Staples et al. (1999), however, did not observe any difference compared to the wild type.

(3) Oestrogenic effects on peripheral T cells

In mammals, in addition to modulate thymopoiesis, E2 modulates Th1-, Th2- and Treg-polarization, *i.e.*, peripheral T cell differentiation: low physiological E2-levels is considered to promote the differentiation of CD4 T cells in Th1 (immunity of type 1, *i.e.,* cell-mediated immunity) with the production of pro-inflammatory cytokines (TNF-α and IFN-γ). And higher E2-concentrations are assigned anti-inflammatory properties by the promotion of Th2 differentiation (type 2 immunity, *i.e.*, humoral immunity) associated with an inhibition of pro-inflammatory cytokine expression (TNF-α, IL-1β) and the stimulation of anti-inflammatory cytokines (IL-4, IL-10, TGF-β; Khan and Ansar Ahmed, 2016; Straub, 2007). Moreover, E2 promotes Treg differentiation and activity (Khan et al., 2012; Khan and Ansar Ahmed, 2016; Straub, 2007). BPA has been reported to entail a similar effect with, notably, the modulation of Th1/Th2 polarization (Jochmanova et al., 2015). Associated to the modulation of T cell polarization, oestrogens have been described to modulate numerous T cell properties, such as cytotoxicity, trafficking, proliferation and humoral response to T cell dependent antigen (Ádori et al., 2010; Forsberg, 1984; Mo et al., 2005; Takao, 2005).

Furthermore, B cell activity appears to be also affected by E2 in a dose dependent manner resulting in a stimulation of (2) antibody producing cell numbers, (2) proliferation and (3) antibody production. It is considered that E2 inhibits B lymphopoiesis and stimulates B cell differentiation and function (Bernardi et al., 2014; Roved et al., 2017; Straub, 2007). Straub (2007) specified that E2 stimulates B cell antibody production, probably by inhibiting T cell-mediated suppression of B cells.

In non-mammalian species, such as birds and teleosts, the state of knowledge is much less developed, but similar effects as those characterised for mammals have been observed. In birds, several oestrogen compounds have been tested *in vivo*. In pigeon and chick oestradiol-exposure has shown to modulate B and T cell response after immunization (Kondo et al., 2004; Selvaraj and Pitchappan, 1985). Moreover, EEDC-exposure (E2, phtalates and BPA in mixture) impact T cell-mediated hypersensitivity and increases the number of B and T cells in nestling starlings (*Sturnus vulgaris*) and chicken respectively (Finger and Gogal, 2013). Oestrogens are likely to directly regulate T cell activity in birds, because *in vitro* E2-exposure of blood leucocytes modulates PHA-induced proliferation and IL-2 production with an stimulating and inhibitory action at low and high concentration respectively (Kondo et al., 1994). Similar E2-effect has been observed in mouse (Priyanka et al., 2013). This result suggests that the biphasic effect of E2 with a pro- and anti-inflammatory activity at low and high concentration respectively may be conserved from bird to mammals (Khan et al., 2012; Straub, 2007).

Similarly in teleost, EE2-injection of unvaccinated gilthead sea bream increased the proliferation and abundance of T cell in the head-kidney (Rodenas et al., 2017a). The same research team also observed that the exposure to EE2, tamoxifen (a non-specific ESR-antagonist) or G-1 of sea bream also modulate B cell abundance, proliferation and the production of both unspecific (natural) and Keyhole limpet hemocyanin (KLH)-specific antibodies (Rodenas et al., 2017a, 2016, 2015). KLH is commonly referred as a T cell dependent antigen (Manning and Nakanishi, 1996). In other teleost species, E2 was also found to modulate mitogen-stimulated B and T cell proliferation *in vivo* and *in vitro* (Iwanowicz et al., 2014; Shelley et al., 2013, 2012).

Altogether these data suggest that E2 directly and similarly modulates T cell activity in birds and teleost as in mammals. However, because the E2-mediated effect on humoral response against T cell dependent antigen does not imply that E2 modulates T cell function, it is not

known if E2 modulates the immune response by acting on Th1/Th2/Treg differentiation in non-mammalian species.

D) The European sea bass as experimental model

The European sea bass (*Dicentrachus labrax,* Linnaeus 1758; Fig. 17) is named "bar commun" in French (or "bar européen" in the Atlantic and "loup" in the Mediterranean), "lubina" in Spanish and "Spigola" in Italian. *D. labrax* belongs to the class Actinopterygii, *i.e.*, the ray-finned fish, the order Perciformes, and the family Moronidae (Families). The European sea bass is euryhaline because it tolerates a wide range of salinity from 3‰ to full strength sea water. This species is as well as eurythermic because it can cope with temperatures ranging from 8 to 24°C (fishbase.org; Pérez-Ruzafa and Concepción, 2015). Its physiological adaptability allows this species to have a wide geographical repartition from the North of Norway to Senegal, the Mediterranean and the Black sea (Fig. 18).

Figure 17 : European sea bass, *Dicentrarchus.labrax* (www.msc.org)

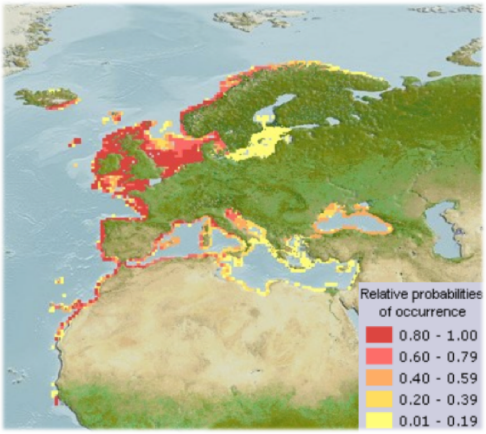


Figure 18 : Native distribution map for the European sea bass (http://www.fishbase.se/summary/63)

Besides its ecological interest, *D. labrax* is a species of high economic importance, notably for commercial, but also for recreational fisheries in numerous countries, such as UK, France and Netherland. Furthermore, *D. labrax* is produced in aquaculture. Because of it high commercial value (Angling fish: 18-26 €/kg) and the world-wide increase of fish consumption, mass-production techniques for juveniles have been developed during the late 1960's. Accordingly, sea bass production has grown massively in the two last decades (Fig. 19). To date, it is the most important commercial fish cultured in the Mediterranean countries (FAO, 2017). Consequently, *D. labrax* has been intensively studied in order to face the numerous diseases, to decrease juvenile mortality and to limit stress that may affect the quality of commercialised fish (FAO, 2017).

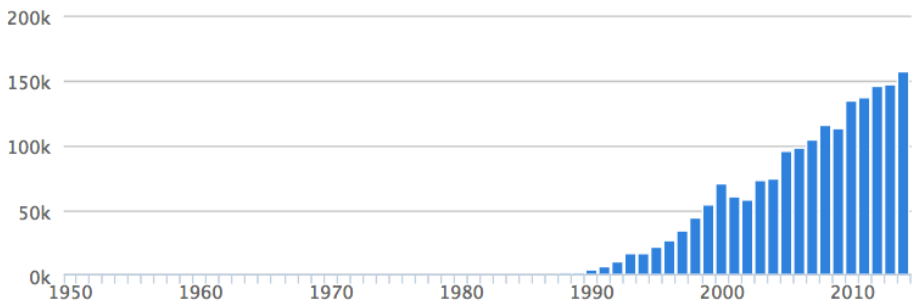


Figure 19 : Evolution of farmed sea bass production (in tones). The main producers are localized in Greece, Turkey, Spain, Croatia and Italy (FAO).

i) Life cycle

The European sea bass is a migratory marine fish. From 1970 to 1984, Pawson et al. (1987) investigated the movement of *D. labrax* by capture-recapture of tagged sea bass in England and Wales. They observed that juveniles (<32 cm, up to four years old) essentially remain within 80 km of the release area, *i.e.*, their nurseries within the estuaries and along the coastal zones. Adolescent *D. labrax* (≥32 cm and <42 cm, four to six years old), which enter their sexual maturation, have a similar pattern of dispersal. However, they increase movement, which is likely to anticipate the adult migration. Adults (>42 cm, older than six years old), were found to migrate to the winter pre-spawning area about 100 km and even up to 800 km, depending on the area between October and December. In spring, they return to the coastal areas for summer feeding. Winter migration appeared to take place when adult sea bass sought warmer temperature (Pawson et al., 1987). This migration pattern was confirmed by several other studies (Fig. 20). And from February to May, mature sea bass spawn offshore and in coastal waters in the western English Channel and eastern Celtic sea (Kelley, 1988; Pawson et al., 2007; Pérez-Ruzafa and Concepción, 2015): One to two months after offshore spawning, postlarval fingerlings of 10-15 mm arrive in estuaries from May to June (Kelley, 1988). During their first summer, juveniles preferentially stay in freshwater and shallow creeks, channels, marsh pools and tributary streams (Kelley, 1988). Larger juveniles move to the deeper parts of the estuary in October (Kelley, 1988). At around four to five years of age, juveniles migrate from their nurseries into the open waters of the English Channel (Pawson et al., 2007). More recently, using acoustic tracking, Doyle et al., (2017) detected adult sea bass (>54 cm) within the Cork harbour, a large estuary notably surrounded by large area of

intertidal mudflat. This study again confirmed the pattern of migration, with adult sea bass residing in their coastal foraging areas (≈3 km) from mid-April to mid-October, showing a fine spatial scale of inter-annual site fidelity. Because of this strong site fidelity and the long coastal residency, Doyle et al. (2017) suggest that the European sea bass is particularly at risk to become locally depleted.

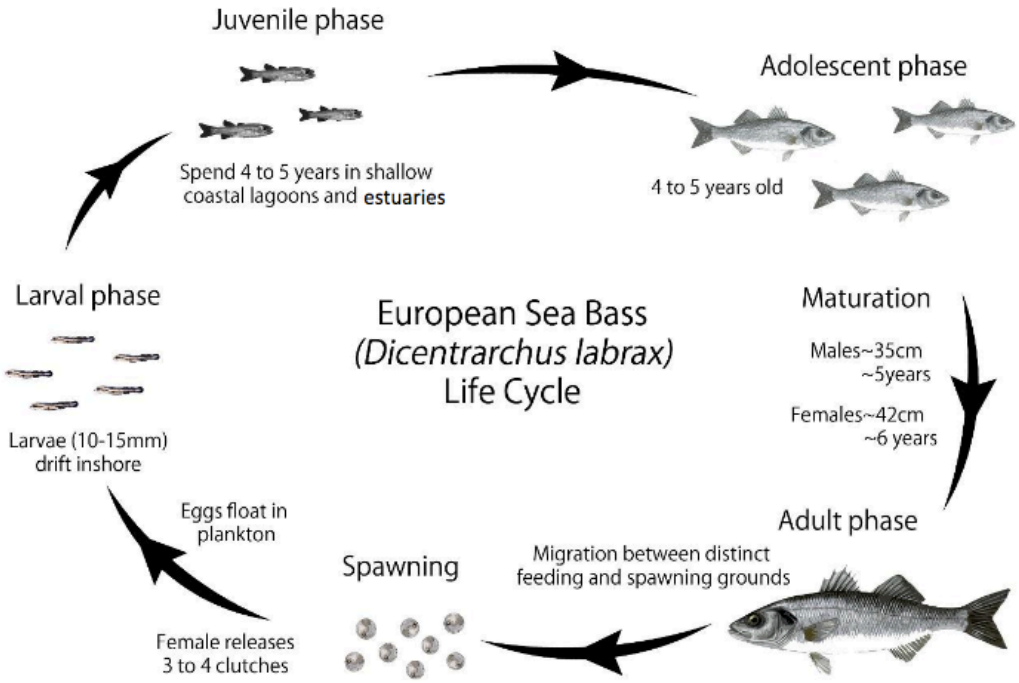


Figure 20 : The European sea bass life cycle (Abi, 2014)

ii) Reproductive strategy

Basically, two extreme life history strategies have been described: the r- and the K-strategy (Pérez-Ruzafa and Concepción, 2015). The r-strategy, on the one hand, implies little care for the offspring, but high expenditure in the reproduction with early sexual maturity, high fecundity, small and abundant offspring, short lifespan and small adult body size. The K-strategy, on the other hand, implies longer life spans with slow maturation, low number of offspring but extensive parental care.

D. labrax is situated in-between the r- and the K-strategy, *i.e.*, shows mixed traits of either reproductive strategy: it is a long-leaving species with a common length of 50 cm and a maximum reported age 30 years (fishbase.org). In the English Channel, females and males start to reproduce when they reach six to seven years of age (>42 cm) for female and four to five years-old (>32 cm) for male (Pawson and Pickett, 1996). Females spawn small pelagic

eggs (1.02-1.39 mm; FAO, 2017), with a high fecundity of about 200,000 eggs/kg body mass (Pérez-Ruzafa and Concepción, 2015). Sea bass are not considered territorial, *i.e.*, they do not take care of the eggs. Hence, much of the energy is allocated by the females into gamete production (Pérez-Ruzafa and Concepción, 2015).

iii) Feeding

The sea bass is a highly adaptable and opportunistic top predator. Its feeding behaviour depends on its age and the seasonal availability of the prey (Doyle et al., 2017; Morin et al., 1999; Pérez-Ruzafa and Concepción, 2015). Juveniles preferentially feed on Mysidacea (small shrimp-like crustacean, 5-25 mm), copepods and isopods and, to a lesser extent, on bivalves and decapods (Pérez-Ruzafa and Concepción, 2015). The food of fish larger than 20 cm is mainly comprised of crabs, shrimps and other fish (Pérez-Ruzafa and Concepción, 2015).

iv) Wild stock

In 2012, the International Council for the Exploitation of the Sea (ICES) warned that sea bass stock should be protected in the Northeast Atlantic by (1) a larger minimum landing size (36 cm), (2) an increase of the net-mesh size, (3) a higher landing limitation for commercial and recreational fisheries and (4) a seasonal/area closure to protect the spawning area where sea bass aggregates (ICES, 2012). Two years late, in 2015, a new ICES report warned that the European sea bass is endangered because of stock decline in the central and South North Sea as well as in the Irish Sea, English Channel, Bristol Channel and Celtic Sea. The ICES report advises that the fish landing, including recreational and commercial fisheries, should be rapidly limited to a sustainable exploitation, thus avoiding population collapse. In fact, the ICES observed very poor recruitment since 2008 and a declining spawning stock biomass since 2010, which is now below “safe biological limits” ($MSYB_{trigger}$; Fig. 21 ; ICES, 2015).

Consequently, the European commission has announced several measures for Sea bass conservation: in 2015, the minimum size was fixed to 42 cm (850/98/EU); in 2015 and again in 2017, the European commission maintained the prohibition of fishing sea bass in the area reported by the ICES, exempting, however, fisheries using hooks and lines (2017/127/EU).

And in addition, recreational fisherman and the incidental by-catches are respectively restricted to one daily fish and 3 % of the weight of the total catch (<400 kg per month).

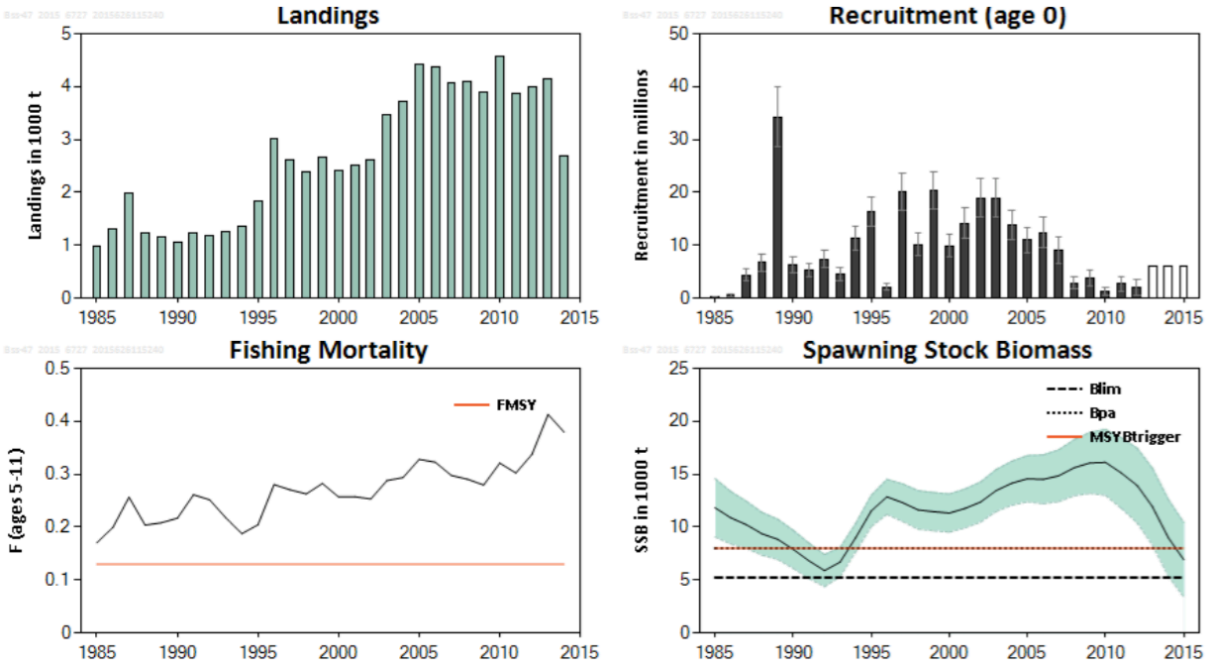


Figure 21 : Stock assessment (weight in thousand tones) in Central and South North Sea, Irish Sea, English Channel, Bristol Channel, Celtic Sea. Landings are from the commercial fishery only. Fishing mortality combines commercial and recreational fisheries. Predicted recruitment values are non-shaded. FMSY: Fishing mortality consistent with achieving maximum sustainable Yield (MSY); Blim: Limit reference point for spawning stock biomass Bpa: Precautionary reference point for spawning stock biomass; MSYBtrigger: A biomass reference point that triggers a cautious response within the ICES MSY framework. From rapport of ICES (2015).

v) Estuary importance

Postlarvae rapidly spread within the estuary according to their physiological optimums (Kelley, 1988). Juvenile and larvae show a preference for low salinity (5-10 ‰), which has been shown to improve survival rate and juvenile swim bladder inflation (Pérez-Ruzafa and Concepción, 2015). Therefore, estuaries which offer refuge and abundant food constitute a key habitat for larvae recruitment and juvenile nursery (Kelley, 1988; Morin et al., 2015, 1999).

This is illustrated by the observations of Kelley (1988), who found postlarvae (0-group) in almost all of the 28 estuaries sampled in UK waters from 1933 to 1987. The importance of a

given estuary as a sea bass nursery most likely depends on its size, which determinates the capacity to support fish (Kelley, 1988). Besides the type of estuary, the weather conditions in winter determinate the duration for which the juveniles remain within the estuary. Accordingly, Kelley (1988) has defined 3 types of estuary:

- Large estuaries (>4 km^2 of water area at low tide), deep with muddy floor. In such large estuaries, the juveniles remain up to 4-5 years, even throughout winter. However, in the case of important precipitation or prolonged cold, older juveniles may temporarily quit the estuary.

- Medium estuaries (>1 km^2 and <4 km^2), mostly shallow water with both muddy and sandy floor. In these estuaries, the juveniles remain the first and probably the second year, including the winter period. The 4 to 5 years old juveniles will, however, be present only in the warmer months.

- Small estuaries (<1 km^2), shallow with sandy floor. In these estuaries, juvenile presence will be limited mostly to the first year. In the second year, the animals are likely to migrate to larger estuaries.

At the same time, all of these estuary types may be visited by adolescent fish from other areas as well as adults (Kelley, 1988).

Detailed knowledge on the presence of juveniles in the Seine estuary is rather scarce. Two studies conducted by Morin et al. (2015, 1999) reported juveniles of one year (G0) and less than two years (G1) in the shallow waters near the Normandy Bridge, on muddy floor (see Fig. 22) and, to a lesser extent, along the Calvados coast. The abundance G0 and G1 had no apparent seasonal variation as far as their localisation is concerned. Also, no significant inter-annual variation in the G0 abundance was observed in 1995 and 2010 (Morin et al., 2015, 1999).

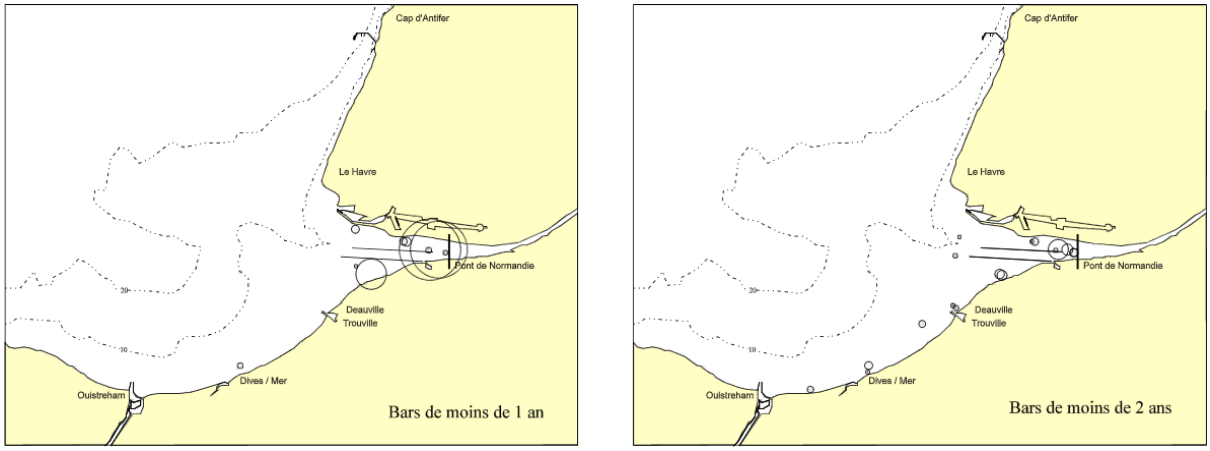


Figure 22 : Juvenile G0 and G1 sea bass relative distribution in the Seine estuary (Autumn 1996 and 1997). From Morin et al., 1999.

vi) Ecotoxicology with a special focus on the English Channel and the Seine estuary

The English Channel is subject to various anthropogenic pressures, comprising intense maritime transport, tourism, nuclear reprocessing, thermal discharge and dredging (Tappin and Millward, 2015). Because of population density and industries, estuarine and coastal waters are particularly subject to contamination from diffuse and point sources (Tappin and Millward, 2015). The fluvial discharges from the adjacent catchment of the English Channel is dominated by the Seine river and its tributaries which represent 41 % of the total long-term mean river flow (Tappin and Millward, 2015). The Seine river is highly contaminated by agriculture, domestic and industrial discharges, receiving wastewater effluents from a catchment comprising one third of the French population, making the Seine estuary one of the most polluted estuary in Europe (Minier et al., 2006; Tappin and Millward, 2015). The chemical contamination consists of, notably, halogenated (PCBs, polychlorinated-p-dibenzo-dioxines, PCDD) and polybrominated compounds (Hexabromocyclododecanes, HBCDDs), pesticides (Lindane), Polycyclic Aromatic Hydrocarbons (PAHs), and numerous compounds with a well-known oestrogen activity have been reported (Gaspéri et al., 2010; Minier et al., 2006; Tappin and Millward, 2015), such as:

- Alkyphenols (commonly represented by nonylphenol, NP, and NP-ethoxylate, NPE), the concentrations of which were in the range of 399-2214 ng/L in the water and 405-9636 ng/g in the suspended particular matter, respectively, for both NP and NPE combined (Tappin and Millward, 2015).

- Phthalates, with the major concern being raised by ethylhexylphthalate (DEHP), dibutylphthalate (DBP) and butylbenzylphthalate (BBP) the concentrations of which in the Seine estuary were 160-314 ng/L for DEHP, 71-181 ng/L for DEP, and 67-319 ng/L for DnBP; BBP and DnOP had very low concentrations or were not detected (Tappin and Millward, 2015).

Numerous other xenoestrogens were also retrieved in the Seine river between 2007 and 2010 (Gaspéri et al., 2010). PBA was abundant, with concentrations around 327 ng/L between Paris and Rouen (Gaspéri et al., 2010). Gaspéri et al., 2010 also quantified synthetic and natural oestrogens (E1, E2, E3 and EE2). E1 and E2 were the most abundant oestrogens amongst the systematically detected ones, with E1 representing 80 % of total measured oestrogens. Total hormone concentrations were in the rang 0.5-21 ng/L depending on the season and flow conditions (Gaspéri et al., 2010).

Furthermore, from May to November 2011 the oestrogenic activity was measured at five sites along the lower Seine between Poses (Upstream of Rouen) and Le Havre using a reporter cell line (MCF-7) that endogenously expresses the oestrogen receptor (ERE) and transfected with the luciferase reporter gene expressed in tandem with ERE (Budzinski et al., 2012). Oestrogenic activity was detected in all samples mostly at 1.6 to 7 ng/L; a maximum of 78 ng/L was measured at the Tancarville bridge in summer (Budzinski et al., 2012).

The impact of anthropogenic activity and pollution on sea bass nursery and its role in the decline of fish stock has, however, been only poorly investigated (Pérez-Ruzafa and Concepción, 2015). One of the exception is the report of Kelley (1988), who found postlarvae (0-group) in almost all the estuaries sampled in UK waters from 1933 to 1987 with the exception of the Mersey likely because of high anthropogenic pollution. In regards to its high trophic position, sea bass will be particularly subject to bioaccumulation of contaminants, with biomagnification *via* feeding being considered the principal route of contamination for this species (Pérez-Ruzafa and Concepción, 2015). As a matter of fact, *D. labrax* has been reported to bioaccumulate, notably, PCB, organochloride and perfluoroalkyl substances (Barbarossa et al., 2016; Loizeau et al., 2001; Schnitzler et al., 2011).

E) Objectives of the thesis

As outlined in the introduction, a body of evidence from the gene-, to the cellular- and organ-level substantiates the idea that teleost immunity shares numerous features with mammalian models. These range from basic immune cells, such as macrophages to avoid infection by killing pathogens to the complex processes for adaptive immune cell development. In fact, most of the newer findings in teleosts arouse from investigations on homologous/analogous genes, cells, mechanisms or proprieties that were first identified in mammals. This approach allowed extending our knowledge on fish immunology in particular and vertebrate immunology in general. The discovery of the first vertebrate phagocyting B cells in teleost fish is a striking example of this progress. In this context, the thymus and its associated T cell differentiation appear to be a fascinating subject for any comparative immunologist.

An endocrine system with sexual steroid hormones, such as oestrogen, represents an evolutionary invention of the vertebrate lineage. The fundamental physiological function of oestrogen, as well as the cellular mechanisms of action have been widely conserved in vertebrates, but the reproductive strategies are highly diverse. The complex interaction between the immune and endocrine system have long since been investigated in mammals. As a matter of fact, this relationship between sex steroid hormone levels and the immune system is well known in humans, where general sexual dimorphisms in immunity exist. Furthermore, the mammalian immune status changes during pregnancy and women also have a higher prevalence for autoimmune diseases. It has however been given far less attention in most other vertebrates, including fish. As the effects of varying oestrogen-levels on the thymus volume and thymopoiesis are relatively well-studied for mammals, it suggests itself to question whether similar phenomena of thymic plasticity and function could be observed in more basic vertebrates with quite different life cycles and, accordingly, different reproductive traits, such as fish. To this end, it is important to know to which extent this important primary lymphoid organ, the thymus, is similar between mammals and fish. On the basis of the functional anatomy of the thymus, one then may be able to identify the cells and regions that can be regulated by oestrogens given the presence and nature of the respective receptors for this steroid hormone, *i.e.*, EsrR1, Esr2a, Esr2b, Gpera and Gperb.

To this effect, the histological structure of the sea bass thymus was examined *in fine* and the distribution of the various oestrogen receptor subtypes within the thymic microenvironment was localized in order to identify the privileged cellular targets. The resulting information should improve the understanding of the cellular action of oestrogen on thymic T cell development in *D. labrax* (and likely other fish). If significant similarities in oestrogen receptor distribution with the mammalian model could be confirmed, the evolution of the oestrogenic modification of the thymus in vertebrates would become more evident. As Segner and co-workers stated (2017): in spite of a growing number of studies demonstrating oestrogenic modulation of the immune system across all vertebrate classes, the knowledge base is still too scarce to fully circumstantiate its evolutionary conservation. The results from this study, which represents the main body of the thesis, are presented in the first article (Paiola et al. 2017). They provide a basis from which to extrapolate on the general connection between oestrogen and the immune system across vertebrates.

Emanating from the examination of the functional thymus anatomy and the face of the wide distribution of oestrogen receptors in the different thymic microenvironments detected in the first study, we questioned how and at which steps of lymphopoiesis pleiotropic oestrogen regulation becomes manifest. Adolescent sea bass exposed *in vivo* to E2, were examined for changes of the expression of several gene markers-related to thymic function and T cell development. In order to better understand the circulation of the T cell within the organism, the expression of T cell gene markers was also investigated in two major secondary lymphoid organs: the head-kidney and the spleen. The gene expression analysis was related to the measurement of several innate and adaptive immune parameters (oxidative burst, oxidative stress, number/proportions of leucocyte populations, viability of leucocytes) on leucocytes isolated from the head-kidney and spleen by flow cytometry. In fact, innate and adaptive immune system work synergistically and are intimately connected, for instance, effector T cells are known to stimulate and amplify the immune response involving innate immune cells. Differentially, T cells can have anti-inflammatory activity and being involved into the resolution phase after inflammation. The second article (Paiola *et al.*, under revision), describes the effects of physiologically elevated E2-levels on thymic T cell development and peripheral T cell differentiation. Notably, the results point to changes in peripheral T cells, which may be associated with a modulation of innate immune cell activity.

To better understand how the innate immune system may be affected by oestrogens and to elucidate, whether regulation of innate immunity is indirect, *via* T cell regulation, or by direct action of oestrogen on innate immune cells, microbicide activity-related to oxidative burst was assessed by *in vitro* exposure of primary leucocytes from thymus, head-kidney and spleen exposed to E2. Because elevated oestrogen-levels following 17β-oestradiol injection specifically altered oxidative burst in leucocytes from the spleen, but had no effect on those from the head-kidney of adolescent sea bass (Article 2; Paiola *et al*., under revision), the oestrogen receptor expression and the phagocyte activity of isolated leucocytes was investigated to identify the underlying mechanisms of this apparent difference in the innate immune response of the two lymphoid organs. Specifically, the question was, whether the induction of immune tolerance in the spleen is due to the anti-inflammatory action of Tregs, or a result of differences in oestrogen receptor disposition and sensitivity of the leucocytes of either organ. To further link the oestrogenic action on adaptive and innate immunity in a comprehensive view, leucocytes isolated from the thymus were compared to the immune cells of spleen and head-kidney. The results of this research are presented in the third article (Paiola *et al*., *in preparation*).

The degree of resemblance is not only interesting from an evolutionary point of view, but also provides information on how xenoestrogens can interfere with the oestrogen-mediated regulation of thymic and immune-function. This may help to acknowledge the principal risk that derives from the environmental exposure of fish to the widespread xenoestrogens. In fact, the background of the research presented in this thesis is the worrisome continuous exposure of humans and wild life to oestrogenic disrupting chemicals (EEDCs). This heterogeneous group of compounds is continuously released into the environment and widely retrieved in the different water bodies. Not only do chemical species with oestrogenic activity affect sexual and reproductive physiology but, related to these, many other physiological functions, amongst them immune system performance. In fish, negative consequences of xenoestrogen-exposure for sex determination and fish reproduction have been proven in numerous teleost species. The implication of such exposure for the immune competence is, however, not well studied and far from being understood. Indeed, our present knowledge with regards to the possible adverse effects that EEDCs may exert on the immune system of non-mammalian vertebrates remains very scant, especially when it comes to aquatic wild life. A slow-growing and late maturing fish, such as the European sea bass which performs seasonal migration, is

exposed to contrasting environments during its life-cycle. Its habitats change in terms of micro-organismal communities and physicochemical conditions. Notably, the coastal areas and estuaries, in which it resides temporarily, are under high anthropogenic pressure. In these conditions, an effective immune response/pathogen resistance must be considered a key element for population survey and fitness, but may itself be affected by anthropogenic pollution. An improved knowledge on the immunomodulatory function of E2 provides information for a better risk assessment-related to EEDC-exposure and could outline possibilities for applied immunotoxicological investigation.

2) Results

Article 1: Oestrogen receptor distribution related to functional thymus anatomy of the European sea bass, *Dicentrarchus labrax* (2017)

Article 2: Oestrogen, an evolutionary conserved regulator of T cell differentiation and immune tolerance in jawed vertebrates? (2018)

Article 3: Oestrogen differentially modulates oxidative burst capacity and ROS-signaling in adaptive and innate immune cells of three major lymphoid organs in European sea bass, *Dicentrarchus labrax* (L.) (*In preparation*)

Additional results in supplementary data

2) Results

A) Article 1

Oestrogen receptor distribution related to functional thymus anatomy of the European sea bass, *Dicentrarchus labrax* (2017)

Developmental and Comparative Immunology 77 (2017) 106–120

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

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

Matthieu Paiola [a], Thomas Knigge [a], Simona Picchietti [b], Aurélie Duflot [a], Laura Guerra [b], Patricia I.S. Pinto [c], Giuseppe Scapigliati [b], Tiphaine Monsinjon [a, *]

[a] Normandy University, FR CNRS 3730 SCALE, UMR-I 02 SEBIO, Université Le Havre Normandie, F-76600 Le Havre, France
[b] Department for Innovation in Biological, Agro-food and Forest Systems, Tuscia University, 01100 Viterbo, Italy
[c] Laboratory of Comparative Endocrinology and Integrative Biology, CCMAR – Centre of Marine Sciences, University of Algarve, 8005-139 Faro, Portugal

ARTICLE INFO

Article history:
Received 3 April 2017
Received in revised form
24 July 2017
Accepted 24 July 2017
Available online 26 July 2017

Keywords:
Immune system
Endocrine system
Lymphopoiesis
T-cells
Teleost

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 localization of oestrogen receptors and suggests that oestrogens modulate 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 express 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 tissues (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-

Abbreviations: Ab, antibody (mAb, monoclonal Ab, pAb, polyclonal Ab); CD, cluster of differentiation; Ct, connective tissue; Ck, cytokeratin; E2, 17-β-oestradiol; 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).

* Corresponding author. UMR-I 02 INERIS-URCA-ULH SEBIO, Unité Stress Environnementaux et BIOsurveillance des milieux aquatiques, FR CNRS 3730 SCALE, Université Le Havre Normandie, 25 rue Philippe Lebon, F-76063 Le Havre Cedex, France.
E-mail address: tiphaine.monsinjon@univ-lehavre.fr (T. Monsinjon).

http://dx.doi.org/10.1016/j.dci.2017.07.023
0145-305X/

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 cells (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 the idea that the immunomodulatory role of E2 probably exists across all vertebrates (reviewed in Burgos-Aceves et al., 2016; Segner et al., 2017; Szwejser et al., 2017b), 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 localised 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., 2017a). It may, therefore, be hypothesized that the different receptors may have a similar distribution in the fish thymus. The morpho-functional organisation 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 localization 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., 2017a). 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 1800 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. Leukocyte 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 1200 *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 1200 *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 1200 *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 5000 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 1 μl 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 °C 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 well detect 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 1 h, followed by 1 h 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 expressed in dendritic cells, neurons, fibroblasts, lymphocytes, smooth and skeletal muscle cells (Picchietti et al., 2015; Zimmer et al., 1995). These 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.

2.5. 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 (HES) or Pappenheim or Periodic Acid-Schiff (PAS) solutions whilst others were used for immunohistochemistry (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)

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		
	sbEsr2b 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; Szwejser et al., 2017b)
	Human GPER conserved 2s extracellular		Rabbit pAb	6 μg/ml	7.5 μg/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

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.

2.6. Flow cytometry

Leucocytes from four fish were adjusted to 1×10^6 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 1200 *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 1200 *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 Fig. 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 (LTECs) 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 and b). Moreover, the cortex was characterized by higher density of proliferating Pcna- positive cells, which were localised mainly within the subcapsular zone (Fig. 2c and 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

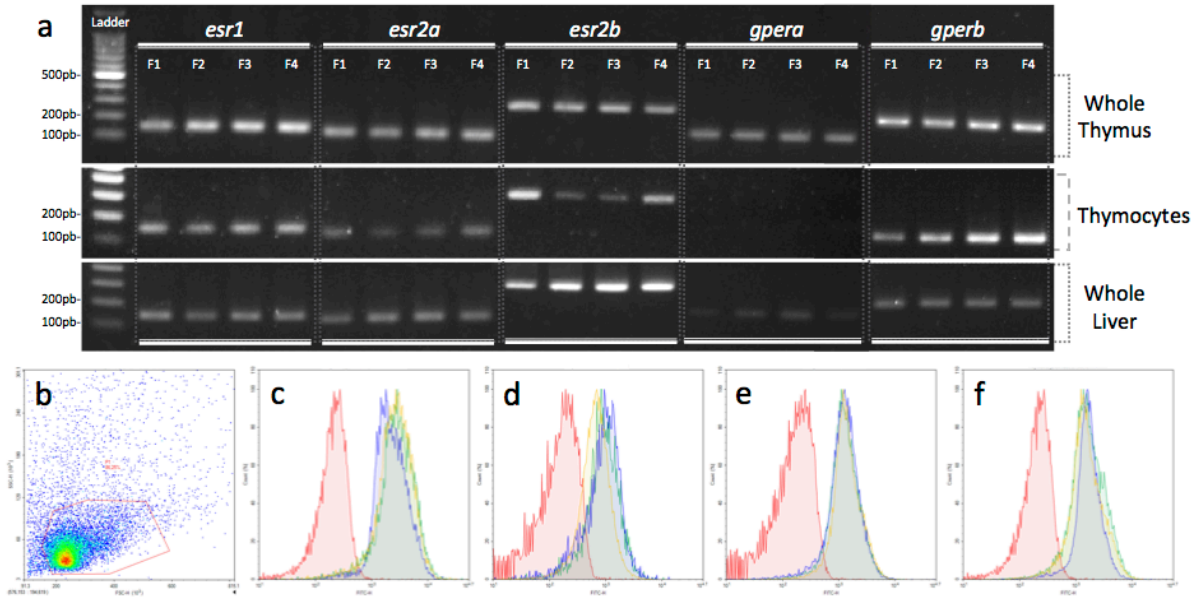


Fig. 1. a, PCR gel for the detection of Esr (*esr1, esr2a, esr2b*) and Gper (*gpera* and *gperb*) transcripts in the whole thymus, isolated thymocytes and liver of one-year-old European sea bass, *Dicentrarchus labrax*, for four different fish (F1–F4); b, Flow cytometric analysis of isolated thymocytes 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 for one-and-a-half-year-old male and female fish, in the thymic parenchyma and the connective tissue, lymphoid cells were immunopositive for 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 localization 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 with flow cytometry (Fig. S8).

In the medulla, and to a lesser extent in the cortex, large pyknic cells (Fig. 6a and b) of a size of *ca.* 17.5 × 15.5 μm (±3.2 × 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 and h) > Esr2a (Fig. 6k) > Esr1 (Fig. 6i and 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 × 19.7 μm (±9.1 × 4.2 s.d.) reminding of Hassall's Corpuscles (HCs) were observed with variable frequency between individuals (Fig. 7). Basically, three structural types could be distinguished: firstly, structures of a generally smaller size with a solid centre and eosinophilic staining (Fig. 7a and 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 and 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 (MCs), previously described in the thymic Ct of perciform fish (Mulero et al., 2007), were identified by eosinophilic staining and a size of 9.4 × 6.7 μm (±1.3 × 0.85 s.d.) (Fig. 4a and 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,

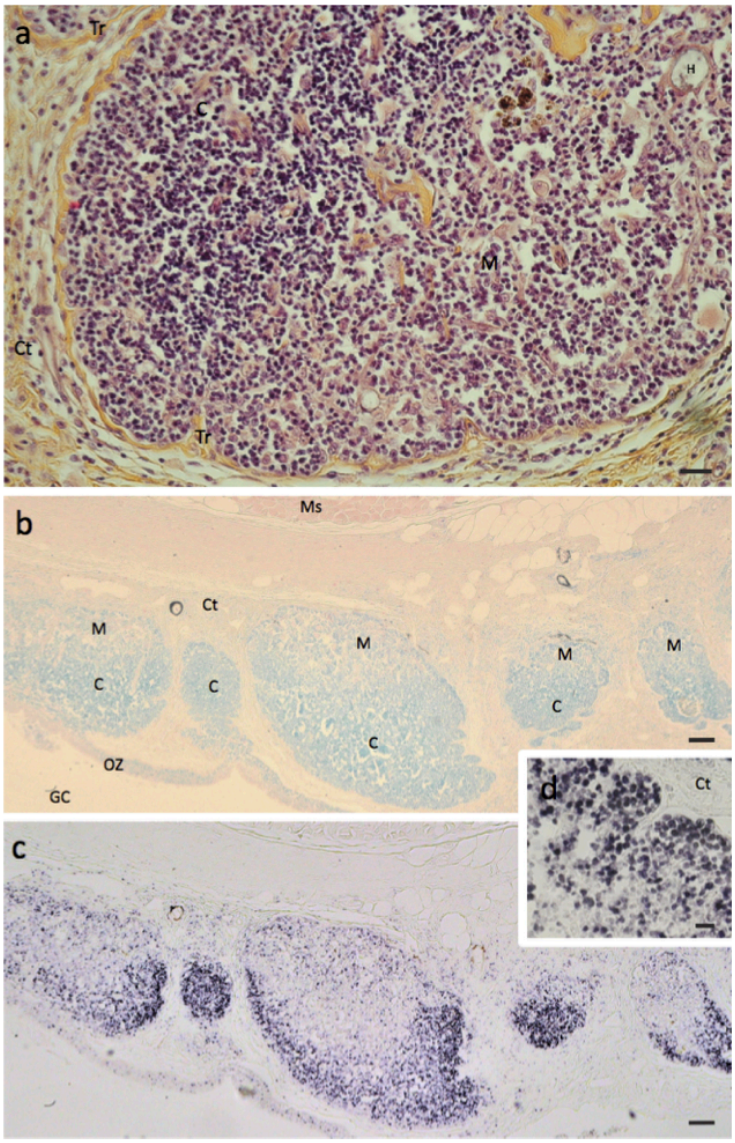


Fig. 2. Representative histological section of a thymus from adult *Dicentrarchus labrax* showing thymic lobules and their regionalization in the cortex (C) and medulla (M) with HES (a) and Pappenheim staining (b). The thymic parenchyma is partially encapsulated in a connective tissue (Ct) forming the 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, Ms: muscle, H: degenerated HC-like. Scale bar: 50 μm in a, b, c and 10 μm in d.

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 immunopositive 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 presents for the first time a concurrent expression of membrane and nuclear oestrogen

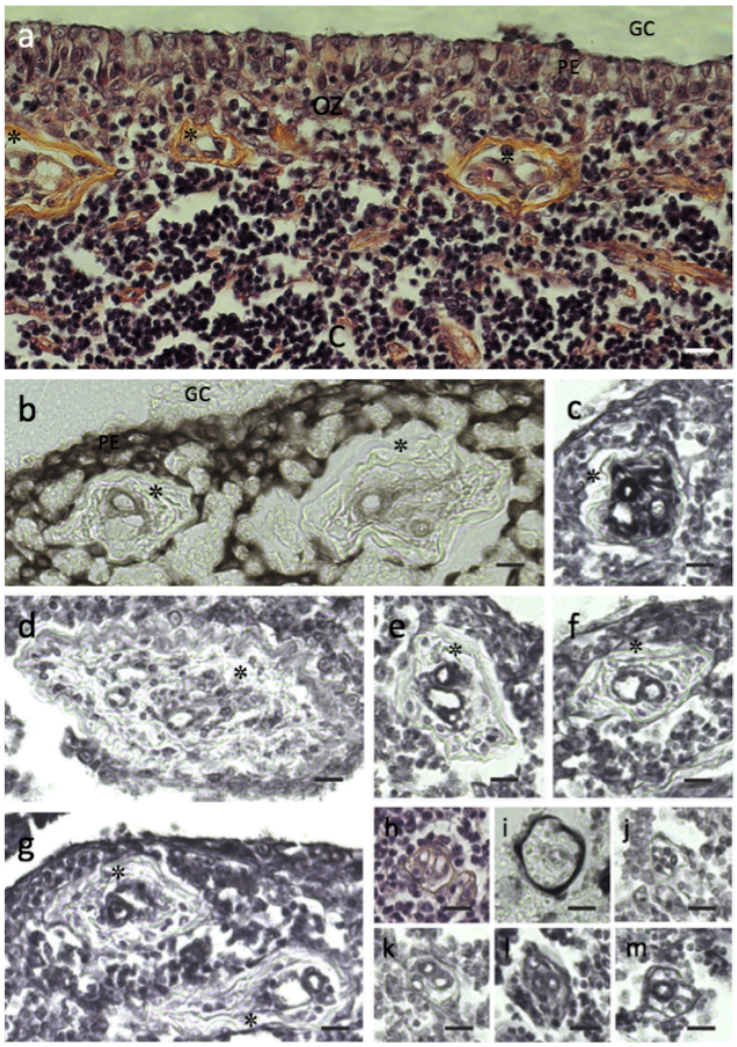


Fig. 3. Histological sections of the thymus of *Dicentrarchus labrax* representing the thymic perivascular system with HES staining (a, h) and IHC using anti-Ck (b, i), anti-S100 (c) and the five different antibodies against oestrogen receptors (d-g, j-m); for details see text. a, histological organisation 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 recognized 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.

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., 2017a) 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., 2017a) and the expression of *esr2* could also be retrieved in the thymus of human, rat, mouse and common carp (Mor et al., 2001; Mosselman et al., 1996; Szwejser et al., 2017a). 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., 2017a). However, for teleost *gper*-isoforms no expression data for a primary lymphoid organ were 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., 2017a). Together these data suggest a role of oestrogens in regulating thymus and T cell development.

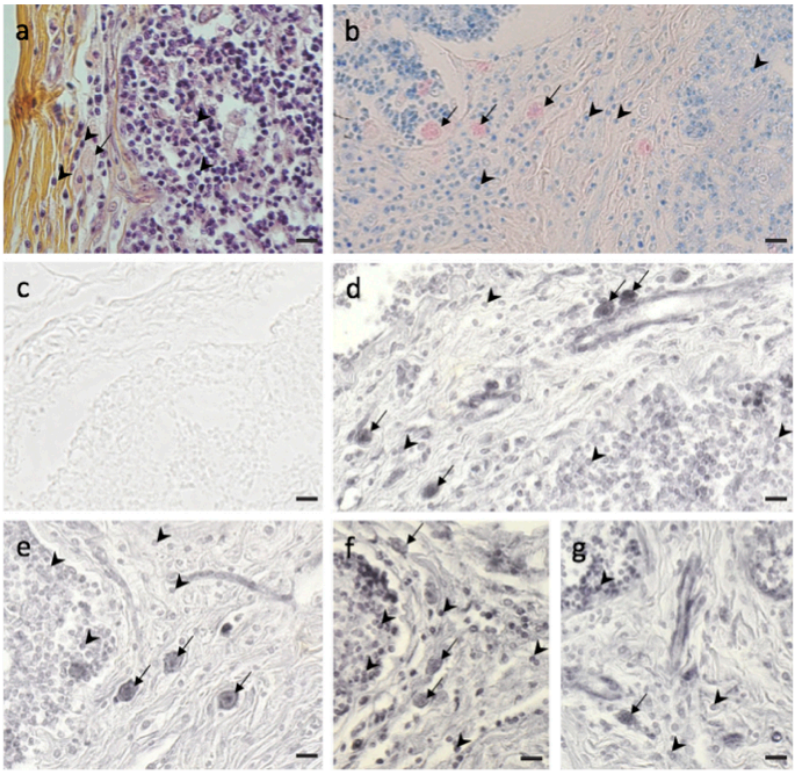


Fig. 4. Histological sections of the thymus of *Dicentrarchus labrax* stained with either HES (a) or Pappenheim (b), respectively showing mast cells with an eosinophilic staining (arrow) in the connective tissue and lymphoid cells (head-arrows) in the connective tissue (left) and in the thymic parenchyma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

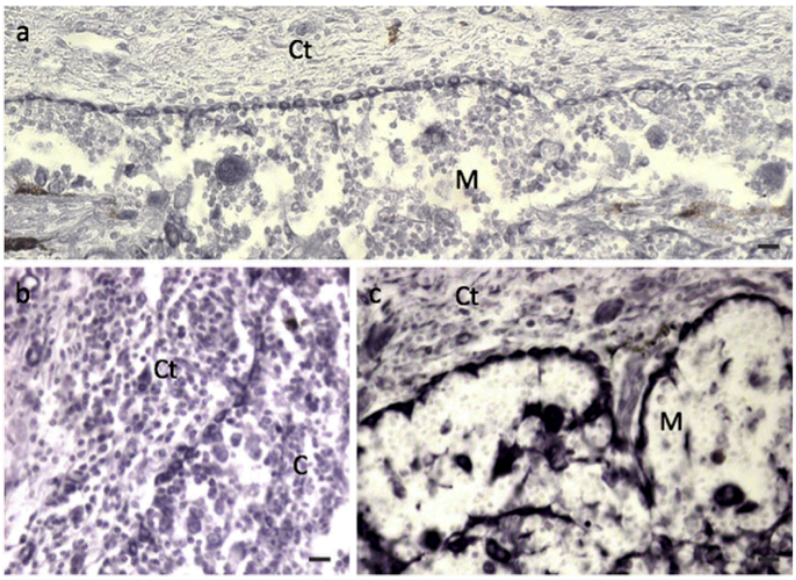


Fig. 5. IHC of histological sections of the thymus of *Dicentrarchus labrax* stained with anti-Gper (a), anti-Esr1 (b) and anti-Ck (c). Cells situated in the subcapsular zone between the connective tissue and the thymic parenchyma with the same morphology as the Ck-positive limiting epithelial cells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

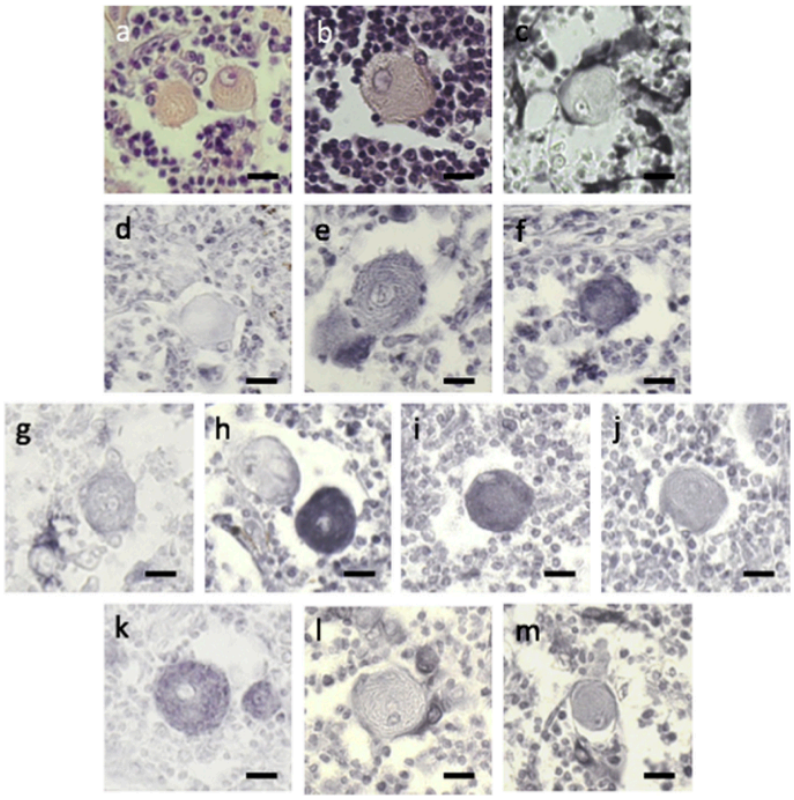


Fig. 6. Histological sections showing myoid cell-like structures in the thymus of *Dicentrarchus labrax*, as rounded cells with an eosinophilic staining with HES both in the medulla (a) and the cortex (b). IHC against Ck (c), S100 (d–f) and the five different oestrogen receptors (g–m) shows that Ck-negative myoid cell-like structures 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.

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 receptor transcripts and proteins were detected in peripheral blood and head-kidney leucocytes of several teleost species (Iwanowicz et al., 2014; Liarte et al., 2011b; Szwejser et al., 2017a). 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 the sea bass pan-T cell antibody (Romano et al., 1997; Scapigliati et al., 1995). We, therefore, conclude that T cells of sea bass express all the nuclear Esr and Gper isoforms. In mammals, mice and rats, 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 ESR1 to be present in thymocytes of chicken (Katayama et al., 2014).

Regarding Gper-isoform expression, the absence of the *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., 2017a). 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 (TECs), 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).

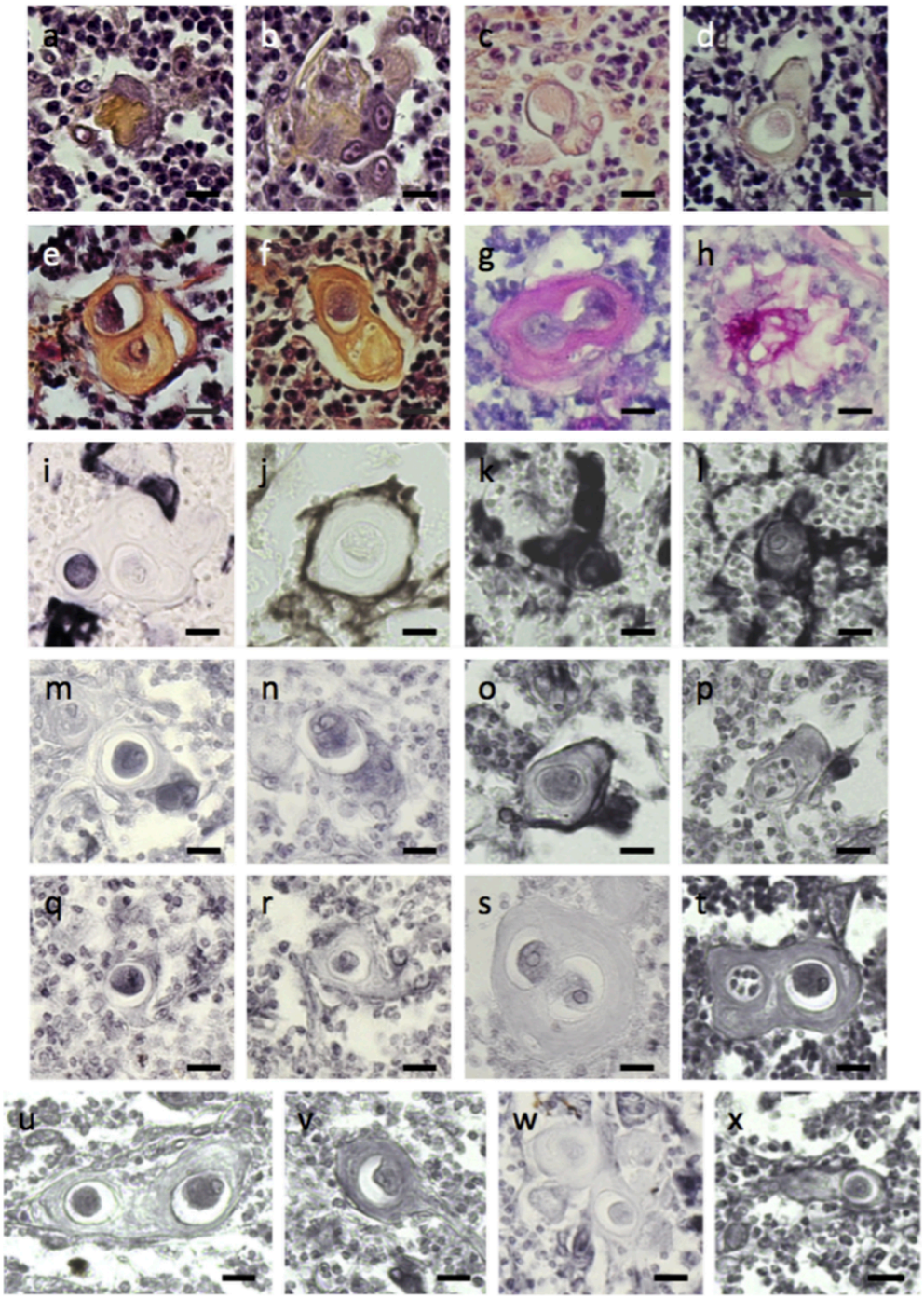


Fig. 7. Histological sections showing Hassall's corpuscle-like structures (HC-like) in the thymus of *Dicentrarchus labrax*, with HES (a–f) and PAS (g, h) staining, IHC using anti-Ck (g–j), anti-S100 (k–n) and five different anti-oestrogen receptors (o–y). a, b, immature HC-like with a solid centre; 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), Esr2*b* (t) and Gper (w, x). Scale bar: 10 μm.

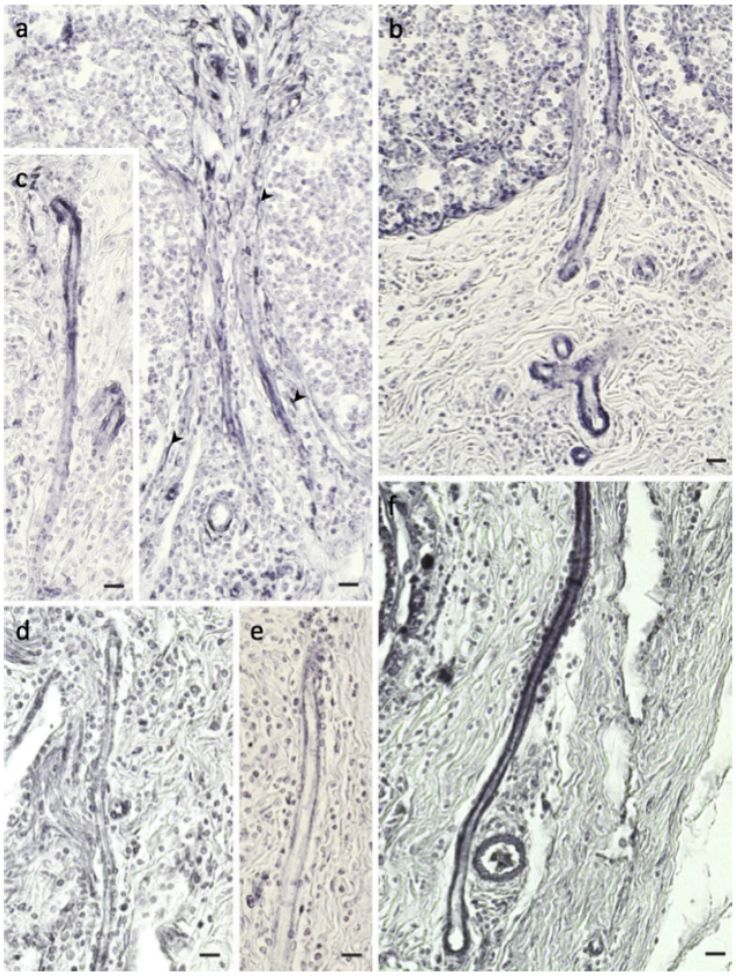


Fig. 8. Histological sections showing the vascular system of the thymus of *Dicentrarchus labrax*; IHC against S100 (a, c) and the five oestrogen receptors (d–f). S100 immunostaining shows S100-positive vessels inside the trabeculae (a, c) formed by S100 positive fibroblasts (arrowheads). IHC against 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 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, 1997
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 and Rat Chicken (ESR1)	Seiki and Sakabe, 1997, 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.

In vitro and *in vivo* studies have shown that oestrogen inhibits thymosin α-1 and the SDF-1α/CXCL12 production in cortical TECs, 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 indicated a high organ activity in T cell formation. Although, the functional significance of MyCs is still uncertain (reviewed by Zapata et al., 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 localization in the medulla, one may assume that oestrogenic regulation of MyCs 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 HCs described in human foetuses and new-born 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 humans is not fully elucidated and remains controversial. In sea bass, as in other vertebrates, HC-like immunoreactivity (CK and S100) indicates that TECs and MyCs are likely to be responsible for HC-like formation (Bodey and Kaiser, 1996). Our study demonstrated immunostaining of all oestrogen receptor 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 mice (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 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. (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 MCs in the testis of lizards, the proliferation and the differentiation of MCs in the gonad of toads and frogs (Lutton and Callard, 2006) and the number of MCs 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 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 MCs) 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 MCs or MyCs) in teleosts that call for further investigation of their role in thymus function.

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.

Acknowledgements

This research was supported by the ANR financed project ETaT (ANR-15-CE32-0014). M.P. was supported by FR CNRS 3730 SCALE. The authors thank Matthieu Bonnet and the staff at Aquacaux for fish hatchery. We would like to thank Salima Aroua, Sabrina Jolly, Stéphanie Olivier, Agnès Poret and Béatrice Rocher for their assistance throughout the exposures and samplings. Special thanks are due to Helmut Segner for help with the manuscript and Adelino Canário for producing and providing the anti-Esr1 and anti-Esr2a antisera.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2017.07.023.

References

Attia, H.F., El-Zoghby, I.M.A., Hussein, M.N., Bakry, H.H., 2010. Seasonal changes in the thymus gland of *Tilapia nilotica* fish. Minufiya Vet. J. 7.

Avilés-Trigueros, M., 1993. Identificación y organización de los componentes celulares del timo de lubina (*Dicentrarchus labrax*) (PhD thesis). Universidad de Murcia.

Bajoghli, B., Aghaallaei, N., Hess, I., Rode, I., Netuschil, N., Tay, B.-H., Venkatesh, B., Yu, J.-K., Kaltenbach, S.L., Holland, N.D., Diekhoff, D., Happe, C., Schorpp, M., Boehm, T., 2009. Evolution of genetic networks underlying the emergence of thymopoiesis in vertebrates. Cell 138, 186–197. http://dx.doi.org/10.1016/j.cell.2009.04.017.

Bajoghli, B., Kuri, P., Inoue, D., Aghaallaei, N., Hanelt, M., Thumberger, T., Rauzi, M., Wittbrodt, J., Leptin, M., 2015. Noninvasive *in toto* imaging of the thymus reveals heterogeneous migratory behavior of developing T cells. J. Immunol. 195, 2177–2186. http://dx.doi.org/10.4049/jimmunol.1500361.

Bernardi, A.I., Andersson, A., Stubelius, A., Grahnemo, L., Carlsten, H., Islander, U., 2015. Selective estrogen receptor modulators in T cell development and T cell dependent inflammation. Immunobiology 220, 1122–1128. http://dx.doi.org/10.1016/j.imbio.2015.05.009.

Bodey, B., Kaiser, H.E., 1996. Development of Hassall's bodies of the thymus in humans and other vertebrates (especially mammals) under physiological and pathological conditions: immunocytochemical, electronmicroscopic and *in vitro* observations. Vivo Athens Greece 11, 61–85.

Boehm, T., Hess, I., Swann, J.B., 2012. Evolution of lymphoid tissues. Trends Immunol. 33, 315–321. http://dx.doi.org/10.1016/j.it.2012.02.005. Special focus: Structure and function of lymphoid tissues.

Boehm, T., Swann, J.B., 2014. Origin and evolution of adaptive immunity. Annu. Rev. Anim. Biosci. 2, 259–283. http://dx.doi.org/10.1146/annurev-animal-022513-114201.

Bornemann, A., Kirchner, T., 1998. Thymic myoid cell turnover in myasthenia gravis patients and in normal controls. Virchows Arch. 432, 357–361. http://dx.doi.org/10.1007/s004280050178.

Bowden, T.J., Cook, P., Rombout, J.H.W.M., 2005. Development and function of the thymus in teleosts. Fish. Shellfish Immunol. Fish. Larval Immun. 19, 413–427. http://dx.doi.org/10.1016/j.fsi.2005.02.003.

Burgos-Aceves, M.A., Cohen, A., Smith, Y., Faggio, C., 2016. Estrogen regulation of gene expression in the teleost fish immune system. Fish. Shellfish Immunol. 58, 42–49. http://dx.doi.org/10.1016/j.fsi.2016.09.006.

Cabas, I., Rodenas, M.C., Abellan, E., Meseguer, J., Mulero, V., Garcia-Ayala, A., 2013. Estrogen signaling through the G protein-coupled estrogen receptor regulates granulocyte activation in fish. J. Immunol. 191, 4628–4639. http://dx.doi.org/10.4049/jimmunol.1301613.

Cao, J., Chen, Q., Lu, M., Hu, X., Wang, M., 2017. Histology and ultrastructure of the thymus during development in tilapia, *Oreochromis niloticus*. J. Anat. 230, 720–733. http://dx.doi.org/10.1111/joa.12597.

Castillo, A., López-Fierro, P., Zapata, A., Villena, A., Razquin, B., 1991. Post-hatching development of the thymic epithelial cells in the rainbow trout *Salmo gairdneri*: an ultrastructural study. Am. J. Anat. 190, 299–307. http://dx.doi.org/10.1002/aja.1001900310.

Chapman, J.C., Chapman, F.M., Michael, S.D., 2015. The production of alpha/beta and gamma/delta double negative (DN) T-cells and their role in the maintenance of pregnancy. Reprod. Biol. Endocrinol. 13 http://dx.doi.org/10.1186/s12958-015-0073-5.

Chilmonczyk, S., 1983. The thymus of the rainbow trout (*Salmo gairdneri*) light and electron microscopic study. Dev. Comp. Immunol. 7, 59–68. http://dx.doi.org/10.1016/0145-305X(83)90055-1.

Cooper, M.D., Alder, M.N., 2006. The evolution of adaptive immune systems. Cell 124, 815–822. http://dx.doi.org/10.1016/j.cell.2006.02.001.

Crivellato, E., Nico, B., Battistig, M., Beltrami, C.A., Ribatti, D., 2005. The thymus is a site of mast cell development in chicken embryos. Anat. Embryol. (Berl.) 209, 243–249. http://dx.doi.org/10.1007/s00429-004-0439-5.

Danel, L., Souweine, G., Monier, J.C., Saez, S., 1983. Specific estrogen binding sites in human lymphoid cells and thymic cells. J. Steroid Biochem. 18, 559–563. http://dx.doi.org/10.1016/0022-4731(83)90131-0.

DeWitt, J.C., Peden-Adams, M.M., Keil, D.E., Dietert, R.R., 2012. Current status of developmental immunotoxicity: early-life patterns and testing. Toxicol. Pathol. 40, 230–236. http://dx.doi.org/10.1177/0192623311427709.

Do, Y., Ryu, S., Nagarkatti, M., Nagarkatti, P.S., 2002. Role of death receptor pathway in estradiol-induced t-cell apoptosis *in vivo*. Toxicol. Sci. 70, 63–72. http://dx.doi.org/10.1093/toxsci/70.1.63.

Ebbesen, P., Christensen, H.E., 1972. Foa-Kurloff cells in the Hassal bodies of oestrogenized Guinea-pigs. Acta Pathol. Microbiol. Scand. [A] 80, 600–602. http://dx.doi.org/10.1111/j.1699-0463.1972.tb00321.x.

Erlandsson, M.C., Ohlsson, C., Gustafsson, J.-A., Carlsten, H., 2001. Role of oestrogen receptors α and β in immune organ development and in oestrogen-mediated effects on thymus. Immunology 103, 17–25. http://dx.doi.org/10.1046/j.1365-2567.2001.01212.x.

Esteban, M.Á., Cuesta, A., Chaves-Pozo, E., Meseguer, J., 2015. Phagocytosis in teleosts. Implications of the new cells involved. Biology 4, 907–922. http://dx.doi.org/10.3390/biology4040907.

Forsberg, J.-G., 1996. The different responses of the female mouse thymus to estrogen after treatment of neonatal, prepubertal, and adult animals. Cells Tissues Organs 157, 275–290. http://dx.doi.org/10.1159/000147890.

Franchini, A., Ottaviani, E., 1999. Immunoreactive POMC-derived peptides and cytokines in the chicken thymus and bursa of Fabricius microenvironments: age-related changes. J. Neuroendocrinol. 11, 685–692. http://dx.doi.org/10.1046/j.1365-2826.1999.00385.x.

Galindo-Villegas, J., Garcia-Garcia, E., Mulero, V., 2016. Role of histamine in the regulation of intestinal immunity in fish. Dev. Comp. Immunol. Spec. Issue Intest. Immun. 64, 178–186. http://dx.doi.org/10.1016/j.dci.2016.02.013.

Glucksmann, A., Cherry, C.P., 1968. The effect of castration, oestrogens, testosterone and the oestrous cycle on the cortical epithelium of the thymus in male and female rats. J. Anat. 103, 113–133.

Gorgollon, P., 1983. Fine structure of the thymus in the adult cling fish *Sicyases sanguineus* (Pisces, Gobiesocidae). J. Morphol. 177, 25–40. http://dx.doi.org/10.1002/jmor.1051770103.

Gould, K.A., Shull, J.D., Gorski, J., 2000. DES action in the thymus: inhibition of cell proliferation and genetic variation. Mol. Cell. Endocrinol. 170, 31–39. http://dx.doi.org/10.1016/S0303-7207(00)00336-1.

Gulino, A., Screpanti, I., Torrisi, M.R., Frati, L., 1985. Estrogen receptors and estrogen sensitivity of fetal thymocytes are restricted to blast lymphoid cells*. Endocrinology 117, 47–54. http://dx.doi.org/10.1210/endo-117-1-47.

He, W., Sakabe, K., Sakai, N., Seiki, K., 1996. Sex steroid modulation of thymic hormone production in thymus epithelial cell culture. Pathophysiology 3, 169–174. http://dx.doi.org/10.1016/0928-4680(96)00011-9.

Hince, M., Sakkal, S., Vlahos, K., Dudakov, J., Boyd, R., Chidgey, A., 2008. The role of sex steroids and gonadectomy in the control of thymic involution. Cell. Immunol. 252, 122–138. http://dx.doi.org/10.1016/j.cellimm.2007.10.007.

Iwanowicz, L.R., Stafford, J.L., Patiño, R., Bengten, E., Miller, N.W., Blazer, V.S., 2014. Channel catfish (*Ictalurus punctatus*) leukocytes express estrogen receptor isoforms ERα and ERβ2 and are functionally modulated by estrogens. Fish. Shellfish Immunol. 40, 109–119. http://dx.doi.org/10.1016/j.fsi.2014.06.021.

Jin, C., Fu, W.-X., Xie, L.-P., Qian, X.-P., Chen, W.-F., 2003. SDF-1α production is negatively regulated by mouse estrogen enhanced transcript in a mouse thymus epithelial cell line. Cell. Immunol. 223, 26–34. http://dx.doi.org/10.1016/S0008-8749(03)00152-7.

Kamo, I., Tada-Kikuchi, A., Furukawa, S., Iwasaki, Y., Nonaka, I., Satoyoshi, E., Itoh, T., 1985. Effects of thymic myoid cell culture supernatant on cells from lymphatic tissues. Cell. Immunol. 94, 587–597. http://dx.doi.org/10.1016/0008-8749(85)90282-5.

Kannan, T.A., Ramesh, G., Ushakumary, S., Dhinakarraj, G., Vairamuthu, S., 2015. Thymic Hassall's corpuscles in Nandanam chicken - light and electronmicroscopic perspective (*Gallus domesticus*). J. Anim. Sci. Technol. 57 http://dx.doi.org/10.1186/s40781-015-0064-2.

Katayama, M., Fukuda, T., Hatabu, T., Narabara, K., Abe, A., Kondo, Y., 2014. Changes in estrogen receptor expression in the chick thymus during late embryonic development. Anim. Sci. J. 85, 277–285. http://dx.doi.org/10.1111/asj.12114.

Kawashima, I., Sakabe, K., Akatsuka, A., Seiki, K., 1995. Effects of estrogen on female mouse thymus, with special reference to ER-mRNA and T cell subpopulations. Pathophysiology 2, 235–241. http://dx.doi.org/10.1016/0928-4680(95)00047-2.

Klein, S.L., Flanagan, K.L., 2016. Sex differences in immune responses. Nat. Rev. Immunol. http://dx.doi.org/10.1038/nri.2016.90.

Kondo, Y., Goto, C., Abe, A., 2004. Effects of estrogen treatment during the embryogenic period on chick antibody production. J. Poult. Sci. 41, 85–93.

Laan, M., Haljasorg, U., Kisand, K., Salumets, A., Peterson, P., 2016. Pregnancy-induced thymic involution is associated with suppression of chemokines essential for T-lymphoid progenitor homing. Eur. J. Immunol. 46, 2008–2017. http://dx.doi.org/10.1002/eji.201646309.

Lafont, A.-G., Rousseau, K., Tomkiewicz, J., Dufour, S., 2016. Three nuclear and two membrane estrogen receptors in basal teleosts, *Anguilla sp.*: identification, evolutionary history and differential expression regulation. Gen. Comp. Endocrinol. 235, 177–191. http://dx.doi.org/10.1016/j.ygcen.2015.11.021.

Langenau, D.M., Zon, L.I., 2005. The zebrafish: a new model of T-cell and thymic development. Nat. Rev. Immunol. 5, 307–317. http://dx.doi.org/10.1038/nri1590.

Le Panse, R., Berrih-Aknin, S., 2005. Thymic myoid cells protect thymocytes from apoptosis and modulate their differentiation: implication of the ERK and Akt signaling pathways. Cell Death Differ. 12, 463–472. http://dx.doi.org/10.1038/sj.cdd.4401611.

Liarte, S., Cabas, I., Chaves-Pozo, E., Arizcun, M., Meseguer, J., Mulero, V., García-Ayala, A., 2011a. Natural and synthetic estrogens modulate the inflammatory response in the gilthead seabream (*Sparus aurata* L.) through the activation of endothelial cells. Mol. Immunol. 48, 1917–1925. http://dx.doi.org/10.1016/j.molimm.2011.05.019.

Liarte, S., Chaves-Pozo, E., Abellán, E., Meseguer, J., Mulero, V., García-Ayala, A., 2011b. 17β-Estradiol regulates gilthead seabream professional phagocyte responses through macrophage activation. Dev. Comp. Immunol. 35, 19–27. http://dx.doi.org/10.1016/j.dci.2010.07.007.

Lutton, B., Callard, I., 2006. Evolution of reproductive–immune interactions. Integr. Comp. Biol. 46, 1060–1071. http://dx.doi.org/10.1093/icb/icl050.

Martín, A., Casares, F., Alonso, L., Nieuwenhuis, P., Vicente, A., Zapata, A.G., 1995. Changes in the blood-thymus barrier of adult rats after estradiol-treatment. Immunobiology 192, 231–248. http://dx.doi.org/10.1016/S0171-2985(11)80100-2.

Massart, S., Milla, S., Kestemont, P., 2014. Expression of gene, protein and immunohistochemical localization of the estrogen receptor isoform ERα1 in male rainbow trout lymphoid organs; indication of the role of estrogens in the regulation of immune mechanisms. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 174, 53–61. http://dx.doi.org/10.1016/j.cbpb.2014.06.001.

Mor, G., Munoz, A., Redlinger Jr., R., Silva, I., Song, J., Lim, C., Kohen, F., 2001. The role of the Fas/Fas ligand system in estrogen-induced thymic alteration. Am. J. Reprod. Immunol. 46, 298–307. http://dx.doi.org/10.1034/j.1600-0897.2001.d01-16.x.

Moreno, A.M., Zapata, A.G., 1991. *In situ* effects of estrogens on the stroma of rat thymus. Lymphat 81–87. Tissues Vivo Immune Responses Dekker N. Y.

Mosselman, S., Polman, J., Dijkema, R., 1996. ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett. 392, 49–53. http://dx.doi.org/10.1016/0014-5793(96)00782-X.

Mulero, I., Sepulcre, M.P., Meseguer, J., García-Ayala, A., Mulero, V., 2007. Histamine is stored in mast cells of most evolutionarily advanced fish and regulates the fish inflammatory response. Proc. Natl. Acad. Sci. 104, 19434–19439.

Nakanishi, T., Shibasaki, Y., Matsuura, Y., 2015. T cells in fish. Biology 4, 640–663. http://dx.doi.org/10.3390/biology4040640.

Nancy, P., Berrih-Aknin, S., 2005. Differential estrogen receptor expression in autoimmune *myasthenia gravis*. Endocrinology 146, 2345–2353. http://dx.doi.org/10.1210/en.2004-1003.

Narita, S., Goldblum, R.M., Watson, C.S., Brooks, E.G., Estes, D.M., Curran, E.M., Midoro-Horiuti, T., 2006. Environmental estrogens induce mast cell degranulation and enhance IgE-mediated release of allergic mediators. Environ. Health Perspect. 115, 48–52. http://dx.doi.org/10.1289/ehp.9378.

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15–24. http://dx.doi.org/10.1016/j.ygcen.2013.03.032, 7th International Symposium on Fish Endocrinology.

Okasha, S.A., Ryu, S., Do, Y., McKallip, R.J., Nagarkatti, M., Nagarkatti, P.S., 2001. Evidence for estradiol-induced apoptosis and dysregulated T cell maturation in the thymus. Toxicology 163, 49–62. http://dx.doi.org/10.1016/S0300-483X(01)00374-2.

Olde, B., Leeb-Lundberg, L.M.F., 2009. GPR30/GPER1: searching for a role in estrogen physiology. Trends Endocrinol. Metab. 20, 409–416. http://dx.doi.org/10.1016/j.tem.2009.04.006.

O'Neill, J.G., 1989. Thymic development in two species of marine teleost; an Antarctic silverfish, *Pleuragramma antarcticum* Boulenger, and a warmer-water sea bass, *Dicentrarchus labrax* (LINNAEUS). Proc. NIPR Symp Polar Biol. 2, 83–93.

Öner, H., Ozan, E., 2002. Effects of gonadal hormones on thymus gland after bilateral ovariectomy and orchidectomy in rats. Arch. Androl. 48, 115–126. http://dx.doi.org/10.1080/01485010231726742 7.

Picchietti, S., Abelli, L., Guerra, L., Randelli, E., Proietti Serafini, F., Belardinelli, M.C., Buonocore, F., Bernini, C., Fausto, A.M., Scapigliati, G., 2015. MHC II-β chain gene expression studies define the regional organization of the thymus in the developing bony fish *Dicentrarchus labrax* (L.). Fish. Shellfish Immunol. 42, 483–493. http://dx.doi.org/10.1016/j.fsi.2014.11.012.

Picchietti, S., Guerra, L., Buonocore, F., Randelli, E., Fausto, A.M., Abelli, L., 2009. Lymphocyte differentiation in sea bass thymus: CD4 and CD8-α gene expression studies. Fish. Shellfish Immunol. 27, 50–56. http://dx.doi.org/10.1016/j.fsi.2009.04.003.

Picchietti, S., Guerra, L., Selleri, L., Buonocore, F., Abelli, L., Scapigliati, G., Mazzini, M., Fausto, A.M., 2008. Compartmentalisation of T cells expressing CD8α and TCRβ in developing thymus of sea bass *Dicentrarchus labrax* (L.). Dev. Comp. Immunol. 32, 92–99. http://dx.doi.org/10.1016/j.dci.2007.04.002.

Pinto, P., Estêvão, M., Power, D., 2014. Effects of estrogens and estrogenic disrupting compounds on fish mineralized tissues. Mar. Drugs 12, 4474–4494. http://dx.doi.org/10.3390/md12084474.

Pinto, P.I.S., Estêvão, M.D., Andrade, A., Santos, S., Power, D.M., 2016. Tissue responsiveness to estradiol and genistein in the sea bass liver and scale. J. Steroid Biochem. Mol. Biol. 158, 127–137. http://dx.doi.org/10.1016/j.jsbmb.2015.12.023.

Pinto, P.I.S., Estêvão, M.D., Redruello, B., Socorro, S.M., Canário, A.V.M., Power, D.M., 2009. Immunohistochemical detection of estrogen receptors in fish scales. Gen. Comp. Endocrinol. 160, 19–29. http://dx.doi.org/10.1016/j.ygcen.2008.10.011.

Plotkin, J., Prockop, S.E., Lepique, A., Petrie, H.T., 2003. Critical role for CXCR4 signaling in progenitor Localization and T Cell differentiation in the postnatal thymus. J. Immunol. 171, 4521–4527. http://dx.doi.org/10.4049/jimmunol.171.9.4521.

Prossnitz, E.R., Barton, M., 2014. Estrogen biology: new insights into GPER function and clinical opportunities. Mol. Cell. Endocrinol. 389, 71–83. http://dx.doi.org/10.1016/j.mce.2014.02.002.

Raica, M., Cîmpean, A.M., Nico, B., Guidolin, D., Ribatti, D., 2010. A comparative study of the spatial distribution of mast cells and microvessels in the foetal, adult human thymus and thymoma. Int. J. Exp. Pathol. 91, 17–23. http://dx.doi.org/10.1111/j.1365-2613.2009.00689.x.

Raica, M., Encică, S., Motoc, A., Cîmpean, A.M., Scridon, T., Bârsan, M., 2006. Structural heterogeneity and immunohistochemical profile of Hassall corpuscles in normal human thymus. Ann. Anat. - Anat. Anz 188, 345–352. http://dx.doi.org/10.1016/j.aanat.2006.01.012.

Ribatti, D., Crivellato, E., 2016. The role of mast cell in tissue morphogenesis. Thymus, duodenum, and mammary gland as examples. Exp. Cell Res. 341, 105–109. http://dx.doi.org/10.1016/j.yexcr.2015.11.022.

Rijhsinghani, A.G., Thompson, K., Bhatia, S.K., Waldschmidt, T.J., 1996. Estrogen blocks early T cell development in the thymus. Am. J. Reprod. Immunol. N. Y. N. 1989 (36), 269–277. http://dx.doi.org/10.1111/j.1600-0897.1996.tb00176.x.

Rodenas, M.C., Tamassia, N., Cabas, I., Calzetti, F., Meseguer, J., Cassatella, M.A., García-Ayala, A., Mulero, V., 2017. G protein-coupled estrogen receptor 1 regulates human neutrophil functions. Biomed. Hub. 2 http://dx.doi.org/10.1159/000454981, 2–2.

Romano, N., Baldassini, M.R., Terribili, F.R., Abelli, L., Mastrolia, L., Mazzini, M., 1997. Histological observations on lymphomyeloid organs of the Antarctic fish *Trematomus bernacchii* (Teleostei: nototheniidae). Polar Biol. 18, 358–362. http://dx.doi.org/10.1007/s003000050200.

Romano, N., Taverne-Thiele, A.J., Fanelli, M., Baldassini, M.R., Abelli, L., Mastrolia, L., Van Muiswinkel, W.B., Rombout, J.H.M.W., 1999. Ontogeny of the thymus in a teleost fish, *Cyprinus carpio* L.: developing thymocytes in the epithelial microenvironment. Dev. Comp. Immunol. 23, 123–137. http://dx.doi.org/10.1016/S0145-305X(98)00053-6.

Ross, M.A., Korenchevsky, V., 1941. The thymus of the rat and sex hormones. J. Pathol. Bacteriol. 52, 349–360.

Sailendri, K., Muthukkaruppan, V., 1975. Morphology of lymphoid organs in a cichlid teleost, *Tilapia mossambica* (Peters). J. Morphol. 147, 109–121. http://dx.doi.org/10.1002/jmor.1051470108.

Sakabe, K., Seiki, K., Kawashima, I., Urano, R., Akatsuka, A., Nakano, M., 1993. Expression of estrogen receptor cells, with special reference to thymulin (FTS)-producing cells in human fetal thymus during mid-pregnancy. Med. Sci. Res. 21, 337–341.

Savino, W., Bartoccioni, E., Homo-Delarche, F., Gagnerault, M.C., Itoh, T., Dardenne, M., 1988. Thymic hormone containing cells—IX. Steroids in vitro modulate thymulin secretion by human and murine thymic epithelial cells. J. Steroid Biochem. 30, 479–484. http://dx.doi.org/10.1016/0022-4731(88)90148-3.

Scapigliati, G., Mazzini, M., Mastrolia, L., Romano, N., Abelli, L., 1995. Production and characterisation of a monoclonal antibody against the thymocytes of the sea bass *Dicentrarchus labrax* (L.)(Teleostea, Percicthydae). Fish. Shellfish Immunol. 5, 393–405. http://dx.doi.org/10.1006/fsim.1995.0039.

Schneider, A.E., Kárpáti, É., Schuszter, K., Tóth, E.A., Kiss, E., Kulcsár, M., László, G., Matko, J., 2014. A dynamic network of estrogen receptors in murine lymphocytes: fine-tuning the immune response. J. Leukoc. Biol. 96, 857–872. http://dx.doi.org/10.1189/jlb.2A0214-080RR.

Screpanti, I., Meco, D., Morrone, S., Gulino, A., Mathieson, B.J., Frati, L., 1991. *In Vivo* modulation of the distribution of thymocyte subsets: effects of estrogen on the expression of different T cell receptor Vβ gene families in CD4−, CD8− thymocytes. Cell. Immunol. 134, 414–426. http://dx.doi.org/10.1016/0008-8749(91)90314-2.

Seemann, F., Knigge, T., Duflot, A., Marie, S., Olivier, S., Minier, C., Monsinjon, T., 2016. Sensitive periods for 17β-estradiol exposure during immune system development in sea bass head kidney: sensitivity of sea bass head kidney development to E2. J. Appl. Toxicol. 36, 815–826. http://dx.doi.org/10.1002/jat.3215.

Seemann, F., Knigge, T., Olivier, S., Monsinjon, T., 2015. Exogenous 17-β-oestradiol (E2) modifies thymus growth and regionalization in European sea bass *Dicentrarchus labrax*. J. Fish. Biol. 86, 1186–1198. http://dx.doi.org/10.1111/jfb.12626.

Segner, H., Verburg-van Kemenade, B.M.L., Chadzinska, M., 2017. The immunomodulatory role of the hypothalamus-pituitary-gonad axis: proximate mechanism for reproduction-immune trade offs? Dev. Comp. Immunol. 66, 43–60. http://dx.doi.org/10.1016/j.dci.2016.07.004.

Seiki, K., Sakabe, K., 1997. Sex hormones and the thymus in relation to thymocyte

proliferation and maturation. Arch. Histol. Cytol. 60, 29–38.
Selvaraj, P., Pitchappan, R.M., 1985. Effect of oestradiol dipropionate on the immune system of the pigeon, Columba livia. Dev. Comp. Immunol. 9, 669–677. http://dx.doi.org/10.1016/0145-305X(85)90031-X.
Sfacteria, A., Brines, M., Blank, U., 2015. The mast cell plays a central role in the immune system of teleost fish. Mol. Immunol. 63, 3–8. http://dx.doi.org/10.1016/j.molimm.2014.02.007.
Staples, J.E., Gasiewicz, T.A., Fiore, N.C., Lubahn, D.B., Korach, K.S., Silverstone, A.E., 1999. Estrogen receptor alpha is necessary in thymic development and estradiol-induced thymic alterations. J. Immunol. Balt. Md 1950 (163), 4168–4174.
Sufi, B.G., Mori, K., Nomura, I., 1980. Involution of thymus in relation to sexual and steroid hormone treatments in salmonid fish. Tohoku J. Agri. Res. 31, 97–105.
Szwejser, E., Maciuszek, M., Casanova-Nakayama, A., Segner, H., Verburg-van Kemenade, B.M.L., Chadzinska, M., 2017a. A role for multiple estrogen receptors in immune regulation of common carp. Dev. Comp. Immunol. 66, 61–72. http://dx.doi.org/10.1016/j.dci.2016.04.003.
Szwejser, E., Verburg-van Kemenade, B.M.L., Maciuszek, M., Chadzinska, M., 2017b. Estrogen-dependent seasonal adaptations in the immune response of fish. Horm. Behav. 88, 15–24. http://dx.doi.org/10.1016/j.yhbeh.2016.10.007.
Tamaki, M., Konno, Y., Kobayashi, Y., Takeda, M., Itoga, M., Moritoki, Y., Oyamada, H., Kayaba, H., Chihara, J., Ueki, S., 2014. Expression and functional roles of G-protein-coupled estrogen receptor (GPER) in human eosinophils. Immunol. Lett. 160, 72–78. http://dx.doi.org/10.1016/j.imlet.2014.03.012.
Tatner, M.F., 1996. Natural changes in the immune system of fish. In: The Fish Immune System: Organism, Pathogen, and Environment, pp. 255–287.
Usselman, C.W., Stachenfeld, N.S., Bender, J.R., 2016. The molecular actions of oestrogen in the regulation of vascular health: oestrogen and vascular health. Exp. Physiol. 101, 356–361. http://dx.doi.org/10.1113/EP085148.
van de Wijngaert, F.P., Kendall, M.D., Schuurman, H.-J., Rademakers, L.H., Kater, L., 1984. Heterogeneity of epithelial cells in the human thymus. Cell Tissue Res. 237, 227–237. http://dx.doi.org/10.1007/BF00217140.
Wang, C., Dehghani, B., Magrisso, I.J., Rick, E.A., Bonhomme, E., Cody, D.B., Elenich, L.A., Subramanian, S., Murphy, S.J., Kelly, M.J., Rosenbaum, J.S., Vandenbark, A.A., Offner, H., 2008. GPR30 contributes to estrogen-induced thymic atrophy. Mol. Endocrinol. 22, 636–648. http://dx.doi.org/10.1210/me.2007-0359.
Watanabe, N., Wang, Y.-H., Lee, H.K., Ito, T., Wang, Y.-H., Cao, W., Liu, Y.-J., 2005. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. Nature 436, 1181–1185. http://dx.doi.org/10.1038/nature03886.
Yonezawa, Y., Shin, Y., Abe, A., Kondo, Y., 2008. Estrogen receptor alpha expression in the thymus of chick embryos during late stages of embryogenesis. Anim. Sci. J. 79, 270–273. http://dx.doi.org/10.1111/j.1740-0929.2008.00526.x.
Yurchinskij, V.J., 2016. Age-related morphological changes in Hassall's corpuscles of different maturity in vertebrate animals and humans. Adv. Gerontol. 6, 117–122. http://dx.doi.org/10.1134/S2079057016020156.
Zaitsu, M., Narita, S.-I., Lambert, K.C., Grady, J.J., Estes, D.M., Curran, E.M., Brooks, E.G., Watson, C.S., Goldblum, R.M., Midoro-Horiuti, T., 2007. Estradiol activates mast cells *via* a non-genomic estrogen receptor-α and calcium influx. Mol. Immunol. 44, 1977–1985. http://dx.doi.org/10.1016/j.molimm.2006.09.030.
Zapata, A.G., Chiba, A., Varas, A., 1996. Cell and tissues of the immune system of fish. In: The Fish Immune System: Organism, Pathogen, and Environment, pp. 255–287.
Zimmer, D.B., Cornwall, E.H., Landar, A., Song, W., 1995. The S100 protein family: history, function, and expression. Brain Res. Bull. 37, 417–429. http://dx.doi.org/10.1016/0361-9230(95)00040-2.
Zoller, A.L., Kersh, G.J., 2006. Estrogen induces thymic atrophy by eliminating early thymic progenitors and inhibiting proliferation of -selected thymocytes. J. Immunol. 176, 7371–7378. http://dx.doi.org/10.4049/jimmunol.176.12.7371.
Zoller, A.L., Schnell, F.J., Kersh, G.J., 2007. Murine pregnancy leads to reduced proliferation of maternal thymocytes and decreased thymic emigration. Immunology 121, 207–215. http://dx.doi.org/10.1111/j.1365-2567.2006.02559.x.

Supplementary materials:

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
```

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
```

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

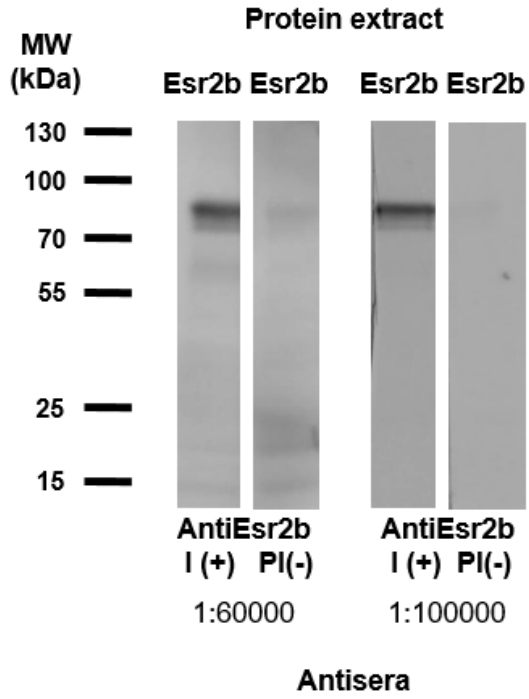


Fig. S5. Western blot of the recombinant sea bream Esr2b protein incubated with the produced anti-dlEsr2b (I, immune, (+) reaction) or with the respective pre-immune serum (PI or (-) control) at two different dilutions, detected by the ECL Plus Western Blotting Chemiluminescent Detection System. Molecular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

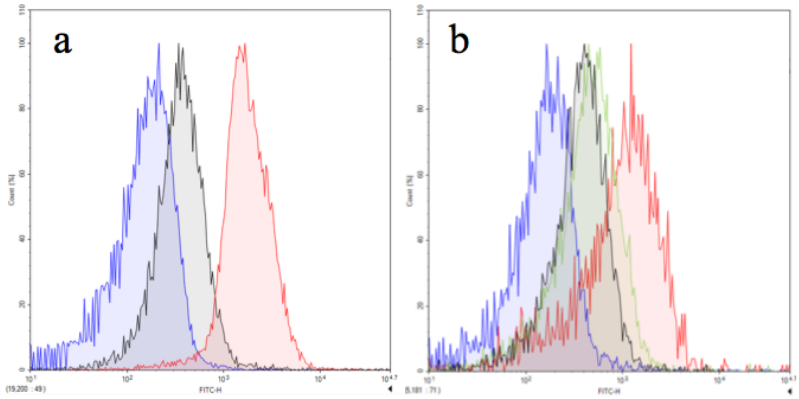


Fig. S7: Flow cytometric analysis of isolated thymocytes in green fluorescence (FITC-H) from two independent experiments (a-b). Histogram overlays from thymocytes incubated without primary antibody in blue, with the isotype-matched control in black, with the blocking peptide in green and with the respective anti-GPER (N-15) 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 (N-15) pre-incubated with the specific blocking peptide. Scale bar: 10 µm

B) Article 2

Oestrogen, an evolutionary conserved regulator of T cell differentiation and immune tolerance in jawed vertebrates? (2018)

Developmental and Comparative Immunology 84 (2018) 48–61

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Oestrogen, an evolutionary conserved regulator of T cell differentiation and immune tolerance in jawed vertebrates?

Matthieu Paiola [a], Thomas Knigge [a], Aurélie Duflot [a], Patricia I.S. Pinto [b], Emilie Farcy [c], Tiphaine Monsinjon [a, *]

[a] Normandy University, FR CNRS 3730 SCALE, UMR-I 02 INERIS-URCA-ULH Environmental Stress and Aquatic Biomonitoring (SEBIO), University of Le Havre Normandy, 76600 Le Havre, France
[b] Laboratory of Comparative Endocrinology and Integrative Biology, CCMAR – Centre of Marine Sciences, University of Algarve, 8005-139 Faro, Portugal
[c] Montpellier University, UMR MARBEC (UM, CNRS, Ifremer, IRD), 34095 Montpellier, France

ARTICLE INFO

Article history:
Received 25 September 2017
Received in revised form
18 January 2018
Accepted 18 January 2018
Available online 31 January 2018

Keywords:
Thymus
Spleen
Head-kidney
Teleost
Regulatory T cell
Gamma-delta T cell

ABSTRACT

In teleosts, as in mammals, the immune system is tightly regulated by sexual steroid hormones, such as oestrogens. We investigated the effects of 17β-oestradiol on the expression of several genes related to T cell development and resulting T cell subpopulations in sea bass, *Dicentrarchus labrax*, for a primary lymphoid organ, the thymus, and two secondary lymphoid organs, the head-kidney and the spleen. In parallel, the oxidative burst capacity was assessed in leucocytes of the secondary lymphoid organs. Apoptosis- and proliferation-related genes, indicative of B and T cell clonal selection and lymphoid progenitor activity, were not affected by elevated oestrogen-levels. Sex-related oestrogen-responsiveness in T cell and antigen-presenting cell markers was observed, the expression of which was differentially induced by oestrogen-exposure in the three lymphoid organs. Remarkably, in the spleen, oestrogen increased regulatory T cell-related gene expression was associated with a decrease in oxidative burst capacity. To the best of our knowledge, this study indicates for the first time that physiological levels of oestrogen are likely to promote immune tolerance by modulating thymic function (*i.e.*, T cell development and output) and peripheral T cells in teleosts, similar to previously reported oestrogenic effects in mammals.

1. Introduction

In all jawed vertebrates, T cells constitute the central component of the adaptive immunity, which develops in the thymus. The thymus represents an evolutionary innovation of the vertebrate lineage related to the appearance of somatic gene recombination and the production of a high diversity of receptors that recognize and fend off abnormal cells (Boehm and Swann, 2014; Rauta et al., 2012). Despite its early evolutionary appearance, the number of thymic glands per animal, their anatomical location, developmental origin and functional processes may differ in some detail (Bajoghli et al., 2015; Boehm and Swann, 2014; Rodewald, 2008).

The vertebrate immune system has been recognised to interact with the endocrine system (*e.g.*, Lutton and Callard, 2006; Segner et al., 2017). This crosstalk is, notably, characterized by a sexual dimorphism in immune system performance extensively described for mammals, but also occasionally reported for birds, reptiles, amphibians and teleosts (Segner et al., 2017). In mammals, oestrogens are key immunomodulatory hormones known to modulate T cell development and function (Hince et al., 2008; Straub, 2007). Although oestrogens appear to be evolutionary ancient immunomodulators (Burgos-Aceves et al., 2016; Segner et al., 2017; Straub, 2007; Szwejser et al., 2016), knowledge about their effects on T cells in fish is scarce.

Abbreviations: AIRE, autoimmune regulator; APC, antigen-presenting cell; E2, 17β-oestradiol; EE2, 17α-ethinylestradiol; ETP, early thymic progenitor; FOXN1, forkhead-box n1; FOXP3, forkhead-box p3; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; GSI, gonado-somatic index; HSI, hepato-somatic index; *K*, Fulton's condition factor; MFI, Mean Fluorescent Intensity; MHC, major histocompatibility complex; P_i, inorganic phosphorus; PCNA, proliferating cells nuclear antigen; PMA, phorbol 12-myristate 13-acetate; RAG1, recombination activating gene 1; ROS, reactive oxygen species; SSI, spleno-somatic index; TCR, T cell receptor; TEC, thymic epithelial cell; Treg, regulatory T cell; TSA, tissue specific antigen.

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

https://doi.org/10.1016/j.dci.2018.01.013
0145-305X/

T cell-function is based on the specificity of a T cell receptor (TCR), formed by a membrane heterodimer composed either of TCRγ and δ chains or of TCRα and β chains, defining two fundamentally different T cell lineages: γδ T cells have innate-like properties and bind antigens directly, while αβ T cells, or conventional T cells, recognise antigen peptides presented by the major histocompatibility complex (MHC) of type I or II (Boehm and Swann, 2014; Buonocore et al., 2012; Chien et al., 2014; Wan et al., 2017). In the thymus, αβ T cells differentiate into single positive CD8 and CD4 T cells, also referred to as cytotoxic and helper T cells, respectively, by recognition of the peptide-MHC-I or II complex (Klein et al., 2014; Nakanishi et al., 2015). MHC-I is expressed by all cell types and MHC-II by specialised antigen-presenting cells (APCs), such as dendritic cells, macrophages and B cells (Esteban et al., 2015; Lewis et al., 2014; Wilson, 2017).

APCs are important for immune tolerance, because they must perform several steps of αβ T cell selection, including central (positive and negative selection) as well as peripheral selection in order to screen clones able to discriminate self from nonself (Audiger et al., 2017; Boehm and Swann, 2014; Li and Rudensky, 2016). APCs also promote specific activation and differentiation of helper T cells, or regulatory T cells (Tregs), another type of CD4 single positive T cells that are of great importance for immune tolerance. In fact, Tregs regulate innate and adaptive immune cell activity and maintain self- and nonself-tolerance (Audiger et al., 2017; Li and Rudensky, 2016; Steinman and Banchereau, 2007).

The thymus provides, maintains and restores the peripheral T cell-repertoire (Boehm and Swann, 2014; Manning and Nakanishi, 1996). The teleost thymic and peripheral T cell-differentiation is depicted in Fig. 1, providing a conceptual framework for the choice of gene markers used in this study. The transcriptional factor Ikaros is expressed in early T cell progenitors (ETP) deriving from the head-kidney (Fig. 1.2A; Bajoghli et al., 2009; Lam et al., 2004). ETP homing, T cell-commitment and -maturation are accomplished by thymic epithelial cells (TEC) under the regulation of the transcription factor forkhead-box n1 (FOXN1, Bajoghli et al., 2009; Žuklys et al., 2016). Recombination-activating genes (RAG) 1 and 2 allow for the rearrangement of TCRδ, γ, β and α gene segments assuring commitment to αβ or γδ T cell subtypes (Fig. 1.2B; Bajoghli et al., 2015; Muñoz-Ruiz et al., 2017; Yui and Rothenberg, 2014). T cell fate involves MHC-II+ cells (APCs), such as medullary thymic epithelial cells (mTEC), which are regulated by the transcription factor AIRE (autoimmune regulator) (Fig. 1.2C and 1.3; Anderson and Su, 2016; Bajoghli et al., 2015; Saltis et al., 2008). Treg-differentiation and -function is regulated by the forkhead-box p3 (FOXP3) transcription factor (Fig. 1.2C and 1.3; Li and Rudensky, 2016; Quintana et al., 2010; Sugimoto et al., 2017). Immune cell homeostasis and tolerance may involve steps of apoptosis mediated by (1) the extrinsic pathway *via* Caspase-8 and (2) the intrinsic pathway *via* Caspase-9 (Audiger et al., 2017; Bouillet and O'Reilly, 2009; Daley et al., 2017; Luzio et al., 2013).

In teleosts, the head-kidney and spleen constitute the major secondary lymphoid organs (Parra et al., 2013; Rauta et al., 2012; Zwollo, 2016). The spleen is present in all jawed vertebrates, whereas the head-kidney is only found in teleost fish (Boehm and Swann, 2014; Parra et al., 2013). Because lymphopoiesis as well as T cell-activation are highly dependent on proliferation (Boehm and Swann, 2014; Chien et al., 2014; Nakanishi et al., 2015), the proliferating cells nuclear antigen (PCNA) gene was also analysed in all three lymphoid organs.

To elucidate the effects of 17β-oestradiol (E2) on central and peripheral T cells, European sea bass (*Dicentrarchus labrax*) were injected with E2. The resulting changes in E2-levels, were confirmed by several known plasmatic parameters, such as calcium, phosphorus, or vitellogenin (Vtg). The relative expression of *ikaros*, *rag1*, *foxn1* and *aire* were analysed in the thymus (Fig. 1) because they represent thymus-specific function and steps of T cell development. In addition, *caspase8*, *caspase9*, *mhc2α chain* (*mhc2a*), *tcra*, *foxp3* and *tcrg* were analysed in the thymus, in the head-kidney and in the spleen. To corroborate the changes in activity and differentiation of T cells with innate immune cell competence, the capacity of oxidative burst was measured in leucocytes isolated from the spleen and the head-kidney.

2. Materials and methods

2.1. Animals

A cohort of fingerlings of *D. labrax* was obtained from the hatchery "L'écloserie marine de Gravelines" (Gravelines, France) and raised in the facilities of "Aquacaux" (Octeville, France) in 1800 L tanks with a continuous flow of filtered and aerated marine seawater at environmental temperatures until about three years of age. At this time, the population comprised adolescent males and females. A few males showed signs of beginning sexual maturity, but the majority of animals were still sexually immature (see GSI in Table 1). 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).

2.2. Treatment and sampling

Two successive exposure experiments were carried out, within one week of interval (Fig. S1). The fish were allotted randomly to either of the experiments and did not differ significantly with respect to the different physiological and developmental biometric indices (see supplementary data, Fig. S2), so that the data of both experiments were combined. For details on the experimental setup and validation of the experimental combination see supplementary materials and methods. Experimental conditions were the same as for hatchery (Table 1) and monitored at each injection time using a Multiparameter Meter (HI 9828, HANNA instruments, Woonsocket, USA). Fish received three consecutive intra-peritoneal injections of 0.5 mg E2/kg, prepared according to Pankhurst et al. (1986), or vehicle alone (*i.e.*, organic colza oil) at day 1, day 3 and day 6 (for details on E2-solution preparation see supplementary materials and methods). At day 7, all fish were anesthetized with tricaine methanesulfonate (MS 222; Sigma, St. Louis, USA) before weight and total length were recorded. A blood sample was taken from the caudal vein with a heparinised syringe, centrifuged at 1000 *g* and 4 °C for 10 min, followed by a subsequent centrifugation of the supernatant at 3000 *g* and 4 °C for another 10 min. The plasma was snap frozen and stored at −80 °C until analyses of Vtg-, E2-, cortisol-, calcium- (Ca^{2+}) and inorganic phosphorus- (P_i) levels were carried out; sample sizes for each treatment group and measurement are specified in Table 2. The fish were sacrificed by an overdose of MS 222 and subsequent decapitation. Thymus, head-kidney, spleen, liver and gonads were dissected. Individual tissue samples of thymus, head-kidney and spleen were immediately frozen in liquid nitrogen and stored at −80 °C for later RNA-extraction. A part of head-kidney and spleen was gently passed through a 100 μm cell strainer adding Leibovitz medium (L15, Sigma) and kept two hours at 4 °C for leucocyte isolation and flow cytometry. Gender was determined by macroscopic observation and confirmed *post hoc* by gonad histology. The total length and weight of the fish body, liver, gonads and spleen were used to establish the Fulton's condition factor (*K*), the hepato-somatic (HSI), the gonado-somatic (GSI) and the spleno-somatic (SSI) indices according to Hadidi et al. (2008), Robinson et al. (2008) and

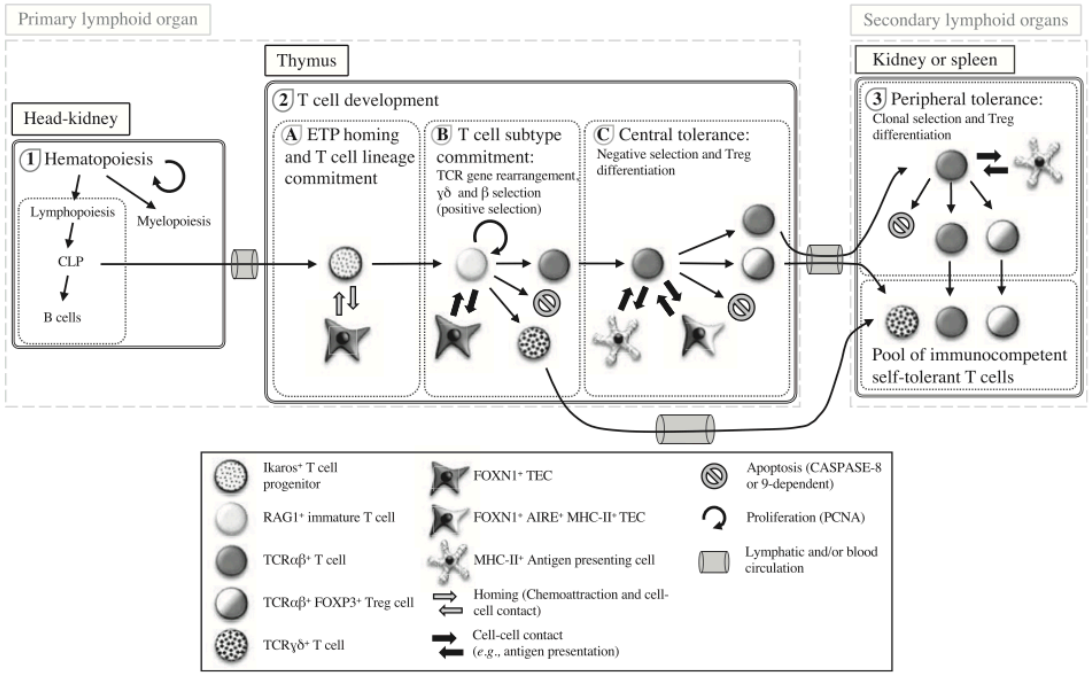


Fig. 1. Successive steps of teleost thymic and peripheral T cell differentiation in steady-state with their genetic markers used in the present study. **1**, The head-kidney (*i.e.*, bone marrow equivalent) contains hematopoietic stem cells providing both myeloid and lymphoid progenitors (Boehm and Swann, 2014; Parra et al., 2013). The common lymphoid progenitor (CLP) differentiates into the B cell lineage or migrates to the thymus. **2 A**, Following homing, early thymic progenitors (ETP) commit to the T cell lineage after interacting with thymic epithelial cells (TEC) (Bajoghli et al., 2009; Yui and Rothenberg, 2014). **2 B**, TCR gene recombination determines the immature T cell fate: apoptosis (death by neglect) or αβ and γδ T cell-differentiation following β or γδ selection. Committed αβ T cells undergo positive selection, resulting in CD4 or CD8 SP differentiation (Klein et al., 2014; Muñoz-Ruiz et al., 2017; Turchinovich and Pennington, 2011). **2 C**, selected αβ T cells can undergo negative selection or Treg-differentiation, two processes important for central tolerance. Negative selection allows the elimination of potentially harmful autoreactive T cells with a high TCR affinity for the self-peptide-MHC complex by apoptosis (Klein et al., 2014; Li and Rudensky, 2016). **3**, Naïve and mature T cells migrate into the secondary lymphoid organs, where they undergo a new selection process important for peripheral tolerance, including clonal selection and Treg-differentiation (Audiger et al., 2017; Li and Rudensky, 2016). The selected self-tolerant T cells constitute a pool of immunocompetent T cells able to elaborate and coordinate a specific immune response (Boehm and Swann, 2014; Nakanishi et al., 2015).

Zha et al. (2007), respectively: K (g/cm^3) = body weight (g)/total length (cm)^3 x 100; HSI (%) = liver weight (g)/body weight (g) x 100; GSI (%) = gonad weight (g)/body weight (g) x 100. Sample sizes per measurement and treatment group are specified in Table 2.

2.3. Hormone and mineral plasma levels

E2-, cortisol-, Ca^{2+}- and P_i-levels were quantified in duplicate for each fish as previously described (Pinto et al., 2016). The E2-and cortisol-levels were analysed by radioimmunoassay using specific antisera against E2 (Guerreiro et al., 2002) and cortisol (Rotllant et al., 2005). Free hormones of the heat-denaturated plasma samples were separated using dextran-coated charcoal. Total plasma Ca^{2+}- and P_i-levels were quantified using *o*-cresolphthalein and phosphomolybdate colorimetric assays, respectively (Spinreact 1001060 and 1001150, Barcelona, Spain).

2.4. Vitellogenin plasma levels

Plasmatic Vtg-levels were determined as described by Pinto et al. (2016). Briefly, 10 µL of plasma were diluted 1/10 in Tris buffer, pH 7.8, and mixed with an equal volume of SDS-PAGE sample buffer, boiled for 5 min, centrifuged at 1700 *g* for 1 min and run on an 8% SDS-PAGE with a prestained, dual-color SDS-PAGE molecular weight marker (ref. 161–0374, Bio-Rad Laboratories, Hercules, USA). Gels were stained with Coomassie blue, scanned and digital images captured using Alpha Imager System (Alpha Innotech, San Leandro, USA). Vtg at 180 kDa was quantified using Image J (v.1.48). Total plasma protein was determined after dilution (1:75) using the Bradford method (Bradford, 1976) with bovine serum albumin (Sigma) as a standard. The relative plasma level of Vtg was expressed as band density (pixels/mm^2/µg total protein) of the respective sample.

2.5. Gene expression

Relative gene expression of *ikaros, rag1, foxn1 and aire* was assessed in the thymus only. Gene expression of *tcra, foxp3, tcrg, pcna, mhc2a, caspase8, caspase9*, the elongation factor α1 (*ef1a*), the ribosomal protein L13a gene (*l13a*) and the 40S ribosomal protein S30 (*fau;* for sequence determination procedure see supplementary materials and methods) was assessed in the thymus, the head-kidney and the spleen. For sample sizes per treatment group and analysis the reader is referred to Table 2. Details on the respective primers are given in Table S3. Total RNA was extracted using Tri Reagent® (Sigma) according to the supplier's instructions. Tissue samples were homogenised twice in Precellys® tubes (CK14; Bertin instruments, Montigny-le-Bretonneux, France) for 10 s at 5000 rpm and subsequently centrifuged at 12,000 *g* for 15 min at 4 °C to eliminate debris. After RNA-extraction, possible DNA contamination was removed by digestion with the TURBO DNA-*free* Kit

Table 1
Experimental and physicochemical conditions with somatic indices for each experimental group (for details and experimental setup see text and supplementary materials and methods). Values are means and standard deviation (s.d.). GSI, gonado-somatic index; HSI, hepato-somatic index; *K*, Fulton's condition factor; SSI, spleno-somatic index.

Tank volume			1800 L			
Water flow			continuous			
Exposure route			intraperitoneal injection			
E2 doses			0.5 mg/kg injected 3 times			
Exposure duration			1 week			
Physicochemical conditions	November 2014 (Experiment 1)					
	CTR		s.d.	E2		s.d.
Temperature (°C)	13.70	±	0.26	13.67	±	0.18
pH	8.13	±	0.52	8.16	±	0.11
Conductivity (mS/cm)	46.54	±	5.96	47.36	±	4.03
Salinity (g/kg)	30.65	±	3.19	32.09	±	0.78
Fish density per tank g/L	7.35			7.52		
Somatic indices						
K	1.32	±	0.11	1.36	±	0.11
GSI	0.50	±	0.31	0.601	±	0.62
SSI	0.10	±	0.04	0.09	±	0.03
HSI	1.21	±	0.27	1.29**	±	0.35
Physicochemical conditions	December 2014 (Experiment 2)					
	CTR		s.d.	E2		s.d.
Temperature (°C)	11.58	±	0.58	11.92	±	0.46
pH	8.68	±	0.58	8.84	±	0.67
Conductivity (mS/cm)	37.55	±	0.53	38.07	±	0.48
Salinity (g/kg)	32.98	±	0.07	33.16	±	0.19
Fish density per tank g/L	9.12			8.80		
Somatic indices						
K	1.41	±	0.21	1.36	±	0.11
GSI	0.74	±	0.65	1.02	±	1.14
SSI	0.11	±	0.03	0.09	±	0.03
HSI	1.52	±	0.34	1.68+	±	0.32

**and +, significantly different between CTR- and E2-groups of the respective experiment at $p \leq 0.001$ (*t*-test or *U*- test, respectively).

Table 2
Sample sizes analysed in each treatment group. GSI, gonado-somatic index; HSI, hepato-somatic index; *K*, Fulton's condition factor; SSI, spleno-somatic index.

	CTR-F	E2-F	CTR-M	E2-M
Biometric index				
K	59	55	18	22
GSI	59	54	18	21
SSI	30	27	10	13
HSI	59	55	18	22
Gene expression				
Thymus	15	13	5	7
Head-kidney	14	11	9	11
Spleen	12	11	10	8
Plasmatic parameters				
E2	26	25	9	10
VTG	8	12	7	6
Ca	26	24	9	10
Pi	26	23	9	10
Cortisol	26	25	9	11
Flow cytometry				
Head-kidney	10	9	5	6
Spleen	7	4	4	5

(Invitrogen-Ambion, Carlsbad, USA) according to the supplier's instructions. RNA quality was assessed on 1% agarose gels and the yield was quantified with a Nanodrop One (ThermoFisher, Waltham, USA) at 260 nm and 280 nm. Absorbance ratios 260/280 between 1.93 and 2.01 indicated pure RNA samples. Samples were stored at −80 °C until further processing.

Reverse transcription was performed using 1 μg of RNA, 0.5 μg of Oligo(dT), dNTP and M-MLV RT (Promega, Fitchburg, USA) with an incubation of 5 min at 70 °C and 60 min at 42 °C at a final volume of 25 μL and cDNA was stored at −20 °C. Real-time qPCR was performed using three technical replicates per individual sample (*i.e.*, biological replicate corresponding to the n given in Table 2) by separately adding cDNA diluted 1:20 in DNase/RNase free water. Different reaction volumes were employed according to the respective gene and thermocycler combinations used for qPCR (Table S3). Two genes (*tcrg* and *ikaros*) were analysed in 10 μL using the Rotor-Gene Q instrument (Qiagen, Hilden, Germany) with QuantiTect SYBR® Green PCR Kit 5 (Qiagen). The twelve remaining genes were analysed in either 5 μL, or 1.5 μL with the LightCycler® 480 Instrument II (Roche Molecular Diagnostics, Pleasanton, USA) with LightCycler® 480 SYBR Green I Master (Roche). Different reaction volumes were used in order to adjust the cycle threshold between different genes. A plate linker sample was run on all plates for each given gene. For the final volume of 1.5 μL reaction mix, 0.5 μL of cDNA and 1 μL of PCR mix (enzyme, dNTP and primers) were loaded on a 384-microwell plate using a Labcyte Acoustic Automated Liquid Handler (Echo® 525, Labcyte™, San Jose, USA; GenomiX platform, Montpellier University). The use of the Labcyte Echo enables miniaturization of qRT-PCR assay (Agrawal et al., 2016; Green et al., 2016). Oligonucleotides were used at a final concentration of either 0.5 μM or 0.75 μM as optimized for each gene (Table S3). Negative controls were performed with DNA free water. For LightCycler® 480 SYBR Green I Master qPCR conditions were as follows: initial incubation at 95 °C for 10 min followed by 45 cycles at 95 °C for 10 s, 60 °C for 10 s (for primer specific hybridization temperature see Table S3) and 72 °C for 10 s. The QuantiTect qPCR conditions were: 95 °C for 15 min, 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, with the three last steps being repeated for 40 times. PCR-products were evaluated on 2% agarose gels and assessed with the qPCR melting curves. Cycle thresholds were determined either with LightCycler 1.5480 or with Rotor-Gene Q software version 2.0.2 (Build 4). For each organ and primer pair, the PCR amplification efficiency was calculated by serial dilutions of cDNA pooled with samples of all conditions (experiment, treated, control and gender; see Table S3).

For the quantification of relative gene expression, the reference genes: *ef1a*, *l13a* and *fau* (Table S3) emerged to be stably expressed between control and treatments in males and females for each organ and experiment (Supplementary data, Fig. S3). The primers for *fau* and *ef1a* were previously utilized as reference genes in sea bass by Mitter et al. (2009). Furthermore, *l13a* and *ef1a* were validated as suitable references genes with xenoestrogen exposure in teleost fish (Lorin-Nebel et al., 2014; McCurley and Callard, 2008). The relative expression ratio of a target gene was calculated based on the formula of Pfaffl (2001) using the efficiency of each primer pair calculated in each organ (Table S3) and the geometric mean of the three reference genes as recommended by Vandesompele et al. (2002). Henceforth, the normalized expression of each analysed gene is referred to as 'gene expression'. Furthermore, as a control, normalization with each reference gene (*ef1a, l13a, fau*) was checked independently.

2.6. Leucocyte isolation and, assessment of the viability and population distribution

The cell solutions from head-kidney and spleen (see 2.2, "sampling") were loaded on Histopaque®-1077 (Sigma) and leucocytes were separated by centrifugation for 30 min at 400 *g* and 4 °C. The leucocytes were collected at the interface and washed twice at 1200 *g* and 4 °C for 5 min. The cell concentration was determined with a hemocytometer and adjusted to 1×10^6 cells per mL. Cell

mortality was assessed as described by Seemann et al. (2016). Briefly, the cells were incubated with 50 µg/mL of iodide propidium at room temperature for 10 min in obscurity. The different cell types were defined by their granularity/internal complexity (SSC, side scatter) and their electronic volume. In agreement with previous work (Granja et al., 2015; Seemann et al., 2016), two populations were identified: the lymphoid and myeloid cells, the former being of larger size and granularity. The mortality was assessed as the percentage of lymphoid and myeloid cells with high red fluorescence. The population distribution corresponded to the percentage of events in either of the gates for each cell type. Each flow cytometric measurement was conducted with 25,000 events in the gate "cells" comprising both cell types. The analyses of flow cytometry were conducted using the Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter) and the software FlowJo® (version 8.7, Ashland, Oregon, USA).

2.7. Respiratory burst

Respiratory burst was assessed in leucocytes isolated from the head-kidney and spleen as described by Bado-Nilles et al. (2014). The analysed sample sizes in each treatment group are specified in Table 2. Briefly, for each sample two aliquots of cells were incubated with 5 µM of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, ThermoFisher) for 30 min. Subsequently, one aliquot was stimulated for 30 min using 2 µg/mL of phorbol 12-myristate 13-acetate (PMA, Sigma). The membrane permeant H_2DCFDA is converted by intracellular oxidases and esterases to a non-fluorescent product, which reacts with reactive oxygen species (ROS) and converts it to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). The green fluorescence of the unstimulated and stimulated cells was measured for each gate (lymphoid and myeloid cells). Three different values were determined: the stimulation index of respiratory burst as the ratio of the mean fluorescent intensity (MFI) of stimulated cells to unstimulated cells and the MFI (expressing the level of ROS) of the stimulated and unstimulated cells.

2.8. Statistical analysis

All statistical analyses were conducted using SigmaPlot (version 12.0, Systat Software Inc., San Jose, USA), unless otherwise stated. Gene expression data are reported as normalised expression and visualized as box-and-whisker plots, indicating the median, the 25 and 75 percentiles as well as the minimum and maximum values. The results of flow cytometry are depicted as histograms with means and standard errors (s.e.). Prior to analyses, outliers were eliminated using the Grubb's outlier test (for combined data from all groups, GraphPad Software Inc., La Jolla, USA). The datasets were checked for normality and equal variances using the Shapiro-Wilk test and the Levene Median test, respectively. Subsequently, pairwise analysis was conducted to compare E2- and CTR-groups for both genders separately. Were significant differences between treatments absent, E2- and CTR-groups were combined to check for possible gender differences. If normal distribution and homoscedasticity could be confirmed, an independent Student *t*-test was used for pairwise parametric hypothesis testing; otherwise non-parametric Mann-Whitney *U* test was conducted. Pairwise linear correlations were assessed for each grouped data (CTR-F, E2-F, CTR-M and E2-M with experiments combined) for plasmatic E2, cortisol and gene-expression levels using the non-parametric Spearman rank order correlation. The *p*-values were adjusted for multiple hypotheses testing by the False Discovery Rate procedure (Benjamini and Hochberg, 1995), which was set to 15%. The correlations were considered significant at an α-level of 0.4% ($p < 0.004$) as determined by the False Discovery Rate.

3. Results

3.1. Exposure validation

The efficacy of the E2-treatment was validated by the changes in the plasmatic levels of E2, Ca^{2+} and P_i as well as by the changes in the HSI for CTR-M, CTR-F, E2-M and E2-F from both experiments combined (the exact sample sizes of which are displayed in Table 2). When comparing males and females from the CTR- and E2-groups (Fig. 2, Table 1), a significant difference in the plasmatic E2-level (*Kruskal-Wallis*, $H_{3,60}$=49.54, $p < 0.001$) was revealed. Also, the HSI values (*Kruskal-Wallis*, $H_{3,61}$=36.75, $p < 0.001$), Ca^{2+} (*Kruskal-Wallis*, $H_{3,61}$=37.58, $p < 0.001$) and P_i (*Kruskal-Wallis*, $H_{3,68}$=15.38, $p = 0.001$) were significantly different. The Tukey-Kramer *post-hoc* analyses demonstrated that the E2-exposure significantly increased plasmatic concentrations of E2 from 0.040 ng/mL ± 0.023 s.d. to 2.640 ng/mL ± 1.943 s.d. in females and from 0.044 ng/mL ± 0.029 s.d. to 3.560 ng/mL ± 0.432 s.d. in males (both $p < 0.001$; all *p*-values are listed in Table S10). The Tukey-Kramer *post-hoc* analyses indicated a significant increase for the HSI following E2-treatment in females and males (both $p < 0.001$; all *p*-values are listed in Table S11). The Ca^{2+}- and the P_i-levels of the E2-exposed animals were significantly increased in females only (both $p < 0.001$, Tables S12 and S13).

Comparing the CTR and the E2-treatments for males and females combined (Fig. 2, Table 1), the E2-exposure significantly increased the plasmatic concentrations of E2 (*U* test, $p < 0.001$). Significant increases of Ca^{2+} (*U* test, $p < 0.001$) P_i (*U* test, $p < 0.001$) as well as Vtg (*U* test, $p = 0.018$) could also be observed.

3.2. Gene expression

Relative gene expression was compared for CTR-M, CTR-F, E2-M and E2-F from both experiments combined with sample sizes ranging from n = 5 to 15 (exact sample sizes per group and organ are provided in Table 2).

3.2.1. Thymic homing, T cell maturation and TEC function

E2-treatment did not modulate *ikaros*-expression in females (*t*-test, $p = 0.848$; Fig. 3), which, however, tended to increase in males (*t*-test, $p = 0.393$). The E2-treatment decreased the *rag1*-expression level in E2-F, but this was not statistically significant (*U* test, $p = 0.238$; Fig. 3). No significant change of *rag1*-expression could be detected in E2-M (*t*-test, $p = 0.770$).

The E2-treatment did neither significantly decrease *foxn1*-expression (Fig. 3) in females (*U* test, $p = 0.696$), nor in males (*t*-test, $p = 0.963$). However, the E2-treatment affected the significantly positive correlation of *foxn1* with *ikaros* and *foxp3* (Table 3 and Table S14), which was observed in CTR, but not in E2-treated females.

3.2.2. Thymic and peripheral T cell selection

The E2-treatment significantly increased *aire*-expression levels (Fig. 3) in females (*U* test, $p = 0.007$), but not in males (*t*-test, $p = 0.822$).

E2-treatment significantly increased *mhc2a*-expression in the thymus of males, but not in females (*t*-test, $p = 0.038$ and $p = 0.386$, respectively; Fig. 4). This was, however, not the case in the head-kidney of E2-exposed males (Fig. 4), where a trend to increased *mhc2a*-expression level in females was observed (*t*-test, $p = 0.253$ and *t*-test, $p = 0.096$, respectively). In the spleen, E2-treatment did neither affect *mhc2a*-expression in males, nor in females (*t*-test, $p = 0.476$ and *t*-test, $p = 0.890$; Fig. 4).

E2-treatment did not significantly modulate the expression-levels of *caspase8* and *caspase9* (Fig. 4), neither in the thymus of

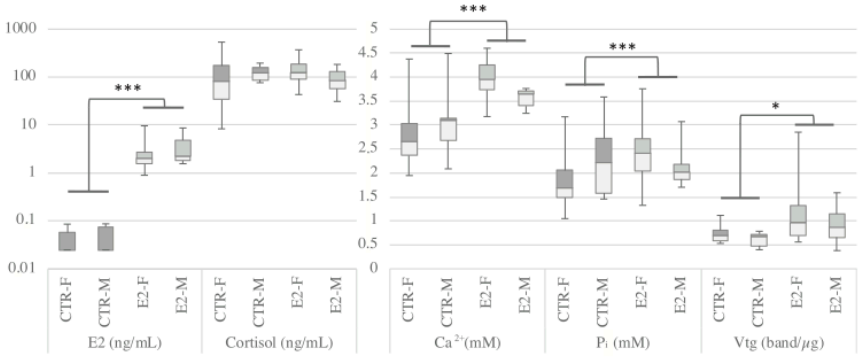


Fig. 2. Circulating plasma levels of 17β-oestradiol (E2), cortisol, calcium (Ca^{2+}), inorganic phosphorus (P_i) and vitellogenin (Vtg) in sea bass, *Dicentrarchus labrax*, observed in females (F) and males (M) of E2-exposed (E2) and control (CTR) fish. Circulating plasma levels are represented by box-and-whisker plots. The horizontal line inside each box indicates the median, top lines of the box are the 25 and 75 percentiles and the whisker limits represent extreme values. Asterisks indicate significant differences between the E2 and CTR group at the level *, $0.05 \geq p > 0.01$ and ***, $p < 0.001$, respectively (Mann-Whitney *U* test).

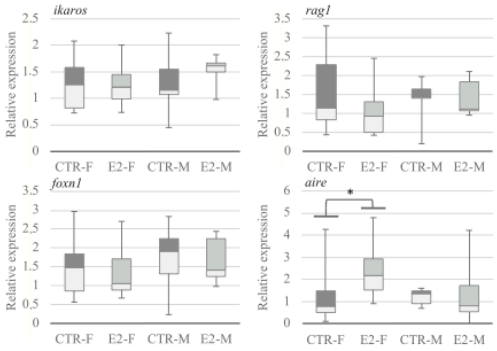


Fig. 3. Relative expression of thymic function-related genes, *ikaros*, *rag1*, *foxn1* and *aire*, in the thymus of sea bass, *Dicentrarchus labrax*, in females (F) and males (M) of E2-exposed (E2) and control (CTR) fish. Relative expression-levels are represented by box-and-whisker plots. The horizontal line inside each box indicates the median, top lines of the box are the 25 and 75 percentiles and the whisker limits depict extreme values. *, significantly different at $p = 0.007$ (Mann-Whitney *U* test).

females (*U* test, $p = 0.800$ and $p = 0.781$, respectively), nor that of males (*t*-test, $p = 0.923$ and $p = 0.141$, respectively). The same was observed in the head-kidney of females (*t*-test, $p = 0.947$ and $p = 0.856$, respectively) and males (*t*-test, $p = 0.136$ and *U* test, $p = 0.704$) as well as in the spleen of females (*t*-test, $p = 0.999$ and $p = 0.341$, respectively) and males (*U* test, $p = 0.661$ and $p = 0.950$, respectively).

The correlations of *caspase9* and *caspase8* with *ikaros*, *foxn1*, as well as *pcna* in the thymus of females are presented in Table 3 and Table S14. In CTR-F, the expression-level of *caspase9* was significantly correlated with *ikaros* and *foxn1*. Also in CTR-F, *capsase8*-expression was significantly correlated with *pcna*. Similar correlations were not observed in E2-F.

Correlations of the expression-levels of *caspase9* and *caspase8* with *foxp3*, *tcra* and *tcrg* as well as *mhc2a* in the head-kidney and spleen are presented in Tables 3 and 4 as well as Tables S15–18. In the head-kidney, *caspase9*-expression showed a significantly positive correlation with *foxp3* in CTR-F, but not in E2-F (Table 3 and Table S15), whereas this positive correlation was not altered in E2-M, as compared to CTR-M (Table 4 and Table S16).

3.2.3. Cell proliferation

Following E2-treatment, *pcna*-expression (Fig. 4) appeared to decrease in females, but this decrease was not statistically significant for any of the three lymphoid organs (*U*-tests for thymus, head-kidney, spleen: $p = 0.200$, $p = 0.366$, $p = 0.079$; respectively), whereas no effect occurred in E2-M (*t*-tests for thymus and head-kidney, $p = 0.981$, $p = 0.351$, respectively; *U* test for spleen, $p = 0.885$).

The *pcna*-expression in the head-kidney was positively and significantly correlated (Table 3 and Table S15) with *mhc2a* in CTR-F. This positive correlation was lost in E2-F.

3.2.4. T cell subtypes

In the thymus, E2-treatment tended to decrease the expression-level of *tcra* (Fig. 5) in E2-F (*t*-test, $p = 0.154$), whereas it did not affect E2-M (*t*-test, $p = 0.286$).

In the head-kidney, E2-treatment increased the expression-level of *tcra* (Fig. 5) in females, but did not modulate the *tcra*-expression in males (*t*-test, $p = 0.023$ and $p = 0.430$, respectively). In the head-kidney of CTR-F and E2-F, *tcra*- and *pcna*-expression showed no significant correlation (Table 3 and Table S15). In the head-kidney, E2-M showed a significantly positive correlation between *tcra* and *mhc2a* (Table 4 and Table S16); a similar correlation was not observed in CTR-M.

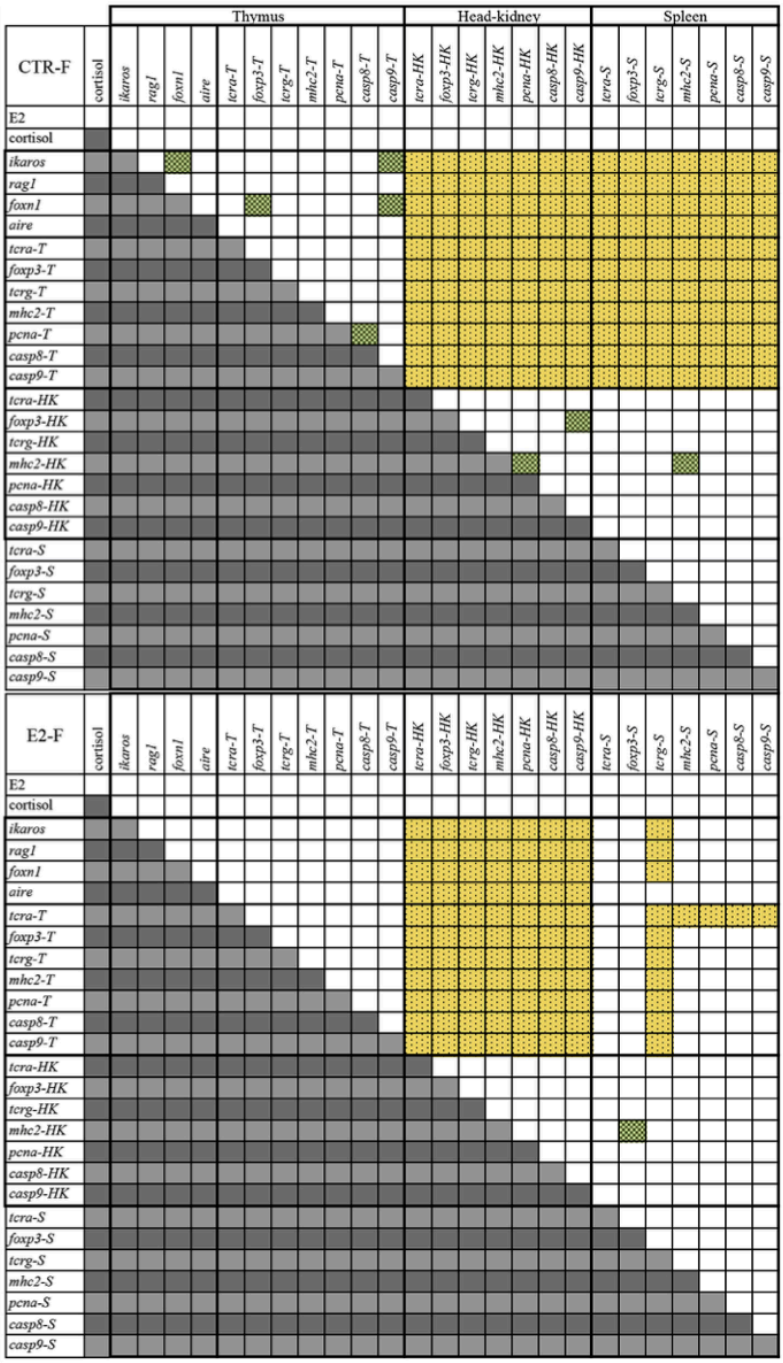
In the spleen, E2-treatment did not increase the expression-level of *tcra* in females (*t*-test, $p = 0.605$), but it tended to increase in males, albeit not significantly (*U* test, $p = 0.083$).

In the E2-treated fish (Fig. 5), the expression-level of *foxp3* increased significantly in the thymus of E2-M (*t*-test, $p = 0.036$), but did not in E2-F ($p = 0.258$, *t*-test).

In the head-kidney, *foxp3*-expression was neither modulated in males, nor in females (*t*-test, $p = 0.642$ and $p = 0.268$, respectively; Fig. 5). In the spleen, the expression-level of *foxp3* was increased in females, but not in males (*t*-test, $p = 0.021$ and $p = 0.860$, respectively). In the male spleen, the E2-treatment resulted in a significantly positive correlation of *foxp3* with *mhc2a* and *pcna* (Table 4 and Table S18). Similar correlations were not observed in the spleen and head-kidney of CTR-F, CTR-M and E2-F (Tables 3 and 4 and Tables S15–18).

The E2-treatment did neither modify the expression-level of *tcrg* in the thymus of E2-F (*U* test, $p = 0.452$; Fig. 5), nor in E2-M (*t*-test, $p = 0.998$). However, female thymuses generally had a lower *tcrg*-expression than those of males (*U* test CTR-F + E2-F *vs.* CTR-M + E2-M combined, $p = 0.020$). In head-kidney and spleen, the E2-

Table 3
Matrices of Spearman Rank Order correlations between the plasmatic E2-, cortisol- and gene-expression levels in control (CTR-F) and E2-injected females (E2-F) in the thymus (T), the head-kidney (HK) and the spleen (S). Significantly positive correlations ($p < 0.004$) are marked as green, chequered squares. Yellow, dotted squares signify insufficient replicate number ($n \leq 5$) and, therefore, absence of correlation. Redundant data are depicted as shaded squares. Casp, Caspase; other abbreviations as in text.



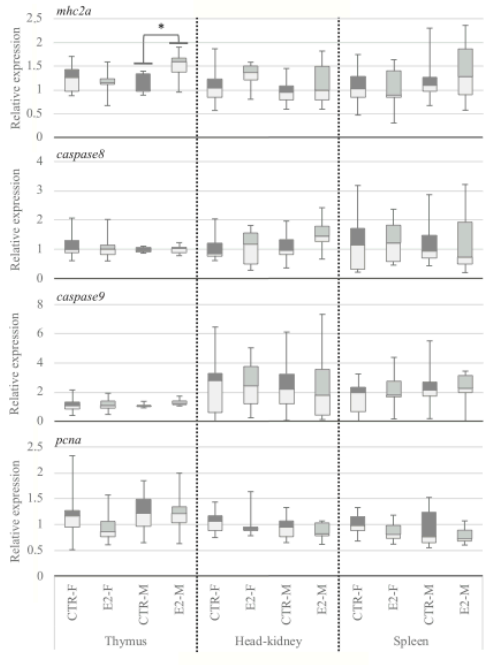


Fig. 4. Relative expression of antigen-presenting cell and cellular homeostasis-related genes, *mhc2a*, *caspase8*, *caspase9* and *pcna*, in thymus, head-kidney and spleen of sea bass, *Dicentrarchus labrax*, in females (F) and males (M) of E2-exposed (E2) and control (CTR) fish. Relative expression-levels are represented by box-and-whisker plots with the median, 25% and 75% quartiles as well as extreme values depicted by the bars. *, significantly different at $p = 0.038$ (*t*-test).

treatment increased the expression-level of *tcrg* in E2-M (*t*-test, $p = 0.037$ and $p = 0.030$, respectively), but had no effect in E2-F (*U* test, $p = 0.869$, *t*-test, $p = 0.170$). In spleen, the E2-M showed a positive and strong correlation (Table 4B and Table S18) between *tcrg*, *mhc2a* and *pcna*. Similar correlations were not observed in the spleen of CTR-M, CTR-F and E2-F, nor in the head-kidney of E2-M, CTR-M and E2-F (Tables 3–4A and Tables S15–18).

In CTR-M, but not E2-M, the expression of *tcrg* in the head-kidney and spleen was positively and significantly correlated ($r = 0.850$ $p < 0.001$; $r = 0.393$, $p = 0.341$).

3.3. Flow cytometry

Respiratory burst was assessed by flow cytometry for CTR-M, CTR-F, E2-M and E2-F from both experiments combined with sample sizes ranging from n = 4 to 10 (exact sample sizes per group and organ are displayed in Table 2).

3.3.1. Leucocyte mortality and distribution

E2-treatment did neither significantly alter the mortality of the lymphocytes and myeloid cells isolated from the head-kidney (*t*-test, $p = 0.126$ and $p = 0.961$, respectively), nor from the spleen (*t*-test, $p = 0.601$ and $p = 0.485$, respectively) (Fig. 6b). In addition, no effect of E2-treatment was observed for the population distribution of isolated leucocytes from the head-kidney and spleen.

3.3.2. Oxidative burst

E2-treatment did not affect the basal ROS-level (unstimulated, *i.e.*, without PMA) of isolated leucocytes from the head-kidney or the spleen (Fig. 6c). Similarly, no statistically significant effect of E2-treatment on the ROS-levels of stimulated (*i.e.*, with PMA) lymphocytes and myeloid cells isolated from the head-kidney could be observed (*t*-test, $p = 0.914$; *U* test, $p = 0.361$, respectively). On the contrary, E2-treatment significantly decreased the ROS-levels of stimulated cells (with PMA) from the spleen: the MFI in lymphocytes of E2-treated animals was less than half as compared to controls (44.03 ± 11.41 s.e. *vs.* 94.38 ± 14.98 s.e.; *U* test, $p = 0.016$) and about the same was observed in myeloid cells (223.6 ± 54.77 s.e. *vs.* 446.9 ± 17.85 s.e.; *U* test, $p = 0.016$). The oxidative burst index for lymphocytes of the head-kidney remained unaffected by E2-treatment (CTR 3.89 ± 0.46 s.e. *vs.* E2 3.90 ± 0.40; *U* test, $p = 0.836$), as was the case for myeloid cells (CTR 6.315 ± 0.736 s.e. *vs.* E2 7.824 ± 1.039; *U* test, $p = 0.333$). In the spleen, E2-treatment significantly decreased the oxidative burst index in lymphocytes with 3.205 ± 0.424 s.e. in CTR *vs.* 1.854 ± 0.420 in E2-treated fish (*U* test, $p = 0.022$). A similar effect seemed to occur in the myeloid cells from the spleen with 4.770 ± 0.467 in CTR *vs.* 3.488 ± 0.756 in E2-treated animals, but without this difference being statistically significant (*t*-test, $p = 0.168$).

4. Discussion

The three intra-peritoneal injections with E2 over one week lead to clearly elevated E2-plasma levels, which were similar to concentrations observed in female sea bass during the spawning season (Mañanós et al., 1997; Navas et al., 1998). Hence, the E2-treatment was both efficient and situated within the physiological range of E2-titers. The effectiveness of the E2-treatment, was further underscored by a significant increase of Ca^{2+}-, P_i- and Vtg-levels in the serum, all of which are known to be induced by E2 (Nelson and Habibi, 2013; Pinto et al., 2016). As the liver produces Vtg, the increased HSI is coherent with an E2-stimulated Vtg-synthesis. Similarly, an increase of the HSI has been observed after 17α-ethynylestradiol (EE2) treatment in other teleost species (Rodenas et al., 2016; Zha et al., 2007).

According to the oestrogenic modulation of the adaptive immune system and the thymus plasticity described for teleosts and other vertebrates (Lutton and Callard, 2006; Seemann et al., 2015; Segner et al., 2017; Szwejser et al., 2016), this study suggests that E2 modulates thymic and peripheral T cell-maturation in a complex manner, as outlined in the following.

4.1. Thymic T cell development and release

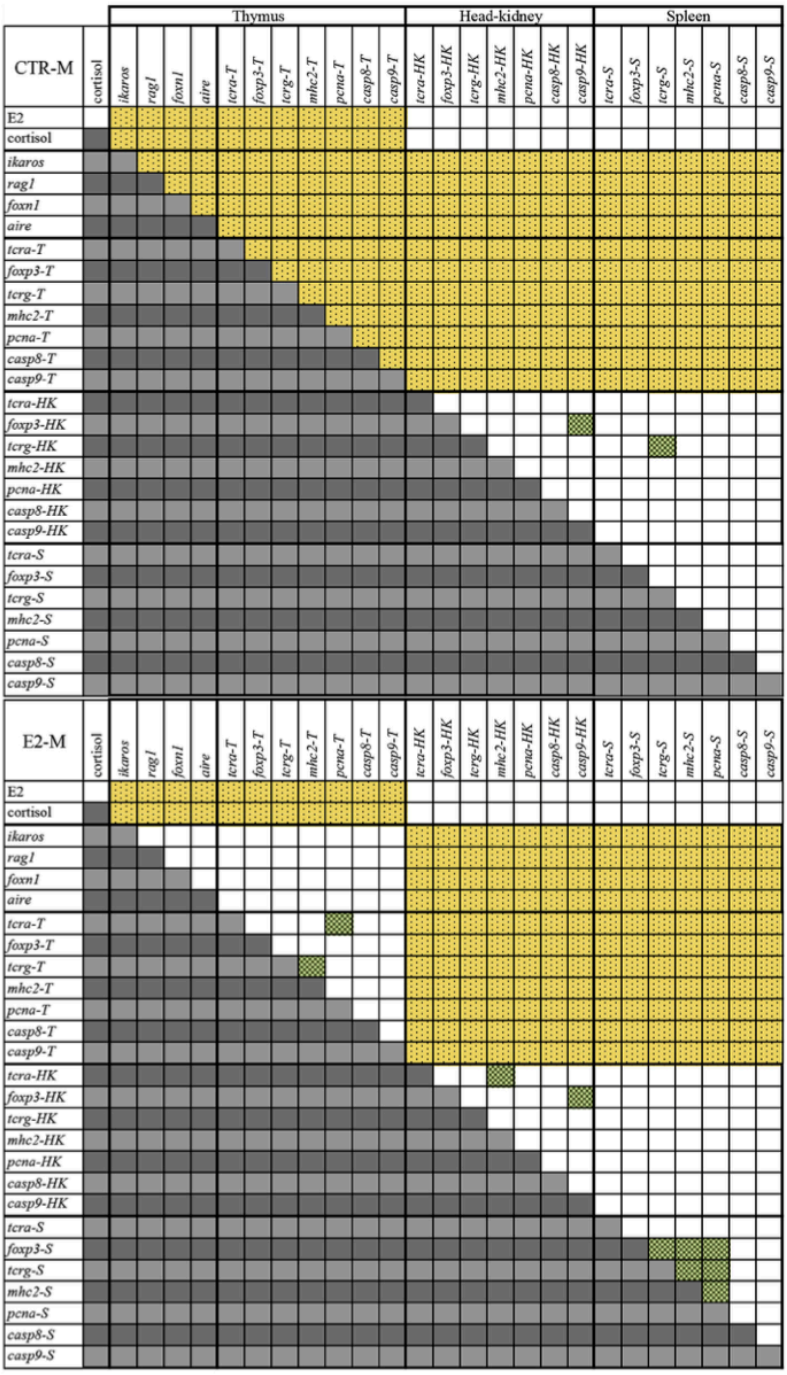
4.1.1. Thymic homing

The ETPs are represented by *ikaros*-expression, which, in zebrafish, is up-regulated before ETPs begin to express *rag1*. This probably occurs after progenitor cells are committed to the T cell lineage, just like in mammals (Fig. 1.2A; Lam et al., 2004; Yui and Rothenberg, 2014). E2-treatment of sea bass did not significantly affect the expression-level of *ikaros* in our experiments. This suggests that the colonization of the thymus by ETPs was not modulated by physiological E2-levels, as opposed to the rapid decline of ETPs observed in mice after E2-injection or during pregnancy (Zoller et al., 2007; Zoller and Kersh, 2006).

4.1.2. T cell-maturation and TEC function

The T cell-maturation, -selection and -differentiation involves the transcription factors FOXN1 and AIRE expressed in TEC (Fig. 1.2; Abramson and Anderson, 2017; Anderson and Su, 2016; Bajoghli et al., 2015, 2009; Saltis et al., 2008; Žuklys et al., 2016). In the thymus of sea bass, E2-treatment did not significantly affect the expression-level of *foxn1*. However, the E2-treatment (1) increased

Table 4
Matrices of Spearman Rank Order correlations between the plasmatic E2-, cortisol- and gene-expression levels in control (CTR-M) and E2-injected males (E2-M) in the thymus (T), the head-kidney (HK) and the spleen (S). Significantly positive correlations ($p < 0.004$) are marked as green, chequered squares. Yellow, dotted squares signify insufficient replicate number ($n \leq 5$) and, therefore, absence of correlation. Redundant data are depicted as shaded squares. Casp, Caspase; other abbreviations as in text.



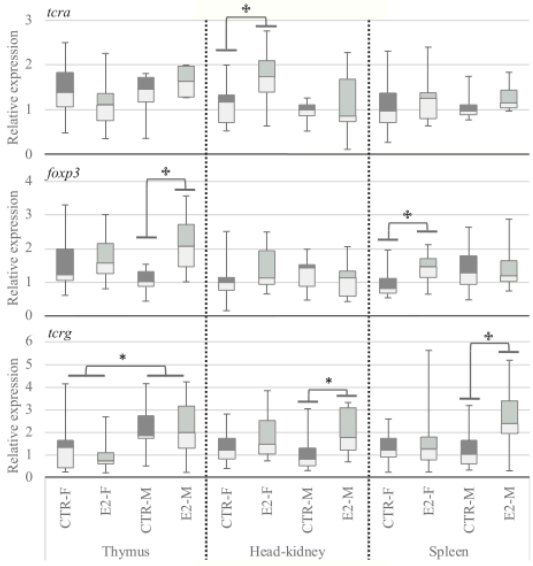


Fig. 5. Relative expression of T cell-related genes, *tcra*, *foxp3* and *tcrg*, in thymus, head-kidney and spleen of sea bass, *Dicentrarchus labrax*, in females (F) and males (M) of E2-exposed (E2) and control (CTR) fish. Relative expression-levels are represented by box-and-whisker plots with the median, 25% and 75% quartiles as well as extreme values depicted by the bars. Asterisks indicate significant differences at $p < 0.05$ (*, *U* test, ✣, *t*-test).

the *aire*-expression level, and (2) eliminated the significant correlation of *foxn1*-expression with *ikaros* and *foxp3* in females, which suggests that E2 modulated TEC function and, hence, thymic T cell maturation (Bajoghli et al., 2015; Klein et al., 2014). These observations are in good agreement with the mammalian oestrogenic modulation of TEC function demonstrated both *in vivo* and *in vitro* (Dragin et al., 2016; Jin et al., 2003; Martín et al., 1995a).

4.1.3. T cell lineage subtype commitment

Early RAG1/2+ T cell lineage committed progenitors possibly differentiate into γδ or αβ T cells (Fig. 1.2B). In sea bass, E2 tended to decrease the expression of *rag1* in the thymus of females. This suggests that E2 does not halt early T cell-maturation in teleosts, as proposed for mammals (Bernardi et al., 2015; Rijhsinghani et al., 1996). The γδ-selection is characterized by the expression of TCRγδ and, to some extent, by a lower proliferation in comparison to the β-selected cells (Muñoz-Ruiz et al., 2017; Turchinovich and Pennington, 2011; Yui and Rothenberg, 2014). In the present study, expression of *tcrg* in the thymus was not affected by E2-treatment, neither in males, nor in females. Female sea bass, however, expressed significantly lower levels of *tcrg*. In addition, E2 tended to decrease the relative expression of *pcna* in female sea bass. Overall, these results suggest that sex steroids, such as E2, have the potential to modulate T cell subtype commitment. Because E2-treatment did not change the expression-level of *tcra* in the thymus of males and females, there was no indication of E2 affecting the αβ T cell lineage. This suggests that the main thymic T cell lineage, *i.e.*, the αβ T cells, would remain weakly affected after one week of elevated physiological E2-levels.

In the mammalian thymus, E2-exposure increased the proportion of T cells with mature phenotypes CD3, CD4 or CD8 SP. Therefore, E2 was presumed to stimulate an alternative intrathymic pathway of CD4 and CD8 double negative T cell maturation including αβ and γδ T cells (Abo, 2001; Chapman et al., 2015; Rijhsinghani et al., 1996; Screpanti et al., 1991). Except for the γδ T cells, this intrathymic pathway could not be directly evidenced for the sea bass thymus, because, although sex-depended differences existed, no treatment effect became visible. Any confirmation of whether such an alternative intrathymic pathways exists is hampered by the lack of specific markers, which can distinguish unconventional T cells (Cheroutre et al., 2011).

4.1.4. Central tolerance

The increase of *aire*- and *mhc2a*-expression in male and female sea bass following E2-treatment could indicate an increased central tolerance (Fig. 1.2 C). Interestingly, *aire*-expression in mammals is also regulated by oestrogen, where it was, however, reported to decrease the number of AIRE+ TECs and AIRE-expression in TECs, resulting in a decrease of AIRE-dependent tissue specific antigen-production and an increase in the prevalence of autoimmune diseases (Anderson and Su, 2016; Dragin et al., 2016; Klein et al., 2014). Given the potential functional conservation of AIRE and MHC-II in T cell selection of jawed vertebrates (Bajoghli et al., 2015; Lewis et al., 2014; Saltis et al., 2008), our results suggest that E2 enhanced central tolerance in female and in male sea bass.

The putative increase of central tolerance may result in increased immature T cell-apoptosis in the thymus, as it has been reported for mice or lizard (Do et al., 2002; Hareramadas and Rai, 2006; Wang et al., 2008). In sea bass, however, the E2-treatment did not affect the *caspase*-expressions. On the one hand, this suggests that in teleosts, E2 does not strongly affect T cell-apoptosis. On the other hand, the lack of an obvious E2-induced apoptosis is in agreement with the observations of Chapman et al. (2015), who proposed that E2 does not enhance thymic T cell-apoptosis at physiological concentrations.

The increase of central tolerance may also result in an increase of Treg-differentiation. For instance, the proportion of thymus-derived Treg increased with elevated E2-levels during early pregnancy in mice (Teles et al., 2013). Interestingly, in male sea bass, the expression of *foxp3* increased concomitantly to the increase in *mhc2a*-expression, suggesting that E2 induced Treg-differentiation. This observation provides additional support for the E2-induced central tolerance in sea bass.

4.1.5. T cell migration

Considering the peripheral αβ T cells (Fig. 1.3), we observed that E2-treatment significantly increased the expression-level of *tcra* in the head-kidney of females. It also tended to increase *tcra* in the spleen of males. These changes could derive from T cell-activation, characterized by a stimulation of proliferation. As no significant increase of *pcna*-expression was observed and no correlation between the expression-levels of *pcna* and *tcra* could be determined, one may hypothesise that the increase of *tcra*-expression is due to an increased αβ T cell input in the head-kidney. These αβ T cells are probably coming from the thymus, which is the main source of T cells (Boehm and Swann, 2014; Manning and Nakanishi, 1996). This interpretation is in line with the rapid increase of the number of T cells in the head-kidney of gilthead sea bream following EE2-exposure observed by Rodenas et al. (2017). Likewise, these authors could not detect any effect of EE2 on T cell proliferation.

As for the peripheral γδ T cells, E2-treatment resulted in an (1) increase of *tcrg*-expression in the head-kidney and the spleen of male sea bass, and a (2) strongly positive correlation of *tcrg* with *pcna* and *mhc2a* in spleen, but not in head-kidney. Because mammalian γδ T cells can show clonal expansion (Chien et al.,

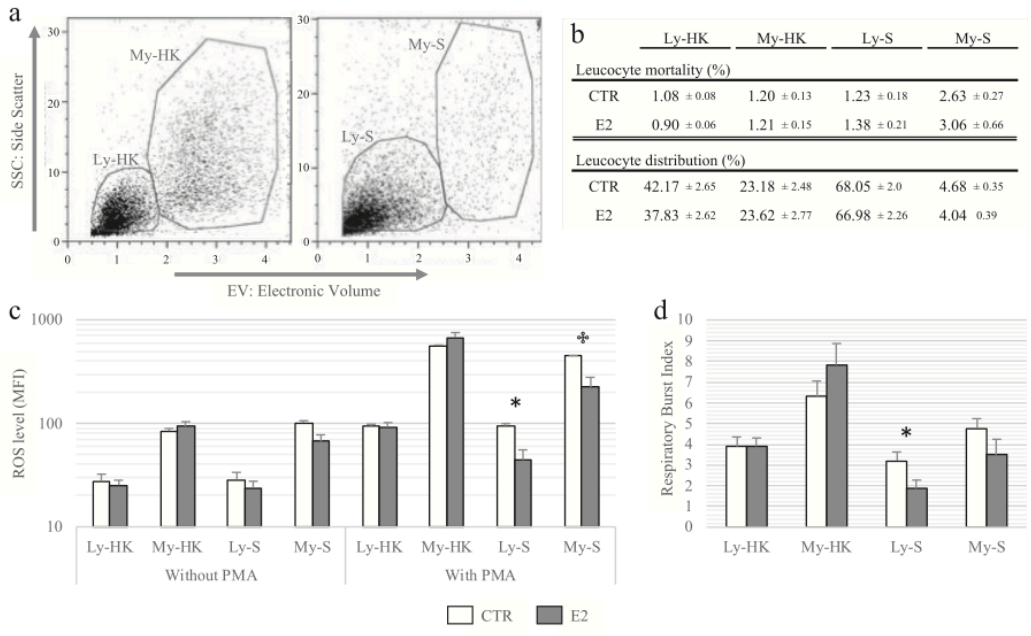


Fig. 6. Flow cytometric analysis of sea bass leucocytes isolated from the head-kidney (HK) and spleen (S) of control (CTR) and E2-exposed (E2) fish. a, Side scatter (SSC)/Electronic volume (EV) profiles with the gates for lymphoid (Ly) and myeloid cells (My) for each organ; b, leucocyte mortality and distribution; c, ROS-levels of stimulated and unstimulated cells, *i.e.*, with and without PMA, respectively, depicted as Mean Fluorescent Intensity (MFI); d, Respiratory Burst Index. Values are means ± standard error. Asterisks indicate significant differences between control and E2-treated fish at *, $p < 0.05$, (*U* test) and ⁺, $p = 0.02$ (*t*-test).

2014), and because γδ T cells express MHC-II (Chien et al., 2014; Wan et al., 2017), our observations suggest that E2 induced proliferation and entry of γδ T cells in the spleen and the head-kidney, respectively. Furthermore, it may be assumed that γδ T cells of the head-kidney and the spleen represent two distinct populations.

These interpretations, together with the lower expression of *tcrg* in the thymus of females as well as the potentially E2-induced increase of αβ T cells in the head-kidney of females, suggest that E2 induced thymic T cell output in sea bass, similar to observations made in mammals (Chapman et al., 2015; Martín et al., 1995a). This T cell-release is believed to be promoted by an E2-mediated vasodilatation, which is induced by thymic mast cell-activation (Chapman et al., 2015). A similar mechanism may be assumed in sea bass, as Paiola et al. (2017) detected nuclear and membrane oestrogen receptor expression in the thymic vessels and mast cells of *D. labrax*. In mammals, these E2-mediated changes of T cell-output have been associated with a stimulation of an alternative intrathymic pathway of T cell-maturation (Abo, 2001; Chapman et al., 2015). Similar effects are, therefore, likely to occur in the thymus of sea bass.

4.1.6. Thymic APC migration and differentiation

Because E2 increased *mhc2a*-expression in the thymus of males, one may assume that E2 stimulated antigen presentation in the thymus of male sea bass, as it was described for mammals (Wira et al., 2003). Correspondingly, in the mammalian thymus, E2 increases thymic migration and/or differentiation of several potential APCs, such as B cells, plasma cells, macrophages and granulocytes (Martín et al., 1994, 1995b; Ross and Korenchevsky, 1941). The increase of *foxp3* in male sea bass, which occurred concomitantly to the increase of *mhc2a*-expression, corroborates this interpretation. As a matter of fact, an increased number of APCs (both mTEC and DC) has been shown to stimulate thymus-derived Treg-generation (Abramson and Anderson, 2017; Lin et al., 2016). This would confirm that E2 modulates T cell fate in the thymus of sea bass.

4.2. Peripheral tolerance and T cell differentiation

In the periphery, naïve and mature T cells undergo supplementary selection and differentiation (Fig. 1.3). At first sight, E2 did not alter B and T cell clonal selection, because *caspase*-expression was not significantly modulated by E2-treatment. This interpretation is further underscored by an unaltered leucocyte viability in the spleen and head-kidney following E2-treatment. At closer examination, however, E2-treatment strongly increased the *foxp3*-expression in the spleen of females, suggesting an E2-related induction of peripheral T cell-differentiation or a migration of thymus-derived Tregs. Interestingly, a highly significant and positive correlation of *foxp3* with *mhc2a* as well as *pcna* was observed in the spleen of males following E2-treatment. By presenting the peptide-MHC-II complex, APCs induce Treg-differentiation followed by a high level of proliferation (Li and Rudensky, 2016). Accordingly, one may assume that in the spleen of sea bass, E2 induced Treg-differentiation in females and, to a lesser extent, in males. Interestingly, pregnancy as well as *in vitro* and *in vivo* E2-treatment both increased the proportion of Treg and *foxp3*-expression and FOXP3-levels in the spleen of female mice (Polanczyk et al., 2004, 2005; Tai et al., 2008). This is likely to enhance the suppressive activity of Tregs (Polanczyk et al., 2005). In teleosts, FOXP3 is believed to have a similar function in Tregs (Quintana et al., 2010; Sugimoto et al., 2017; Wen et al., 2011). Therefore, the E2-mediated increase of *foxp3*-levels in the spleen suggests that E2 promotes tolerance and anti-inflammatory activity through an expansion of FOXP3+ αβ Treg cells in the spleen of sea

bass, similar to the situation described for mammals. This interpretation is further corroborated by the fact that the inhibition of oxidative burst capacity occurred solely in the spleen, but not in the head-kidney.

Furthermore, in the male spleen, the apparent E2-mediated anti-inflammatory response could also result from the increase of γδ T cells with a regulatory phenotype. Indeed, high E2-levels promote immune tolerance during pregnancy in mammals, by increasing peripheral γδ Tregs (Chapman et al., 2015). One may speculate that this also the case in fish, although the corresponding γδ T cell subtypes have not yet been identified in teleosts.

5. Conclusion

The results of this study covering T cell-related gene expression in the sea bass provide evidence for E2-induced immune tolerance by qualitatively and quantitatively modulating the peripheral T cell subsets in *D. labrax*. Two mechanisms are likely to be involved: (1) a modulation of the thymic function, such as the T cell lineage commitment and release, *i.e.*, the stimulation of an intrathymic alternative pathway of T cell maturation, and (2) a modulation of the differentiation and/or the activity of mature/peripheral T cells. Similar mechanisms of E2-action on the thymus and peripheral lymphoid organs have been identified in mammals (Abo, 2001; Chapman et al., 2015; Polanczyk et al., 2005). The gender-specific behaviour of the different T cell subsets in the various lymphoid organs underscores the implication of oestrogens in these processes.

During mammalian pregnancy, the expansion of Treg, double negative αβ and γδ T cells in peripheral organs, including the spleen, is believed to be important to avoid a detrimental immune response against the semiallogenic foetus (Chapman et al., 2015; Clark, 2016). Our study suggests that the E2-induced increase of T cell-mediated peripheral tolerance is evolutionarily conserved in jawed vertebrates. In sea bass, higher plasmatic levels of E2 are associated with seasonal events of reproduction, migration and a switch to nocturnal feeding and locomotor behaviour (del Pozo et al., 2014; Mañanós et al., 1997). Consequently, the promotion of immune tolerance by E2 could be related to changes in diet, energy budget and reserves as well as environmental pathogens.

Compliance with ethical standards

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

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This research was supported by the ANR financed project ETaT (ANR-15-CE32-0014). M.P. was supported by the Normandy region through the FR CNRS 3730 SCALE scholarship. P.I.S.P. was supported through the Foundation for Science and Technology of Portugal (FCT), through the projects PTDC/AAG-GLO/4003/2012 and CCMAR/Multi/04326/2013 and the fellowship SFRH/BPD/84033/2012. The authors thank Matthieu Bonnet and the staff at Aquacaux for fish hatchery, Elsa Couto and Soraia Santos (CCMAR) for carrying out sea bass plasma analyses and Sandrine Crochemore for her technical support in gene expression measurements. We would like to thank Salima Aroua, Sabrina Jolly, Stéphanie Olivier, Agnès Poret and Béatrice Rocher for their assistance throughout the exposures and samplings.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2018.01.013.

References

Abo, T., 2001. Extrathymic pathways of T-cell differentiation and immunomodulation. Int. Immunopharmacol 1, 1261–1273. https://doi.org/10.1016/S1567-5769(01)00057-1.

Abramson, J., Anderson, G., 2017. Thymic epithelial cells. Annu. Rev. Immunol. 35, 85–118. https://doi.org/10.1146/annurev-immunol-051116-052320.

Agrawal, S., Cifelli, S., Johnstone, R., Pechter, D., Barbey, D.A., Lin, K., Allison, T., Agrawal, S., Rivera-Gines, A., Milligan, J.A., Schneeweis, J., Houle, K., Struck, A.J., Visconti, R., Sills, M., Wildey, M.J., 2016. Utilizing low-volume aqueous acoustic transfer with the Echo 525 to enable miniaturization of qRT-PCR assay. J. Lab. Autom. 21, 57–63. https://doi.org/10.1177/2211068215609315.

Anderson, M.S., Su, M.A., 2016. AIRE expands: new roles in immune tolerance and beyond. Nat. Rev. Immunol. 16, 247–258. https://doi.org/10.1038/nri.2016.9.

Audiger, C., Rahman, M.J., Yun, T.J., Tarbell, K.V., Lesage, S., 2017. The importance of dendritic cells in maintaining immune tolerance. J. Immunol. 198, 2223–2231. https://doi.org/10.4049/jimmunol.1601629.

Bado-Nilles, A., Techer, R., Porcher, J.M., Geffard, A., Gagnaire, B., Betoulle, S., Sanchez, W., 2014. Detection of immunotoxic effects of estrogenic and androgenic endocrine disrupting compounds using splenic immune cells of the female three-spined stickleback, *Gasterosteus aculeatus* (L.). Environ. Toxicol. Pharmacol. 38, 672–683. https://doi.org/10.1016/j.etap.2014.08.002.

Bajoghli, B., Aghaallaei, N., Hess, I., Rode, I., Netuschil, N., Tay, B.-H., Venkatesh, B., Yu, J.-K., Kaltenbach, S.L., Holland, N.D., Diekhoff, D., Happe, C., Schorpp, M., Boehm, T., 2009. Evolution of genetic networks underlying the emergence of thymopoiesis in vertebrates. Cell 138, 186–197. https://doi.org/10.1016/j.cell.2009.04.017.

Bajoghli, B., Kuri, P., Inoue, D., Aghaallaei, N., Hanelt, M., Thumberger, T., Rauzi, M., Wittbrodt, J., Leptin, M., 2015. Noninvasive in toto imaging of the thymus reveals heterogeneous migratory behavior of developing T cells. J. Immunol. 195, 2177–2186. https://doi.org/10.4049/jimmunol.1500361.

Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B Methodol 57, 289–300.

Bernardi, A.I., Andersson, A., Stubelius, A., Grahnemo, L., Carlsten, H., Islander, U., 2015. Selective estrogen receptor modulators in T cell development and T cell dependent inflammation. Immunobiology 220, 1122–1128. https://doi.org/10.1016/j.imbio.2015.05.009.

Boehm, T., Swann, J.B., 2014. Origin and evolution of adaptive immunity. Annu. Rev. Anim. Biosci. 2, 259–283. https://doi.org/10.1146/annurev-animal-022513-114201.

Bouillet, P., O'Reilly, L.A., 2009. CD95, BIM and T cell homeostasis. Nat. Rev. Immunol. 9, 514–519. https://doi.org/10.1038/nri2570.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Buonocore, F., Castro, R., Randelli, E., Lefranc, M.-P., Six, A., Kuhl, H., Reinhardt, R., Facchiano, A., Boudinot, P., Scapigliati, G., 2012. Diversity, molecular characterization and Expression of T Cell receptor γ in a teleost fish, the sea bass (*Dicentrarchus labrax*, L). PLoS One 7, e47957. https://doi.org/10.1371/journal.pone.0047957.

Burgos-Aceves, M.A., Cohen, A., Smith, Y., Faggio, C., 2016. Estrogen regulation of gene expression in the teleost fish immune system. Fish Shellfish Immunol. 58, 42–49. https://doi.org/10.1016/j.fsi.2016.09.006.

Chapman, J.C., Chapman, F.M., Michael, S.D., 2015. The production of alpha/beta and gamma/delta double negative (DN) T-cells and their role in the maintenance of pregnancy. Reprod. Biol. Endocrinol. 13. https://doi.org/10.1186/s12958-015-0073-5.

Cheroutre, H., Lambolez, F., Mucida, D., 2011. The light and dark sides of intestinal intraepithelial lymphocytes. Nat. Rev. Immunol. 11, 445–456. https://doi.org/10.1038/nri3007.

Chien, Y., Meyer, C., Bonneville, M., 2014. γδT cells: first line of defense and beyond. Annu. Rev. Immunol. 32, 121–155. https://doi.org/10.1146/annurev-immunol-032713-120216.

Clark, D.A., 2016. The importance of being a regulatory T cell in pregnancy. J. Reprod. Immunol. 116, 60–69. https://doi.org/10.1016/j.jri.2016.04.288.

Daley, S.R., Teh, C., Hu, D.Y., Strasser, A., Gray, D.H.D., 2017. Cell death and thymic tolerance. Immunol. Rev. 277, 9–20. https://doi.org/10.1111/imr.12532.

del Pozo, A., Falcon, J., Sanchez-Vazquez, F.J., 2014. The biological clock and dualism. In: Biology of European Sea Bass. CRC Press, pp. 34–56.

Do, Y., Ryu, S., Nagarkatti, M., Nagarkatti, P.S., 2002. Role of death receptor pathway in estradiol-induced t-cell apoptosis *in vivo*. Toxicol. Sci. 70, 63–72. https://doi.org/10.1093/toxsci/70.1.63.

Dragin, N., Bismuth, J., Cizeron-Clairac, G., Biferi, M.G., Berthault, C., Serraf, A., Nottin, R., Klatzmann, D., Cumano, A., Barkats, M., Le Panse, R., Berrih-Aknin, S., 2016. Estrogen-mediated downregulation of AIRE influences sexual dimorphism in autoimmune diseases. J. Clin. Invest. 126, 1525–1537. https://doi.org/10.1172/JCI81894.

Esteban, M.Á., Cuesta, A., Chaves-Pozo, E., Meseguer, J., 2015. Phagocytosis in teleosts. Implications of the new cells involved. Biology 4, 907–922. https://doi.org/10.3390/biology4040907.

Granja, A.G., Leal, E., Pignatelli, J., Castro, R., Abos, B., Kato, G., Fischer, U., Tafalla, C., 2015. Identification of teleost skin CD8 + dendritic-like cells, representing a potential common ancestor for mammalian cross-presenting dendritic cells. J. Immunol. 195, 1825–1837. https://doi.org/10.4049/jimmunol.1500322.

Green, T.J., Vergnes, A., Montagnani, C., de Lorgeril, J., 2016. Distinct immune responses of juvenile and adult oysters (*Crassostrea gigas*) to viral and bacterial infections. Vet. Res. 47 (72). https://doi.org/10.1186/s13567-016-0356-7.

Guerreiro, P.M., Fuentes, J., Canario, A.V., Power, D.M., 2002. Calcium balance in sea bream (*Sparus aurata*): the effect of oestradiol-17beta. J. Endocrinol. 173, 377–385.

Hadidi, S., Glenney, G.W., Welch, T.J., Silverstein, J.T., Wiens, G.D., 2008. Spleen size predicts resistance of rainbow trout to *Flavobacterium psychrophilum* challenge. J. Immunol. 180, 4156–4165. https://doi.org/10.4049/jimmunol.180.6.4156.

Hareramadas, B., Rai, U., 2006. Cellular mechanism of estrogen-induced thymic involution in wall lizard: caspase-dependent action. J. Exp. Zoolog. A Comp. Exp. Biol. 305A, 396–409. https://doi.org/10.1002/jez.a.260.

Hince, M., Sakkal, S., Vlahos, K., Dudakov, J., Boyd, R., Chidgey, A., 2008. The role of sex steroids and gonadectomy in the control of thymic involution. Cell. Immunol. 252, 122–138. https://doi.org/10.1016/j.cellimm.2007.10.007.

Jin, C., Fu, W.-X., Xie, L.-P., Qian, X.-P., Chen, W.-F., 2003. SDF-1α production is negatively regulated by mouse estrogen enhanced transcript in a mouse thymus epithelial cell line. Cell. Immunol. 223, 26–34. https://doi.org/10.1016/S0008-8749(03)00152-7.

Klein, L., Kyewski, B., Allen, P.M., Hogquist, K.A., 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat. Rev. Immunol. 14, 377–391. https://doi.org/10.1038/nri3667.

Lam, S.H., Chua, H.L., Gong, Z., Lam, T.J., Sin, Y.M., 2004. Development and maturation of the immune system in zebrafish, *Danio rerio*: a gene expression profiling, in situ hybridization and immunological study. Dev. Comp. Immunol. 28, 9–28. https://doi.org/10.1016/S0145-305X(03)00103-4.

Lewis, K.L., Del Cid, N., Traver, D., 2014. Perspectives on antigen presenting cells in zebrafish. Dev. Comp. Immunol. 46, 63–73. Zebrafish Immunity and Infection Models. https://doi.org/10.1016/j.dci.2014.03.010.

Li, M.O., Rudensky, A.Y., 2016. T cell receptor signalling in the control of regulatory T cell differentiation and function. Nat. Rev. Immunol. 16, 220–233. https://doi.org/10.1038/nri.2016.26.

Lin, J., Yang, L., Silva, H.M., Trzeciak, A., Choi, Y., Schwab, S.R., Dustin, M.L., Lafaille, J.J., 2016. Increased generation of Foxp3+ regulatory T cells by manipulating antigen presentation in the thymus. Nat. Commun. 7. https://doi.org/10.1038/ncomms10562.

Lorin-Nebel, C., Budzinski, H., Le Ménach, K., Devier, M.H., Charmantier, G., Gros, R., Grousset, E., Blondeau-Bidet, E., Farcy, E., 2014. 4-Nonylphenol disrupts osmoregulation in the European sea-bass *Dicentrarchus labrax*. J. Xenobiotics 4. https://doi.org/10.4081/xeno.2014.4905.

Lutton, B., Callard, I., 2006. Evolution of reproductive–immune interactions. Integr. Comp. Biol. 46, 1060–1071. https://doi.org/10.1093/icb/icl050.

Luzio, A., Monteiro, S.M., Fontaínhas-Fernandes, A.A., Pinto-Carnide, O., Matos, M., Coimbra, A.M., 2013. Copper induced upregulation of apoptosis related genes in zebrafish (*Danio rerio*) gill. Aquat. Toxicol. 128–129, 183–189. https://doi.org/10.1016/j.aquatox.2012.12.018.

Mañanós, E.L., Zanuy, S., Carrillo, M., 1997. Photoperiodic manipulations of the reproductive cycle of sea bass (*Dicentrarchus labrax*) and their effects on gonadal development, and plasma 17β-estradiol and vitellogenin levels. Fish Physiol. Biochem. 16, 211–222.

Manning, M.J., Nakanishi, T., 1996. The specific immune system: cellular defenses. In: The Fish Immune System: Organism, Pathogen, and Environment. Academic Press, Inc, pp. 159–205.

Martín, A., Alonso, L.M., Del Moral, M.G., Zapata, A.G., 1994. Ultrastructural changes in the adult rat thymus after estradiol benzoate treatment. Tissue Cell 26, 169–179. https://doi.org/10.1016/0040-8166(94)90092-2.

Martín, A., Casares, F., Alonso, L., Nieuwenhuis, P., Vicente, A., Zapata, A.G., 1995a. Changes in the blood-thymus barrier of adult rats after estradiol-treatment. Immunobiology 192, 231–248. https://doi.org/10.1016/S0171-2985(11)80100-2.

Martín, A., Vicente, A., Torroba, M., Moreno, C., Jiménez, E., Zapata, A., 1995b. Increased numbers of CD5+ B cells in the thymus of estradiol benzoate-treated rats. Thymus 24, 111–127.

McCurley, A.T., Callard, G.V., 2008. Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. BMC Mol. Biol. 9 (102). https://doi.org/10.1186/1471-2199-9-102.

Mitter, K., Kotoulas, G., Magoulas, A., Mulero, V., Sepulcre, P., Figueras, A., Novoa, B., Sarropoulou, E., 2009. Evaluation of candidate reference genes for QPCR during ontogenesis and of immune-relevant tissues of European seabass (*Dicentrarchus labrax*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 153, 340–347. https://doi.org/10.1016/j.cbpb.2009.04.009.

Muñoz-Ruiz, M., Sumaria, N., Pennington, D.J., Silva-Santos, B., 2017. Thymic determinants of γδ T cell differentiation. Trends Immunol. https://doi.org/10.1016/j.it.2017.01.007.

Nakanishi, T., Shibasaki, Y., Matsuura, Y., 2015. T cells in fish. Biology 4, 640–663. https://doi.org/10.3390/biology4040640.

Navas, J.M., Mañanós, E., Thrush, M., Ramos, J., Zanuy, S., Carrillo, M., Zohar, Y., Bromage, N., 1998. Effect of dietary lipid composition on vitellogenin, 17β-estradiol and gonadotropin plasma levels and spawning performance in captive sea bass (*Dicentrarchus labrax* L.). Aquaculture 165, 65–79. https://doi.org/10.1016/S0044-8486(98)00246-4.

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15–24, 7th International Symposium on Fish Endocrinology. https://doi.org/10.1016/j.ygcen.2013.03.032.

Paiola, M., Knigge, T., Picchietti, S., Duflot, A., Guerra, L., Pinto, P.I.S., Scapigliati, G., Monsinjon, T., 2017. Oestrogen receptor distribution related to functional thymus anatomy of the European sea bass, *Dicentrarchus labrax*. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2017.07.023.

Pankhurst, N.W., Stacey, N.E., Peter, R.E., 1986. An evaluation of techniques for the administration of 17β-estradiol to teleosts. Aquaculture 52, 145–155. https://doi.org/10.1016/0044-8486(86)90034-7.

Parra, D., Takizawa, F., Sunyer, J.O., 2013. Evolution of B Cell immunity. Annu. Rev. Anim. Biosci. 1, 65–97. https://doi.org/10.1146/annurev-animal-031412-103651.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29 e45–e45. https://doi.org/10.1093/nar/29.9.e45.

Pinto, P.I.S., Estêvão, M.D., Andrade, A., Santos, S., Power, D.M., 2016. Tissue responsiveness to estradiol and genistein in the sea bass liver and scale. J. Steroid Biochem. Mol. Biol. 158, 127–137. https://doi.org/10.1016/j.jsbmb.2015.12.023.

Polanczyk, M.J., Carson, B.D., Subramanian, S., Afentoulis, M., Vandenbark, A.A., Ziegler, S.F., Offner, H., 2004. Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment. J. Immunol. 173, 2227–2230. https://doi.org/10.4049/jimmunol.173.4.2227.

Polanczyk, M.J., Hopke, C., Huan, J., Vandenbark, A.A., Offner, H., 2005. Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. J. Neuroimmunol. 170, 85–92. https://doi.org/10.1016/j.jneuroim.2005.08.023.

Quintana, F.J., Iglesias, A.H., Farez, M.F., Caccamo, M., Burns, E.J., Kassam, N., Oukka, M., Weiner, H.L., 2010. Adaptive autoimmunity and foxp3-based immunoregulation in zebrafish. PLoS One 5, e9478. https://doi.org/10.1371/journal.pone.0009478.

Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. Immunol. Lett. 148, 23–33. https://doi.org/10.1016/j.imlet.2012.08.003.

Rijhsinghani, A.G., Thompson, K., Bhatia, S.K., Waldschmidt, T.J., 1996. Estrogen blocks early T cell development in the thymus. Am. J. Reprod. Immunol. N. Y. N 1989 (36), 269–277. https://doi.org/10.1111/j.1600-0897.1996.tb00176.x.

Robinson, M.L., Gomez-Raya, L., Rauw, W.M., Peacock, M.M., 2008. Fulton's body condition factor K correlates with survival time in a thermal challenge experiment in juvenile Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*). J. Therm. Biol. 33, 363–368. https://doi.org/10.1016/j.jtherbio.2008.05.004.

Rodenas, M.C., Cabas, I., García-Alcázar, A., Meseguer, J., Mulero, V., García-Ayala, A., 2016. Selective estrogen receptor modulators differentially alter the immune response of gilthead seabream juveniles. Fish Shellfish Immunol. 52, 189–197. https://doi.org/10.1016/j.fsi.2016.03.041.

Rodenas, M.C., Cabas, I., Gómez-González, N.E., Arizcun, M., Meseguer, J., Mulero, V., García-Ayala, A., 2017. Estrogens promote the production of natural neutralizing antibodies in Fish through G Protein-coupled estrogen receptor 1. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.00736.

Rodewald, H.-R., 2008. Thymus organogenesis. Annu. Rev. Immunol. 26, 355–388. https://doi.org/10.1146/annurev.immunol.26.021607.090408.

Ross, M.A., Korenchevsky, V., 1941. The thymus of the rat and sex hormones. J. Pathol. Bacteriol. 52, 349–360.

Rotllant, J., Guerreiro, P.M., Anjos, L., Redruello, B., Canario, A.V.M., Power, D.M., 2005. Stimulation of cortisol release by the N Terminus of teleost parathyroid hormone-related protein in interrenal cells *in vitro*. Endocrinology 146, 71–76. https://doi.org/10.1210/en.2004-0644.

Saltis, M., Criscitiello, M.F., Ohta, Y., Keefe, M., Trede, N.S., Goitsuka, R., Flajnik, M.F., 2008. Evolutionarily conserved and divergent regions of the Autoimmune Regulator (Aire) gene: a comparative analysis. Immunogenetics 60, 105–114. https://doi.org/10.1007/s00251-007-0268-9.

Screpanti, I., Meco, D., Morrone, S., Gulino, A., Mathieson, B.J., Frati, L., 1991. *In vivo* modulation of the distribution of thymocyte subsets: effects of estrogen on the expression of different T cell receptor Vβ gene families in CD4−, CD8− thymocytes. Cell. Immunol. 134, 414–426. https://doi.org/10.1016/0008-8749(91)90314-2.

Seemann, F., Knigge, T., Duflot, A., Marie, S., Olivier, S., Minier, C., Monsinjon, T., 2016. Sensitive periods for 17β-estradiol exposure during immune system development in sea bass head kidney: sensitivity of sea bass head kidney development to E2. J. Appl. Toxicol. 36, 815–826. https://doi.org/10.1002/jat.3215.

Seemann, F., Knigge, T., Olivier, S., Monsinjon, T., 2015. Exogenous 17-β-oestradiol (E2) modifies thymus growth and regionalization in European sea bass *Dicentrarchus labrax*. J. Fish. Biol. 86, 1186–1198. https://doi.org/10.1111/jfb.12626.

Segner, H., Verburg-van Kemenade, B.M.L., Chadzinska, M., 2017. The immunomodulatory role of the hypothalamus-pituitary-gonad axis: proximate mechanism for reproduction-immune trade offs? Dev. Comp. Immunol. 66, 43–60. https://doi.org/10.1016/j.dci.2016.07.004.

Steinman, R.M., Banchereau, J., 2007. Taking dendritic cells into medicine. Nature 449, 419–426. https://doi.org/10.1038/nature06175.

Straub, R.H., 2007. The complex role of estrogens in inflammation. Endocr. Rev. 28, 521–574. https://doi.org/10.1210/er.2007-0001.

Sugimoto, K., Hui, S.P., Sheng, D.Z., Nakayama, M., Kikuchi, K., 2017. Zebrafish FOXP3

is required for the maintenance of immune tolerance. Dev. Comp. Immunol. 73, 156–162. https://doi.org/10.1016/j.dci.2017.03.023.
Szwejser, E., Verburg-van Kemenade, B.M.L., Maciuszek, M., Chadzinska, M., 2016. Estrogen-dependent seasonal adaptations in the immune response of fish. Horm. Behav. https://doi.org/10.1016/j.yhbeh.2016.10.007.
Tai, P., Wang, J., Jin, H., Song, X., Yan, J., Kang, Y., Zhao, L., An, X., Du, X., Chen, X., Wang, S., Xia, G., Wang, B., 2008. Induction of regulatory T cells by physiological level estrogen. J. Cell. Physiol. 214, 456–464. https://doi.org/10.1002/jcp.21221.
Teles, A., Thuere, C., Wafula, P.O., El-Mousleh, T., Zenclussen, M.L., Zenclussen, A.C., 2013. Origin of Foxp3+ cells during pregnancy. Afr. J. Clin. Exp. Immunol. 2, 222–233.
Turchinovich, G., Pennington, D.J., 2011. T cell receptor signalling in γδ cell development: strength isn't everything. Trends Immunol. 32, 567–573. https://doi.org/10.1016/j.it.2011.09.005.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 research0034–1. https://doi.org/10.1186/gb-2002-3-7-research0034.
Wan, F., Hu, C., Ma, J., Gao, K., Xiang, L., Shao, J., 2017. Characterization of γδ T cells from zebrafish provides insights into their important role in adaptive humoral immunity. Front. Immunol. 7. https://doi.org/10.3389/fimmu.2016.00675.
Wang, C., Dehghani, B., Magrisso, I.J., Rick, E.A., Bonhomme, E., Cody, D.B., Elenich, L.A., Subramanian, S., Murphy, S.J., Kelly, M.J., Rosenbaum, J.S., Vandenbark, A.A., Offner, H., 2008. GPR30 contributes to estrogen-induced thymic atrophy. Mol. Endocrinol. 22, 636–648. https://doi.org/10.1210/me.2007-0359.
Wen, Y., Fang, W., Xiang, L.-X., Pan, R.-L., Shao, J.-Z., 2011. Identification of Treg-like cells in Tetraodon: insight into the origin of regulatory T subsets during early vertebrate evolution. Cell. Mol. Life Sci. 68, 2615–2626. https://doi.org/10.1007/s00018-010-0574-5.
Wilson, A.B., 2017. MHC and adaptive immunity in teleost fishes. Immunogenetics 1–8. https://doi.org/10.1007/s00251-017-1009-3.
Wira, C.R., Fahey, J.V., Abrahams, V.M., Rossoll, R.M., 2003. Influence of stage of the reproductive cycle and estradiol on thymus cell antigen presentation. J. Steroid Biochem. Mol. Biol. 84, 79–87. https://doi.org/10.1016/S0960-0760(03)00002-5.
Yui, M.A., Rothenberg, E.V., 2014. Developmental gene networks: a triathlon on the course to T cell identity. Nat. Rev. Immunol. 14, 529–545. https://doi.org/10.1038/nri3702.
Zha, J., Wang, Z., Wang, N., Ingersoll, C., 2007. Histological alternation and vitellogenin induction in adult rare minnow (*Gobiocypris rarus*) after exposure to ethynylestradiol and nonylphenol. Chemosphere 66, 488–495. https://doi.org/10.1016/j.chemosphere.2006.05.071.
Zoller, A.L., Kersh, G.J., 2006. Estrogen induces thymic atrophy by eliminating early thymic progenitors and inhibiting proliferation of -selected thymocytes. J. Immunol. 176, 7371–7378. https://doi.org/10.4049/jimmunol.176.12.7371.
Zoller, A.L., Schnell, F.J., Kersh, G.J., 2007. Murine pregnancy leads to reduced proliferation of maternal thymocytes and decreased thymic emigration. Immunology 121, 207–215. https://doi.org/10.1111/j.1365-2567.2006.02559.x.
Žuklys, S., Handel, A., Zhanybekova, S., Govani, F., Keller, M., Maio, S., Mayer, C.E., Teh, H.Y., Hafen, K., Gallone, G., Barthlott, T., Ponting, C.P., Holländer, G.A., 2016. Foxn1 regulates key target genes essential for T cell development in postnatal thymic epithelial cells. Nat. Immunol. 17, 1206–1215. https://doi.org/10.1038/ni.3537.
Zwollo, P., 2016. The humoral immune system of anadromous fish. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2016.12.008.

Supplementary data

A-Materials and Methods:

E2 solution preparation: E2-solutions of 0.5 mg/mL, 1 mg/mL and 2mg/mL were freshly prepared before each experiment, allowing for minimal volumes to be injected. Briefly, 17β-oestradiol (E8875; Sigma, St. Louis, USA) was dissolved at 50 mg/mL in absolute ethanol, dispersed in organic colza oil (La Vie Claire, Montagny, France) and incubated overnight in obscurity at 30 °C to evaporate the solvent. The vehicle control was prepared in exactly the same way, but without E2. During each experiment, the solutions were conserved in obscurity at 4 °C.

Statistical analysis: To evaluate if the eight experimental groups including control (CTR), exposed (E2), females (F) and males (M) from the experiment of November (1) and December (2) (*i.e.*, CTR-F-1, CTR-F-2, E2-F-1, E2-F-2, CTR-M-1, CTR-M-2, E2-M-1 and E2-M-2) could be combined (See figure S1), a Kruskal-Wallis rank sum-test was applied to compare the different biometric indices (*K,* GSI, SSI and HSI) and the plasmatic levels of cortisol, E2, Ca^{2+}, P_i and Vtg followed by a Tukey-Kramer (Nemenyi) *post-hoc* test (see Table S1 for the overall sample size analysis). Afterwards, the biometric indices and the plasmatic parameters were compared between the male and female treatment groups (E2 and CTR) from both experiments combined. Subsequently, pairwise analysis was conducted to compare E2 and CTR groups for both genders. Furthermore, for different parameters E2- and CTR-groups were combined to detect possible gender differences. For pairwise analysis, if normal distribution and homoscedasticity could be confirmed, an independent Student *t*-test was used for parametric hypothesis testing; otherwise non-parametric Mann-Whitney *U*-test was conducted.

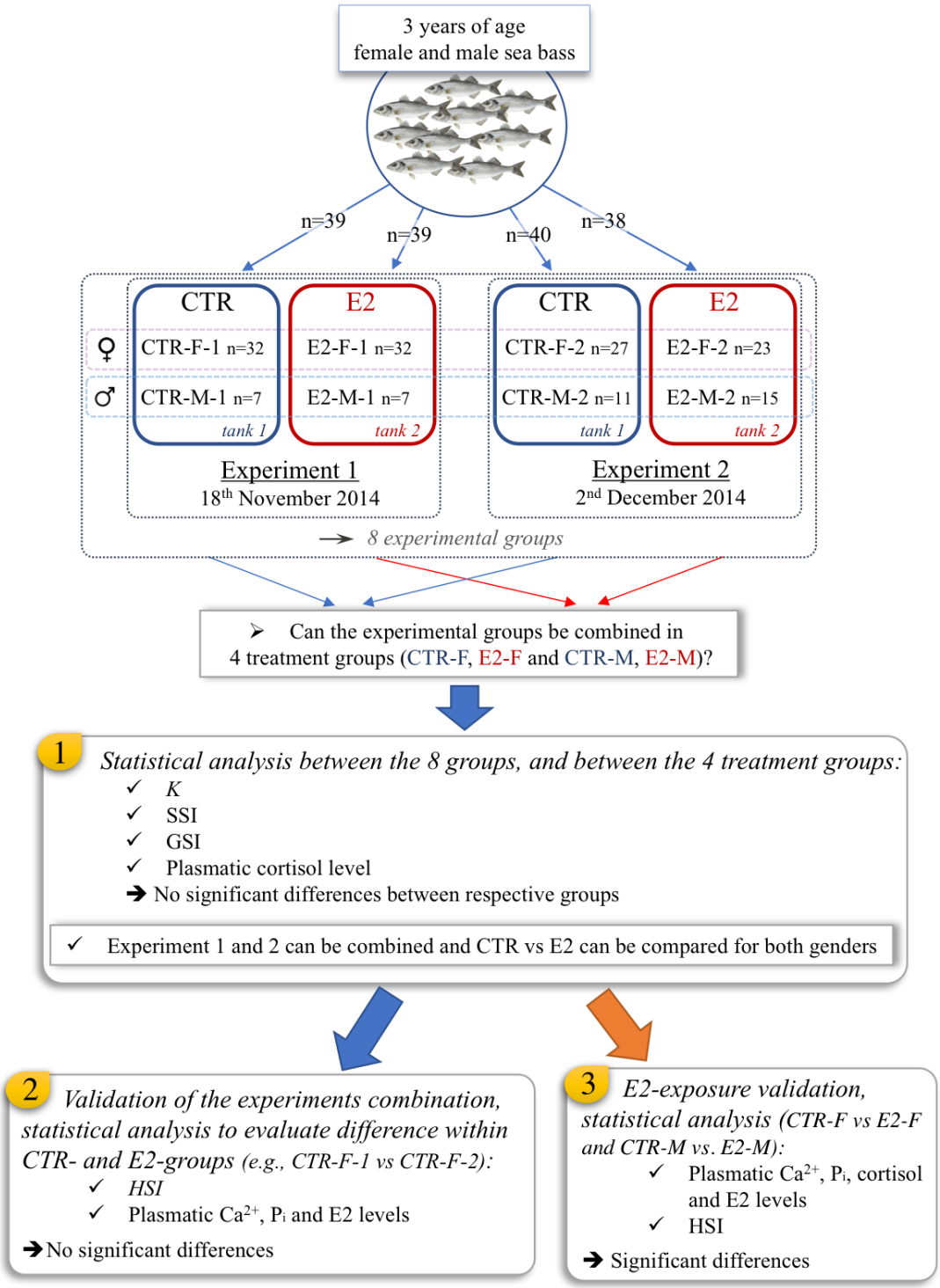


Figure S1: Experimental setup and procedure to validate the pooling of the two experiments. Two successive E2 exposures were conducted leading to 8 experimental groups corresponding to the control and treated fish from both genders and both experiments, i.e., CTR-F-1, CTR-F-2, E2-F-1, E2-F-2, CTR-M-1, CTR-M-2, E2-M-1 and E2-M-2. To validate that both experiments can be combined in four treatment groups including the control and treated groups of both genders (CTR-F, E2-F, CTR-M and E2-M), Fulton's condition factor (K), spleno-somatic index (SSI), gonado-somatic index (GSI) and plasmatic cortisol-level were compared. Because no statistically significant difference could be detected when comparing between the treatment groups for both sexes, the experiments were combined. As a confirmation, no statistically significant differences were observed when comparing: the hepato-somatic index (HSI) and the plasmatic level of Ca^{2+}, P_i and E2 between the respective treatment groups. Subsequently, the E2-exposure was validated with both experiments combined using the plasmatic Ca^{2+}-, P_i- and cortisol-levels as well as the HSI. The different groups were compared using a Kruskal-Wallis rank sum-test followed by a Tukey-Kramer (Nemenyi) post-hoc test.

Table S1: Sample sizes analysed in each experimental group.

	Experiment 1 (November 2014)					Experiment 2 (December 2014)			
Biometric index	CTR-F-1	CTR-M-1	E2-F-1	E2-M-1	Biometric index	CTR-F-2	CTR-M-2	E2-F-2	E2-M-2
K	32	7	32	7	*K*	27	11	23	15
GSI	32	7	32	7	GSI	27	11	22	14
SSI	16	4	16	4	SSI	14	6	11	9
HSI	32	7	32	7	HSI	27	11	23	15
Gene expression					Gene expression				
Thymus	6	2	7	2	Thymus	9	3	6	5
Head-kidney	8	4	6	4	Head-kidney	6	5	5	7
Spleen	8	4	6	3	Spleen	4	6	5	5
Plasmatic parameters					Plasmatic parameters				
E2	13	3	13	3	E2	13	6	12	7
VTG	4	2	7	2	VTG	4	5	5	4
Ca	13	3	13	3	Ca	13	6	11	7
P_i	13	3	12	3	P_i	13	6	11	7
Cortisol	13	3	13	3	Cortisol	13	6	12	8
Flow cytometry					Flow cytometry				
Head-kidney	4	2	4	2	Head-kidney	6	3	5	4
Spleen	4	2	3	2	Spleen	4	1	1	3

Partial gene sequences determination: For *foxn1* and *ikaros* transcript quantification, we first designed primers for conserved sequences by multi-sequence alignments of the following sequences: *Danio rerio*, Accession no. AF092175.1; *Homo sapiens*, BC018349.1; *Xenopus tropicalis,* 001015698.1; *Gallus gallus,* 205088.1; *Larimichtys crocea*, 010734525.1 ; *Salmo salar*, 001173899.1 for *ikaros* and *Larimichthys crocea,* 010735826.1; *Danio rerio,* 212573.1, *Oryzias latipes,* AB274724.1, *Stegastes partitus,* 008295040.1, *Poecilia Formosa,* 007563077.1 for *foxn1*. Partial gene sequences of *D. labrax ikaros* and foxn1 were amplified using the primers specific for the conserved parts of the sequence (Table S2). PCR was performed with cDNA from the thymus of sea bass, specific primers and the Purple Taq (Ozyme, Montigny-le-Bretonneux, France). Negative controls were performed with DNA free water. PCR conditions were as follows: initial incubation at 95°C for 2min followed by 72°C for 5min. For all genes, 40 cycles at 95° for 30s, 60° for 30s (the hybridization temperature is primer specific, see Table S2) and 72°C for 45s were carried out for the Purple Taq. The size of the various amplicons was determined on 2% agarose gels. The PCR product were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Fitchburg, USA) and following the manufacturer instructions. Purified PCR products were then sequenced to validate the amplification specificity and to design specific primers for qPCR (Table S3).

* decrease of 1°C at each cycle over 10 consecutive cycles followed by constant hybridation temperature.

Table S2: Oligonucleotide sequences used to amplify and sequence partial D. labrax sequences

Genes	Primers 5'-3'	Amplicon pb	Hybridation Temperature
foxn1	F: GCATCTTGATCTTCTTGGCTCTG R: CCACTTGTGCAGCTCCTCC	260	60°C
ikaros	F: AGTGTGGTGCCTCTTTCACC R: TCTGTGGCATAGTGCTCTTAC	396	70°C*

Table S3: Oligonucleotide sequences and qPCR conditions for the selected thymic function-related genes (ikaros, rag1, foxn1 and aire), homeostasis-related genes (caspase8, caspase9 and pcna) as well as antigen-presenting-cell- and T cell-related genes (mhc2a, tcra, foxp3 and tcrg). Normalised relative gene expression was calculated by geometric means (for details see text) using three reference genes (ef1a, l13a, fau).

Genes	Primers 5'-3'	Amplicon pb	Hybridation Temperature	Amount of Primer	Reaction volume qPCR	Efficiency			Gene accession numbers
						Thymus	HK	Spleen	
ikaros	F: TTTCACCCAGAAGGGCAACC R: CCGAATGGGTGCGTAAATGG	131	57.5°C	0.5 µM	10µl	1.97			DLAgn_000 10890
rag1	F: CACTGGCTCTACACCTCCAA R: CTCATCTTCCAGGCTCTCCAC	108	60°C	0.75 µM	5µl	1.98			AF137181.1
foxn1	F: CCCTATCTACAGCACCACCT R: GAATAGTGTGGACGGCGAAT	84	60°C	0.5 µM	5µl	2.00			DLAgn_000 31910
aire	F: ACGTCGGTGGGGAGTTTTAC R: TCCGTCCTTACACACTGCAC	158	60°C	0.5 µM	5µl	2.18			DLAgn_561 0
tcra	F: GGCCACTGGTTTCAGCAGAT R: AGAGCCATGAGGTTCACCAT	213	60°C	0.5 µM	1.5µl	1.99	1.99	1.90	AY831387. 1
foxp3	F: GGAGCAGTATTGTGGGCACT R: TCGTCTGGAAGCTGTTTGGG	80	60°C	0.5 µM	1.5µl	1.99	1.83	2.10	KJ818328
tcrg	R: CCAACTGCACACATGCTGAC R: TTGAGGCACTTTATCAAACCTGT	164	60°C	0.5 µM	10µl	1.96	1.94	1.97	EU853841.1
pcna	F: GCTGGGTACAGGAAACGTCA R: GCGTGGCTTTGGTGAAGAAG	137	60°C	0.5 µM	1.5µl	1.919	1.98	1.77	JQ755266.1
mhc2a	F: GGTCTGACTGTCGGTCTGCT R: TGCACTCGTTTCCTTTGATG	67	60°C	0.5 µM	1.5µl	2.04	1.98	1.90	AM113466. 1
caspase8	F: TGTCAGGGAAGCCTCTACCA R: CATCCCCAGCAGGAAGTCAG	150	60°C	0.5 µM	5µl	2.28	1.98	2.00	FJ225665.1
caspase9	F: CGAATGCAACCGAGCACAAA R: ACTAACGACCGCCAATGAGG	193	60°C	0.5 µM	5µl	2.09	2.13	1.80	DQ345775. 1
l13a	F: TCTGGAGGACTGTCAGGGGCATGC R: AGACGCACAATCTTGAGAGCAG	145	60°C	0.5 µM	1.5µl	2.08	2.10	2.04	DLAgn_000 23060
fau	F: GACACCCAAGGTTGACAAGCAG R: GGCATTGAAGCACTTAGGAGTTG	149	60°C	0.5 µM	1.5µl	1.96	2.04	1.97	DLAgn_000 67980
ef1a	F: GGCTGGTATCTCTAAGAACG R: CCTCCAGCATGTTGTCTCC	239	60°C	0.5 µM	1.5µl	1.60	2.00	2.03	AJ866727.1

B-Results:

Female and male fish had total lengths of 30.07 cm ± 2.16 s.d. and 29.72 cm ± 2.25 s.d., respectively, and weighed 377.4 g ± 87.63 s.d. and 380.9 g ± 97.82 s.d.. When comparing the biometric indices between all eight experimental groups (see figure S2), the Fulton's condition factor *K* indicated significant differences between the experimental groups (*Kruskal-Wallis*, $H_{7,146}=20.35$, $p=0.005$). The *post-hoc* analysis revealed a significant difference between CTR-F-1 and E2-M-2 ($p=0.021$) and between CTR-M-1 and E2-M-2 ($p=0.010$) (overall *p*-values are reported in Table S4). The GSI (*Kruskal-Wallis*, $H_{7,146}=7.87$, $p=0.344$) did not differ significantly across all experimental groups, neither did the SSI (*Kruskal-Wallis*, $H_{7,72}=9.06$, $p=0.248$), nor the plasmatic level of cortisol (*Kruskal-Wallis*, $H_{7,63}=13.27$, $p=0.066$).

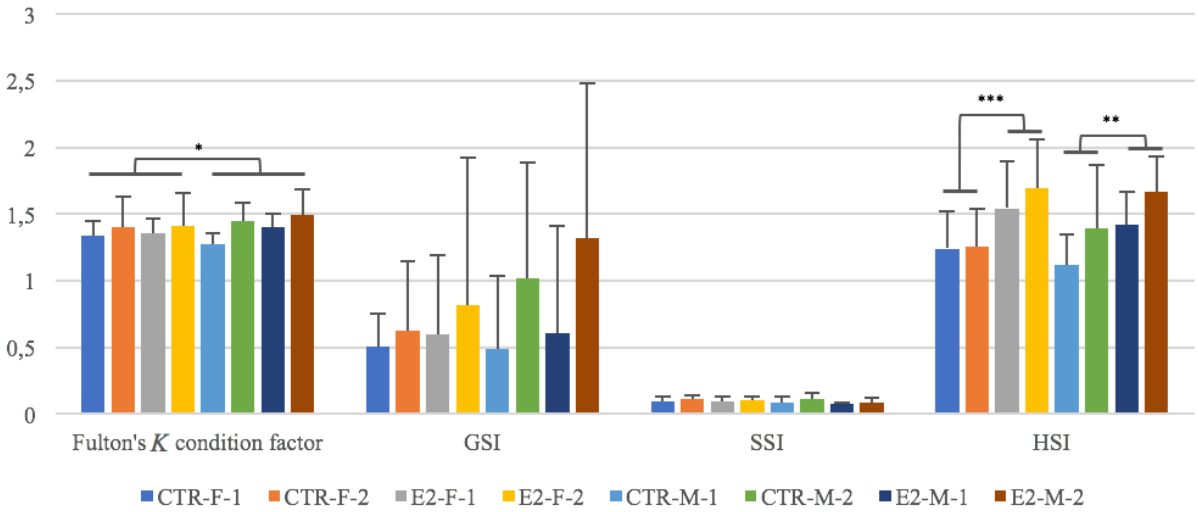


Figure S2: Biometric indices (Fulton's K condition factor, gonado-somatic index (GSI), spleno-somatic index (SSI) and hepato-somatic index (HSI)) of the different experimental groups including control (CTR), exposed (E2), females (F) and males (M) from the experiment of November (1) and December (2). Indices are means and standard deviation. *, significantly different at 0.05≥p>0.01 for males and females from both experiments and treatments combined; **, significantly different at 0.01≥p>0.001 for males from both experiments combined and ***, significantly different at p<0.001 for females from both experiments combined.

Table S4: p values obtained with the Tukey-Kramer (Nemenyi) post-hoc test corresponding to the Fulton's condition factor K pairwise analysis of the experimental groups. Values indicate significant differences at p<0.05

	CTR-F-1	CTR-F-2	CTR-M-1	CTR-M-2	E2-F-1	E2-F-2	E2-M-1
CTR-F-2	0.974						
CTR-M-1	0.861	0.492					
CTR-M-2	0.299	0.804	0.082				
E2-F-1	0.994	1.000	0.584	0.662			
E2-F-2	0.818	1.000	0.304	0.961	0.994		
E2-M-1	0.878	0.997	0.387	1.000	0.987	0.999	
E2-M-2	0.021	0.237	0.010	1.000	0.123	0.547	0.970

When both experiments were combined to compare females and males from the two treatment groups (CTR *vs*. E2), *K* values were significantly different (*Kruskal-Wallis*, $H_{3,150}$=9.53, p=0.023), with *post-hoc* analysis revealing a significant difference between CTR-F and E2-M (p=0.011; overall p-values are listed in Table S5). When combining the two experiments, the GSI (*Kruskal-Wallis*, $H_{3,150}$=0.87, p=0.832), the SSI (*Kruskal-Wallis*, $H_{3,74}$=0.87, p=0.198) and the plasmatic level of cortisol (*Kruskal-Wallis*, $H_{3,67}$=5.81, p=0.121; Fig. 2) did not reveal any significant difference between CTR-F, CTR-M, E2-F and E2-M.

Table S5: p values obtained with the Tukey-Kramer (Nemenyi) post-hoc test corresponding to the Fulton's K condition factor pairwise analysis with the two experiments confounded. Values in red correspond to p<0.05

	CTR-F	CTR-M	E2-F
CTR-M	0.951		
E2-F	0.824	1.000	
E2-M	0.011	0.203	0.077

Comparing E2 *vs*. CTR with all invidious, *i.e.*, both genders and both experiments combined, *K* (*U*-test, p=0.062), GSI (*U*-test, p=0.380), SSI (*U*-test, p=0.092) and cortisol-levels (*U*-test, p=0.367) were not statistically different.

Fish from control and treatment groups of the two experiments combined, revealed a significant difference between the *K* of females with 1.37 g/cm^3 ± 0.18 s.d., which was slightly lower than that of males, 1.42 g/cm^3 ± 0.17 s.d. (*U*-test, p=0.026). The GSI between males and females, all treatment groups and both experiments confounded, was not statistically different (*U*-test, p=0.380), neither was the SSI (*U*-test, p=0.092), nor the cortisol-level (*U*-test, p=0.578).

Considering the plasmatic E2-levels in all eight experimental groups, the statistical analysis revealed significant differences (*Kruskal-Wallis*, $H_{7,60}$=54.07, p<0.001), in the HSI values (*Kruskal-Wallis*, $H_{7,146}$=44.94, p<0.001) as well as in the plasmatic level of Ca^{2+} (*Kruskal-Wallis*, $H_{7,61}$=39.10, p<0.001). The *post-hoc* analysis indicated a significant difference of E2-levels, HSI and Ca^{2+}-levels between the CTR and the E2 group for all pairs, except the males of the first experiment (see *p*-values in Table S6-8). Considering the plasmatic level of P_i, the statistical analysis with all experimental groups revealed a significant difference (*Kruskal-Wallis*, $H_{7,61}$=39.10, p=0.011), with the *post-hoc* analysis indicating a significant difference between CTR and E2 group for female of the second experiment only (see *p* value in Table S9).

Table S6: p values obtained with the Tukey-Kramer (Nemenyi) post-hoc test corresponding to the E2 values pairwise analysis of the experimental groups. Values in red correspond to p<0.05

	CTR-F-1	CTR-F-2	CTR-M-1	CTR-M-2	E2-F-1	E2-F-2	E2-M-1
CTR-F-2	0.974						
CTR-M-1	1.000	1.000					
CTR-M-2	1.000	0.999	1.000				
E2-F-1	0.003	4E-05	0.147	0.025			
E2-F-2	0.008	1.41E-04	0.212	0.048	1.000		
E2-M-1	0.258	0.076	0.388	0.286	1.000	1.000	
E2-M-2	0.008	2.58E-04	0.133	0.031	1.000	1.000	1.000

Table S7: p values obtained with the Tukey-Kramer (Nemenyi) post-hoc test corresponding to the HSI values pairwise analysis of the experimental groups. Values in red correspond to p<0.05

	CTR-F-1	CTR-F-2	CTR-M-1	CTR-M-2	E2-F-1	E2-F-2	E2-M-1
CTR-F-2	0.999						
CTR-M-1	0.988	0.963					
CTR-M-2	0.864	0.953	0.645				
E2-F-1	0.009	0.042	0.052	0.942			
E2-F-2	1.610E-04	0.001	0.005	0.445	0.910		
E2-M-1	0.946	0.984	0.761	1.000	0.976	0.647	
E2-M-2	6.720E-04	0.003	0.006	0.416	0.874	1.000	0.596

Table S8: p values obtained with the Tukey-Kramer (Nemenyi) post-hoc test corresponding to the Ca^{2+} values pairwise analysis of the experimental groups. Values in red correspond to p<0.05

	CTR-F-1	CTR-F-2	CTR-M-1	CTR-M-2	E2-F-1	E2-F-2	E2-M-1
CTR-F-2	0.940						
CTR-M-1	0.951	0.100					
CTR-M-2	0.985	1.000	1.000				
E2-F-1	3.4E-05	0.006	0.591	0.065			
E2-F-2	9.6E-05	0.010	0.615	0.083	1.000		
E2-M-1	0.493	0.911	0.998	0.944	0.977	0.979	
E2-M-2	0.493	0.641	0.992	0.805	0.897	0.913	1.000

Table S9: p values obtained with the Tukey-Kramer (Nemenyi) post-hoc test corresponding to the Pi values pairwise analysis of the experimental groups. Values in red correspond at p<0.05

	CTR-F-1	CTR-F-2	CTR-M-1	CTR-M-2	E2-F-1	E2-F-2	E2-M-1
CTR-F-2	1.000						
CTR-M-1	0.555	0.684					
CTR-M-2	0.979	0.996	0.968				
E2-F-1	0.193	0.350	1.000	0.966			
E2-F-2	0.019	0.049	1.000	0.651	0.988		
E2-M-1	1.000	1.000	0.930	1.000	0.932	0.655	
E2-M-2	0.601	0.768	1.000	0.997	1.000	0.966	0.985

In summary, except the Fulton's K condition factor, which presents a gender difference, the pertinent biometric and physiological parameters did not differ significantly between the groups from the two experiments. Hence, the results from both experiments were combined to increase the statistical power for detecting a possible oestrogenic modulation of T cell-related gene expression in males and female sea bass. Importantly, the parameters crucial for the experimental increase of E2-levels, *i.e.*, plasmatic E2-, Ca^{2+}- and P_i-levels as well as HSI were not different detected between the respective treatment groups of both experiments, *e.g.,* CTR-F-1 vs. CTR-F-2 (Tables S6-9) which confirmed that the experiments can be combined.

Table S10: p-value obtained with the Tukey-Kramer (Nemenyi) post-hoc test corresponding to E2-level pairwise analysis of the treatment group from both gender with experiment 1 and 2 combined. Values in red correspond to p<0.05

	CTR-F	CTR-M	E2-F
CTR-M	0.996		
E2-F	2.056E-08	2.76E-04	
E2-M	3.836E-05	0.003	1.000

Table S11: p-value obtained with the Tukey-Kramer (Nemenyi) post-hoc test corresponding to the HSI factor pairwise analysis of the treatment group from both gender with experiment 1 and 2 combined. Values in red correspond to p<0.05

	CTR-F	CTR-M	E2-F
CTR-M	9.744E-01		
E2-F	6.183E-07	0.006	
E2-M	3.007E-04	0.024	1.000

Table S12: p-value obtained with the Tukey-Kramer (Nemenyi) post-hoc test corresponding to the Ca^{2+}-level pairwise analysis of the treatment group from both gender with experiment 1 and 2 combined. Values in red correspond to p<0.05

	CTR-F	CTR-M	E2-F
CTR-M	0.851		
E2-F	2.152E-08	0.003	
E2-M	2.396E-02	0.371	0.356

Table S13: p-value obtained with the Tukey-Kramer (Nemenyi) post-hoc test corresponding to the Pi-level pairwise analysis of the treatment group from both gender with experiment 1 and 2 combined. Values in red correspond to p<0.05

	CTR-F	CTR-M	E2-F
CTR-M	0.319		
E2-F	5.590E-04	0.654	
E2-M	0.360	1.000	0.535

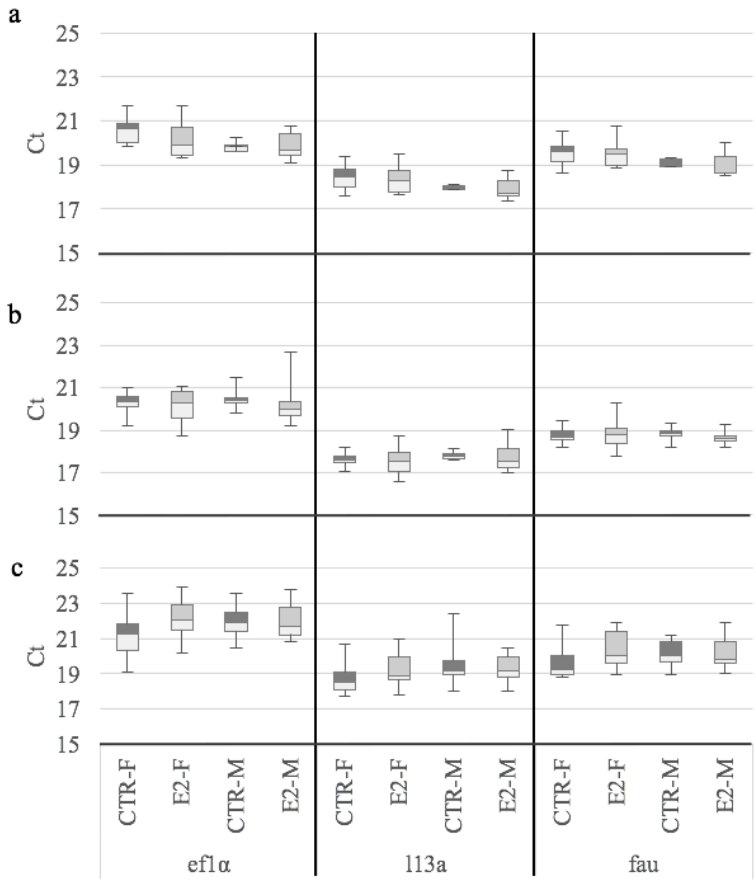


Figure S3: Cycle thresholds (Ct) for the reference genes assayed obtained in qPCR in three lymphoid organs of sea bass, Dicentrarchus labrax, in males (M) and females (F) of E2-exposed (E2) and control (CTR) fish for thymus (a), head-kidney (b) and spleen (c). No significant differences between groups were observed.

Table S14: Matrice of Spearman Rank Order correlation coefficients between the different variables measured in control females (A) and E2-injected females (B) in the thymus (T). For each correlation is the r, p and n values are shown (in this order).

A	*rag1*	*foxn1*	*aire*	*tcr-a-T*	*foxp3-T*	*tcrg-T*	*mhc2-T*	*pcna-T*	*Csp8-T*	*Csp9-T*
ikaros	0.626	**0.732**	0.0179	0.578	0.514	0.242	0.468	0.418	0.379	**0.707**
	0.0158	**0.00145**	0.944	0.0291	0.048	0.414	0.0873	0.117	0.158	**0.00278**
	14	**15**	15	14	15	13	14	15	15	**15**
rag1		0.516	0.13	0.665	0.451	0.0879	0.434	0.27	0.178	0.534
		0.056	0.648	0.0122	0.101	0.764	0.132	0.34	0.532	0.047
		14	14	13	14	13	13	14	14	14
foxn1			-0.129	0.587	**0.811**	-0.225	0.468	0.075	0.321	**0.757**
			0.639	0.0262	**0.2E-6**	0.447	0.0873	0.783	0.235	**0.000575**
			15	14	**15**	13	14	15	15	**15**
aire				-0.253	0.0821	-0.137	-0.2	-0.404	-0.239	-0.0786
				0.373	0.763	0.643	0.482	0.131	0.381	0.773
				14	15	13	14	15	15	15
tcra-T					0.358	0.011	0.64	0.235	0.116	0.64
					0.201	0.964	0.0131	0.407	0.682	0.0131
					14	13	14	14	14	14
foxp3-T						-0.412	0.385	-0.107	0.293	0.654
						0.154	0.167	0.695	0.281	0.00785
						13	14	15	15	15
tcrg-T							-0.137	0.159	-0.412	-0.187
							0.643	0.591	0.154	0.528
							13	13	13	13
mhc2-T								0.424	0.266	0.534
								0.125	0.348	0.047
								14	14	14
pcna-T									**0.711**	0.454
									0.00256	0.0861
									15	15
Csp8-T										0.682
										0.00471
										15

B	*rag1*	*foxn1*	*aire*	*tcr-a-T*	*foxp3-T*	*tcrg-T*	*mhc2-T*	*pcna-T*	*Csp8-T*	*Csp9-T*
ikaros	0.594	0.467	0.407	0.545	0.544	0.308	0.0275	0.313	0.456	0.335
	0.0387	0.102	0.16	0.0623	0.0517	0.295	0.921	0.286	0.111	0.252
	12	13	13	12	13	13	13	13	13	13
rag1		-0.21	0.301	0.164	0.364	-0.217	-0.035	-0.189	0.161	0.042
		0.498	0.329	0.614	0.233	0.484	0.904	0.542	0.603	0.886
		12	12	11	12	12	12	12	12	12
foxn1			0.0549	0.175	0.555	0.341	0.0879	0.33	0.516	0.67
			0.849	0.572	0.0464	0.244	0.764	0.261	0.067	0.0113
			13	12	13	13	13	13	13	13
aire				0.392	0.396	-0.126	0.313	-0.198	-0.011	0.198
				0.197	0.173	0.669	0.286	0.504	0.964	0.504
				12	13	13	13	13	13	13
tcra-T					0.196	-0.126	0.49	0.406	0.049	-0.189
					0.527	0.683	0.0998	0.181	0.869	0.542
					12	12	12	12	12	12
foxp3-T						-0.176	0.396	-0.115	0.72	0.643
						0.553	0.173	0.696	0.00473	0.0167
						13	13	13	13	13
tcrg-T							-0.368	0.0604	0.0769	0.363
							0.206	0.835	0.792	0.214
							13	13	13	13
mhc2-T								-0.121	-0.022	-0.011
								0.682	0.935	0.964
								13	13	13
pcna-T									0.187	-0.022
									0.528	0.935
									13	13
Csp8-T										0.681
										0.00948
										13

Table S15: Matrice of Spearman Rank Order correlation coefficients between the different variables measured in control females (A) and E2-injected females (B) in the head-kidney (HK). For each correlation is the r, p and n values are shown (in this order).

A	*foxp3-HK*	*tcrg-HK*	*mhc2-HK*	*pcna-HK*	*Csp8-HK*	*Csp9-HK*
tcra-HK	0.618	0.336	0.468	0.284	0.297	0.415
	0.0178	0.231	0.0873	0.316	0.293	0.134
	14	14	14	14	14	14
foxp3-HK		0.134	0.618	0.543	0.152	**0.758**
		0.637	0.0178	0.0429	0.594	**0.00102**
		14	14	14	14	**14**
tcrg-HK			0.38	0.292	-0.0418	0.187
			0.173	0.301	0.88	0.511
			14	14	14	14
mhc2-HK				**0.719**	0.134	0.345
				0.0032	0.637	0.219
				14	14	14
pcna-HK					0.341	0.464
					0.225	0.0907
					14	14
Csp8-HK						0.305
						0.279
						14

B	*foxp3-HK*	*tcrg-HK*	*mhc2-HK*	*pcna-HK*	*Csp8-HK*	*Csp9-HK*
tcra-HK	0.583	0.115	0.648	-0.0909	0.285	0.05
	0.0874	0.733	0.0377	0.785	0.404	0.878
	9	10	10	10	10	9
foxp3-HK		0.167	0.367	-0.0667	0.3	0.533
		0.643	0.308	0.844	0.407	0.124
		9	9	9	9	9
tcrg-HK			-0.136	0.3	0.0424	0.05
			0.673	0.353	0.892	0.878
			11	11	10	9
mhc2-HK				-0.0273	-0.0909	0.35
				0.924	0.785	0.331
				11	10	9
pcna-HK					-0.139	0.117
					0.681	0.742
					10	9
Csp8-HK						0.0833
						0.809
						9

Table S16: Matrice of Spearman Rank Order correlation coefficients between the different variables measured in control males (A) and E2-injected males (B) in the head-kidney (HK). For each correlation is the r, p and n values are shown (in this order).

A	*foxp3-HK*	*tcrg-HK*	*mhc2-HK*	*pcna-HK*	*Csp8-HK*	*Csp9-HK*
tcra-HK	0.6 0.0769 9	0.7 0.0301 9	0.533 0.124 9	-0.267 0.462 9	-0.4 0.264 9	0.75 0.0158 9
foxp3-HK		0.65 0.0501 9	0.433 0.223 9	0.117 0.742 9	0.25 0.491 9	**0.883** **0.2E-6** **9**
tcrg-HK			0.65 0.0501 9	0.35 0.331 9	-0.183 0.612 9	0.733 0.02 9
mhc2-HK				0.417 0.243 9	0.217 0.55 9	0.733 0.02 9
pcna-HK					0.333 0.356 9	0.233 0.52 9
Csp8-HK						0.167 0.643 9

B	*foxp3-HK*	*tcrg-HK*	*mhc2-HK*	*pcna-HK*	*Csp8-HK*	*Csp9-HK*
tcra-HK	0.382 0.233 11	0.394 0.243 10	**0.9** **0.2E-6** **11**	-0.127 0.693 11	-0.164 0.614 11	0.0727 0.818 11
foxp3-HK		0.588 0.0665 10	0.391 0.221 11	0.327 0.31 11	0.191 0.557 11	**0.782** **0.00285** **11**
tcrg-HK			0.43 0.199 10	0.212 0.535 10	-0.00606 0.973 10	0.527 0.107 10
cmh2-HK				-0.282 0.384 11	-0.136 0.673 11	0.0909 0.776 11
pcna-HK					0.473 0.132 11	0.518 0.0948 11
Csp8-HK						0.127 0.693 11

Table S17: Matrice of Spearman Rank Order correlation coefficients between the different variables measured in control females (A) and E2-injected females (B) in the spleen (S). For each correlation is the r, p and n values are shown (in this order).

A	*foxp3-S*	*tcrg-S*	*mhc2-S*	*pcna-S*	*Csp8-S*	*Csp9-S*
tcra-S	0.145 0.653 11	-0.145 0.653 11	0.0909 0.776 11	-0.2 0.559 10	-0.0182 0.946 10	-0.127 0.693 11
foxp3-S		0.622 0.0285 12	0.126 0.683 12	0.118 0.714 11	0.182 0.575 11	-0.021 0.939 12
tcrg-S			-0.0909 0.766 12	-0.382 0.233 11	0.0909 0.776 11	-0.0699 0.817 12
mhc2-S				-0.373 0.245 11	0.364 0.257 11	-0.517 0.0795 12
pcna-S					-0.479 0.148 10	0.582 0.0555 11
Csp8-S						-0.664 0.0234 11

B	*foxp3-S*	*tcrg-S*	*mhc2-S*	*pcna-S*	*Csp8-S*	*Csp9-S*
tcra-S	0.58 0.0446 12	0.627 0.0354 11	0.028 0.921 12	-0.497 0.0944 12	0.126 0.683 12	0.315 0.306 12
foxp3-S		0.709 0.0127 11	0.469 0.117 12	-0.035 0.904 12	0.0699 0.817 12	0.51 0.0843 12
tcrg-S			0.0818 0.797 11	0.00909 0.968 11	0.264 0.416 11	0.173 0.595 11
mhc2-S				0.119 0.699 12	-0.322 0.295 12	0.51 0.0843 12
pcna-S					0.0909 0.766 12	-0.105 0.733 12
Csp8-S						-0.559 0.0547 12

Table S18: Matrice of Spearman Rank Order correlation coefficients between the different variables measured in control males (A) and E2-injected males (B) in the spleen (S). For each correlation is the r, p and n values are shown (in this order).

A	*foxp3-S*	*tcrg-S*	*mhc2-S*	*pcna-S*	*Csp8-S*	*Csp9-S*
tcra-S	0.0909	0.515	0.0909	-0.286	-0.248	-0.2
	0.785	0.116	0.785	0.46	0.468	0.559
	10	10	10	8	10	10
foxp3-S		-0.103	0.261	-0.429	-0.588	0.612
		0.759	0.446	0.26	0.0665	0.0537
		10	10	8	10	10
tcrg-S			0.212	0.0952	0.188	-0.127
			0.535	0.794	0.583	0.707
			10	8	10	10
mhc2-S				0.119	0.103	0.0909
				0.749	0.759	0.785
				8	10	10
pcna-S					0.69	-0.595
					0.0474	0.102
					8	8
Csp8-S						-0.37
						0.275
						10

B	*foxp3-S*	*tcrg-S*	*mhc2-S*	*pcna-S*	*Csp8-S*	*Csp9-S*
tcra-S	0.75	0.524	0.548	0.607	0.214	0.714
	0.0384	0.16	0.139	0.121	0.602	0.0545
	7	8	8	7	7	7
foxp3-S		**0.929**	**0.964**	**0.964**	0.679	0.571
		0.2E-6	**0.2E-6**	**0.2E-6**	0.0735	0.15
		7	**7**	**7**	7	7
tcrg-S			**0.976**	**0.964**	0.607	0.643
			0.2E-6	**0.2E-6**	0.121	0.0956
			8	**7**	7	7
mhc2-S				**1**	0.75	0.5
				0.2E-6	0.0384	0.217
				7	7	7
pcna-S					0.75	0.5
					0.0384	0.217
					7	7
Csp8-S						-0.0357
						0.905
						7

C) Article 3

Oestrogen differentially modulates oxidative burst capacity and ROS-signaling in adaptive and innate immune cells of three major lymphoid organs in European sea bass, *Dicentrarchus labrax* (L.) (*In preparation*)

Oestrogen differentially modulates oxidative burst capacity and ROS-signalling in lymphoid and myeloid cells of three major lymphoid organs in European sea bass, *Dicentrarchus labrax* (L.)

Matthieu Paiola, Catarina Moreira, Aurélie Duflot, Thomas Knigge, Tiphaine Monsinjon*

Normandy University, FR CNRS 3730 SCALE, UMR-I 02 INERIS-URCA-ULH Environmental Stress and Aquatic Biomonitoring (SEBIO), Université Le Havre Normandie, F-76600 Le Havre, France

* author for correspondance:

Tiphaine Monsinjon

UMR-I 02 INERIS-URCA-ULH, Unité Stress Environnementaux et Biosurveillance des milieux aquatiques, UFR Sciences et Techniques, Université Le Havre Normandie, 25 rue Philippe Lebon, 76063 Le Havre, France.

E-mail address: tiphaine.monsinjon@univ-lehavre.fr

Keywords: Lymphocytes, myeloid cells, thymus, spleen, head-kidney, reactive oxygen species, reactive nitrogen species

Abbreviations: E2, 17β-oestradiol; FSC, forward scatter; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; H_2O_2, hydrogen peroxide; MFI, mean fluorescent intensity; NO, nitric oxide; $O_2^{\bullet -}$, superoxide anion; $OH^{\bullet}$, hydroxyl radical; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; RNS, reactive nitrogen species; SSC, side scatter

Abstract:

Besides their obvious role in sex determination and reproduction, oestrogens display a complex immunomodulatory function, which appears to be widely conserved across vertebrates. However, to date, our knowledge is insufficient to understand and predict the adverse effects of exogenous oestrogens, extensively introduced into the aquatic environments, on teleost immunocompetence. Therefore, as an integrative measure, we assessed the *in vitro* effects of 17β-oestradiol on isolated leucocytes from the head-kidney, the spleen and the thymus together with their respective oestrogen receptor expression. Given the importance of reactive oxygen species as a signalling/defence component in both innate and adaptive immunity of mammals, we investigated the effect of 17β-oestradiol on the oxidative burst capacity, the redox status and the viability on both adaptive and innate immune cells by flow cytometry. Leucocytes from both head-kidney and spleen expressed nuclear and membrane oestrogen receptors, as is the case in the thymus. 17β-oestradiol-treatment differentially modulated both innate and adaptive immune cells derived from the three lymphoid organs. Our results suggest that B cells have respiratory burst capacity in sea bass as they do in other teleost species and mammals. Intriguingly, our study suggest that 17β-oestradiol stimulates immature T cell apoptosis and modulates lymphocyte and myeloid function/differentiation. As 17β-oestradiol apparently affects the redox status in immune cells, we propose that oestrogens modulate immune cell function in teleosts by acting on non-genomic signalling pathway, which further trigger the reactive oxygen/nitrogen species signalling.

1. Introduction

To eliminate pathogenic agents and abnormal cells, a potent immune system has developed in the vertebrate lineage, which is commonly divided in two components: the innate and adaptive system, also referred to as specific and non-specific immune system (Boehm and Swann, 2014; Pasman and Kasper, 2017). Commonly, those two components are associated by the myeloid and lymphoid cell lineages. These cells have distinct progenitors and distinct antigen binding receptors that determine their function: innate immune cells express a germline-encoded antigen-binding receptor on their plasmatic membrane, the so-called pathogen recognition receptor, whereas lymphocyte antigen recognition is based on clonally expressed antigen receptor genes the elements of which are assembled in various combinations (Boehm and Swann, 2014; Pasman and Kasper, 2017). Both types of immune cells are, however, essential for the immune system, as they have complementary and non-redundant functions. Indeed, innate immune cells constitute sentinel cells. During an invasion of pathogenic agents, they rapidly react to generate the immune response and eliminate the pathogenic agent. As a second line of defence, innate immune cells and specially antigen-presenting cells activate lymphocytes to trigger a specific immune response (Esteban et al., 2015; Riera Romo et al., 2016).

In many aspects, the innate and adaptive immune system of teleosts bear resemblance to that of mammals at the gene-, cellular- and organ-level. The adaptive component is based on two lineages of lymphocytes with distinct functions, the T and B cells. Basically, T cells coordinate the immune response and the elimination of transformed cells. B cells, on the other hand, are involved in the specific and unspecific humoral defence by the secretion of specific and natural antibodies (Boehm and Swann, 2014; Parra et al., 2013; Tafalla et al., 2016). For the innate component, numerous immune cells, such as macrophages, mast cells, granulocytes, and dendritic cells, initially characterised in mammals, have also been found in teleost fish (Esteban et al., 2015; Havixbeck and Barreda, 2015; Riera Romo et al., 2016; Sfacteria et al., 2015). Moreover, major immune organs appear to be evolutionary conserved. The thymus, which is the primary site of thymopoiesis, and the spleen, a major secondary lymphoid organ, are present in all vertebrate classes and may be considered evolutionary ancient immune organs (Boehm and Swann, 2014; Litman et al., 2010). Differences exist, however, as well. The bone marrow, for instance, is the site of myelopoiesis and B cell development in mammals, whereas in teleost these cells are developed in the head-kidney (Boehm and Swann, 2014; Litman et al., 2010).

In both, teleosts and mammals, the immune system is closely regulated by the endocrine system (Burgos-Aceves et al., 2016; Milla et al., 2011; Segner et al., 2013, 2017; Szwejser et al., 2016). The crosstalk between the endocrine and immune system has been extensively characterized in mammals. Typically, this crosstalk becomes obvious in sexual dimorphisms of immune system performance. For instance, the prevalence of many autoimmune diseases in women illustrates the connection between the endocrine and immune system (Klein and Flanagan, 2016; Segner et al., 2017). Especially the female sex hormone oestrogen has been investigated for its immunoregulatory function. Indeed, this steroid hormone is well known to modulate innate and adaptive immune cell function across all vertebrate classes (Burgos-Aceves et al., 2016; Milla et al., 2011; Segner et al., 2013, 2017; Szwejser et al., 2016). The cellular action of oestrogen is mediated through both, genomic and non-genomic signalling pathways. These pathways either imply the nuclear oestrogen receptors (Esrs), which belong to the super family of the ligand-dependent transcription factors, or a membrane-associated oestrogen receptor comprising membrane Esrs and G-protein-coupled oestrogen receptors (Gpers). A broad and varied expression of these oestrogen receptors can be found in the immune organs and immune cells of fish, a situation quite similar to that observed in mammals (Burgos-Aceves et al., 2016; Milla et al., 2011; Segner et al., 2013; Szwejser et al., 2016). Albeit an increasing body of evidence confirms the conserved immunomodulatory function of oestrogen, the physiological context and the evolutionary role are likely to be different between the different vertebrate classes and, therefore, require further examination (Segner et al., 2017).

Reactive oxygen species (ROS) are commonly measured as a bioindicator of physiological stress. Indeed, ROS are a by-product of cellular respiration and numerous other biological reactions that involve electron transport. ROS are, however, also essential for numerous cellular and physiological functions, including cell survival, cell growth, proliferation and differentiation (Nathan and Cunningham-Bussel, 2013; Yang et al., 2013). The ROS, but also the reactive nitrogen species (RNS), are groups of highly reactive chemical species which rapidly react with macromolecules, such as nucleic acids, lipids and proteins. ROS encompass the commonly described superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), or the hydroxyl radical ($OH^{\bullet -}$). The RNS are notably represented by the nitric oxide (NO), the nitrogen dioxide ($NO_2^{\bullet -}$), or the peroxinitrite $ONOO^-$ (Gostner et al., 2013; Havixbeck and Barreda, 2015; Nathan and Cunningham-Bussel, 2013). ROS and RNS may be part of intracellular signalling pathways by serving as second messengers and by activating transcription factors,

protein tyrosine kinases or phosphatases (Gostner et al., 2013; Nathan and Cunningham-Bussel, 2013; Yang et al., 2013). Moreover, in professional phagocytes (granulocytes and macrophages) of vertebrates, ROS production represents a major means to kill invading microorganisms (Esteban et al., 2015; Riera Romo et al., 2016). In teleosts and mammals, phagocytes utilise membrane-bound NADPH-oxidase and inducible NO-synthase to generate a large amount of $O_2^{\bullet-}$ and NO, respectively. This process is commonly referred to as oxidative or respiratory burst (Gostner et al., 2013; Havixbeck and Barreda, 2015; Yang et al., 2013). Moreover, in macrophages of mammals, mitochondrial-derived ROS also participate in the bactericidal activity (Yang et al., 2013). In the mammalian immune system, ROS and RNS production from different sources and the associated signalling are particularly important for the differentiation, activation and/or apoptosis of T and B cells, but also of natural killer cells, dendritic cells, and macrophages (Gostner et al., 2013; Karpuzoglu and Ahmed, 2006; Yang et al., 2013).

In a previous study, the *in vivo*-effects of 17β-oestradiol (E2) on the expression of genes-related to different T cells in the thymus, head-kidney and spleen of European sea bass was evaluated, coupled to oxidative burst analysis in myeloid and lymphoid cells of head-kidney and spleen (Paiola et al., *under review*). In the spleen, the E2-modulated stimulation of Treg-related gene expression was connected to an inhibition of oxidative burst. Hence, E2 could promote the Treg differentiation and function in the spleen of sea bass, as it does in mammals. To test this hypothesis and to determine if E2 directly modulates phagocyte activity in the head-kidney and the spleen, we investigated (1) the oestrogen receptor expression of both immune organs and associated immune cells, and (2) the E2-effect on the oxidative burst capacity of the isolated leucocytes from the head-kidney and spleen *in vitro*. In addition, the oxidative level and the viability was also analysed on leucocytes of the head-kidney, spleen and thymus. The aim for investigating different immune cell populations was to shed light on the E2-mediated effects on the immune system of teleost fish by providing a more integrated view of oestrogen immunomodulatory function.

2. Material and Methods

2.1. Animals and sampling

Sea bass (*Dicentrarchus labrax)* fingerlings from the hatchery at Gravelines, France were raised at Aquacaux, Octeville, France, in 1,800 L tanks with a continuous flow of filtered and aerated marine seawater at environmental temperatures. The fish were daily fed *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 batches of fish were employed for this study as characterised in the following: five fish of one-and-a-half-year of age with a total length of 21.8 cm ± 2.9 standard deviation (s.d.) and a weight of 97.8 g ± 10 s.d. (group 1) for molecular biology as well as 15 fish of two-years of age for the isolation of leucocytes from head-kidney, spleen and thymus used in the *in vitro* exposures. These fish had a total length of 25.8 cm ± 2.9 s.d. and a weight of 252 g ± 36 s.d. (group 2). Although both populations generally consisted of sexually immature females and males, the gender of some of the older fish (group 2) could be determined by macroscopic observation, resulting in a sex ratio of 1.5 (F/M). Specimens of group 1 were sacrificed in June 2015 and specimens of group 2 at the end of November 2016.

The fish were anesthetized with tricaine methanesulfonate (MS 222; Sigma, St. Louis, USA) in order to measure weight and total length before sacrificing them by an overdose of MS 222. Following decapitation, thymus, head-kidney and spleen were dissected. For group 1, part of the head-kidney and spleen were snap frozen in liquid nitrogen and stored at -80 °C for subsequent RNA extraction, whereas another part was kept for leucocyte isolation. For group 2, both thymuses as well as the entire spleen and head-kidney were used for leucocyte isolation.

2.2. Leucocyte isolation

All solutions for leukocyte preparation were adjusted to 360 mOsm/kg. Dissected thymuses, head-kidney and spleen were immersed in cold Leibovitz medium (L15; Sigma), and forced through a 100 µm cell strainer. The cell suspension was centrifuged at 1,200 *g* for 5 min at 4 °C. The supernatant was discarded and the cells were resuspended in L-15 and filtered

through a 40 µm mesh before loading on a Ficoll gradient (Pancoll, PAN^{TM} BIOTECH, Germany) at a density of 1.077 g/mL followed by a centrifugation at 400 *g* for 30 min at 4 °C. The leucocytes were collected at the interface between the L-15 and the Ficoll, washed and centrifuged twice with L15 at 1,200 *g* for 5 min and 4 °C. For PCR-analyses, erythrocytes were lysed before leucocytes were loaded on the Ficoll gradient in order to ensure cell purity. To this end, leucocytes were incubating with ammonium chloride-Tris solution for 30 min at room temperature under constant stirring. Subsequently, leucocytes were concentrated by centrifugation at 1,200 *g* for 8 min and washed once with L15 centrifugation at 1,200 *g* for 5 min at 4 °C. For flow cytometry, this step was not necessary. Therefore, erythrocyte lysis was omitted for all organs in the exposure experiments. The cells were stored at 4°C in Leibovitz medium (L15, Sigma) overnight before cell exposure.

2.3. RNA extraction and polymerase chain reaction

Four biological replicates were performed for each PCR. Extraction of RNA was conducted with standard procedures as described previously (Paiola et al., 2017). Total RNA was extracted from whole tissue utilizing the Tri Reagent (Sigma) by homogenisation in Precellys® tubes (CK14; Bertin instruments, Montigny-le-Bretonneux, France) twice for 10 s at 5,000 rpm and subsequent centrifugation at 12,000 *g* for 15 min at 4 °C to eliminate debris. RNA extraction from isolated leucocytes was performed with the RNeasy kit (Qiagen, Hilden, Germany) according to the supplier's instructions. After RNA extraction, possible DNA contamination was removed by digestion with the TURBO DNA-*free* Kit (Invitrogen-Ambion, Carlsbad, USA) according to the supplier's recommendation. RNA quality was assessed on 1% (w/v) agarose gels and the yield was quantified with a Nanodrop One (Thermo Fisher Scientific, Waltham, USA). Samples were stored at -80 °C until further processing.

Reverse transcription and PCR were performed as in Paiola et al. (2017). The M-MLV Reverse Transcriptase H$^-$ (Promega, Madison, USA) was used with 1 µg of total RNA and an oligo(dT)15 primer for 10 min of incubation at 40 °C, followed by 60 min at 45 °C and 15 min at 70 °C. The cDNA was stored at -20 °C until performing PCR using oestrogen receptor subtype specific primers. For *esr1*, *esr2a* and *gpera*, Purple Taq (Ozyme, Montigny-le-Bretonneux, France) and for *esr2b* and *gperb*, Platinum® Taq DNA Polymerase (Thermo Fisher Scientific) was used according to (Paiola et al., 2017). Negative controls were

performed with DNA free water. The size of the various amplicons was verified on 2 % agarose gels.

2.4. Leucocyte exposure

Isolated leucocytes were adjusted to $2.5x10^6$ cells/mL and cultured in 96 wells plates with 250,000 cells/well in modified L-15 with 10% of heat-inactivated foetal bovine serum (Sigma) and 1% of penicillin/streptomycin (Gibco, Thermo Fisher Scientific). Depending on the condition, leucocytes were cultured in modified L-15 without the addition of solvent and E2 (CTR), with solvent alone (0.00005%, v/v of ethanol) as a solvent control (SC) as well as with nominal concentrations of 10 nM and 100 nM of E2 (E8875; Sigma) in 0.00005% (v/v) ethanol for 2 h, 24 h and 48 h at 16 °C. The ethanol was reduced to a minimum of 0.00005% v/v, as solvents, such as ethanol, have been reported to modulate immune cell parameters like oxidative burst capacity (Timm et al., 2013).

2.5. Flow cytometry

For the physiological parameter measurements, *i.e.*, viability, population distribution and the ROS production, the cellular concentration was adjusted to $1x10^6$ cells/mL after the culture period, by adding modified L-15 supplemented with the corresponding compound. The cells were mixed by gentle pipetting. The cell mortality was estimated with 50 µg/mL propidium iodide (Sigma), in obscurity over 10 min, at room temperature. The mortality of each subpopulation represents the proportion of lymphoid or myeloid cells with high red fluorescence. All samples with a mortality exceeding 10% were excluded from the analyses. The different cell types were defined by their granularity, *i.e.*, their internal complexity corresponding to the side scatter (SSC) and their size corresponding to the forward scatter (FSC). In agreement with previous work (Granja et al., 2015; Seemann et al., 2016), two populations were identified (Fig. 1): lymphoid and myeloid cells, the former being of larger size (FSC) and granularity (SSC), the latter being smaller and less complex. Each flow cytometric measurement was conducted with 25,000 events in the gate "cells" comprising both cell types. The population distribution for each cell type corresponded to the percentage of events in either of the two gates. The ROS-levels and respiratory burst were assessed using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, ThermoFisher) and the phorbol 12-myristate 13-acetate (PMA, Sigma) as described by Bado-Nilles et al. (2014) and Haugland et al., 2014.. H_2DCFDA is an unspecific oxidative-sensitive fluorescent dye, which reacts with both RNS and ROS. It is commonly used to measure intracellular ROS and oxidative burst

capacity in flow cytometry (Chen et al., 2010; Dupre-Crochet et al., 2013; Peluso et al., 2014). For stimulation, PMA, which is an analogue of diacylglycerol (DAG), an activator of the protein kinase C (PKC) that stimulates NADPH oxidase and provokes oxidative burst in phagocytes (Bado-Nilles et al., 2014; Bylund et al., 2010; Havixbeck and Barreda, 2015). The cells were incubated with 5 µM of H_2DCFDA for 30 min followed by a stimulation using 2 µg/mL of PMA for 30 min. The basal-levels of combined ROS and RNS was measured for each sample by omitting the PMA stimulation. In comparison, the fluorescence with PMA derives from ROS only. The capacity of oxidative burst was measured for each gate (lymphoid and myeloid cells) as performed by Peluso et al., 2012. Four distinct parameters were analysed: the MFI in basal condition, the MFI under stimulated condition, the ROS-level index and proportion of oxidative burst positive cells. The ROS-level index is based on the population mean fluorescent intensity (MFI, arbitrary units) and was previously used to evaluate the oxidative burst capacity on total leucocyte by Bado-Nilles et al. (2014). The ratio of the MFI of unstimulated and stimulated cells is then used to calculate the ROS-level index. The proportion of oxidative burst positive cells was estimated using gates, which select "oxidative burst positive cells" with a high green fluorescence intensity (in the FL1 channel; Fig. 1), as previously performed by Haugland et al., 2014. To quantify the "oxidative burst positives" cells only, the proportion of gated myeloid/lymphoid cells in unstimulated condition was subtracted from the proportion of "oxidative burst positives" myeloid/lymphoid cells in the stimulated condition, *e.g.*, percent of lymphocytes with high fluorescent intensity in the gate "oxidative burst positive" in stimulated condition minus the same parameter in unstimulated condition. All flow cytometric measurements and their corresponding data analyses were carried out with NovoCytes™ (ACEA Biosciences Inc., San Diego, USA) and NovoExpress® software (ACEA Biosciences Inc.).

2.6. Statistical analysis

Statistical analyses were conducted with SigmaPlot (version 12.0, Systat Software Inc., San Jose, USA). The results of flow cytometry are reported as histograms with means and standard errors (s.e.). Prior to analyses, outliers were eliminated using the Grubb's outlier test (for combined data from CTR- and E2-group, ©2017 GraphPad Software Inc., La Jolla, USA). The datasets were checked for normality and equal variances using the Shapiro-Wilk test and the Median-Levene's test, respectively. For comparing gender differences, if normal distribution and homoscedasticity could be confirmed, one-way analysis of variance (ANOVA) was used for parametric hypothesis testing; otherwise non-parametric one-way

ANOVA on ranks (Kruskall-Wallis) was conducted. To compare culture conditions at each incubation time, one way repeated measures ANOVA or, alternatively, non-parametric one way repeated measures ANOVA on ranks (Friedman) was conducted. A repeated measures ANOVA was performed for each related individual to detect differences between conditions for each incubation time. The ANOVA test was followed by a Tukey's *post-hoc* test and the Kruskall-Wallis test was followed by Dunn's test. The results were considered significantly different at an α-level of 5 % ($p<0.05$).

3. Results

3.1. Polymerase chain reaction

Generally, the head-kidney and spleen expressed all transcripts of the nuclear receptors *esr1, esr2a, esr2b* (Fig. 2). As for the membrane receptors, *gpera* produced a weak band in the spleen, whereas no expression was detected in the head-kidney. Contrariwise, the *gperb* displayed a well detectable level of expression in both organs (Fig. 2).

In the isolated leucocytes from the head-kidney, the transcript of *esr1* and *gperb* were well detected. For *esr2a* and *esr2b,* a weak band was visible in all samples. No amplification for *gperb* could be observed for the majority of the fish. The splenocytes produced well-defined *esr1* and *esr2a* amplicons. By contrast, *esr2b* amplicons were well detected in only half of the fish, but barely detectable in the other specimens. Furthermore, *gperb* amplification resulted in a weak, but well detectable band, whereas *gpera* transcripts produced merely very weak bands.

3.2. Effect of E2 on immune cell parameters

Despite the reduced solvent volume, we observed minor, yet significant effects on the measured immune parameters when comparing non-treated cells to the solvent controls. Indeed, ethanol increased the respiratory burst of leucocytes isolated from the head-kidney (Fig. 3A and 4), but also slightly decreased the proportion of macrophages (Fig. 6). Nevertheless, the E2-effects on immune cells parameters were assessed by comparing the E2-exposed conditions to the solvent control, so that the results remained unaffected by the solvent effects.

3.2.1 Redox status

Two hours: In the head-kidney, E2-treatment at 10 nM significantly decreased the ROS-level indices in both lymphoid and myeloid cells (from 62.2 ± 9.7 s.e to 28.9 ± 3.5 s.e., $p<0.001$, and from 430.0 ± 60.6 s.e. to 331.0 ± 39.8 s.e., $p=0.046$, respectively), as shown in figure 3A. At 100 nM the treatment equally decreased the ROS-level index of the lymphocytes (from 62.2 ± 9.7 s.e. to 47.4 ± 7.6 s.e., $p=0.025$) but had no effect on myeloid cells of the head-kidney ($p=0.997$). The diminished ROS-level index is likely due to a significant E2-mediated decrease of PMA-stimulated ROS-levels (Fig. 3 C) at 10 nM (from 153,299 ± 20,462 s.e. to 67,327 ± 10,993 s.e., $p<0.001$ for the lymphoid cells and from 2,974,950 ± 287,733 s.e. to 2,074,639 ± 164,368 s.e. $p<0.05$ for the myeloid cells) and at 100 nM (from 153,299 ± 20,462 s.e. to 106,737 ± 14,567 s.e., $p<0.001$ for the lymphoid cells only). E2 did not significantly modulate the basal ROS/RNS-level in both leucocyte populations of the head-kidney (Fig. 3 B). Using the gates that quantify the oxidative burst positives cells (Fig. 4), the E2-treatment at 10 nM decreased the proportion of oxidative burst positive cells from 10.05 % ± 1.55 to 5.42 % ± 0.98 s.e. for lymphoid cells ($p<0.001$) and from 56.47 % ± 3.86 s.e. to 48.18 % ± 3.05 s.e. for myeloid cells ($p<0.001$). At 100 nM, E2-treatment also decreased the proportion of oxidative burst positive cells from 10.05 % ± 1.55 s.e. to 6.84 % ± 1.38 s.e. in lymphoid cells ($p<0.001$) and 56.47 % ± 3.86 s.e. to 53.72 % ± 4.11 s.e. for myeloid cells. The latter was, however, not statistically significant ($p<0.161$).

In the spleen, E2 did not significantly affect the ROS-level or the respiratory burst capacity (Fig. 3 A and 4). However, 10 nM E2, but not 100 nM, significantly decreased the basal ROS/RNS-level of the myeloid cells (from 14,639 ± 1,053 s.e. to 10,561 ± 1,146 s.e., $p=0.009$); no such effect was observed in lymphoid cells (Fig. 3 B). Considering the stimulated ROS-level (Fig. 3 C), E2 at 10 nM had a significant effect on the myeloid cells only, in which it decreased the MFI from 511,711 ± 55,094 s.e. to 403,690 ± 48,028 s.e. ($p=0.008$).

In the thymus, E2-exposure did not significantly modulate the basal ROS/RNS-level of leucocytes (Fig. 3D).

24 hours: In the head-kidney, 10 and 100 nM E2 significantly decreased the ROS-level indices of lymphoid cells (from 6.0 ± 0.5 s.e. to 4.1 ± 0.3 s.e., $p=0.023$ and to 4.4 ± 0.6 s.e., $p=0.029$; respectively) as well as of myeloid cells (from 20.7 ± 2.8 s.e. to 8.2 ± 1.1 s.e. and to 8.1 ± 1.4 s.e., $p<0.001$ for both concentrations). In this case, a significant increase of the basal

ROS/RNS-level in both leucocyte populations was observed (lymphoid cells: from 4,240 ± 495 s.e. to 6,827 ± 794 s.e. (10nM) and to 6,162 ± 535 s.e. (100nM); myeloid cells: from 85,005 ± 13,191 s.e. to 150,281 ± 22,645 s.e. (10nM) and to 155,601 ± 22,284 s.e. (100nM), $p<0.001$ for both cell types and both concentrations). The stimulated ROS-level, however, remained unchanged. Considering the proportion of oxidative burst positive cells, the E2-treatment had no effect on lymphoid cells, but decreased the proportion of oxidative burst positive myeloid cells at 10 nM from 47.23 % ± 3.72 to 39.95 % ± 2,89 ($p<0.05$), but not at 100 nM.

In the spleen, E2 significantly increased the ROS-level index of lymphoid cells at 100 nM (from 3.27 ± 0.28 s.e. to 9.54 ± 2.25 s.e.; $p<0.05$), but not at 10 nM. For the myeloid cells, E2 significantly decreased the ROS-level index at both concentrations (from 15.59 ± 2.5 s.e. to 4.5 ± 1.1 s.e., $p<0.001$, for 10 nM, and to 8.0 ± 0.8 s.e., $p=0.012$, at 100nM). Furthermore, E2 significantly increased the basal ROS/RNS-level of myeloid cells at both concentrations (from 82,855 ± 12,453.6 s.e. to 145,616 ± 19,987 s.e., $p=0.004$ for 10 nM and to 149,968 ± 21,355 s.e., $p=0.006$ for 100 nM). For the stimulated ROS-level, at 100 nM the treatment tended to increase from 6,358 ± 824 s.e. to 7,433 ± 1,068 s.e., although not statistically significant, whereas no effect occurred at 10 nM (6,925 ± 988 s.e.).

In the thymus, 100 nM E2 significantly and slightly increased the basal ROS/RNS-level in the lymphocytes (from 499.9 ± 22.5 s.e. to 553.4 ± 21.6 s.e., $p<0.001$), but had no effect at 10 nM. No effect on myeloid cells was observed.

48 hours: In the head-kidney, E2 significantly decreased the ROS-level indices of myeloid cells at both concentrations (from 7.4 ± 1.2 s.e. to 4.1 ± 0.6 s.e., $p<0.001$ for 10 nM and to 3.2 ± 0.5 s.e., $p=0.013$ for 100 nM), but did not affect the lymphoid cells. As for the basal and stimulated ROS-levels, E2 significantly decreased the stimulated ROS-level of myeloid cells at 10 nM only (from 1,114,941 ± 125,334 s.e. to 747,460 ± 87,226 s.e., $p<0.001$).

In the spleen, E2 had neither any significant effect on the ROS-level index, nor on the respiratory burst capacity of lymphoid cells, or their basal ROS/RNS-levels (Fig. 3 and 4). However, E2 significantly decreased the stimulated ROS-level of the myeloid cells at 10 nM (from 859,273 ± 105,905 s.e. to 578,953 ± 73,045 s.e., $p=0.039$).

In the thymus, 10 nM E2 significantly decreased the basal ROS/RNS-level in lymphoid cells from 953.1 ± 163.5 to 752.4 ± 99.5 and myeloid cells from 1,055.5 ± 188.3 to 837.8 ± 114.5 (p<0.05 for both cell types).

3.2.2 Leucocytes distribution and mortality

In the head-kidney and spleen, E2-exposure at both concentrations had no significant effect on leucocyte mortality and distribution (Fig. 5 and 6).

In the thymus, E2 significantly increased the mortality of lymphoid cells at 10 nM after 48h (from 2.07 % ± 0.25 s.e. to 2.88 % ± 0.26 s.e., p=0.006), but had no effect on the myeloid cells (Fig. 5). The higher concentration of 100 nM E2 left, however, mortality of either cell type unaffected. On the other hand, 100 nM E2 significantly and slightly decreased the proportion of lymphoid cells (Fig. 6) after 24h and after 48h (from 70.57 % ± 2.31 to 69.01 % p<0.05 and from 64.01 ± 2.45 s.e. to 63.19 ± 2.79 s.e., p=0.006, respectively), resulting in an increased proportion of myeloid cells after 48 h of E2-exposure at 100 nM (from 27.4 % ± 2.12 s.e. to 29.4 % ± 0.02 s.e. p=0.009).

4. Discussion

Oestrogen receptors appear widely expressed at the transcript level in primary and secondary lymphoid organs of the European sea bass. Practically all Esrs and Gperb were expressed in whole head-kidney and spleen as well as in the leucocytes from these organs. This expression pattern is similar to the situation in another major lymphoid organ of sea bass, the thymus and the leucocytes isolated from the thymus, where the Esrs are generally strongly expressed (Paiola et al., 2017). Our results corroborate findings from other teleost species (Lafont et al., 2016; Segner et al., 2013; Szwejser et al., 2017). The expression of oestrogen receptors by different immune cells and immune organs has been reported for both, teleosts and mammals (Cabas et al., 2013a; Rodenas et al., 2017b; Szwejser et al., 2017). Our results suggest that E2 is able to directly modulate the immune cells of the head-kidney and the spleen of sea bass as well.

Commonly, the oxidative burst capacity of immune cells is measured in isolated myeloid cells (Cabas et al., 2013a; Iwanowicz et al., 2014; Szwejser et al., 2017) or in the total leucocyte populations (Bado-Nilles et al., 2014; Haugland et al., 2014; Peluso et al., 2012). Mammalian

and teleost B lymphocytes, however, can also have innate immune cell-like properties, such as antimicrobial, phagocytic and oxidative burst capacities (Kovacs et al., 2015; Riera Romo et al., 2016; Zhang et al., 2017). Moreover, because ROS are involved in both lymphoid and myeloid cell function and differentiation, we analyzed ROS-levels of lymphoid and myeloid cells separately as proposed by Peluso et al. (2012) and Rabesandratana et al. (1992). Similar to Rabesandratana et al. (1992), we detected that PMA increased the MFI of both lymphoid and myeloid cells. Both authors observed that the intracellular increase of ROS-level arise from myeloid cells, but not from lymphocytes in human blood (Peluso et al., 2012; Rabesandratana et al., 1992). In neutrophils, PMA stimulates intracellular (possibly inside the granule) and extracellular production of $O_2^{\bullet-}$ (Bylund et al., 2010). Therefore, in other phagocytes and B cells, PMA is likely to stimulate extracellular $O_2^{\bullet-}$ mainly, which is rapidly converted into H_2O_2, either by superoxide dismutase or spontaneously (Bylund et al., 2010; Peluso et al., 2014). H_2O_2 does not diffuse freely across bilayer membranes. Its diffusion can, however, be facilitated by different aquaporin subtypes (Bienert and Chaumont, 2014). Interestingly, aquaporin water channels and H_2O_2 are both involved in phagocyte and B and T lymphocyte function as well as their differentiation (Bertolotti et al., 2016; Bienert and Chaumont, 2014; Tyteca et al., 2015; Zhu et al., 2011). The observed increase of ROS-level in stimulated lymphocytes most likely results from extracellularly H_2O_2-produced by the phagocytes, which enters the cells by facilitated diffusion *via* the aquaporins. Similarly, van Reyk et al. (2001) observed that PMA-stimulation of lymph node leucocytes increased ROS-level in T cells by stimulating the NADPH oxidase of phagocytes. Consequently, to measure the oxidative burst capacity and to distinguish it from other sources, such as H_2O_2 derived from phagocytes, we used two methods of measurement and normalization. In agreement with this diffusion observed in mammals, in the present work, E2 appeared to have similar effects on the ROS-level index. Effectively, E2-stimulated ROS-levels of both lymphoid and myeloid cells for most times points. This convergence could indeed result from H_2O_2-diffusion across lymphocytes. With the method using gates in the FL1 channel, we could quantify a small fraction of lymphoid cells in the head-kidney, but not in the spleen, which have apparently oxidative burst capacity and, therefore, may correspond to a B cell population with innate-like properties. Consequently, the increase of the ROS-level index observed at 24 h of 100 nM E2-exposure in lymphoid cells from the spleen does not result from a stimulation of oxidative burst capacity, but rather from an E2-mediated stimulation of H_2O_2-diffusion through the plasmatic membrane. This underscores the importance to measure the immune cell

populations separately as well as to utilise different methods of quantification and normalisation.

The E2-mediated increase of ROS-level in the lymphoid cells of the spleen at 24 h of culture suggests, therefore, that E2 stimulates aquaporin expression and thus stimulate lymphocyte differentiation and function. In fact, recently it was proposed by Bertolotti *et al.,* (2016) that B-cell-activation and differentiation is dependent on the stimulation of aquaporin expression, allowing for the entry of extracellular H_2O_2 produced by B cells, *i.e.,* autocrine action of H_2O_2 (Bertolotti et al., 2016). Our interpretation that E2 modulates lymphocyte differentiation is firstly corroborated by the time point at which the stimulation occurs and, secondly, by observations in sea bream head-kidney and in mammals (Rodenas et al., 2017a; Straub, 2007). Indeed, Rodenas et al. (2017a) found a boost in B lymphocyte differentiation following *in vivo* exposure to ethinylestradiol and G-1, a GPER-specific agonist. In mammals, E2 also enhances peripheral splenic B cell differentiation and activity (Bernardi et al., 2014; Roved et al., 2017; Straub, 2007). As far as the T cells are concerned, extracellularly produced H_2O_2 also displays an autocrine/paracrine action, which is involved in T cell activation, apoptosis and differentiation (Belikov et al., 2015). Therefore, the E2-mediated increase of ROS-level after stimulation, most likely deriving from H_2O_2, may also result from a modulation of T cell function through facilitated diffusion by aquaporins, which has been shown to regulate T cell function (Hara-Chikuma et al., 2012). Numerous examples for the modulation of T cell differentiation, activity and function by E2 exist across jawed vertebrates (Khan and Ansar Ahmed, 2016; Kondo et al., 1994; Paiola et al., *under review*). That this E2-mediated modulation of T cells implies ROS-signalling is exemplified by the 24 h and 48 h of *in vitro* E2-exposure of leucocytes from the thymus in the present study. Indeed, E2-exposure increased the ROS/RNS-level in thymic lymphocytes, which is followed by an increased mortality of thymic lymphoid cells only as well as a decreased proportion of lymphocytes. This suggests that E2 stimulates apoptosis by increasing ROS-levels in immature T cells of sea bass. As a confirmation, in teleosts, oestradiol benzoate-injection has been reported to increase the number of degenerating lymphocytes in masu salmon (Sufi et al., 1980). Similar observations have been made in mammals, in which ROS can trigger the extrinsic or intrinsic pathway of T cell apoptosis (Gostner et al., 2013; Yang et al., 2013) and E2-induced immature T cell apoptosis *in vitro* and *in vivo* for lizard and mouse, respectively (Do et al., 2002; Hareramadas and Rai, 2006; Martín et al., 1994b).

In mammals and teleosts, E2 modulates the function of myeloid cells such as macrophages and granulocytes, both *in vitro* and *in vivo* (Cabas et al., 2013b; Rodenas et al., 2017b; Roved et al., 2017; Szwejser et al., 2016, 2017). In mammals, E2-signalling is also important for dendritic cell differentiation and function (Laffont et al., 2017; Roved et al., 2017). Once more, E2-mediated regulation of immune cells implies ROS/RNS signalling by (1) increasing anti-oxidant enzyme activity, and (2) regulating NO and ROS production by modulating gene expression of NO synthase and NADPH oxidase (Karpuzoglu and Ahmed, 2006; Priyanka et al., 2013; Straub, 2007). Similarly, our results show that E2-exposure transiently increased the basal ROS/RNS-level in head-kidney leucocytes and in myeloid cells of the spleen at 24 h. This suggests that E2 stimulates ROS/RNS production in innate and adaptive immune cells of sea bass *via* a genomic action. In mammals, ROS/RNS signalling is involved in the differentiation, apoptosis and activity of various myeloid cells (Gostner et al., 2013; Karpuzoglu and Ahmed, 2006; Nathan and Cunningham-Bussel, 2013; Yang et al., 2013). Our results confirm that E2 modulates both innate and adaptive immune cell activity/differentiation by acting on the ROS/RNS production/signalling in sea bass as well. Similarly, *in vitro,* bisphenol A-exposure of carp macrophages increased NO activity as well as total ROS/RNS-level after 6 h and 24 h by activating ESR pathways (Yang et al., 2015).

As ROS- and Ca^{2+}-signalling are tightly connected and are both involved in cell signalling regulating differentiation, apoptosis and activity of numerous immune cells (Görlach et al., 2015), and because leucocytes of European sea bass express Gperb, it is possible that E2 also exerts a non-genomic regulation *via* changes in Ca^{2+}-fluxes. Non-genomic E2-signalling would explain why E2-exposure significantly modulated the stimulated ROS-level of head-kidney and spleen-derived leucocytes at 2 h already. The short lapse of time clearly points to a rapid E2-modulation of immune cell activity in sea bass by acting on membrane-associated oestrogen receptors. Such rapid E2-signalling is in line with findings in other teleosts and in mammals, where E2 has been described to modulate the respiratory burst of granulocytes and macrophages by acting on GPER (Cabas et al., 2013a; Rodenas et al., 2017b; Szwejser et al., 2017). For instance, in lymphocytes of mice, oestrogen initiated a rapid, cytosolic pathway of signalisation, which resulted in the stimulation of T and B cell proliferation (Ádori et al., 2010; Schneider et al., 2014). In mammals, E2 also rapidly stimulates NO-production in human granulocytes and macrophages (Straub, 2007).

In our study, E2-exposure (for 2h) resulted in an inhibition of the oxidative burst, in both head-kidney and spleen-derived myeloid cells as well as in lymphoid cells of the head-kidney.

These results suggest that, in sea bass, E2 (rapidly) inhibited ROS-production by decreasing the activity of NADPH oxidase. The stimulation of antioxidant enzyme activity by E2 was demonstrated in the splenocytes of mice following 24h of *in vitro* exposure and may provide an explanation for the observed changes in ROS/RNS-production of sea bass (Priyanka et al., 2013). The effect was still significant after 24 h and 48h of treatment, but only in the myeloid cells of the head-kidney. Although most studies described a stimulation of the respiratory burst by E2 (Bado-Nilles et al., 2014; Cabas et al., 2013b; Iwanowicz et al., 2014; Szwejser et al., 2017), the results are difficult to compare, because of, interspecies differences, seasonal variations, differences in sexual maturation and, most importantly, culture conditions and differences in ROS-level assessment. As for the latter, the ROS-sensitives probes measuring intracellular and/or extracellular ROS are quite diverse (Bylund et al., 2014), as are the time-kinetic evolutions (Cabas *et al.* 2013b). Furthermore, as presented we show that the method of normalization can lead to very different conclusions. This may be exemplified by the results for the leucocytes of the spleen. In fact, based on the ratio, the measure of oxidative burst capacity may be biased if E2 affects the basal RNS/ROS-level: In the case of an the E2-mediated increase of the basal RNS/ROS-level, the overall respiratory burst index decreases, whereas a decrease of the basal RNS/ROS-level would result in an over-estimation of the respiratory burst index. Changes in the basal values are, however, not reported very often, even if intracellular level of ROS is measured (Bylund et al., 2014), which makes it difficult to evaluate the parameters that affected the respiratory burst index, in the first place.

5. Conclusion

The *in vitro* E2-exposure of the leucocytes isolated from the head-kidney and the spleen clearly indicated that E2 modulates either the microbicidal capacity of lymphocytes and myeloid cells of both organs, their ROS-signalling, or both, for that matter. The short-term and longer-term responses of the different leucocytes at time points from 2 h to 48 h point to E2-signalling *via* either genomic and membrane-bound oestrogen receptors. Considering the microbicide activity of the leucocytes, our result indicates that the phagocytes isolated form the head-kidney are more E2-sensitive in comparison to the leucocytes of the spleen. These observations are corroborated by previous findings, which suggest, that E2 promotes an inhibition of the oxidative burst capacity in the spleen of sea bass *in vivo* by promoting

regulatory T cell differentiation (Paiola et al. 2018). Similar effects of E2 on leucocytes have also been reported for mammals (Khan and Ansar Ahmed, 2016; Straub, 2007).

In particular, our results suggest that E2 modulates immune cell activity, apoptosis, and differentiation in sea bass by tightly regulating the ROS/RNS-levels and acting *via* ROS/RNS-signalisation. Notably, E2 appears to differentially modulate lymphoid and myeloid cells from the three lymphoid organs. Consequently, from an ecotoxicological point of view, the immune cells populations and associated immune parameters for *in vitro* based bioassays or for biomonitoring purposes should be carefully chosen, because the different leucocyte populations have intrinsic differences in the E2-responsiveness as well as in the functions modulated by E2.

Acknowledgements: This research was supported by the ANR financed project ETaT (ANR-15-CE32-0014). M.P. was supported by the Normandy region through the FR CNRS 3730 SCALE. P.I.S.P. The authors thank Matthieu Bonnet and the staff at Aquacaux for fish hatchery.

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.

References

Ádori, M., Kiss, E., Barad, Z., Barabás, K., Kiszely, E., Schneider, A., Sziksz, E., Ábrahám, I.M., Matkó, J., Sármay, G., 2010. Estrogen augments the T cell-dependent but not the T-independent immune response. Cell. Mol. Life Sci. 67, 1661–1674. https://doi.org/10.1007/s00018-010-0270-5

Bado-Nilles, A., Techer, R., Porcher, J.M., Geffard, A., Gagnaire, B., Betoulle, S., Sanchez, W., 2014. Detection of immunotoxic effects of estrogenic and androgenic endocrine disrupting compounds using splenic immune cells of the female three-spined

stickleback, Gasterosteus aculeatus (L.). Environ. Toxicol. Pharmacol. 38, 672–683. https://doi.org/10.1016/j.etap.2014.08.002

Belikov, A.V., Schraven, B., Simeoni, L., 2015. T cells and reactive oxygen species. J. Biomed. Sci. 22. https://doi.org/10.1186/s12929-015-0194-3

Bernardi, A.I., Andersson, A., Grahnemo, L., Nurkkala-Karlsson, M., Ohlsson, C., Carlsten, H., Islander, U., 2014. Effects of lasofoxifene and bazedoxifene on B cell development and function. Immun. Inflamm. Dis. 2, 214–225. https://doi.org/10.1002/iid3.37

Bertolotti, M., Farinelli, G., Galli, M., Aiuti, A., Sitia, R., 2016. AQP8 transports NOX2-generated H 2 O 2 across the plasma membrane to promote signaling in B cells. J. Leukoc. Biol. 100, 1071–1079. https://doi.org/10.1189/jlb.2AB0116-045R

Bienert, G.P., Chaumont, F., 2014. Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. Biochim. Biophys. Acta BBA - Gen. Subj. 1840, 1596–1604. https://doi.org/10.1016/j.bbagen.2013.09.017

Boehm, T., Swann, J.B., 2014. Origin and Evolution of Adaptive Immunity. Annu. Rev. Anim. Biosci. 2, 259–283. https://doi.org/10.1146/annurev-animal-022513-114201

Burgos-Aceves, M.A., Cohen, A., Smith, Y., Faggio, C., 2016. Estrogen regulation of gene expression in the teleost fish immune system. Fish Shellfish Immunol. 58, 42–49. https://doi.org/10.1016/j.fsi.2016.09.006

Bylund, J., Björnsdottir, H., Sundqvist, M., Karlsson, A., Dahlgren, C., 2014. Measurement of Respiratory Burst Products, Released or Retained, During Activation of Professional Phagocytes, in: Neutrophil Methods and Protocols, Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 321–338. https://doi.org/10.1007/978-1-62703-845-4_21

Bylund, J., Brown, K.L., Movitz, C., Dahlgren, C., Karlsson, A., 2010. Intracellular generation of superoxide by the phagocyte NADPH oxidase: How, where, and what for? Free Radic. Biol. Med. 49, 1834–1845. https://doi.org/10.1016/j.freeradbiomed.2010.09.016

Cabas, I., Rodenas, M.C., Abellan, E., Meseguer, J., Mulero, V., Garcia-Ayala, A., 2013a. Estrogen Signaling through the G Protein-Coupled Estrogen Receptor Regulates Granulocyte Activation in Fish. J. Immunol. 191, 4628–4639. https://doi.org/10.4049/jimmunol.1301613

Cabas, I., Rodenas, M.C., Abellan, E., Meseguer, J., Mulero, V., Garcia-Ayala, A., 2013b. Estrogen Signaling through the G Protein-Coupled Estrogen Receptor Regulates Granulocyte Activation in Fish. J. Immunol. 191, 4628–4639. https://doi.org/10.4049/jimmunol.1301613

Chen, X., Zhong, Z., Xu, Z., Chen, L., Wang, Y., 2010. 2′,7′-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy. Free Radic. Res. 44, 587–604. https://doi.org/10.3109/10715761003709802

Do, Y., Ryu, S., Nagarkatti, M., Nagarkatti, P.S., 2002. Role of Death Receptor Pathway in Estradiol-Induced T-Cell Apoptosis in Vivo. Toxicol. Sci. 70, 63–72. https://doi.org/10.1093/toxsci/70.1.63

Dupre-Crochet, S., Erard, M., Nusse, O., 2013. ROS production in phagocytes: why, when, and where? J. Leukoc. Biol. 94, 657–670. https://doi.org/10.1189/jlb.1012544

Esteban, M.Á., Cuesta, A., Chaves-Pozo, E., Meseguer, J., 2015. Phagocytosis in Teleosts. Implications of the New Cells Involved. Biology 4, 907–922. https://doi.org/10.3390/biology4040907

Görlach, A., Bertram, K., Hudecova, S., Krizanova, O., 2015. Calcium and ROS: A mutual interplay. Redox Biol. 6, 260–271. https://doi.org/10.1016/j.redox.2015.08.010

Gostner, J.M., Becker, K., Fuchs, D., Sucher, R., 2013. Redox regulation of the immune response. Redox Rep. 18, 88–94. https://doi.org/10.1179/1351000213Y.0000000044

Granja, A.G., Leal, E., Pignatelli, J., Castro, R., Abos, B., Kato, G., Fischer, U., Tafalla, C., 2015. Identification of Teleost Skin CD8 + Dendritic-like Cells, Representing a Potential Common Ancestor for Mammalian Cross-Presenting Dendritic Cells. J. Immunol. 195, 1825–1837. https://doi.org/10.4049/jimmunol.1500322

Hara-Chikuma, M., Chikuma, S., Sugiyama, Y., Kabashima, K., Verkman, A.S., Inoue, S., Miyachi, Y., 2012. Chemokine-dependent T cell migration requires aquaporin-3–mediated hydrogen peroxide uptake. J. Exp. Med. 209, 1743–1752. https://doi.org/10.1084/jem.20112398

Hareramadas, B., Rai, U., 2006. Cellular mechanism of estrogen-induced thymic involution in wall lizard: caspase-dependent action. J. Exp. Zoolog. A Comp. Exp. Biol. 305A, 396–409. https://doi.org/10.1002/jez.a.260

Haugland, G.T., Rønneseth, A., Wergeland, H.I., 2014. Flow cytometry analyses of phagocytic and respiratory burst activities and cytochemical characterization of leucocytes isolated from wrasse (Labrus bergylta A.). Fish Shellfish Immunol. 39, 51–60. https://doi.org/10.1016/j.fsi.2014.04.023

Havixbeck, J., Barreda, D., 2015. Neutrophil Development, Migration, and Function in Teleost Fish. Biology 4, 715–734. https://doi.org/10.3390/biology4040715

Iwanowicz, L.R., Stafford, J.L., Patiño, R., Bengten, E., Miller, N.W., Blazer, V.S., 2014. Channel catfish (Ictalurus punctatus) leukocytes express estrogen receptor isoforms ERα and ERβ2 and are functionally modulated by estrogens. Fish Shellfish Immunol. 40, 109–119. https://doi.org/10.1016/j.fsi.2014.06.021

Karpuzoglu, E., Ahmed, S.A., 2006. Estrogen regulation of nitric oxide and inducible nitric oxide synthase (iNOS) in immune cells: Implications for immunity, autoimmune diseases, and apoptosis. Nitric Oxide 15, 177–186. https://doi.org/10.1016/j.niox.2006.03.009

Khan, D., Ansar Ahmed, S., 2016. The Immune System Is a Natural Target for Estrogen Action: Opposing Effects of Estrogen in Two Prototypical Autoimmune Diseases. Front. Immunol. 6. https://doi.org/10.3389/fimmu.2015.00635

Klein, S.L., Flanagan, K.L., 2016. Sex differences in immune responses. Nat. Rev. Immunol. https://doi.org/10.1038/nri.2016.90

Kondo, Y., Oka, H., Abe, A., Tanabe, A., 1994. Dose-related Effects of. Beta-estradiol on Chicken T Cell Activity. J. pult. sci. 31, 327–334. https://doi.org/10.2141/jpsa.31.327

Kovacs, I., Horvath, M., Lanyi, A., Petheo, G.L., Geiszt, M., 2015. Reactive oxygen species-mediated bacterial killing by B lymphocytes. J. Leukoc. Biol. 97, 1133–1137. https://doi.org/10.1189/jlb.4AB1113-607RR

Laffont, S., Seillet, C., Guéry, J.-C., 2017. Estrogen Receptor-Dependent Regulation of Dendritic Cell Development and Function. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.00108

Lafont, A.-G., Rousseau, K., Tomkiewicz, J., Dufour, S., 2016. Three nuclear and two membrane estrogen receptors in basal teleosts, Anguilla sp.: Identification, evolutionary history and differential expression regulation. Gen. Comp. Endocrinol. 235, 177–191. https://doi.org/10.1016/j.ygcen.2015.11.021

Litman, G.W., Rast, J.P., Fugmann, S.D., 2010. The origins of vertebrate adaptive immunity. Nat. Rev. Immunol. 10, 543–553. https://doi.org/10.1038/nri2807

Martín, A., Alonso, L.M., Del Moral, M.G., Zapata, A.G., 1994. Ultrastructural changes in the adult rat thymus after estradiol benzoate treatment. Tissue Cell 26, 169–179. https://doi.org/10.1016/0040-8166(94)90092-2

Milla, S., Depiereux, S., Kestemont, P., 2011. The effects of estrogenic and androgenic endocrine disruptors on the immune system of fish: a review. Ecotoxicology 20, 305–319. https://doi.org/10.1007/s10646-010-0588-7

Nathan, C., Cunningham-Bussel, A., 2013. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. Nat. Rev. Immunol. 13, 349–361. https://doi.org/10.1038/nri3423

Paiola, M., Knigge, T., Picchietti, S., Duflot, A., Guerra, L., Pinto, P.I.S., Scapigliati, G., Monsinjon, T., 2017. Oestrogen receptor distribution related to functional thymus anatomy of the European sea bass, Dicentrarchus labrax. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2017.07.023

Parra, D., Takizawa, F., Sunyer, J.O., 2013. Evolution of B Cell Immunity. Annu. Rev. Anim. Biosci. 1, 65–97. https://doi.org/10.1146/annurev-animal-031412-103651

Pasman, L., Kasper, D.L., 2017. Building conventions for unconventional lymphocytes. Immunol. Rev. 279, 52–62. https://doi.org/10.1111/imr.12576

Peluso, I., Manafikhi, H., Reggi, R., Palmery, M., 2014. Interference of flavonoids with fluorescent intracellular probes: Methodological implications in the evaluation of the oxidative burst by flow cytometry. Cytometry A 85, 663–677. https://doi.org/10.1002/cyto.a.22490

Peluso, I., Morabito, G., Riondino, S., La Farina, F., Serafini, M., 2012. Lymphocytes as internal standard in oxidative burst analysis by cytometry: A new data analysis approach. J. Immunol. Methods 379, 61–65. https://doi.org/10.1016/j.jim.2012.02.015

Priyanka, H.P., Krishnan, H.C., Singh, R.V., Hima, L., ThyagaRajan, S., 2013. Estrogen modulates in vitro T cell responses in a concentration- and receptor-dependent manner: Effects on intracellular molecular targets and antioxidant enzymes. Mol. Immunol. 56, 328–339. https://doi.org/10.1016/j.molimm.2013.05.226

Rabesandratana, H., Fournier, A.-M., Chateau, M.-T., Serre, A., Dornand, J., 1992. Increased oxidative metabolism in PMA-activated lymphocytes: a flow cytometric study. Int. J. Immunopharmacol. 14, 895–902.

Riera Romo, M., Pérez-Martínez, D., Castillo Ferrer, C., 2016. Innate immunity in vertebrates: an overview. Immunology 148, 125–139. https://doi.org/10.1111/imm.12597

Rodenas, M.C., Cabas, I., Gómez-González, N.E., Arizcun, M., Meseguer, J., Mulero, V., García-Ayala, A., 2017a. Estrogens Promote the Production of Natural Neutralizing Antibodies in Fish through G Protein-Coupled Estrogen Receptor 1. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.00736

Rodenas, M.C., Tamassia, N., Cabas, I., Calzetti, F., Meseguer, J., Cassatella, M.A., García-Ayala, A., Mulero, V., 2017b. G Protein-Coupled Estrogen Receptor 1 Regulates Human Neutrophil Functions. Biomed. Hub 2, 2–2. https://doi.org/10.1159/000454981

Roved, J., Westerdahl, H., Hasselquist, D., 2017. Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. Horm. Behav. 88, 95–105. https://doi.org/10.1016/j.yhbeh.2016.11.017

Schneider, A.E., Kárpáti, É., Schuszter, K., Tóth, E.A., Kiss, E., Kulcsár, M., László, G., Matko, J., 2014. A dynamic network of estrogen receptors in murine lymphocytes: fine-tuning the immune response. J. Leukoc. Biol. 96, 857–872. https://doi.org/10.1189/jlb.2A0214-080RR

Seemann, F., Knigge, T., Duflot, A., Marie, S., Olivier, S., Minier, C., Monsinjon, T., 2016. Sensitive periods for 17β-estradiol exposure during immune system development in sea bass head kidney: Sensitivity of sea bass head kidney development to E2. J. Appl. Toxicol. 36, 815–826. https://doi.org/10.1002/jat.3215

Segner, H., Casanova-Nakayama, A., Kase, R., Tyler, C.R., 2013. Impact of environmental estrogens on fish considering the diversity of estrogen signaling. Gen. Comp. Endocrinol. 191, 190–201. https://doi.org/10.1016/j.ygcen.2013.05.015

Segner, H., Verburg-van Kemenade, B.M.L., Chadzinska, M., 2017. The immunomodulatory role of the hypothalamus-pituitary-gonad axis: Proximate mechanism for reproduction-immune trade offs? Dev. Comp. Immunol. 66, 43–60. https://doi.org/10.1016/j.dci.2016.07.004

Sfacteria, A., Brines, M., Blank, U., 2015. The mast cell plays a central role in the immune system of teleost fish. Mol. Immunol. 63, 3–8. https://doi.org/10.1016/j.molimm.2014.02.007

Straub, R.H., 2007. The Complex Role of Estrogens in Inflammation. Endocr. Rev. 28, 521–574. https://doi.org/10.1210/er.2007-0001

Sufi, B.G., Mori, K., Nomura, I., 1980. Involution of Thymus in Relation to Sexual and Steroid Hormone Treatments in Salmonid Fish. Tohoku J. of Agri. Res. 31, 97–105.

Szwejser, E., Maciuszek, M., Casanova-Nakayama, A., Segner, H., Verburg-van Kemenade, B.M.L., Chadzinska, M., 2017. A role for multiple estrogen receptors in immune regulation of common carp. Dev. Comp. Immunol. 66, 61–72. https://doi.org/10.1016/j.dci.2016.04.003

Szwejser, E., Verburg-van Kemenade, B.M.L., Maciuszek, M., Chadzinska, M., 2016. Estrogen-dependent seasonal adaptations in the immune response of fish. Horm. Behav. https://doi.org/10.1016/j.yhbeh.2016.10.007

Tafalla, C., Leal, E., Yamaguchi, T., Fischer, U., 2016. T cell immunity in the teleost digestive tract. Dev. Comp. Immunol., Special Issue: Intestinal Immunity 64, 167–177. https://doi.org/10.1016/j.dci.2016.02.019

Timm, M., Saaby, L., Moesby, L., Hansen, E.W., 2013. Considerations regarding use of solvents in in vitro cell based assays. Cytotechnology 65, 887–894. https://doi.org/10.1007/s10616-012-9530-6

Tyteca, D., Nishino, T., Debaix, H., Smissen, P.V.D., N'Kuli, F., Hoffmann, D., Cnops, Y., Rabolli, V., Loo, G. van, Beyaert, R., Huaux, F., Devuyst, O., Courtoy, P.J., 2015. Regulation of Macrophage Motility by the Water Channel Aquaporin-1: Crucial Role of M0/M2 Phenotype Switch. PLOS ONE 10, e0117398. https://doi.org/10.1371/journal.pone.0117398

van Reyk, D.M., King, N.J.C., Dinauer, M.C., Hunt, N.H., 2001. The intracellular oxidation of 2′,7′-dichlorofluorescin in murine T lymphocytes. Free Radic. Biol. Med. 30, 82–88. https://doi.org/10.1016/S0891-5849(00)00449-4

Yang, M., Qiu, W., Chen, B., Chen, J., Liu, S., Wu, M., Wang, K.-J., 2015. The In Vitro Immune Modulatory Effect of Bisphenol A on Fish Macrophages via Estrogen Receptor α and Nuclear Factor-κB Signaling. Environ. Sci. Technol. 49, 1888–1895. https://doi.org/10.1021/es505163v

Yang, Y., Bazhin, A.V., Werner, J., Karakhanova, S., 2013. Reactive Oxygen Species in the Immune System. Int. Rev. Immunol. 32, 249–270. https://doi.org/10.3109/08830185.2012.755176

Zhang, X.-J., Wang, P., Zhang, N., Chen, D.-D., Nie, P., Li, J.-L., Zhang, Y.-A., 2017. B Cell Functions Can Be Modulated by Antimicrobial Peptides in Rainbow Trout Oncorhynchus mykiss: Novel Insights into the Innate Nature of B Cells in Fish. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.00388

Zhu, N., Feng, X., He, C., Gao, H., Yang, L., Ma, Q., Guo, L., Qiao, Y., Yang, H., Ma, T., 2011. Defective macrophage function in aquaporin-3 deficiency. FASEB J. 25, 4233–4239. https://doi.org/10.1096/fj.11-182808

Figures

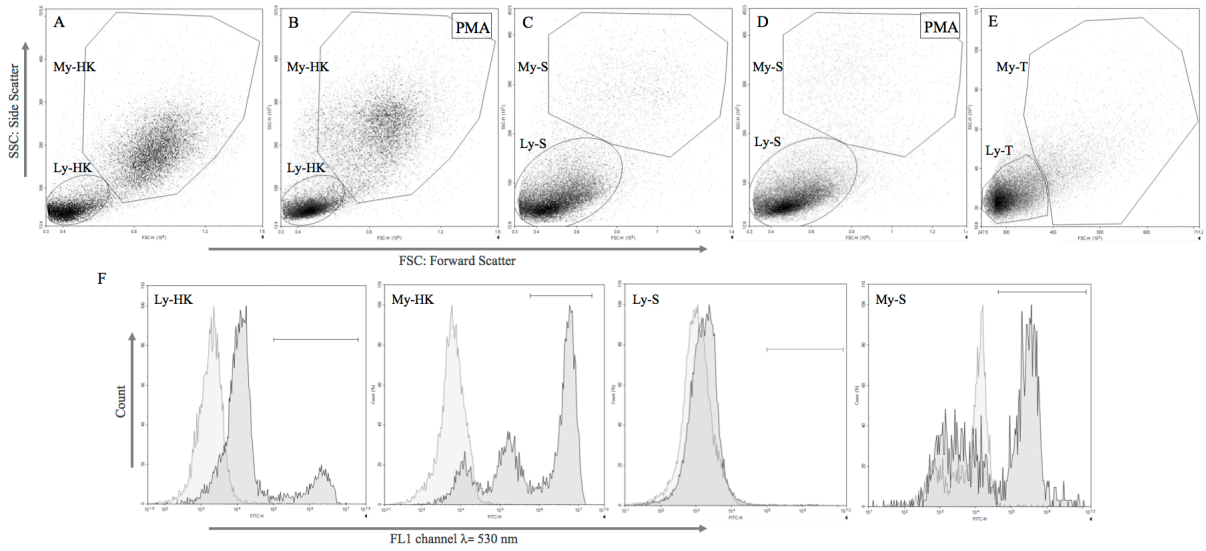


Fig. 1: Representative Side scatter (SSC) / Forward scatter (FSC) flow cytometric profiles of leucocytes isolated from the head-kidney (A, B), spleen (C, D) and thymus (E). Lymphoid (Ly) and myeloid (My) cells were analysed separately by gating cell populations according to size and granularity. A, C and E show profiles of leucocytes isolated from the head-kidney, spleen and thymus in basal condition; B, D show profiles of leucocytes isolated from the head-kidney and spleen in PMA-stimulated condition. F, histogram in the FL1 channel of unstimulated (pale grey) and stimulated (dark grey) lymphoid and myeloid cells from the head-kidney and spleen with gates selecting the "oxidative burst positive cells", i.e. the peaks with the highest fluorescence situated on the right-hand side. HK, head-kidney; S, spleen; T, thymus.

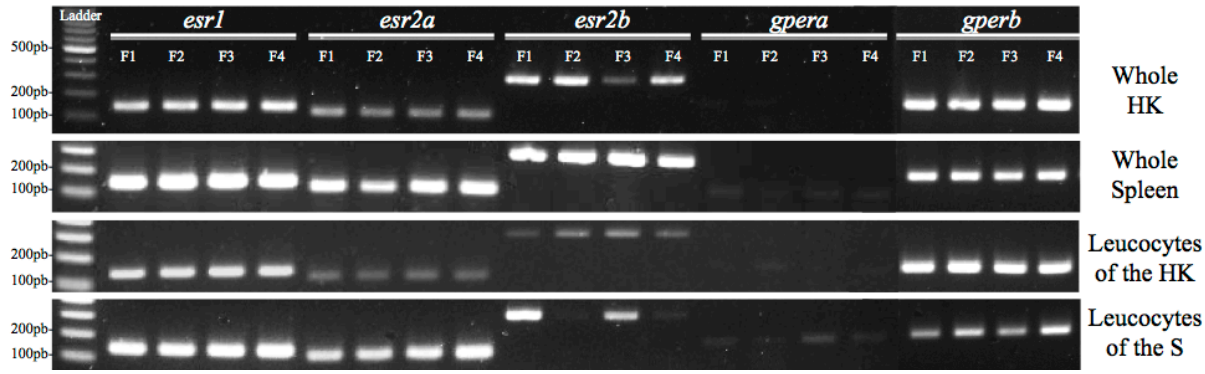


Fig. 2: PCR-amplicons of the *esr1, esr2a, esr2b, gpera* and *gperb* in whole tissue extract of head-kidney (HK) and spleen (S) as well as in isolated leucocytes from the respective organs.

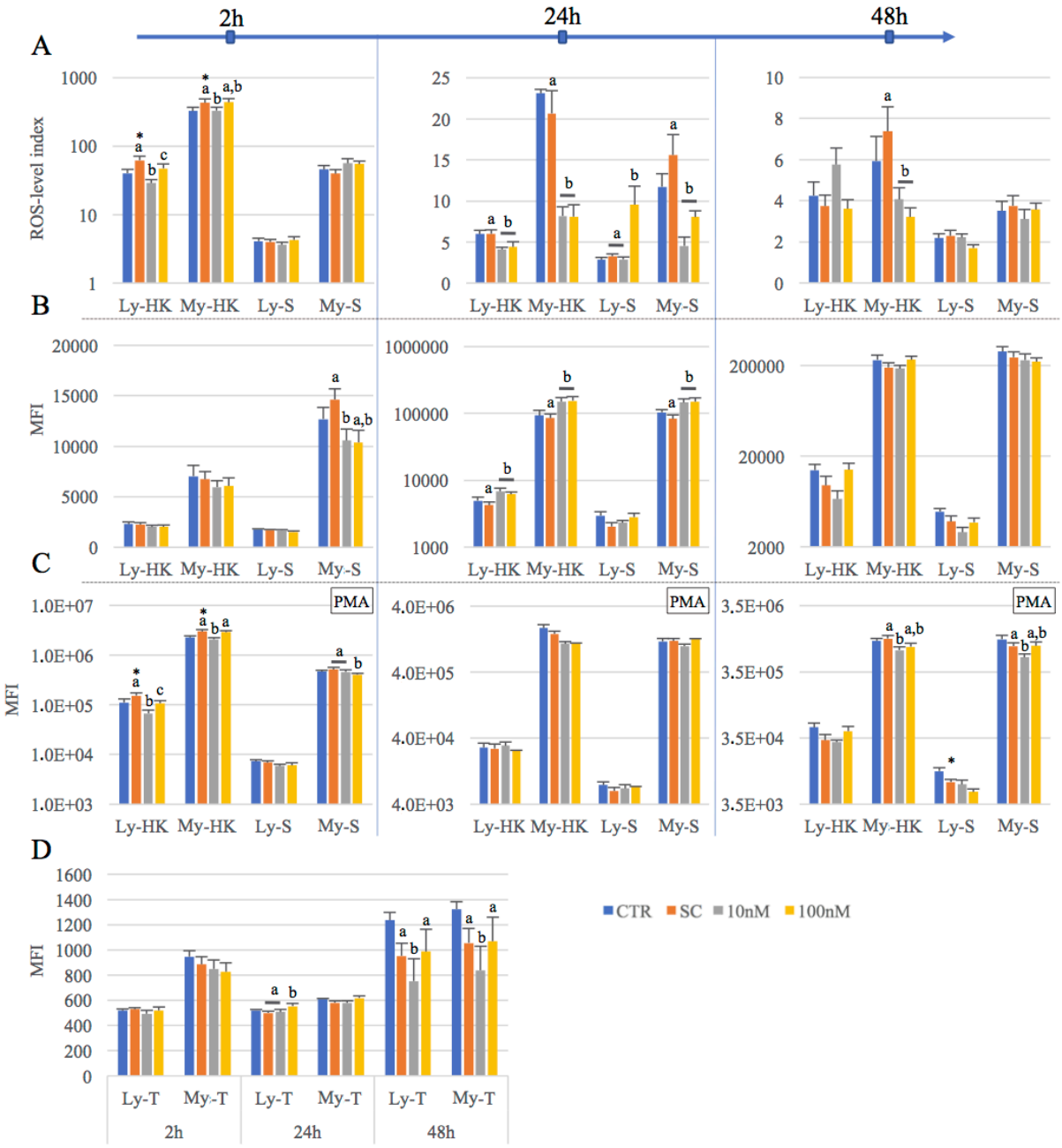


Fig. 3: ROS-level index (A), basal ROS-level (B, D) and PMA-stimulated ROS-level (C) of the leucocytes isolated from the head-kidney (HK), the spleen (S) and the thymus (T), measured in lymphoid (Ly) and myeloid (My) cells after 2 h, 24 h and 48 h of primary cell culture without treatment (CTR), with 0.00005 % ethanol (*i.e.*, solvent control, SC), with 10 nM and 100 nM 17β-oestradiol. MFI, mean fluorescence index (for details see text). Values are means ± standard error of the means; *, significantly different between the CTR and SC cells at $p<0.05$; different letters indicate significant differences between SC, 10nM and 100nM groups at $p<0.05$.

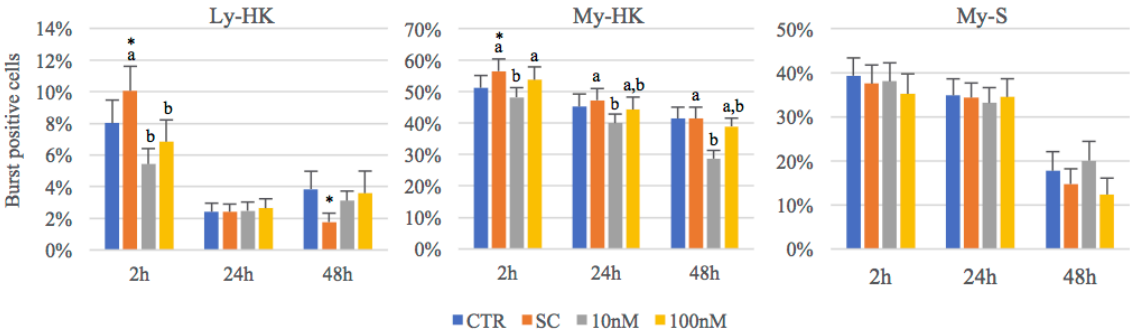


Fig. 4: Proportion of oxidative burst positive lymphoid (Ly) and myeloid (My) cells after 2 h, 24 h and 48 h of culture without treatment (CTR), with 0.00005 % ethanol (*i.e.*, solvent control, SC), with 10 nM and 100 nM 17β-oestradiol. The proportion is established as was cells with a high fluorescence in the FL1 channel as shown in figure 1 and cells. Values are means ± standard error of the means; *, significantly different between the CTR and SC cells at $p<0.05$; different letters indicate significant difference between SC, 10nM and 100nM groups at $p<0.05$.

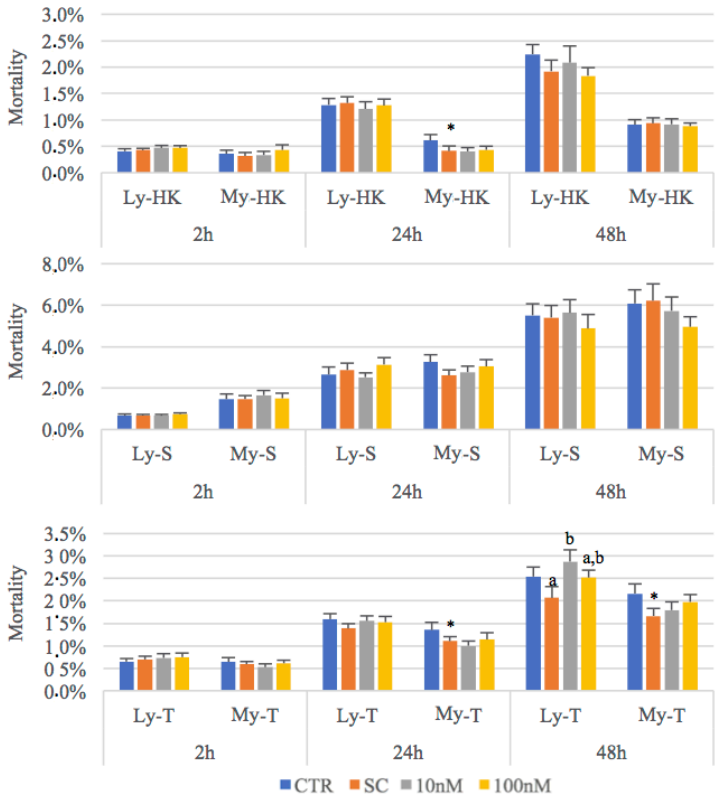


Fig. 5: Mortality of the isolated leucocytes from the head-kidney (HK), spleen (S) and thymus (T) after 2 h, 24 h and 48 h of culture without treatment (CTR), with 0.00005 % ethanol (*i.e.*, solvent control, SC), with 10 nM and 100 nM 17β-oestradiol. *, significantly different between the CTR and SC cells at $p<0.05$; different letters indicate significant difference between SC, 10nM and 100nM groups at $p<0.05$.

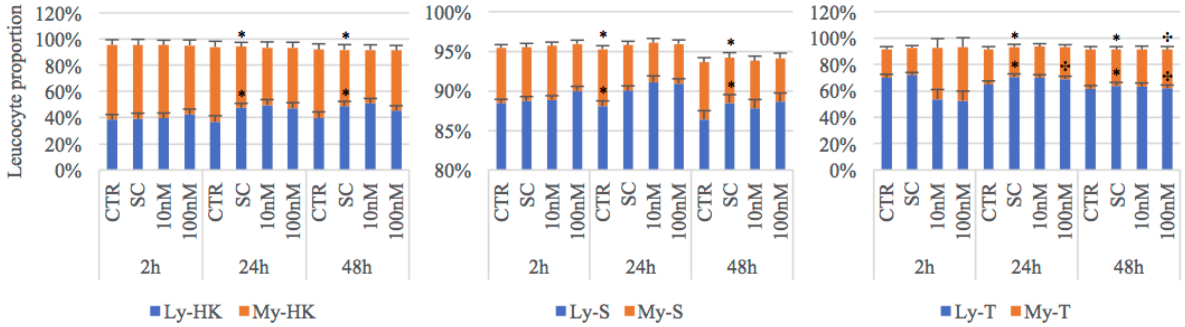


Fig. 6: Leucocyte distribution of the leucocytes derived from the head-kidney (HK), spleen (S) and thymus (T) after 2 h, 24 h and 48 h of culture without treatment (CTR), with 0.0005 % ethanol (*i.e.*, solvent control, SC), with 10 nM and 100 nM 17β-oestradiol. *, significantly different between the CTR and SC cells at $p<0.05$. ✣, significantly different between SC and 100nM groups at $p<0.05$.

3) Discussion

3) Discussion

Steroid hormones, and specifically oestrogens, exert their biological regulation on numerous physiological processes beyond sex determination and reproduction, one of them being the immune system. It is, however, not fully established how this regulation works in different vertebrates and to which extent immune organs and their oestrogenic regulation have been conserved throughout vertebrate evolution. Aside from the fundamental interest this may have for comparative immunology, the degree of conservation would underscore assumptions about the possible effects that environmental oestrogens may have on the immune system of lower vertebrates like fish.

To be able to evaluate whether such conservation of the immune system and its oestrogenic regulation exists, in this study we concentrated on a major type of immune cells, the T cells and its associated primary lymphoid organ the thymus. With respect to the morpho-functional specialisation of the thymus and because specific tools for the identification of the different thymic cells were lacking, the thymic structure was analysed by histology and immunohistochemistry. Our study confirmed the conserved functional anatomy in sea bass (Bajoghli et al., 2015; Picchietti et al., 2015). The cortical region, for instance, provides the microenvironment necessary for multiple proliferation steps of immature T cells (Forsberg, 1996; Picchietti et al., 2015). In addition, we described, for the first time in sea bass HC with a structure close to the small forms found in mammals (Raica et al., 2006). On the basis of this apparent conservation of thymic structure and, putatively, function, we could extrapolate the connection between the different thymic cells and their role in the sequential steps of T cell differentiation. As for oestrogenic regulation, we were able to localise oestrogen receptors in several thymic cells. For the most part, our observations appear to correspond to the oestrogen receptor distribution so far identified in mammals, notwithstanding the fact that the distribution of certain oestrogen receptor subtypes, *i.e.*, GPER, ESR2 has not been yet fully elucidated in the mammalian models. Despite some uncertainties to this respect, our study suggests that all the oestrogen receptors are likely to be involved in the oestrogenic effects on thymus function, as was previously suggested by studies using oestrogen receptor knockout mice (Erlandsson et al., 2001; Staples et al., 1999; Wang et al., 2008). Moreover, we observed, for the first time, that thymic cells supposed to play a key role in the E2-mediated regulation of thymic function, *i.e.*, mast cells and endothelial cells, express oestrogen receptors. Consequently, our data reinforce previous findings in teleosts, suggesting that the

oestrogenic effect on the thymus is conserved across jawed vertebrates. In addition, our findings correspond to the hypothesis of Chapman et al. (2015) concerning the cellular implication of mast cells in oestrogenic regulation of thymus function. Notwithstanding the assumption of a simpler immune system in teleosts, as compared to mammals, the wide distribution of the different oestrogen receptor subtypes observed in our study suggests a highly complex oestrogenic regulation of thymic function. Oestrogens may, therefore, be considered part of the hormonal signalling that regulates the different thymic cells and T cell maturation *in fine*.

This conclusion was further corroborated by experimental *in vivo* E2-exposure. The results of this exposure study provide some evidence for the oestrogenic modulation of both central and peripheral T cell differentiation. Actually, oestrogen appears to stimulate an alternative pathway of T cell maturation, thus promoting immune tolerance. Our results suggest the E2-mediated-splenic immune tolerance to be triggered by the thymic release of γδ and αβ T cells as well as the promotion of central or peripheral Treg differentiation. These processes have been previously described for mammals (Abo, 2001; Chapman et al., 2015; Khan and Ansar Ahmed, 2016; Polanczyk et al., 2005) and seem to exist in fish as well. As a matter of fact, *in vitro* E2-exposure of isolated leucocytes from the thymus, head-kidney and spleen further confirmed the promotion of immune tolerance, because in both, the leucocytes from the head-kidney and from the spleen, E2 inhibited of the oxidative burst capacity. This effect was even more pronounced in the leucocytes of the head-kidney. Furthermore, our results suggest that, in sea bass as in mammals, oestrogens promote immature T cell apoptosis.

In the following, the results described in the three articles will be discussed in more detail. Moreover, complementary unpublished data obtained during the thesis will be commented as far as they substantiate the overall conclusion. The discussion is divided into sections, which represent the assumed underlying cellular mechanisms involved in the oestrogenic regulation of thymic function and T cell development, both in mammals and teleosts. Eventually, the biological and ecotoxicological significance of the oestrogenic effects on T cell differentiation will be discussed.

A) Are oestrogenic effects on T cell differentiation conserved from teleosts to mammals?

In mammals, the first oestrogen receptor subtype ESR1 investigated in the thymic microenvironment by immunohistochemistry appeared to be mainly expressed in TEC (Greene et al., 1986; Seiki and Sakabe, 1997). Later, oestrogen receptors, ESR1 and ESR2 were also detected in leucocytes isolated from the thymus of humans and rodents by molecular biology and binding studies (Danel et al., 1983; Gulino et al., 1985; Kawashima et al., 1995; Mor et al., 2001). More recently, ESR1 was detected by immunofluorescence in immature human T cells (Nancy and Berrih-Aknin, 2005). By combining results from polymerase chain reaction, immunohistochemistry and immunofluorescence, we clearly demonstrated that sea bass T cells notably express Esr1 (Article 1). As in mammals, Esr1 was strongly detected in LTEC. Similar to observations made in chicks (Katayama et al., 2012), Esr1 was also expressed in myoid cells. Furthermore, ESR1 was detected in HC like, in which it was also found to be present in mammals and chicks (Seiki and Sakabe, 1997; Yonezawa et al., 2008). This thymic distribution of ESR1 qualifies this receptor as a major signalling element involved in thymic function. In mice, the E2-induced-thymus atrophy and the associated shift within the CD4/CD8 T cell subpopulation mainly involved the expression of ESR1 in both stromal and hematopoietic cells (radio sensitive and resistant elements(Erlandsson et al., 2001; Staples et al., 1999; Wang et al., 2008). In summary, the Esr1 distribution within the sea bass thymus closely resembles its distribution within the mammalian thymus. One may, therefore, assume that its major regulatory function may also be evolutionary conserved. Esr1 knockout zebrafish lines, might confirm the primordial Esr1 role for thymic function in the future.

Besides ESR1, thymic cells express also ESR2 and GPER at the transcriptional level (Mor et al., 2001; Mosselman et al., 1996; Szwejser et al., 2017; Wang et al., 2008) suggesting diverse signalling pathways (MISS and NISS) in teleosts and mammals (Azenabor and Hoffman-Goetz, 2001; Erlandsson et al., 2001; Wang et al., 2008). Detailed knowledge on the implication of oestrogen receptors other than ESR1 in vertebrates is, however, scarce and exclusively limited to the organ-level or to isolated leucocytes. Nevertheless, it becomes clear that oestrogens target several cell types *via* several receptor subtypes. This is likely to be the case in all vertebrates, as suggested by the similarities between fish, reptiles, birds and

mammals. Hence, the effects of oestrogen on the thymic function are difficult to understand and should be investigated using several approaches and models.

i) Does oestrogen modulate T cell activity directly?

Thymic T cells of mammals, teleosts (Article 1), birds and, probably, of reptiles are known to express oestrogen receptors. The complexity of the oestrogen effects on T cell maturation is likely increased by the variable expression of oestrogen receptors at different stages of differentiation. In fact, in mice, *esr1* is expressed in DN T cells and specially in CD44+CD25- DN1 (DN1>DN2>DN3), while *gper* is mainly present on CD44+ CD25+ DN2, but not on DN1 and DN4 (DN2>DN3) (Wang et al., 2008). In the human thymus, ESR1 and ESR2 are strongly expressed in CD4 SP, to a lesser extent DP and CD8 SP, and at the lowest level in DN T cell (Nancy and Berrih-Aknin, 2005). To better understand E2-related effects on T cells, Wang et al. (2008) investigated the E2 modulated activity of two transcription factors in purified CD25- DN T cells, *i.e.*, nuclear factor of activated T-cells (NFAT) and Nuclear Factor kappa B (NFκB), which are crucial for T cell development (Gerondakis et al., 2013; Macian, 2005; Wang et al., 2008). They observed E2-modulated early T cell maturation by inhibiting NFκB activity *via* ESR1-signalling, whereas no effect on NFAT could be observed (Wang et al., 2008).

The presence of a membrane oestrogen receptor Gper in sea bass leucocytes isolated from the thymus is suggested by the results presented in article 1. Molecular biology analysis, however, only demonstrated the transcript of the teleost *gperb* isoform in leucocytes isolated from the thymus. These findings are corroborated by the observation of Gper in T cells from the thymus and spleen of mice (Brunsing et al., 2013; Schneider et al., 2014; Wang et al., 2008) and by the expression of a functional GPER in teleost and human leucocytes (Cabas et al., 2013; Rodenas et al., 2017b; Szwejser et al., 2016; Tamaki et al., 2014). The modulation of ROS-levels in T cells, which was observed when exposing thymic leucocytes *in vitro* to E2, may be indicative of a transduction pathway associated with Ca^{2+}-signalling (Görlach et al., 2015) [see also section v) part (3)]. This suggests that E2 could induce MISS in sea bass T cells, as it was observed at physiological E2 concentrations in mouse leucocytes isolated from the thymus (Azenabor and Hoffman-Goetz, 2001) and SP CD4/CD8 T cell from the spleen (Benten et al., 1998; Schneider et al., 2014). It would not be unlikely for E2 to modulate T cell Ca^{2+}-signalisation in fish, as this mechanism is involved in numerous T cell functions,

such as proliferation, apoptosis, differentiation, TCR- and chemokine- signalization in mammals (Li and Rudensky, 2016; Oh-hora and Rao, 2008). Overall, our results, as well as those from Hareramadas and Rai (2006), Mor et al., (2001) and Schneider et al., (2014) for lizards and mammals, respectively, point towards an E2-modulated T cell development by directly targeting T cells *via* the genomic and non-genomic pathway.

ii) Does oestrogen modulate thymic T cell proliferation?

In both, teleosts and mammals, E2 has been reported to modulate thymic and peripheral T cell proliferation. In the splenocytes of mice, for instance, E2 appeared to directly activate ESR1 and ESR2 (Priyanka et al., 2013). Similar observations could be made for Esr1 in the PBL of channel catfish (Iwanowicz et al., 2014). The E2-related activation was suppressed by the non-specific ESR-antagonist ICI 182,780. Moreover, in mice, *in vitro* exposure of splenocytes confirmed the implication of both MISS and NISS in the E2-mediated-regulation of T cell proliferation (Schneider et al., 2014).

When using *pcna* expression as a measure of proliferation in the thymus (Article 2), we observed that E2 tended to decrease immature T cell proliferation in female sea bass. As the decrease was no statistically significant, our results suggest that one week of elevated physiological E2-levels do not strongly modulate T cell proliferation in sea bass, which is different to results obtained in mammals (Martin et al., 1994b; Zoller and Kersh, 2006). A direct marker of leucocyte proliferation, however, such as the *in vivo* thymocyte incorporation of 5-ethynyl-2′-deoxyuridine after injection would be better suited to elucidate whether T cells from mammals and fish behave differently with respect to the regulation of their proliferation by oestrogen (Rodenas et al., 2017a).

iii) Does oestrogen modulate mature and progenitor T cell trafficking?

(1) ETP homing

In the connective tissue of the sea bass thymus, vascular endothelial cells appeared to express the oestrogen receptors and especially Esr1, Esr2a, Esr2b and Gper (Article 1). Although the presence of oestrogen receptors in vascular endothelial cells has been described for other tissues and organs (Prossnitz and Barton, 2014; Straub, 2007), it is here described for the first time for the thymus. This result is an important feature, because oestrogen receptor expression

by endothelial cells is tissue specific (Chakrabarti et al., 2014; Isensee et al., 2009), and, therefore, cannot simply be extrapolated from one organ to another. In mice, Buono et al. (2016) showed that thymic vascular endothelial cells expressed KitL, the ligand of c-kit (CD117) localized on early thymic progenitors (ETP). This is essential for ETP homing since KitL deletion results in 50% of thymocyte loss together with an extensive loss in c-kit+ ETP. Interestingly, Buono et al. (2016) also observed that cortical endothelial cells express *delta-like 4* and *cxcl12*, which are also implicated in ETP homing and T cell commitment, both in teleosts and in mammals (Bajoghli, 2013; Halkias et al., 2014). Our results indicate that E2 could modulate ETP homing and T cell commitment by acting on endothelial cells. Similar effects may be mediated by LTEC in teleosts and mammals. In fact, in mammals, these cells express various thymic factors, *e.g.*, GH (Maggiano et al., 1994), thymulin (Savino and Dardenne, 1984; Seiki and Sakabe, 1997), lymphopoeitin, thymosin β4 and α1 (Savino and Dardenne, 1984; van de Wijngaert et al., 1984) and likely stromal cell-derived factor-1 (SDF-1/CXCL12; Suzuki et al., 1999). Thymic growth factors, such as CXCL12, thymulin or GH are important for thymocyte migration, homing and proliferation (Bajoghli et al., 2015; Savino et al., 2012, 2015). Consequently, E2-mediated modulation of ETP homing and T cell maturation is likely to be brought about by an effect on LTECs. This hypothesis is reinforced by the fact that LTEC activity is modulated by E2 (Martín et al., 1995a; Moreno and Zapata, 1991). However, *ikaros* and *rag1* expression were not significantly modulated in the thymus of sea bass by E2-treatment (Article 2). This in turn suggests that ETP homing and early T cell commitment in sea bass are not as strongly modulated by E2, as they are in mammals (Zoller and Kersh, 2006).

(2) T cell output

For the immune response, the T cells that have been educated in the thymus must leave the thymus and act in the periphery. T cell output and fate of the peripheral T cells represent important targets of oestrogenic regulation (Chapman et al., 2015).

In sea bass, thymic MCs appear to express all the oestrogen receptors, but especially Esr1 and Esr2a (Article 1). Oestrogen receptor expression in thymic MC has not yet been described in mammals. However, mouse bone marrow derived MC and human colonic mucosa MC express ESR1 and GPER, respectively (Narita et al., 2006; Qin et al., 2014; Zaitsu et al., 2007). In addition, the thymic vessels of sea bass appear to express the different oestrogen

receptors, which suggests an E2-induced vasodilatation by acting simultaneously on MC and thymic vessels (Article 1). Thymic vasodilatation is believed to facilitate T cell output and has been reported to be subject to modulation by E2 (Chapman et al., 2015; Martín et al., 1995a). Moreover, in immature female chicks, DES-treatment increased the number of T cells in the vagina, ovary and liver (Zheng and Yoshimura, 2001). We assume that elevated E2-levels probably stimulated the T cell release and the migration into the spleen and head-kidney of sea bass (Article 2). Overall, these findings together with those reported for birds and mammals strongly suggest that E2-mediated vasodilatation enhances T cell output from the thymus. Importantly, this enhanced T cell output may also consist of a leakage of immature and "uneducated" T cells (Chapman et al., 2009).

iv) Does oestrogen modulate central tolerance?

(3) Thymic APC number and/or function

The increase of *mhc2a* expression in the male thymus of sea bass following E2-treatment suggests that E2 stimulates antigen presentation. In fact, in mammals, E2 has been reported to increase the number of potential APCs (B cell, plasma cells, DC-like and interdigitated cells). These APCs are likely to come from the periphery, because of their close association to E2-induced-vasodilated vessels (Martín et al., 1994a, 1995a). Alternatively, the increase of *mhc2a* could also be mediated by the induction of thymic APC differentiation and activity, as E2-treatment has also been described to (1) stimulate intrathymic granulopoiesis (Martín et al., 1994a) and to (2) directly modulate peripheral DC differentiation and function in mammals (Kovats, 2015; Laffont et al., 2017). The potential stimulation of thymic DC-function by E2 was confirmed by Selvaraj et al. (2005), who observed that E2-injection increased thymic IL-2 mRNA in the mouse. As a matter of fact, the only described source of IL-2 in the thymus appears to be the DC (Weist et al., 2015). In teleosts, DC-like cells have been described only recently. To the best of our knowledge, it is still unknown if (1) they express the different oestrogen receptors and if (2) they are modulated by E2, as has been shown for mammals (Kovats, 2015; Laffont et al., 2017). Moreover, HCs, which express ESR1 in mammals, chick and sea bass (Article 1), may provide another level of modulation. In mammals, an implication of HCs in the activation of medullary dendritic cells was reported (Watanabe et al., 2005). Hence, E2 may also indirectly stimulate DCs by stimulating HCs. Although the observed increase of *mhc2a* expression in our study could also be mediated by a

direct effect on the TEC, which express both oestrogen receptors and MHC-II, this mechanism is rather unlikely, because, in mammals, MHC-II in TEC was decreased by E2 (Dragin et al., 2017; Moreno and Zapata, 1991). In addition, B cells, which, in mammals, are considered potent thymic APCs, are present in the thymus of teleost fish (Klein et al., 2014; Yamano et al., 2015; Zapata et al., 1996). This is underscored by the expression of both igT+ and IgM+ transcripts in the sea bass thymus. IgM positive cells represent about 5% of the isolated leucocytes (Picchietti et al., 2017; Romano et al., 2017), whereas IgT transcripts are expressed at quite high levels (likewise to the head-kidney; Buonocore et al., 2017). It has been shown by Zhu et al., 2014 that B cells generally exert APC capacity, which suggests that B cells are likely to be involved in the central tolerance of vertebrates. Oestrogen modulates B cell activity, in mammals as well as in teleosts, potentially by acting on their oestrogen receptor (Iwanowicz et al., 2014; Khan et al., 2012; Kovats, 2015; Rodenas et al., 2017a). Indeed, in mice, and most likely in sea bass, B cell activity can be modulated by E2 through MISS (Schneider et al., 2014). Overall, data from the literature and from our study strongly suggest a modulation of central tolerance by E2 by acting on thymic APCs, including B cells, across all jawed vertebrates.

(4) Clonal selection

In mammals, FasL-mediated apoptosis of immature T cells can be stimulated by E2 (Do et al., 2002; Mor et al., 2001). As FasL is localized in mTEC and HC (Bai et al., 2013), it enables apoptosis *via* the modulation of HC activity in humans. Furthermore, in the periphery APCs, such as DCs, play a key role in clonal selection of T cells by inducing of FasL-mediated apoptosis after T cell activation (Audiger et al., 2017; Paulsen and Janssen, 2011). E2 could stimulate the extrinsic pathway of apoptosis involved in clonal selection in jawed vertebrates, either *via* HC or *via* the migration of APCs into the thymus and the function they exert therein. This hypothesis may explain the observed E2-mediated increase of apoptosis in different studies (Okasha et al., 2001; Wang et al., 2008).

In our *in vivo* exposure study, however, *caspase8* and *caspase9* transcript-levels were not strongly modified by E2-treatment, suggesting that oestrogen does not affect immature T cell apoptosis in sea bass (Article 2). In fact, Chapman et al. (2015) suggest that doses of E2 within a physiological range do not induce a strong T cell apoptosis in mammals. Future research should consider other indicators of apoptosis. The focus on *caspase8* and *caspase9* is

certainly insufficient and perhaps these genes are not the most appropriate ones, as Do et al. (2002), for instance, neither observed any change in the *caspase8* mRNA-level, despite clearly demonstrating that E2 induced the extrinsic apoptosis pathway.

v) Does oestrogen modulate T cell lineage commitment?

E2 may stimulate alternative intrathymic pathways of T cell-maturation, *i.e.*, the commitment into γδ T cell and Treg lineages. In mammals, E2 has been described to promote the differentiation of the thymic pIELp as well as iNKT (Abo, 2001; Cheroutre et al., 2011; Narita et al., 1998; Screpanti et al., 1991). The commitment into Treg, pIEL, iNKT and γδ T cells depends on a strong TCR-signalling process (Cheroutre et al., 2011; Hogquist and Jameson, 2014; Lambolez et al., 2006; Li and Rudensky, 2016; Muñoz-Ruiz et al., 2017). This process has been designated as agonist selection for distinction from positive and negative selection (Gascoigne et al., 2016; Hogquist and Jameson, 2014) [see also part (3) of this section]. Indeed, during T cell education not all self-reactive T cell clones are negatively selected. The T cell repertoire is broadly self-reactive and can drive the agonist selection.

The strengh of TCR-signalling, which promotes T cell lineage commitment, is determinated by TCR-affinity as well as by the environmental situation, *e.g.*, cytokines (IL-2, TGF-β) and co-stimulatory receptors (CD28) which is modulating TCR-signalling intensity. As a matter of fact, self-reactivity is believed to trigger pIEL or iNKT differentiation in the cortex of the thymus and Treg differentiation in the medulla (Hogquist and Jameson, 2014).

(1) Treg

(a) Thymus-derived-Treg

As it probably is the case in mammals (Teles et al., 2013b), the E2-mediated increase of *foxp3* expression in the thymus of males indicates that E2 promotes the differentiation of thymus-derived-Treg (tTreg) in sea bass (Article 2), as Foxp3 is a key transcription factor in the control of Treg development. This effect is likely to depend on APCs, as outlined above. In the thymus of female sea bass, the increase of *aire* and the lack of any significant change in the relative expression of the caspases may be indicative of E2-stimulated AIRE-dependent Treg differentiation. However, no significant effect of E2 was observed on the relative

expression of *foxp3*. It might be that, in the adolescent female fish utilized for the E2-exposures, endogenic oestrogen already initiated Treg development. Consequently, the E2-induced-Treg differentiation in the thymus might occur at very low E2-levels. Accordingly, the increase of *foxp3* in the spleen of female sea bass may be mediated by an E2-mediated stimulation tTreg migration to the spleen. Because the majority of plasma samples from the control group had E2-levels below the detection limit, possible gender differences in plasmatic E2-levels of the control group could not be confirmed.

The commitment into the γδ T cell lineage was indicated by E2-mediated changes of *tcrg* expression in the spleen and the head-kidney. However, this was observed solely in male sea bass, which, again may be explained by possible gender differences in endogenous oestrogen-levels. This explanation is further supported by the fact that in the female thymus, *tcrg* expression is significantly lower than in males.

The E2-related modification of tTreg differentiation may also be effectuated by HCs, which appear to be implicated in the differentiation of tTreg *via* the activation of medullary dendritic cells (Watanabe et al., 2005).

Contrariwise, the apparent increase of the number of Tregs in the thymus of male sea bass could result from an E2-stimulated cell trafficking from peripheral lymphoid organs. Paradoxically, a migration of peripheral Tregs back to the thymus may take place. The biological significance of this mechanism, which has been described for mammals, remains poorly understood, but it is believed to represent a feedback regulation inhibiting *de novo* tTreg differentiation (Li and Rudensky, 2016; Ulges et al., 2016). Evidence suggests a similar mechanism in teleosts, because thymic re-entry of T cells has been described for juvenile medaka by Bajoghli et al. (2015).

(b) Peripheral Treg

Our results obtained with *foxp3* as well as the decrease of oxidative burst capacity suggest that E2 induced pTreg differentiation and function or the migration of tTreg towards the spleen (Article 2). In mammals, high physiological E2-levels augment the proportion of Tregs in the periphery, *i.e.*, spleen, blood or lymphoid nods (Polanczyk et al., 2004, 2005; Spanier et al., 2015; Tai et al., 2008). During the menstrual cycle of mice, for instance, increased Treg numbers in the vaginal lumen and uterus coincide with higher E2-levels at oestrus (Teles et

al., 2013a). Moreover, *in vitro* E2-exposure has been found to stimulate Treg differentiation of CD3/CD28-stimulated CD4+ T cells as well as Treg suppressive activity and proliferation in humans and mice (Polanczyk et al., 2005; Prieto and Rosenstein, 2006; Tai et al., 2008). This differentiation did not occur in ERKO mice. It can also be prevented by the oestrogen receptor antagonist (ICI 182,780), on the one hand, and stimulated by an oestrogen receptor agonist (G-1). This suggests that E2-stimulated Treg differentiation *in vitro* is directly triggered in the T cells *via* GPER or ESR1 (Brunsing et al., 2013; Khan et al., 2012; Schneider et al., 2014; Tai et al., 2008). Direct E2-stimulation of Treg differentiation is confirmed by (1) *in vivo* E2-treatment of ESR1-deficient mice, which diminished FOXP3 induction (Polanczyk et al., 2004), (2) the anti-inflammatory effect of G-1 in a mouse autoimmune model and (3) a reduction in the anti-inflammatory effect of E2 under autoimmune conditions in GPER-deficient mice (Khan and Ansar Ahmed, 2016; Wang et al., 2009). Therefore, during pregnancy, E2 is likely to constitute an important accessory signal to promote Treg differentiation. To the best of our knowledge, the process of Treg differentiation has not been investigated in teleosts for the time being. Therefore, no data exist that could corroborate our hypothesis. Nevertheless, if we postulate that Treg differentiation in teleosts is homologous to that in mammals, which may be assumed on the grounds of numerous anatomical and functional similarities of the thymus and T cell between fish and mammalian species, E2 may also represent an accessory signal that promotes Treg differentiation in teleosts by activating membrane and nuclear oestrogen receptors expressed by T cells. In the mammalian thymus, Treg differentiation is determined by TCR-affinity for the pMHC as well as accessory signals, such as cytokines (IL-2, TGF-β) and co-stimulatory receptors (CD28) provided by the APCs (Li and Rudensky, 2016; Ulges et al., 2016; Weist et al., 2015). Therefore, during pregnancy, it is likely that tTreg and pTreg differentiation will be also influenced by an E2-action on APCs, such as DCs. As matter of fact, transfection of E2-exposed splenic DCs *in vitro* into a rat model of autoimmune disease stimulates Treg differentiation *in vivo* (Khan et al., 2012; Straub, 2007). Similarly, in endometriosis, E2 locally stimulates the differentiation of CD3/28-stimulated CD4 T cells into Tregs (Wei et al., 2016). Furthermore, Wei *et al.* (2016) also observed that E2 promotes Treg function by stimulating the expression of IDO1 enzyme in macrophages and endometrial stromal cells (Wei et al., 2016). Because teleost immune cells, such as macrophages, B cells and APCs are susceptible to be modulated by E2 (Iwanowicz et al., 2014; Lewis et al., 2014; Liarte et al., 2011), it is not unlikely that Treg differentiation in teleosts is modulated *via* several pathways.

In vitro, Treg inhibits T cell proliferation in mammals and fish (Plitas and Rudensky, 2016; Polanczyk et al., 2005; Wen et al., 2011) and, *in vivo,* deletion of Treg stimulates T cell proliferation in peripheral tissue (Sugimoto et al., 2017; Wen et al., 2011). Consequently, the E2-mediated Treg differentiation would correspond to an E2-induced inhibition of the PBL proliferation (stimulated with ConA) measured after *in vivo* E2-exposure of rainbow trout (Shelley et al., 2013). However, future investigations should confirm that E2-stimulated Treg differentiation is conserved in teleosts.

(2) Unconventional T cell lineages

In matters of IEL, which are commonly referred to as CD8αα+ αβ or γδ T cells, similar cells probably exist in teleosts as well. Contrariwise to their mammalian counterpart, teleost IELs do not appear to be oligoclonal (Cheroutre et al., 2011; Tafalla et al., 2016). As in mammals, they show cytotoxic activity, but clear characteristics that allow to distinguish them as a specific populations different from systemic T cells have not been yet defined (Salinas, 2015; Tafalla et al., 2016). The origin of teleost IELs, including αβ and γδ T cells, is probably similar to that in mammals, in which IEL arise from the thymus followed by *in situ* differentiation as well as extrathymic differentiation. As a matter of fact, *rag1*+ lymphocytes have been observed in the intestine of juvenile carp (Huttenhuis et al., 2006). Moreover, in sea bass DLT15-purified intestinal T cells expressed *rag1* (Boschi et al., 2011). During ontogenesis, DLT15+*tcrβ*- cells, which most likely correspond to γδ T cells, were simultaneously detected in the thymus and in the intestine at 35 days post-fertilization (Romano et al., 2011). The *tcrβ*+ cells first appeared in the thymus 35 dpf and then, 6 days later, in the intestine (Romano et al., 2011). These observations indicate that during the early ontogenesis of sea bass, αβ T cells are of thymic and γδ T cells are of extrathymic origin. Similarly, in carp T cells first appeared in the intestine during ontogenesis (Huttenhuis et al., 2006). In fact, Huttenhuis *et al*. (2006) observed a decrease of *rag1* expression in the intestine during the ontogenesis. This would confirm that, in fish, the extrathymic origin of T cells is quite important in the early steps of ontogenesis. At later developmental stages, however, the thymus probably becomes the main site of γδ T cell formation, as is clearly shown by the seven fold higher expression of *tcrγ* in the thymus as compared to the intestine and other lymphoid organs of sea bass (Boschi et al., 2011). In mammals, the intestine, liver and, to a lesser extent, the uterus have been identified as the main sites of extrathymic T cell

differentiation. Different physiological conditions are known to stimulate extrathymic T cell differentiation, such as aging, stress, pregnancy and oestrogen-treatment (Abo, 2001). In our study, E2-treatment increased the relative expression of *tcrγ* in the head-kidney and spleen of males (Article 2). In addition, the expression-levels of *tcrγ* were highly correlated to that of *pcna*. These results suggest that E2-stimulated the extrathymic T cell differentiation in the spleen of sea bass. Future work should investigate the effect of E2-treatment on *rag1* expression in the spleen, the liver and the intestine in order to determinate if the E2-mediated stimulation of extrathymic T cell differentiation is evolutionary conserved.

In mammals, mTECs, are believed to play an important role in the development of γδ T cells, pIELs and iNKTs, because their frequencies are altered when mTEC (but not cTEC) are impaired (Abramson and Anderson, 2017; Fujikado et al., 2016). As a matter of fact, IL-17A^{+} γδ T cells (Tγδ17), the majority of thymic iNKT and mTEC are co-localized in the medulla (Abo, 2001; Fujikado et al., 2016). Moreover, Aire+ mTECs regulate γδ T cell-differentiation, both in an Aire-dependent and Aire-independent manner (Fujikado et al., 2016; Roberts et al., 2012). Fujikado et al. (2016) observed that *aire* depletion in mouse as well as *aire* mutation in humans stimulated the development of the peripheral population of quasi-invariant pro-inflammatory Tγδ17, which infiltrate tissues targeted by an autoimmune attack. Hence, AIRE promotes immunologic tolerance by dampening Tγδ17 (Fujikado et al., 2016). We found that, in sea bass, E2-modulated TEC function, which was indicated by an altered *aire* expression. Thus, E2 may trigger γδ T cell differentiation in sea bass by acting on mTEC in a manner comparable to its regulatory action in mammals. E2-treatment, tended to decrease the relative expression of *tcrg* in the thymus of female sea bass, whereas no such effect could be observed in the spleen or the head-kidney. However, these results do not exclude the implication of Aire in the γδ T cell differentiation of sea bass. In fact, in mice *aire*-deficiency had no, or only a weak effect on thymic and peripheral population of γδ T cells, *i.e.*, when all γδ T subpopulations are quantified together (Fujikado et al., 2016). Interestingly, the increase of *aire* expression in E2-treated females appeared to entail, an induction of peripheral immune tolerance in the spleen, as outlined above. This suggests that E2 decreases the differentiation and number of Tγδ17 (or homologous pro-inflammatory T cells) in the spleen.

In mice and in humans, IL-7 drives Tγδ17 differentiation (Fujikado et al., 2016; Vantourout and Hayday, 2013). Fujikado et al. (2016) hypothesised that AIRE regulates Tγδ17 differentiation by repressing IL-7 gene expression. Because IL-7 mRNA has been detected in

human HCs (Watanabe et al., 2005), it may be assumed that E2 regulates Tγδ17 commitment by modulating the production of IL-7 from HCs. In teleosts, IL-7 is important for T cell development (Boehm and Swann, 2014), but it is unknown if this interleukin is involved in γδ T cell commitment, for this process has not been investigated in fish. Hence, future research should determine (1) the appearance of these cells during teleost ontogenesis, (2) if E2 regulates γδ T cell development in fish and (3) if γδ T cells are also represented by several subtypes, *e.g.*, TGF-β+, IFNγ+, IL-17+ γδ T cells, as it is the case in mammals.

Considering iNKTs, in contrast to NK-like cells, homologous/analogous cells have not been identified yet in teleost (Riera Romo et al., 2016; Tafalla et al., 2016). On the contrary, in birds and amphibians, iNKT-like cells have been described, suggesting a considerable conservation amongst vertebrates (Riera Romo et al., 2016). In mammals, iNKT have innate immune cell-like properties and, notably, antimicrobial, antitumoural and antiviral functions. These are conferred by the cytotoxic properties of iNKT cells and their capacity to release chemokines, Th1 and Th2 cytokines (Pasman and Kasper, 2017; Riera Romo et al., 2016).

(3) Oestrogenic regulation of ROS/Ca^{2+}-signalisation: the keystone?

As mentioned above, the commitment of T cells to Treg, pIEL, iNKT and γδ T cell lineages requires agonist selection in mammals. TCR-signalling can be influenced by E2 MISS-activation, which modifys Ca^{2+}-fluxes in T cells (Offner et al., 2000). T cell fate and agonist selection require antigen-initiated signals, which activate downstream signalling pathways tightly regulated by accessory signal such as CD28- or TGF-β-signalling (Li and Rudensky, 2016). Elevated E2-concentrations could, therefore, modulate downstream TCR-signalling and, thereby, promote the differentiation of T cells into Treg, pIELp, iNKT and γδ T cells during the central events of T cell commitment (Fig. 23). In mammals, iNKT as well as pIEL differentiate from DP (Abramson and Anderson, 2017; Gascoigne et al., 2016; Hogquist and Jameson, 2014; Mondoon et al., 2017). Interestingly, it was observed that both membrane receptor-deficiency in GPER-knockout and G-1 injection into wild-type mice increased DP T cell apoptosis (Isensee et al., 2009; Wang et al., 2008). These bewildering results may be explained by the different levels of Ca^{2+} necessary to destine T cell for either lineage commitment or apoptosis (Fig. 23). At low E2-concentrations, GPER-signalling may represent an important co-signalling factor to avoid death by neglect, *i.e.*, to elicit a weak, but lasting Ca^{2+}-signal for positive selection. Elevated E2-levels within the physiological range

activate GPER to produce an intermediate Ca^{2+}-signal of shorter duration, which modifies the TCR-signal in DP T cells towards agonist selection. Supraphysiologically high E2-levels rather provoke a strong GPER-activation (as does high G-1 administration) resulting in a short, but strong Ca^{2+}-signal, which in turn favours negative selection. Thus, distinct combinations of Ca^{2+}-concentrations and induction periods are required for either positive, agonist or negative selection with extremely low or extremely high E2-concentrations leading to apoptosis through death by neglect or through negative selection, respectively (Fig. 23). This modulation of Ca^{2+}-signalling has already been described to modulate positive and agonist selection (Gascoigne et al., 2016; Hogquist and Jameson, 2014). The hypothesis that E2 promotes agonist selection explains the E2- and G-1-mediated promotion of Treg differentiation (Brunsing et al., 2013; Polanczyk et al., 2004).

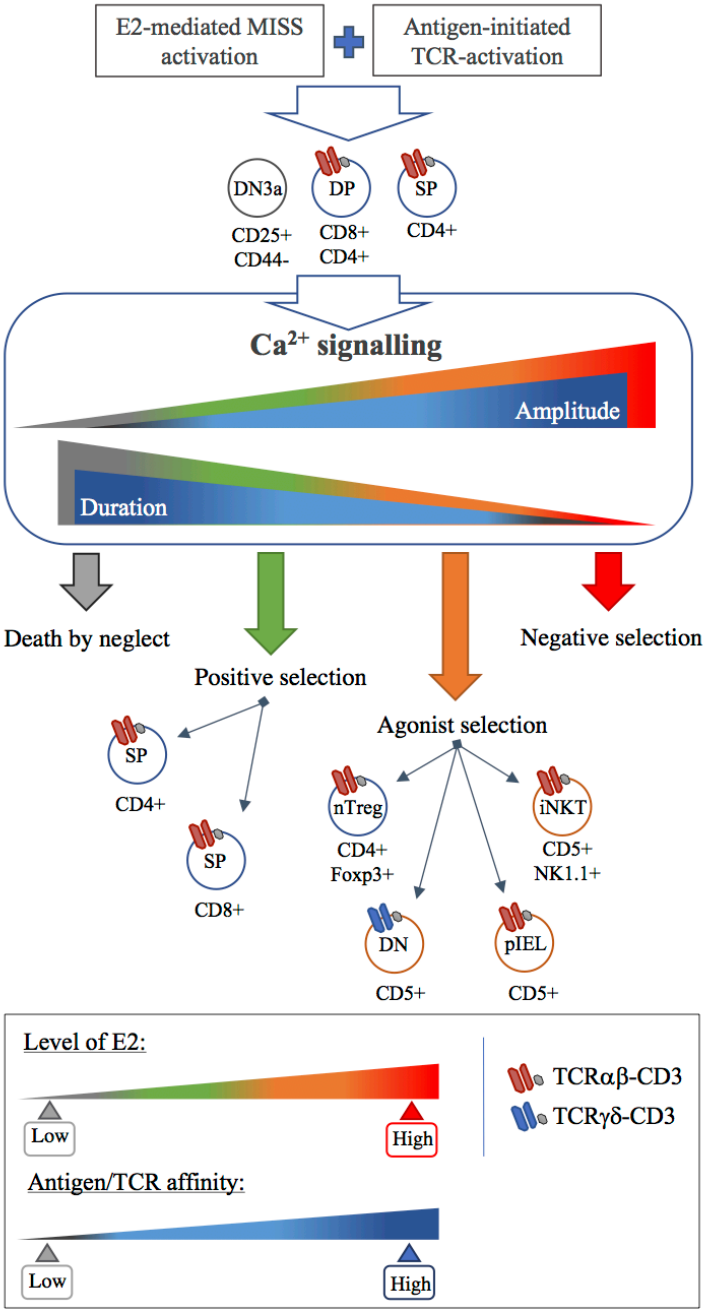


Figure 23: TCR-, Ca^{2+}- and E2-signalling directing T cell fate. Immature (DP, DN3a) and mature (SP CD4+) T cell fate is determined by a TCR transduction signal of a certain amplitude and duration. Antigen-initiated TCR-signalling pathway is mediated by Ca^{2+}-signalling which is dependent of the interaction strength between the antigen and the TCR. By acting through MISS, E2 equally modulates Ca^{2+}-levels, which may influence TCR-signal transduction and thus modulate T cell fate. E2-deficiency or GPER-deficiency promotes death by neglect: the downstream TCR-signalling pathway is too low to provide the pro-survival/differentiation signal and the clone is eliminated by death by neglect (apoptosis). Low E2-levels induce a Ca^{2+}-signalling, which promotes positive selection, *i.e.*, differentiation of conventional T cells (SP CD4 or CD8). High E2-levels induce a stronger but short Ca^{2+}-signalling, which promotes agonist selection, *i.e.*, Treg, pIEL, iNKT and γδ T cells. At very high levels of E2 (*e.g.*, supraphysiological E2-concentrations), E2 induces very strong Ca^{2+}-signalling, which favour negative selection and thus increase the elimination by apoptosis.

As mentioned earlier, the E2-mediated MISS in T cells is likely to be evolutionary conserved amongst vertebrates. We observed that E2 slightly increased ROS-levels in leucocytes of the thymus after 24 h of *in vitro* exposure. Based on the fact that ROS-, Ca^{2+}- and TCR-signaling are intrically linked (Belikov et al., 2015; Gascoigne et al., 2016; Görlach et al., 2015), TCR-activation not only stimulates Ca^{2+}-signalling, but also leads to ROS-production *via* the activation of dual oxidase 1 (DUOX-1) and the NADPH oxidase-2 (NOX-2) leading to the formation of superoxide, which becomes rapdily converted into hydrogen peroxide.

Furthermore, TCR-activation increases the ROS production through the activation of 5-lipoxygenase as well as the release of superoxide from the mitochondria (Belikov et al., 2015). Interestingly, all of these ROS sources are activated by Ca^{2+} and, in mammals, ROS-levels regulate T cell apoptosis, differentiation and proliferation (Belikov et al., 2015; Görlach et al., 2015; Hedi and Norbert, 2004). After 48 h of exposure we observed an increase of T cell mortality, which indicates that E2-induced immature T cell apoptosis in sea bass may follow the same principles as in mammals.

In mammals, FasL expression in whole thymus as well as in isolated thymocytes was induced by E2-treatment (Do et al., 2002; Mor et al., 2001). Furthermore, FasL expression can be stimulated by ROS production and by Ca^{2+} (Hildeman et al., 2003). At supraphysiological E2-concentrations, the E2-mediated stimulation of FasL is likely to trigger immature T cell apoptosis. By contrast, at physiological E2-concentrations, the low-level induction of FasL may stimulate TCR-signalling (Paulsen and Janssen, 2011). Hence, the E2-mediated increase of FasL is likely to represent a supplementary mechanism to increase TCR-signalling and thus trigger agonist selection.

vi) Is oestrogen-mediated atrophy evolutionary conserved?

In mammals, the drastic thymic atrophy brought about by elevated E2-levels is likely to arise from a rapid change in the thymic T cell fate. An important factor, which is probably responsible for this E2-mediated atrophy is the rapid and increased output of early committed T cell progenitors. It remains uncertain if a similar process occurs in teleost. Nevertheless, our results show that E2 could promote agonist selection of γδ T cells and Treg cells. In the mammalian thymic cortex, immature T cells seem to have brief migratory pauses depending on transient Ca^{2+} fluxes that gradually increase the basal Ca^{2+}-level during positive selection. Afterwards, positively selected T cells move much faster towards the medulla. On the contrary, negative selection reduces T cell motility rates in the medulla, leading to migratory arrest and increase cell-to-cell contact with APCs, which is followed by apoptosis (Gascoigne et al., 2016). In fact, high affinity between pMHC and TCR induces a strong TCR-signal that leads to cell arrest. Consequently, positive signalling requires more than 36 h whereas negative selection necessitates just one hour (Gascoigne et al., 2016). Subsequent to positive selection, the following differentiation of conventional T cells in the medulla represents a long process of maturation as well. Therefore, because agonist selection is an intermediate

state of TCR-signalling between positive and negative selection to neither of which the agonist selected T cells are submitted (Lambolez et al., 2006), it is likely that pIEL, iNKT, or γδ T cells rapidly differentiate and migrate to the periphery. As for the γδ T cells, it is unknown whether they indeed undergo negative selection. However, it was suggested that γδ T cells proliferate less and differentiate more rapidly than αβ T cells (Taghon et al., 2006). Consequently, the E2-mediated T cell commitment probably differentiates the cells more rapidly and the resulting lineages migrate more quickly into the periphery than conventional T cells. This hypothesis could, at least partly, explain the promotion of thymic atrophy at high physiological E2-levels. As our results provide evidence for E2 to direct T cell maturation towards unconventional T cells, which develop more rapidly and leave the thymus earlier, this would suggest that the E2-mediated thymic atrophy is evolutionary conserved and, therefore, also applies to teleost fish.

(1) Thymic seasonal variation and involution

The number of isolated leucocytes was measured in at least one of the thymuses of each and every fish sacrificed during the three years of this PhD-thesis, including those of the field catches and those of the hatchery at Aquacaux. If thymus size changes with the age of the fish, which can be roughly estimated by its total length, then a relationship between leucocyte number and length may emerge when organising the collected data into different classes of total length as well as the time point, *i.e.*, month of sampling. The results clearly show that the thymuses of juveniles of one year of age (G0-G1 class, 6-15 cm) up to two and three years of age (G2-G3 class, 15-30 cm) undergo a strong seasonal variation (Fig. 24). This is the first evidence in sea bass of the seasonal variation of thymic function, *i.e.*, seasonal thymus atrophy. In fact, thymus involution or atrophy is commonly referred to as a decrease of T cell content (Hince et al., 2008; Tatner, 1996). Our findings indicate that the thymus atrophy doesn't result from a change of food availability or quality because fish from the hatchery also undergo thymus atrophy. In agreement with our findings, Honma and Tamura (1984) observed similar thymus plasticity in fish caught in wild and fish reared in controlled condition under natural photoperiod and temperature. They reported that in various species of perennial fish seasonal thymus involution occurs several times during their lifespan and that temperature and photoperiod are critical elements of this involution (see below). For instance, fish exposed to complete darkness in June and July show an important involution and, during

winter, fish exposed to high temperatures show a rapid hypertrophy within 10 days. These findings also are in line with the histological observations described in the first article for fish of three years of age, which were sampled in October and in December. Notably, an important connective tissue, numerous myoid cells and a difficult to distinguish regionalisation were observed in some fish. These elements are characteristic of involuted thymuses. Correspondingly, Honma and Tamura (1984) noticed that in fish, the seasonal involution is associated to a relative increase in the amount of connective tissue and lacunae with increasing age.

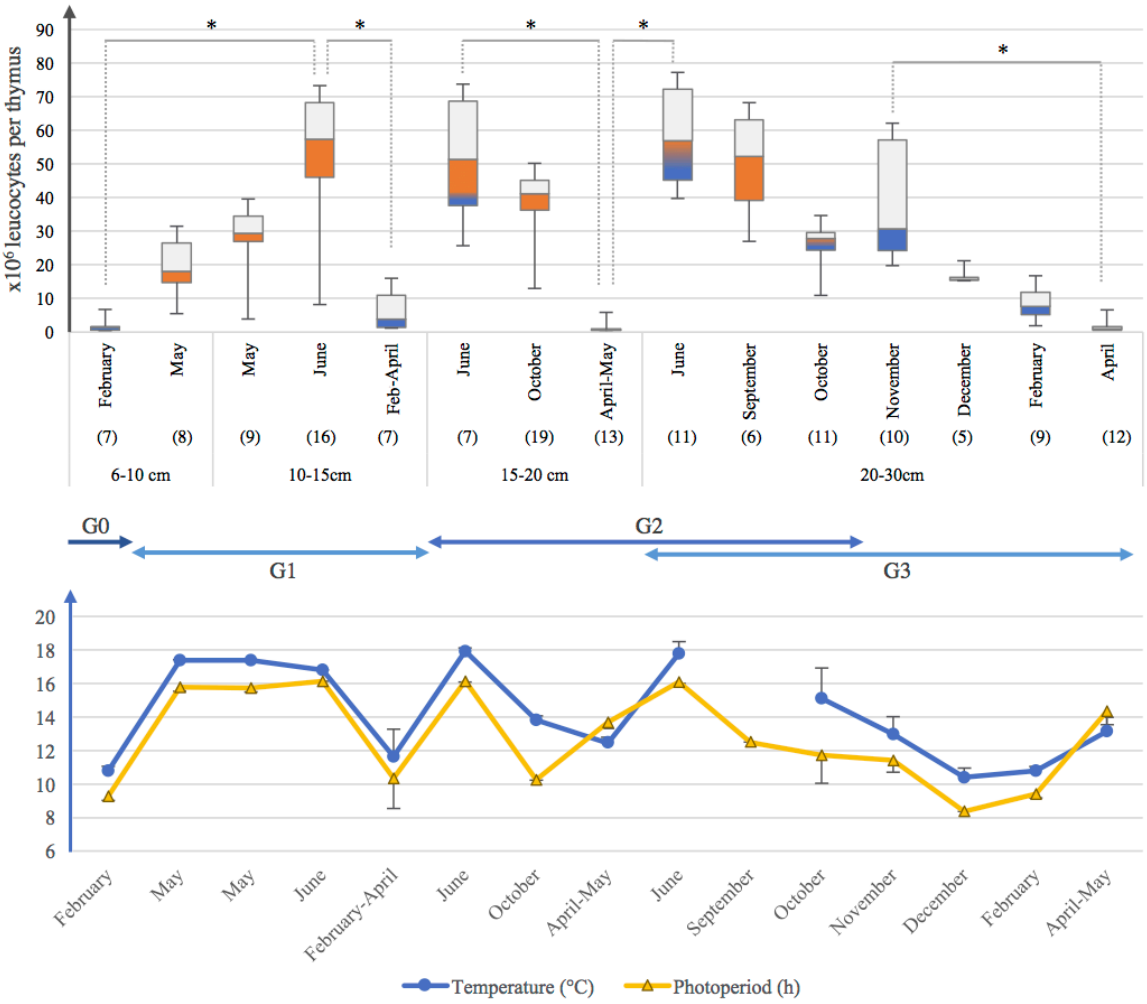


Figure 24: Thymic number of isolated leucocytes per thymus following total length classes as well as the month of the sampling. For the wild fish, the age classes (G1-G3) were estimated with the size of the fish based the work of Claridge and Potter (1983). Evolution of temperature (blue) and photoperiod (yellow) ± standard error associated with the date of the sampling. The box plots show minimum, maximum values, as well as the third and second quartile. Boxes in orange and blue indicate that the fish were sampled in the wild or taken from hatchery, respectively. Fish in the length class of 6-10 cm sampled in February and 20-30 cm sampled in December and April were obtained almost exclusively from the hatchery. The fish of 15-20 cm sampled in April-May were manly obtained from wild. *, significantly different at $p<0.05$ (ANOVA on ranks followed by Dunn's test). Numbers in parentheses correspond to biological replicates. Note: one temperature of sampling is missing for the fish of 20-30 cm caught in the wild.

Recently, the thymic volume evolution including total, cortical and medullary volume has been investigated during the thymus ontogenesis in sea bass from 93 dph from 239 dph (Seemann et al., 2015). The authors observed that the volume increased with the age, *i.e.*, the size until 183 dph. Then, around 210 dph the thymic volume tendentially decreased to reach statistically significant difference in volume at 239 dph. These total volume changes were associated with a change of ratio cortical/medullary volume from 3.15 at 93 dph to 1.31 at 239 dph. Therefore, the putative decrease at about eight months of age could correspond to the first seasonal involution.

Whether this first thymic involution follows an endogenous programme or whether it depends on external abiotic and biotic cues, *i.e.*, a *zeitgeber*, remains to be elucidated. The sea bass of Seemann et al. (2015) appeared to develop a thymus atrophy under constant experimental conditions, *i.e.*, constant photoperiod, temperature and food supply, speaking in favour of a endogen regulation. On the other hand, Honma and Tamura (1984) confirmed a seasonal thymus atrophy, which was independent of the time point of hatching in medaka, and, therefore, must have been triggered by *zeitgeber*. Similarly to our observation on the seasonal involution, these authors also documented a maximum thymus thickness in June and July, *i.e.*, the months with the highest length of day, for several species of annual and perennial fish in Japan. This means that thymus volume reaches a maximum in summer, independent of the time of spawning, the latter of which differs in the species studied.

In mammals and other teleost species, first signs of thymus involution, *i.e.*, decrease of thymic function also occur before puberty: about a few weeks after birth in rats or mice, six months after birth in horses, or 15 wpf in zebrafish (Aw and Palmer, 2012; Lam et al., 2002; Ottaviani et al., 2008). This early occurrence of thymus involution, which is situated in a period of high somatic growth, implies active regulatory processes as opposed to a passive degradation with age or a lowered metabolism during unfavourable environmental conditions.

(2) Endocrine control of thymic involution

Seasonal variation of photoperiod (circadian rhythm) and temperature are, doubtlessly, determinant environmental factors affecting animal physiology. This holds particularly true in poikilothermic animals, such as teleost fish (Bowden et al., 2007; Pérez-Ruzafa and

Concepción, 2015). Daylight and temperature regulate many fish behaviours, for instance related to reproduction (*e.g.,* maturity, spawning and migration), but also immune system performance. As for the latter, fish appear to exhibit seasonal fluctuations in their susceptibility to various pathogenic agents (Bowden et al., 2007; Pérez-Ruzafa and Concepción, 2015; Szwejser et al., 2016).

The synchronisation of seasonal environmental changes with the thymic function, requires to sense these physical changes and to transduce them into a molecular signal and a systemic communication. In both mammals and teleosts, daylight timing depends on melatonin secretion by the pineal gland (Bowden et al., 2007; Vera and Migaud, 2014). Melatonin synthesis is also believed to communicate a temperature signal. In fact, it was hypothesised that temperature regulates the amplitude of the melatonin secretion whereas photoperiod would control its nocturnal rise (Bowden et al., 2007). Indeed, melatonin is considered as time-keeping hormone, which drives circadian (daily) as well as circannual (seasonal) rhythms in vertebrates (Vera and Migaud, 2014). In mammals, melatonin has been reported to modulate T cell differentiation and activity and it was suggested to modulate thymic function with, notably, the promotion of thymus hypertrophy (Ren et al., 2017). Therefore, it is possible that in sea bass, melatonin is involved in the seasonal thymus plasticity, because in winter and autumn the average concentration of circulating melatonin is much lower in comparison to the rest of the year (Vera and Migaud, 2014). As a confirmation, eyeless and pinealectomized fish, presumably displaying only very low melatonin-levels deriving from other sources, showed evidence of thymic involution (Honma and Tamura, 1981).

Other hormones are likely to be involved as well. In vertebrates, stress-related hormones, such glucocorticoid hormone are well known to promote thymus atrophy (Honma and Tamura, 1984; Savino et al., 2015; Sufi et al., 1980). However, in farmed sea bass, the plasmatic level of cortisol showed the lowest level during the coldest period (Pascoli et al., 2011). Consequently, it appears unlikely that the cortisol-level is responsible of the observed seasonal atrophy.

Considering the sexual hormones and the gonadal differentiation, Blázquez and co-workers (2008) monitored the gene expression and enzymatic activity of the gonadal aromatase in juvenile sea bass during the first year. At 150 dph, first signs of sexual differentiation could be noted by histological examination, corresponding to an increase of the gene expression of the gonadal aromatase. Accordingly, the gonadal aromatase activity significantly increased at

200 dph to reach a peak at 250 dph. Although the female group showed a higher enzymatic activity at 200 dph, both groups reached their maximum activity at 250 dph. In teleost fish, oestrogen and gonadal aromatase are involved in gonad differentiation (Blázquez et al., 2008; Guiguen et al., 2010). For masculization and testis differentiation, the oestrogen-level and gonadal aromatase activity subsequently decrease (Guiguen et al., 2010). Because in sea bass, first thymus atrophy is likely to appear around 210 dph and then shows a clear involution at 239 dph (Seemann et al., 2015), these data confirm a major implication of oestradiol in the initial thymus involution.

Moreover, different to the smaller fish examined during this PhD-thesis, the thymus of sea bass with a total length over 15 cm was still involuted in April. Moreover, in fish with total length between 20 and 30 cm, but not in smaller fish, the thymus showed an intermediate level of involution in October (Fig. 24). Because the onset of maturity is related to fish length and because sexual maturity begins in September/October (del Pozo et al., 2014; Taranger et al., 2010), one may speculate that these differences could be due to higher levels of androgen and oestrogen. As a confirmation, fish of 20-30 cm of October, but not of June, which were reared in captivity tend to have a more pronounced involution (See section supplementary results). It is known that juveniles and pubescent fish have a seasonal cycle of sexual hormone production similar to mature fish, but at a much lower level (Taranger et al., 2010). Following our hypothesis that sexual hormones promote thymus atrophy, in mature fish the involution may be more pronounced and thymus hypertrophy may need longer to build up during spring (Fig. 25). Comparing sexes, males tended to have a lower number of thymocytes, but this difference was not statistically significant (Supplementary results). These preliminary results need further confirmation by extending the sample collection on fish from the wild and the aquaculture to confirm the tendencies. Moreover, future investigations should concomitantly evaluate the plasmatic level of the sexual hormones to validate the hypothesis.

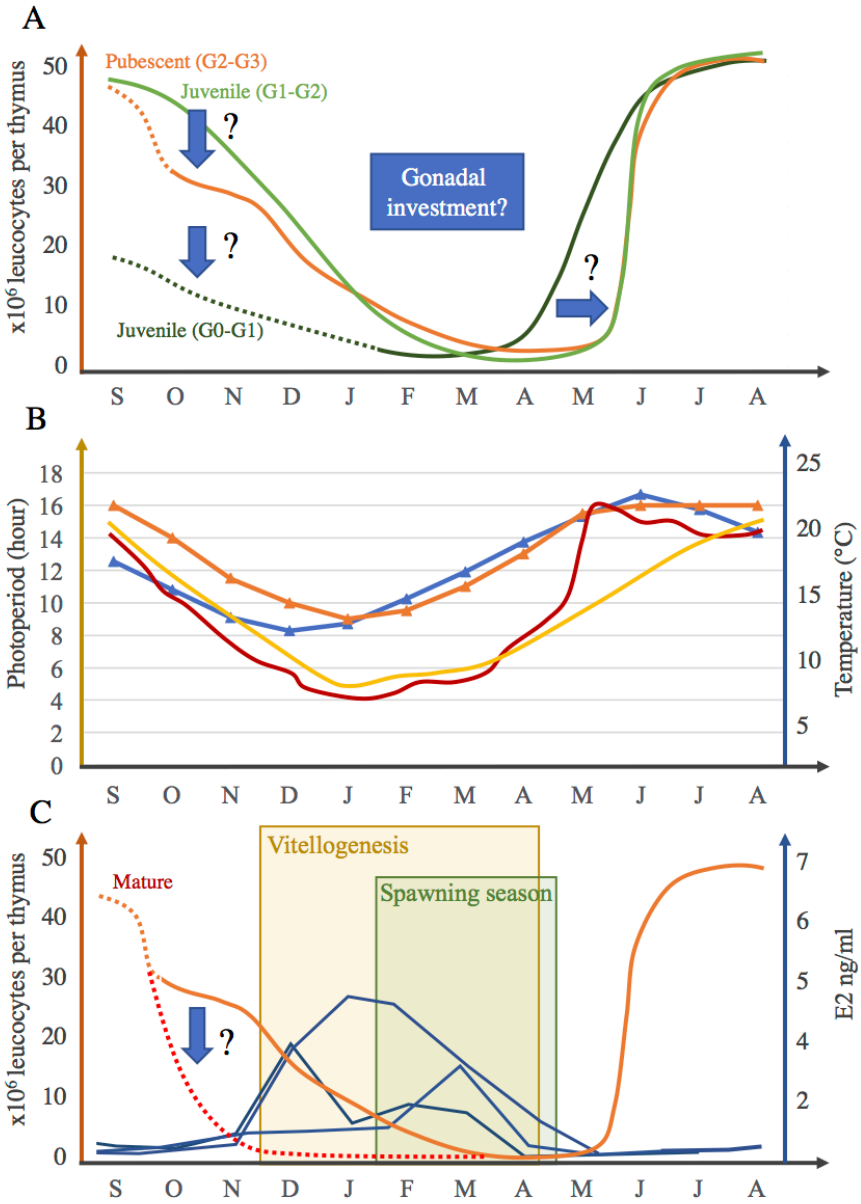


Figure 25: Proposed model for thymus involution in juvenile, pubescent and mature sea bass over a one year cycle. The thymus size is related to the number of isolated leucocyte. A, Seasonal decrease of thymocyte content in juvenile and pubescent sea bass (G0-G3) gradually occurs during autumn and is completed with the winter season. During June, the size is totally recovered. In juvenile and pubescent fish, the seasonal thymus atrophy appears to coincide with the beginning of the gonadal investment/development (October). The delayed thymus recovery from May to June for the older fish likely results from higher E2-levels during gonadal investment. B, Variation of photoperiod and water temperature in the wild and in the fish hatchery of Aquacaux from 2016-2017. Data of the Seine river by courtesy of GIP Seine-Aval. The curves with triangles correspond the natural (Paris, blue) and adjusted photoperiod (orange), respectively. The two other curves correspond to the variation of the temperature in the wild (red) and the fish hatchery of Aquacaux (yellow). C, Patterns of plasmatic E2-levels taken from the literature. Because, those patterns were obtained from sea bass sampled in Torre de la Sal (Spain), the E2-peaks were adjusted based on the fact that in western Europe, the spawning season occurs one month later and expands over three months (Beraud et al. 2017). Similar seasonal fluctuations were observed for the plasma levels of testosterone (Cerdá et al., 1995). Based on these data, we propose a hypothetical seasonal thymus plasticity for mature sea bass (red), with a more pronounced thymus atrophy that occurs earlier in comparison to the pubescent sea bass because of higher level of sexual hormones. The dotted line represents the putative fluctuations in leucocyte number.

B) Biological and ecological significance of an evolutionary conserved oestrogenic regulation on T cells

In mammals, the oestrogen-mediated Treg, IEL, iNKT and γδ T cell differentiation must be essential for pregnancy tolerance. It is believed that foetus "destruction" is avoided thanks to the peripheral induction of specific Tregs derived from conventional T cells and the proliferation of pre-existing Tregs (Clark, 2016; Li and Rudensky, 2016).

i) Mechanism for oestrogen-induced T cell-mediated tolerance

1. Central tolerance

tTregs represent key components of central tolerance, which enable tolerance towards self-peptides necessary to avoid autoimmune diseases. Their implication in tolerance towards non-self-peptides, however, is much less documented (Clark, 2016; Plitas and Rudensky, 2016; Russler-Germain et al., 2017; Samstein et al., 2012) [see following sections]. Oestrogen appears to increase the process of central tolerance by increasing the number of several APCs. More specifically, Martín et al. (1994a) observed an increase number of plasma cells and DC-like in the thymus of rat after E2-administration. And in mammals, plasmacytoid dendritic cells have been described to migrate into the thymus and present antigens collected from the periphery (Klein et al., 2014). Therefore, E2 may stimulate DC migration into the thymus to promote specific Treg differentiation and the elimination of T cells, which are potentially harmful for the semiallogeneic foetus. This process may be particularly important in the early process of pregnancy, as (Teles et al., 2013b) observed that during the pre-implantation period of the blastocyte, the number of tTregs rapidly increased in the thymus, lymph nodes and the uterus. However, after 5 days of pregnancy, the population of thymus derived Tregs decreased and was replaced by Tregs of peripheral origin (Teles et al., 2013b).

Although not generally included in central tolerance, unconventional T cells such as iNKT, γδ T and CD8αα+ αβ T cells are important for mucosal immunity. During pregnancy, their number increases especially in the uterus and in the decidual membranes. This strongly suggests that they are involved in pregnancy tolerance as well (Abo, 2001; Chapman et al., 2015; Kabelitz et al., 2013). Our results from T cell-related gene expression indicate that E2-induced peripheral expansion of γδ T cells with an tolerogenic phenotype, *i.e.*, γδ Treg, in the

spleen of male sea bass. Furthermore, our results suggest that E2 may stimulate γδ T cell proliferation and thus mediates γδ T cell activation in the spleen. To the best of our knowledge, direct oestrogenic action on γδ T cells as well as oestrogen receptor expression in these cells has not been reported yet and, generally, γδ Treg differentiation remains poorly understood in mammals (Kabelitz et al., 2013). As suggested for αβ Tregs, E2 may constitute a co-stimulatory signal that drives γδ Treg differentiation. Further investigations in mammals and teleosts should determine if γδ T cells express oestrogen receptors and if their activity becomes modulated by oestrogen.

2. Peripheral tolerance

Peripheral tolerance, such as peripheral differentiation of Tregs (pTregs) is believed to be highly important to tolerate non-self-peptides and to avoid an immune response against food- or commensal bacteria-derived-antigens as well as paternal alloantigens (Clark, 2016; Plitas and Rudensky, 2016). This appraisal is based on the fact that an impairment of pTreg differentiation in mice decreases the number of intestinal Tregs. This in turn results in allergic and asthma-like inflammations of the gut and/or the lung as well as increased losses of embryos (Chaouat, 2016; Plitas and Rudensky, 2016; Russler-Germain et al., 2017). Therefore, to regulate the peripheral immune response against "non-self" antigens, such as intestinal bacteria, peripheral differentiation of naïve T cells into specific pTreg is triggered by non-self-antigens *via* TCR-signalisation (Li and Rudensky, 2016; Russler-Germain et al., 2017).

ii) Potential significance for teleost fish

(1) Treg, γδ T cell and IEL in teleost

Mucous membranes represent interfaces with the environment. They harbour both, beneficial and pathogenic bacteria. It is therefore mandatory to develop an appropriate immune response and to discriminate pathogenic from normal residential bacteria. In mammals, Treg are required to tolerate intestinal bacteria, they are essential to maintain tissue homeostasis in the intestine, but also in the lung (Plitas and Rudensky, 2016; Russler-Germain et al., 2017). Teleost Treg and IEL, including γδ and αβ T cells, must also be important for mucosal immunity in the digestive tract, the gills and, probably, in the skin (Sugimoto et al., 2017;

Tafalla et al., 2016; Wen et al., 2011). In fact, Treg deletion in zebrafish or pufferfish induces inflammatory lesions in tissues exposed to external stimuli (epidermis of the skin, mucosal tissues of the pharynx, intestines and especially the gills) (Sugimoto et al., 2017; Wen et al., 2011). These data suggest the existence of pTreg homologous to the mammalian form in fish. However, non-mammalian species, such as teleost fish, lack the TGF-β responsive element named conserved noncoding sequence 1 (CNS1) in the promotor region of the FOXP3 gene. The depletion of CNS1 drastically reduces pTreg in mice. Therefore, it was hypothesised that pTregs are an innovation of placental mammals (Chaouat, 2016; Clark, 2016; Li and Rudensky, 2016; Plitas and Rudensky, 2016). A possibility would be that in teleosts non-self antigen specific Tregs would differentiate in the thymus. It is questionable, however, if this would be sufficient to enable peripheral tolerance. Further investigations in teleosts are needed to determine the origin of the mucosa residential Tregs and their mechanism of differentiation. However, this is likely to be challenging, because even in mammals the markers used for tTreg and pTreg identification remain controversial (Russler-Germain et al., 2017). This is because the frontier of specificity between the tTreg and pTreg is in fact not limited to the tolerance for self and non-self, respectively. Indeed, tTregs also have important features during mammalian pregnancy (Chaouat, 2016). Moreover, as outlined above, peripheral DCs can migrate in the thymus and trigger T cell maturation towards non-self-antigens. For instance, injection of peripheral DCs induced a threefold increase of tTreg (Ulges et al., 2016). Moreover, similarly to what we hypothesised previously, Russler-Germain et al., (2017) assumed that colonic bacterial antigens may be transported to the thymus for T cell selection, because a study found a marked overlap of the TCR repertoire between thymic and colonic Tregs. Furthermore, in the mammalian skin, residential Tregs are represented by both tTreg and pTreg, and both populations are important to maintain tolerance for commensal bacteria (Ulges et al., 2016). Interestingly, in a mouse model, neonatal skin colonization by *Staphylococcus epidermidis* provoked a rapid and primordial influx of tTreg into the skin. On the other hand, pTreg in the skin are probably involved in maintaining the immune tolerance during microbial fluctuation or after infection (Ulges et al., 2016).

(2) Endogenic oestrogen and potential immunoregulatory function in sea bass

In adult sea bass, annual reproductive migration involves plasmatic E2-fluctuations. Sea bass from the North Atlantic move from their summer feeding grounds to offshore pre-spawning and spawning areas during October to December, when the water becomes cooler. Spawning, on the other hand, commences along the British islands and in the mid-Western Channel during March. At the end of spawning in April/May, sea bass individually migrate back to their respective feeding grounds (Pérez-Ruzafa and Concepción, 2015). Consequently, the E2-induced peripheral tolerance could be related to changes in diet, energy budget and reserves and/or environmental pathogens [see also previous section and article 2]. An E2-mediated stimulation of IEL, γδ Treg differentiation or specific αβ Treg differentiation may be primordial to avoid an immune response against newly encountered, but not harmful antigens. Similarly, for juvenile and mature fish, winter-time coincides with a change of feeding behaviour when the fish become nocturnal, whereas during the rest of the year, sea bass displays a diurnal locomotor and feeding activity (del Pozo et al., 2014). Consequently, E2-mediated modulation of the immune system may represent an adaption to changing environmental conditions and physiological requirements.

Furthermore, semiallogeneic pregnancy is not a mammalian invention. It may actually have been realised early in the evolution of bony and cartilaginous fish (Chaouat, 2016; Clark, 2016). Although viviparous teleosts are exclusively aplacental, placental viviparity appeared several times during vertebrate evolution and has been described for taxons as different as sharks and reptiles (Chaouat, 2016; Segner et al., 2017). In viviparous sharks, pregnancy can last from about half a year to two years (Chaouat, 2016). At the same time, teleosts and sharks quickly reject allogeneic grafts, which demonstrates that their immune system recognises non-self peptides. Hence, oestrogen-induced T cell immunomodulation may indeed represent an ancient mechanism allowing to tolerate foetus implantation in viviparous placental jawed vertebrates. However, only progesterone, but not yet oestrogen, has been ascribed an immunomodulatory role during pregnancy in various vertebrate groups such as reptiles and mammals (Segner et al., 2017).

Nevertheless, these processes do not explain the E2-mediated immunomodulation observed in male sea bass. In mammals, E2 has been shown to modulate thymus morphology and T cell development in both sexes (Hince et al., 2008; Staples et al., 1999). Indeed, males are

commonly used to investigate oestrogenic immunomodulation, so as to exclude endogenous hormonal fluctuations (Schneider et al., 2014). Moreover, E2 has a critical role in thymus ontogenesis during the neonatal period.

Overall, a body of evidence corroborates the importance of paracrine and autocrine E2-actions for the immunoregulation of T cells. In mice, aromatase and 17β-HSD transcripts and activity have been detected in T cells isolated form the spleen (Samy et al., 2001). In the thymus, however, aromatase is, apparently, not expressed at transcript-level (Li et al., 2002).

(3) Why does the thymus involute?

In mammals, the E2-mediated thymus atrophy results from the activation intrathymic and extrathymic differentiation of unconventional T cells. Interestingly, these cells are also commonly referred to as innate-like lymphocytes, because, different from conventional T cells, (1) their antigen receptors are less diverse, (2) they are early responders during the immune response, due to an shortened maturation and (3) they do not present a memory response (Pasman and Kasper, 2017). Furthermore, they can have APC capacity and can respond *via* either their TCR or PRR, *e.g.*, iNKT or γδ T cells. These "in-between" cells, therefore, bridge innate and adaptive immunity by the early secretion of conventional leucocyte cytokines (Pasman and Kasper, 2017). They are believed to be an important component of the immune system, which co-evolved with conventional B/T lymphocytes in ancient jawed vertebrates. Unconventional lymphocytes are important immune cells during the development of adaptive immunity, as they appear early during ontogenesis and in the mucosa, where they establish a specialized relationship with microbiota; as "first-line" defence lymphocytes, they have tolerating and repressive function towards microbes.

As mentioned earlier, thymus atrophy occurs with aging, but also in numerous other physiological states, such as stress, for instance. In mammals, thymus atrophy notably occurs following various stresses, such as infection, starvation, graft versus host, chemotherapy and radiotherapy (Chaudhry et al., 2016). However, in the case of aging and infection, the significance as well as the underlying cause of the thymic involution remains enigmatic. In the case of infection, it is assumed that this phenomenon may represent a deliberative strategy to avoid the generation of pathogen tolerant T cells (Chaudhry et al., 2016). As for aging, it was proposed that thymic involution may represent an energetic trade-off to favour other

functions, such as reproduction (Chaudhry et al., 2016). Along the lines of these hypotheses, I postulate that seasonal and E2-mediated thymus involution in sea bass results from a switch in the thymic function towards the production of innate-like lymphocytes. In addition, innate-like lymphocytes represent an important component of the mucosa that allows the organism to adapt to the changes in antigens from different microorganism communities during winter. These cells appear to have a more winter adapted physiology, because they do not need clonal expansion and maturation to be effective and, therefore, are untroubled by time and energy consuming proliferation steps. Consequently, the endocrine regulation of thymus function is likely to represent an important component to anticipate wintertime changes as well as energy trade-offs related to the period of reproductive investment.

C) Ecotoxicological significance

Thymus function across all vertebrates seems to be tightly regulated by the endocrine system, notably by oestrogen. However, the coastal waters and especially the estuaries, where sea bass reside frequently, are under high anthropogenic pressure. The latter implies various pollutants, which are partly represented by oestrogenic endocrine disrupting chemicals (EEDC). Therefore, environmental oestrogens may interfere with T cell differentiation and inhibit the adaptive immune system with the promotion of immune tolerance and a less specific innate-like immunity.

i) Consequences of xenoestrogen-exposure for adult fish?

Our results suggest that E2 may represent a co-signal triggering Treg differentiation in teleosts, as it has been described for mammals. These Tregs are particularly important for the homeostasis of the tissues in contact with environmental antigens. However, those interfaces are also the ones, which are most exposed to water pollutants such as EEDCs. Therefore, by promoting Treg differentiation, including both tTreg and pTreg, xenoestrogens could impair the capacity to mount an adaptive immune response against pathogenic agents.

Xenoestrogen-induced Treg-dysregulation could be highly detrimental, as it is known from mammals that Treg function is vital for regulating the inflammation (Plitas and Rudensky, 2016). Indeed, Treg dysfunction is largely implicated in autoimmune disorders, allergy, acute and chronic infection, tissue repair as well as allergy and cancer (Plitas and Rudensky, 2016).

Therefore, by interfering with Treg differentiation, xenoestrogens may be detrimental for the immune homeostasis of fish.

In addition, xenoestrogens are likely to target the thymus activity directly and to alter its function as suggested by our E2-exposure experiment. As a matter of fact, the thymic vascularisation is directly derived from the gill vascular system, which constitutes a major surface in contact with the environment (Fig. 26; Zapata et al., 1996). Therefore, the thymus is one of the first organs that receives environmental oestrogens and oestrogen mimics transported by the blood stream.

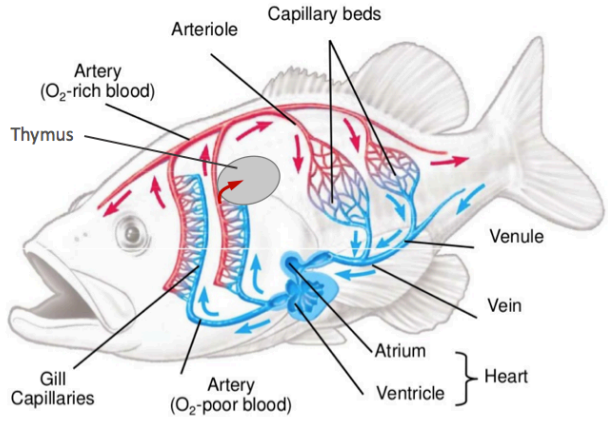


Figure 26: Blood circulation in teleost fish. Modified from © Pearson Education Inc.

Hence, two potential mechanisms can be proposed by which xenoestrogen may induce immune tolerance: (1) by directly acting on mucosal T cells and (2) by acting indirectly on thymus function and T cell maturation, including peripheral T cell differentiation. The latter could be triggering immune tolerance in the spleen, a major secondary lymphoid organ, which is a key component for mounting an adaptive immune response.

In addition, it is likely that xenoestrogens also inhibit the development of conventional T cells and promote the development of innate-like lymphocytes, which would impair the capacity to rise a specific immune response. Such effects could be of minor impact in relatively cold areas of sea bass distribution or during the colder winter periods. When fish are confronted with higher temperatures in the warmer areas of their distribution range, during summertime, or in the context of global warming, they are likely to face a different pathogen pressure that requires a better performing adaptive immune response (Bailey et al., 2017; Makrinos and Bowden, 2016).

ii) Consequences of xenoestrogen-exposure for juvenile fish?

In mammals, perinatal exposure to PCBs and other xenoestrogens (*e.g.*, DES, BPA, phtalates) has been linked to numerous immune diseases in adults, such as allergies, asthma and autoimmune diseases (DeWitt and Keil, 2017; Giusti et al., 1995; Jochmanova et al., 2015;

Winans et al., 2011). Generally, the developing immune system must be considered more sensitive to exogenous stressors, including xenobiotics, than the fully developed immune system. Developmental immunotoxicity is likely to impair proper immune system functioning. As a matter of consequence, the susceptibility to infection could be increased, tumour surveillance may be diminished and allergic, inflammatory and autoimmune diseases are favoured (DeWitt and Keil, 2017). Importantly, the negative effects for organismal health may manifest themselves at later stages in life.

(1) Which are the key stages of early tTreg development and release?

(a) Mammals

In female and male rat, it has been shown that DES injection during the first five days, or a single injection of E2 on the 5th day after birth induced a reversible thymus enlargement in 4 to 8 week-old rat (Forsberg, 1996, 2000; Leceta et al., 1988). In both sexes this was associated with a long term, time-delayed modulation of the T cell dependent immune response named delayed type hypersensitivity (DTH) (Bernardi et al., 2015; Forsberg, 1996, 2000).

In mice, thymectomy before the 3rd day after birth induced autoimmunity caused by effector T cells. Autoimmunity could be prevented by the adoptive transfer of splenic T cells from non thymectomized mouse (Clark, 2016). This critical window, is associated with the generation and release of specific, AIRE-dependent Tregs, which occur early after birth and have a specific function in maintaining T cell self-tolerance (Fujikado et al., 2016; Li and Rudensky, 2016). Expression of AIRE during the embryonic and neonatal period is necessary to establish long-lasting self-tolerance (Fujikado et al., 2016; Roberts et al., 2012). In humans and mice, AIRE expression is down-regulated by E2 through ESR1 (Dragin et al., 2016), which suggests that E2 could modulate tTreg development by acting on AIRE. Therefore, xenoestrogen-exposure during the critical phase of tTreg development and release could disturb this process and thus have the long-term detrimental effects. This assumption is corroborated by oestrogen-exposure during the first week of the neonatal period, which induced polycystic ovarian syndrome in a mouse model by acting on the thymus and likely by affecting Treg development (Chapman et al., 2009).

Moreover, ESR1-deletion in mouse had a detrimental effect on thymus growth in the post-natal period (five day-old and adult mouse), but not at the foetal stage (Yellayi et al., 2000). ESR1-deficient mice showed a reduced medulla, but no alteration of the cortical area (Dragin et al., 2016; Yellayi et al., 2000). These data indicate that E2-signalling has a key role during the neonatal period for the formation of the medulla, the microenvironment of which is fundamental for tTreg maturation (Klein et al., 2014; Li and Rudensky, 2016; Watanabe et al., 2005). Although ESR1-deficiency may not modulate *aire* expression and function (Dragin et al., 2016) and Yellayi et al. (2000) have shown that ESR1 deletion disturbs the peripheral T cell response by stimulating the DTH in adults, which may be induced by a lower or altered Treg content.

Furthermore, in regard to the potential GPER-mediated stimulation of TCR-signalling, it is likely that exogenous oestrogens may modulate T cell fate by favouring agonist selection or negative selection, which, consequently, may alter the Treg repertory. Such a change in the Treg composition may be highly detrimental, especially during the neonatal period.

(b) Fish and birds

Overall the results suggest that the cellular and tissular organisation of the thymus is conserved between teleosts and mammals and that the regulation of T cell development by E2 follows similar traits in all jawed vertebrates. These findings corroborate previous results, which indicated that E2 is involved in thymus ontogenesis and T cell development in teleosts, as it is in birds and in mammals. Our data, together with those of Seemann et al. (2015, 2016) provide convincing reasons to assume that exposure during critical windows of thymus development, would entail long-term effects in adult teleosts and birds, as it has been proven for mammals.

It was shown recently that dietary exposure of 60 dph juvenile sea bream to EE2, G-1 and tamoxifen provoked long term effects at 210 and 333 dph on the antibody production after immunization with the T cell-dependent antigen (KLH). The effects were characterized by an increase or decrease in anti-KLH IgM serum-levels depending on the timing of vaccination (Rodenas et al., 2016). On the contrary, exposure of adult sea bream to EE2 caused only short term effects on the anti-KLH IgM titer after 56 days of depuration, which disappeared after 88 days of depuration (Rodenas et al., 2015).

In chicks, E2-treatment on the 4th day of embryogenesis induced thymus hypertrophy and triggered a long-term effect in the embryos, which showed a stimulated humoral response against T cell-dependent antigen (goat red blood cells, GRBC). The same E2-treatment administered at day 14 of embryogenesis did, however, not modulate thymus morphology and had no effect on humoral response against GRBC (Kondo et al., 2004). These results strongly point to E2-sensitive windows in thymus ontogenesis, as they were described for mammals, and as they exist most likely in teleosts as well (Seemann et al., 2015, 2016, 2017). Obviously, key events in the establishment of T cell-dependent immune system are hormonally regulated and their disturbance by exogenous E2 may produce unwanted and potentially negative effects at later stages of life.

In non-mammalian species such as teleosts, the knowledge on Treg ontogenesis and differentiation is rather scarce. In zebrafish, Sugimoto et al. (2017) investigated *foxp3a* transcripts at various stages from day three to 13 wpf. They observed that *foxp3a* drastically increased between 14 and 28 dpf (Sugimoto et al., 2017). In agreement, Hui et al., (2017) detected *foxp3a*:RFP+ cells in the thymic medulla of zebrafish by 16 dpf which further accumulate during the development (Lam et al., 2002). Interestingly, this increase of *foxp3a* and appearance of *foxp3a*:RFP+ cells corresponded to the period of thymus ontogenesis when the regionalisation and the mature morphology of the medulla are acquired, *i.e.,* between 14 dpf and 21 dpf (Lam et al., 2004). After 28 dpf the relative expression of *foxp3a* continued to increase, but at a lower extent (Sugimoto et al., 2017). These data further suggest that Treg development in teleost takes place in the medulla, likewise to mammalian Tregs. Accordingly, tTreg differentiation and peripheral release in zebrafish would occur after 28 dpf, when the medulla has a mature morphology. Intriguingly, *foxp3a*-deficient zebrafish have an induction of Th1/Th2 and pro-inflammatory cytokines as well as growth retardation at 90 dpf (adults), but not at 30 dpf (juveniles). This emphasises the central role of the thymus in the mucosa specific Treg differentiation of teleosts, but also that juveniles have developed other mechanisms of mucosa-related immune tolerance. Most likely, these imply unconventional T cells, which may be gradually reinforced by tTregs during ontogenesis. In sea bass, the critical window of oestrogen-exposure identified by Seemann et al. (2015) corresponds to the end of the thymic regionalisation, *i.e.*, when the medulla is well formed (95 dpf). Therefore, we may assume that the critical window of thymus ontogenesis identified in sea bass corresponds well to the critical wave of tTreg differentiation.

In chicks, however, Kondo et al. (2004) identified a critical window of thymus development before the colonization of the thymus with T cell progenitors. This critical window opens at 6.5 days of embryonic development. This is way too early to correspond to the critical window of Treg differentiation in fish or mammals, which was considered to be susceptible to oestrogens. Further investigations should determinate if this step of thymus ontogenesis is also sensitive to oestrogen, both in mammals and teleosts.

Nevertheless, our results suggest that in teleosts E2 modulates TEC function, notably *aire* expression and Treg development similar to what is known in mammals. Further investigations are needed to:

- Confirm that oestrogen-exposure during critical windows of the thymus ontogenesis has long-term consequences in adult teleosts as in mammals.

- Determine if tTreg differentiation and release represent the key components.

(2) Which are the key stages of early γδ T cell development and release?

In mammals, unconventional T cells, such as the quasi-invariant population of Tγδ17 cells and other γδ T cells subtypes are mainly produced during the perinatal period (Chien et al., 2014; Muñoz-Ruiz et al., 2017; Pasman and Kasper, 2017). Subsequently, thymus derived γδ T cells take residence in peripheral tissues, as for instance Tγδ17 cells in the mucosa, where they persist throughout life (Chien et al., 2014; Muñoz-Ruiz et al., 2017). Because the activity of TEC, and especially Aire+ mTEC, appears to be modulated by E2, it is likely that perinatal exposure to E2 disturbs γδ T cell differentiation, which could have a persistent detrimental effect in view of their unique function within the immune defence. In teleosts, E2 appears to modulate also γδ T cell differentiation as well. Therefore, further investigations should determine whether E2-exposure during critical windows of immune system ontogenesis changes the peripheral γδ T cell compartment in the long term.

4) Conclusion

4) Conclusion

In view of the oestrogenic regulation of thymic function in a teleost fish, the European sea bass, our results show that, in addition to the morpho-functional organisation, the distribution of the oestrogen receptors appear evolutionary conserved. Our data further indicate that thymic and peripheral T cell maturation is influenced by E2. It has long been considered that oestrogen negatively affects T cell maturation by promoting thymic lymphopenia, associated with a decline in adaptive immune function, a decrease in peripheral naïve T cells and the aging process in mammals. Differently to glucocorticoids, however, at elevated physiological oestrogen-levels the E2-mediated thymus atrophy is not due to increased apoptosis of immature T cells, but rather stems from a stimulation of the alternative pathway of T cell maturation. The unconventional T cells emanating from this alternative pathway have innate-like properties and include γδ T cells, CD8αα+ αβ T cells and iNKT. These unconventional T cells apparently develop more rapidly than conventional T cells (*i.e.*, SP CD8αβ+ and CD4 αβ T cells), because their proliferation is less intense, they bear a less diverse TCR repertoire and, therefore, request no selection within the thymic medulla. The preponderant differentiation into unconventional T cells reduces the overall thymic T cell content, which is further exacerbated by the rapid release of the early committed T cell progenitors that differentiate into γδ T cells and CD8αα+ αβ T cells in the peripheral tissues, *e.g.*, the gut epithelium. The combination of these phenomena, therefore, entails thymus atrophy.

It is believed that thymus atrophy results, at least partly, from energy trade-offs. Indeed, the development of conventional T cells, which discriminate self from non-self is highly energy-consuming, as it involves numerous steps of intensive proliferation with more than 90% of the immature T cells finally being eliminated by apoptosis. Consequently, the E2-mediated thymus atrophy, *i.e.*, the inhibition of conventional T cell differentiation during mammalian pregnancy directs energy to the foetus. Moreover, the E2-mediated promotion of Treg and unconventional T cell development increases peripheral tolerance to avoid the embryos rejection during pregnancy. Notwithstanding, the immune response being essential to the organism's survival, the immune system simply favours a less complex strategy.

In sea bass, our results suggest that oestrogens also turn thymic T cell maturation towards a less energy-demanding formation of unconventional T cells (*i.e.*, γδ T cells) and promotes

Treg differentiation. Endogenous E2-levels of sea bass rise in the middle of autumn and further increase during wintertime. This period is associated with numerous physiological changes including (1) investment into reproduction (*i.e.*, gametogenesis) and spawning (at the beginning of spring), (2) migration to pre-spawning and spawning areas, and (3) adoption of a nocturnal locomotor and feeding behaviour. Consequently, the E2-mediated switch of thymic function to "low cost" T cell differentiation could allocate energy towards the reproductive system. Furthermore, antigen pressure may be altered by the migration-related environmental changes and the shift in feeding behaviour. In this situation, an increase of peripheral immune tolerance would represent an evolutionary advantage to avoid an energy-consuming and over-performing immune response. Firstly, the lower environmental temperatures in winter limit the metabolic rate of poikilothermic fish, which together with potential lower food availability renders energy less available for a powerful and specific immune system. Secondly, the redistribution of resources towards migration and reproduction leaves less energy at the immune systems' disposal. Furthermore, the promotion of peripheral innate-like T cell fraction may kill two birds with one stone: together with a lowered energetic cost, their innate kinetics allow for a quicker response to infection without extensive differentiation and proliferation (See figure 27 for an overview of the E2-effects on thymic and peripheral T cell differentiation in vertebrates).

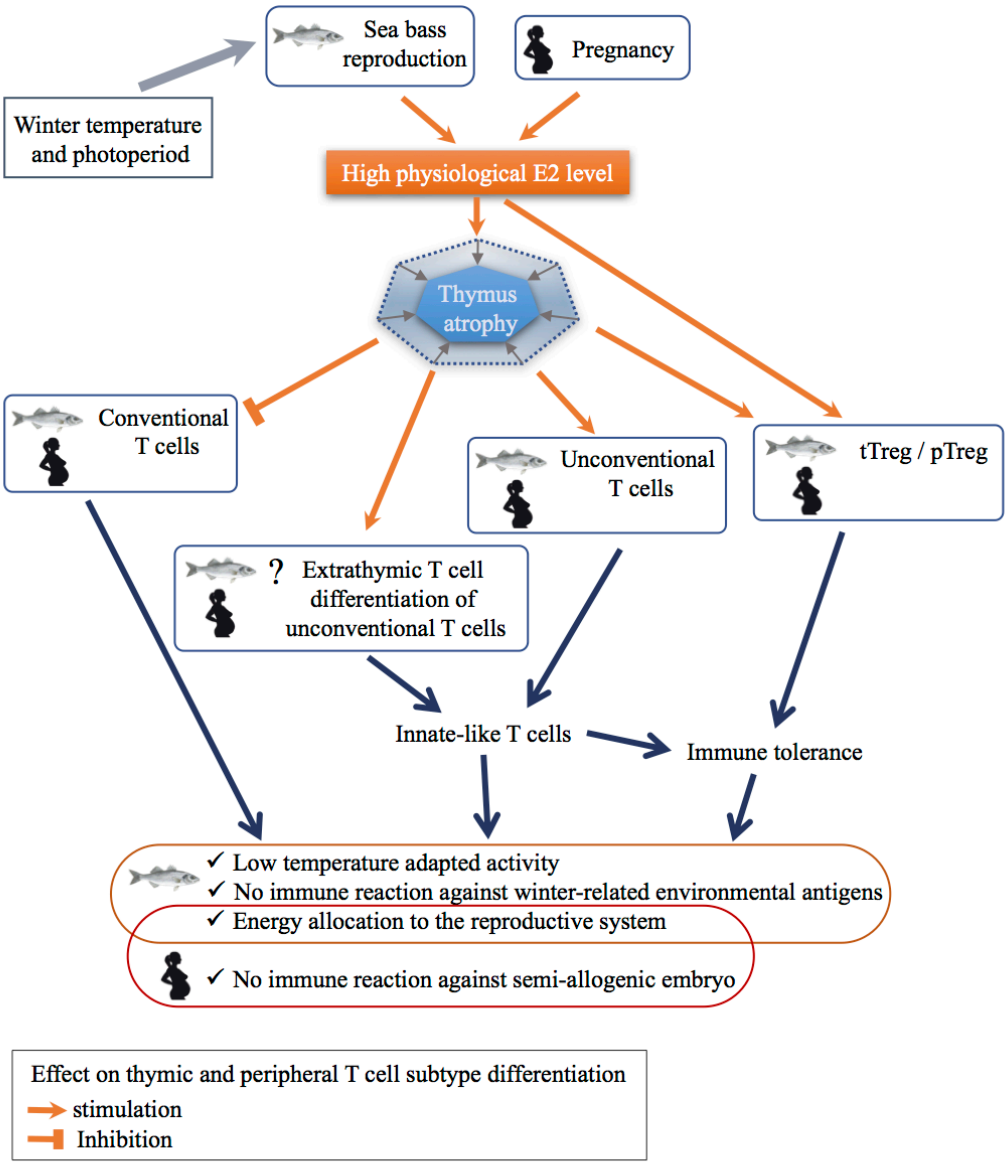


Figure 27 : Graphic account of the oestrogen-mediated modulation of T cell differentiation and its biological significance in sea bass and mammals. In sea bass, seasonal reproduction is triggered by winter-related environmental conditions (*e.g.*, temperature, food availability, etc.). Teleost reproduction and mammalian pregnancy are associated with elevated physiological E2-levels, which modulate peripheral and thymic T cell maturation. The effects of E2 on thymic T cell differentiation result in a strong atrophy of the thymus, because E2 inhibits the intricate processes of conventional T cell differentiation (*i.e.*, SP CD8αβ+ and CD4 αβ T cells) and stimulates the more rapid intrathymic differentiation of unconventional T cells (*i.e.*, γδ T cells, CD8αα+ αβ T cells and iNKT). Additionally, thymus atrophy results from the stimulation of extrathymic γδ T cells and CD8αα+ αβ T cells differentiation, as E2 enhances the peripheral exportation of early committed T cell progenitors. The main question that remains to be answered is whether this maturation pathway is indeed stimulated by E2 in teleost fish. In the thymus and/or the periphery, E2 promotes thymic derived Treg (tTreg) and peripheral Treg (pTreg) differentiation. The E2-mediated increase of Treg and unconventional T cells results in an increase of peripheral immune tolerance. The E2-mediated (1) inhibition of conventional T cell differentiation, (2) increase of peripheral innate-like T cells and (3) enhancement of immune tolerance represent key mechanisms that allow to allocate energy towards the reproductive system. In sea bass, this process is likely to be responsible for adapting the immune system to winter conditions. In mammals, and presumably in teleosts as well, the E2-mediated increase of immune tolerance is important to either tolerate the foetus, or winter-related changes in environmental antigens, *i.e.*, microorganism populations.

The shift in the T cell maturation process is a short term and a tightly regulated strategy, either in the case of pregnancy in mammals, or because of seasonal changes in sea bass. Thymus atrophy is associated to given physiological conditions and environmental factors in sea bass, because, for organisms in seasonally contrasted environments, abiotic factors such as temperature and photoperiod are important *zeitgebers* or synchronizers (del Pozo et al., 2014) that coordinate numerous physiological systems in relation to seasonal environmental constraints. Consequently, the thymus and its T cells appear as the central core element that ties together the somatic- and reproductive-level (Ottaviani et al., 2008). By integrating various signals, such as gonadal or neuroendocrine hormones, the T cell differentiation can turn the immune system towards a low-cost defence system depending on seasonal and reproductive requirements. In line with this hypothesis, lower temperatures have been associated with a predominantly innate and tolerant immunity in teleost fish (Bailey et al., 2017; Makrinos and Bowden, 2016) that allows for resource redistribution, but also assures organism survival. From an ecotoxicological point of view, our results confirm that oestrogenic endocrine disrupting chemicals modulate the immune system towards immune tolerance and innate immunity *via* thymic and peripheral T cell differentiation. Consequently, the permanent presence of estrogenic compounds is likely to results in altered immune system efficiency, with less specificity and lack of memory of unconventional T cells.

5) Outlook

5) Outlook

Future investigations should firstly determine the seasonal variation of plasmatic oestrogen- and androgen-levels in juvenile, pubescent and mature sea bass with respect to the thymic leucocyte content and immune status. The aim would be to confirm that, in sea bass, summer and winter conditions and thus, supposedly, conventional and unconventional T cells are associated with different immune strategy and trigger an adaptive and innate-like immune response, respectively. To confirm whether seasonal changes in oestrogen-levels promote innate-like immunity and increased immunotolerance by Treg differentiation and unconventional T cell expansion, γδ T cell- and Treg-related gene expression as well as oxidative burst capacity should be monitored in the leucocytes from the head-kidney and spleen of sea bass sampled in monthly intervals. To validate the biological significance of the seasonal thymus atrophy and to determine whether it is associated with a stimulation of extrathymic T cell differentiation, T cell subtypes as well as RAG-related genes and cell-mediated cytotoxicity should be monitored in the gut and the gills together with their corresponding leucocytes. Because, the extrathymic pathway of differentiation and expansion of unconventional T cells implies an increase of basal proliferation and a lower clonal expansion/proliferation capacity, the activity and basal level of T cell proliferation as well as the capacity to respond to T cell specific mitogen could be also monitored. Newly produced, sea bass-specific anti-Foxp3 and anti-Tcrγ antibodies that could be employed in immunohistochemistry and flow cytometry, would help to consolidate these investigations.

To go further into the evolutionary questions raised by this thesis, comparative studies in zebrafish using anti-Tcrγ antibodies (Wan et al., 2017) as well as transgenic lines with cell-specific reporter gene expression in T cell (*lck*+), CD4 T cell (*cd4.1*+), Treg cells (*foxp3*+) and *rag1/2*+ cells (Dee et al., 2016; Hui et al., 2017; Jessen et al., 1999) will be helpful. By studies with E2-exposed zebrafish, it could be confirmed whether oestrogen indeed shifts the immune system towards innate or adaptive immunity, by excluding the factor seasonality. In addition, because aryl hydrocarbon receptor activation promotes T cell differentiation in in mouse and zebrafish, future work should determine if Gper and Esr1 are involved in *in vitro* Treg differentiation in sea bass as well (Adurthi et al., 2017; Brunsing et al., 2013). This work may be used to characterize Treg activity, *e.g.,* anti-proliferative activity and interleukin expression. Eventually, the immune reaction toward T cell-dependent antigens, (2) immune

challenges with pathogen agents and (3) an assessment of the immune memory function, should be analysed in sea bass and zebrafish.

The proposed approaches may shed light on the poorly understood vertebrate thymus atrophy and provide a new model to investigate the unconventional T cell development and function as well as thymus regrowth. As a matter of fact, the thymus regeneration is of great interest in biomedical research to provide new strategies to restore thymus function in the case of injury or the toxic insults of chemotherapy (Chaudhry et al., 2016). Improving our knowledge in this field would also provide insight into the ecotoxicological significance of xenoestrogen-exposure for the thymus and T cell function. Particularly, in the context of global warming the supposed xenoestrogen-mediated modulation of the immune system towards an increased tolerance with innate properties could prove to be deleterious, because higher temperatures enhance parasite pressure, which requires a stronger adaptive response in teleost fish (Bailey et al., 2017).

6) Supplementary data

6) Supplementary data

A) Supplementary materials and methods

i) 17β-oestradiol as EEDC-model

The natural ligand E2 was chosen as a model-EEDC due to (1) its high receptor affinity, (2) it is physiologically present in male and female animals and because (3) E2 represents an environmental concern. Indeed, this potent oestrogen is continuously rejected in the environment *via* animal excretion and bacterial degradation of the conjugated forms (Adeel et al., 2017; Elnwishy and Sedky, 2016; Ting and Praveena, 2017). Furthermore, E1 which is frequently the most abundant environmental oestrogen, can be endogenous converted by vertebrates leading to high-plasma level of E2 (Ankley et al., 2017). And as spotted in the introduction, most EEDCs interfere with numerous signalling pathways, therefore, unravel endogen hormone action may represent a first step to understand the immunotoxicological action of EEDC.

Moreover, in an historically point of view, E2 represents the most investigated oestrogenic compounds in human and rodent thymus (See introduction, section C) ii)). Consequently, E2 represents the oestrogen of choice in comparative studies which aim to elucidate the elementary pathways of oestrogenic immunomodulation in teleost.

ii) Flow cytometry

The leucocytes from the head-kidney and spleen were isolated using a ficoll solution. The leucocytes solution was adjusted to $1x10^6$ cells/ml and different physiological parameters were routinely monitored in flow cytometry: the cell viability, the oxidative burst and the phagocytosis. For the analysis, different immune cell populations were discriminated based on their size (forward-scatter, FSC) and their granularity/internal complexity (side-scatter, SSC). In agreement with previous work in rainbow trout (Granja et al., 2015), two populations were distinguished: a population with a smaller size and granularity, which correspond to the lymphoid cells and another population corresponding to the myeloid cells. In peripheral blood leucocytes of human three populations of leucocytes can be distinguished: lymphocytes, monocytes and neutrophils (Peluso et al., 2012). Similarly in zebrafish three populations could be discrminated amongst the leucocytes of the head-kidney (Takizawa et

al., 2016). For analysis lymphoid and myeloid cells were selected drawing gates (Fig. 28-30), *i.e.*, alive cells of each population are analysed separately and the debris are excluded.

The proportion of cells in the two gates constitutes a first immune parameter. The other measured parameters are based on the use of different probes which when they are excited by the laser in the blue (λ=488 nm of wave length) they have the characteristic to emit photon with different wave length.

(1) Viability

All physiological tests were performed with Leibovitz cell culture medium (L15, Sigma, St. Louis, USA) adjusted to 360-370 mOsm/kg. The viability of the leucocyte was assessed using the iodide propidium. After incubation of 50 µg/ml for 10 min at room temperature and in obscurity, the emitted fluorescence was measured with the channel PE-Texas Red, collecting the photon emission between 615 and 620 nm. Cells with a high fluorescence are in late apoptosis or necrosis (Bado-Nilles et al., 2014). To quantify their proportion, a gate was drawn for each immune cell population (Fig. 28 and 29).

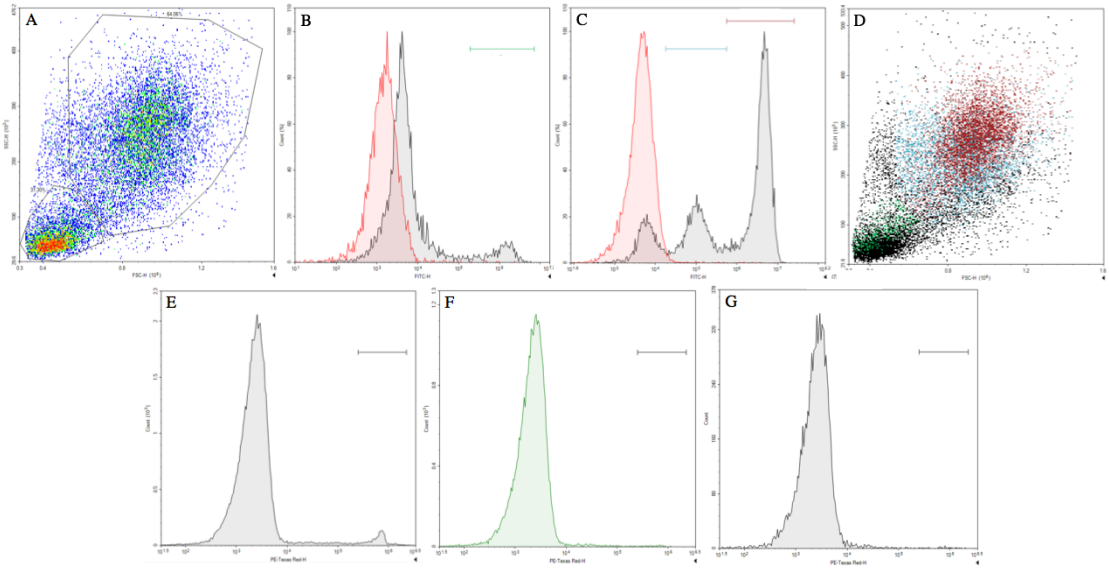


Figure 28: Flow cytometer analysis of the immune cells isolated from the head-kidney with representative cytograms obtained with the tests of viability and oxidative burst. A, forward-scatter (FSC) and side-scatter (SSC) cytogram with the gates used to differentiate lymphoid and myeloid cells. Lymphoid cells correspond to the population of cells with the lowest size (FSC) and complexity (SSC). B and C correspond to the overlapped fluorescence histograms obtained with the FITC channel during the oxidative burst test with PMA stimulated and unstimelated cells in black and red respectively. Gates were drawn to determinate if the different "oxidative burst positive" populations have different size and complexity. D, FSC/SSC cytogram with in colour the "oxidative burst positive" cells gated in B and C with in green the lymphocyte and in blue and red the myeloid cells. E-G, histogram of the viability test proceeded with the iodide propidium and the channel PE-Texas Red-H from ungated events (E), lymphoid (F) and myeloid cells (G). To quantify the "dead cells", gates were formed for lymphoid and myeloid gates.

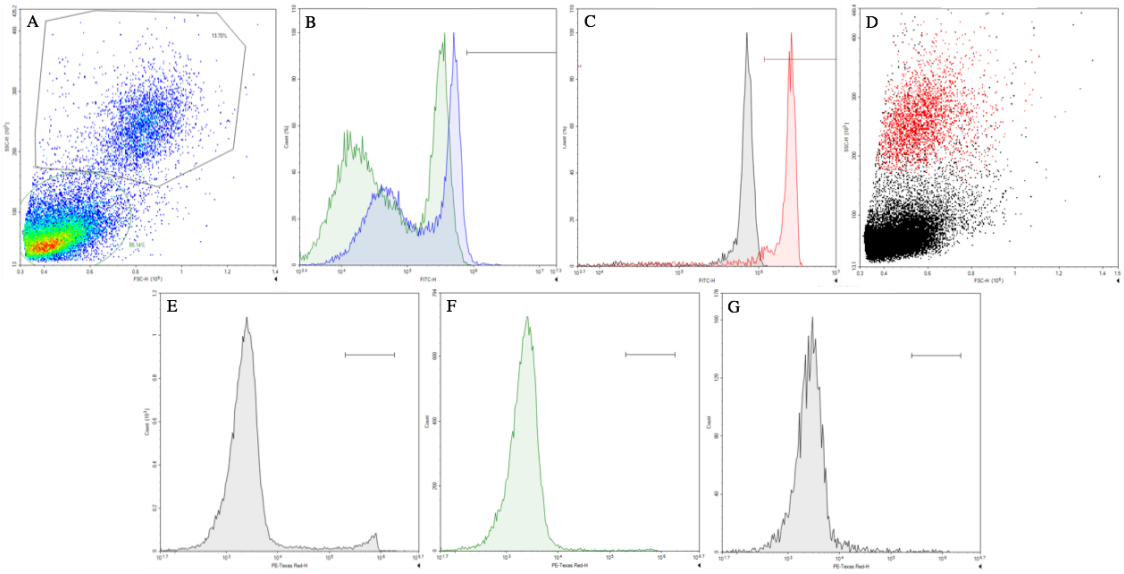


Figure 29: Flow cytometer analysis of the immune cells isolated from the spleen with representative cytograms obtained with the tests of viability and oxidative burst. A, forward-scatter (FSC) and side-scatter (SSC) cytogram with the gates used to differentiate lymphoid and myeloid cells. B and C correspond to the overlapped fluorescence histograms obtained with the FITC channel during the oxidative burst test with PMA stimulated and unstimelated cells in green/black and blue/red respectively. Gates were drawing to determinate if the different "oxidative burst positive" populations have different size and complexity. D, the FSC/SSC cytogram with in colour the "oxidative burst positive" cells gated in C with in red the myeloid cells. E-G, histogram of the viability test proceeded with the iodide propidium and the channel PE-Texas Red-H from ungated events (E), lymphoid (F) and myeloid cells (G). To quantify the "dead cells", gates were formed for lymphoid and myeloid gates.

(2) Oxidative burst

The capacity of oxidative burst was routinely evaluated using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) at 5 µM for 1 hour. After 30 min of incubation, the cells were stimulated with the phorbol 12-myristate 13-acetate (PMA) at a concentration of 2 µg/ml. The fluorescence at 530 nm (FL1 channel) was measured for each leucocyte population (Fig. 28 and 29). The result was compared to the fluorescence of unstimulated cells. Two ways to evaluate the capacity of oxidative burst were used: (1) the ratio of mean fluorescence intensity (stimulated cells/unstimulated cells) and (2) gates which quantified cells that exceed a given fluorescence (Fig. 28 and 29). The second method quantifies only the cells, which are apparently doing a "true" oxidative burst. Furthermore, as the results change with the sensitivity of the detector using the first method, and because the flow cytometer was renewed during the thesis, the gating method allow for comparison between different flow cytometers.

(3) Phagocytosis

Phagocytosis was assessed using fluorescent beads: FluoSpheres® Carboxylate-Modified Microspheres, 1.0 µm, yellow-green fluorescent (505/515) (F8823, Thermo Fisher Scientific, USA). Immune cell solutions at 10^6 cells/ml were incubated in obscurity with 2 µg/ml of PMA and beads (ratio cells/beads:1/50) for 2 hours at 16°C. The fluorescence of the cells was measured in the channel PE-Texas Red-H (Fig. 30). The number of fluorescent peaks with a high fluorescence is related to the number of beads detected within each cell. And the cells detected with more than three beads are considered phagocyting cells (Seemann et al., 2016).

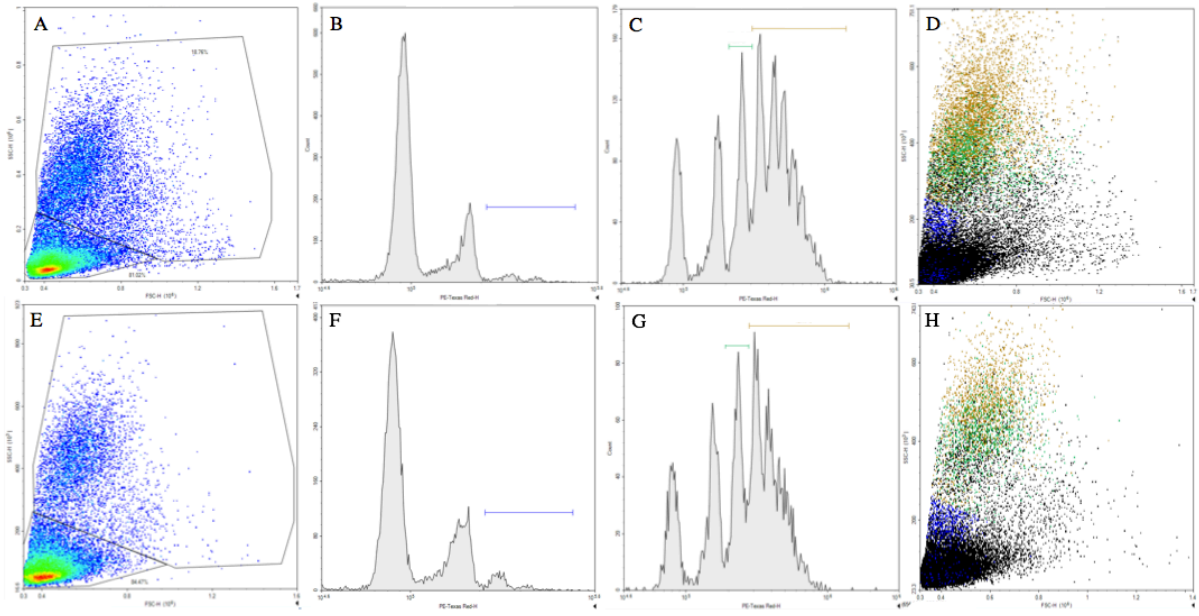


Figure 30: Flow cytometer analysis of the immune cells isolated from the spleen and head-kidney with representative cytograms obtained with the tests of phagocytosis. A and E, cytograms in forward-scatter (FSC) and side-scatter (SSC) with the gates used to differentiate lymphoid and myeloid cells. Lymphoid cells correspond to the population of cells with the lowest size (FSC) and complexity (SSC). B and F correspond to the fluorescence histogram in the channel PE-Texas Red-H of lymphoid cells respectively gated in A and E. For lymphoid cells, a gate quantifying the cells with three and more beads was drawn. C and G, correspond to the same parameters but for the myeloid cells gated in A and E. D and C, FSC/SSC cytograms with in blue the events corresponding to the phagocyting lymphocytes and in green and yellow myeloid cells with respectively 3 and at least 4 beads gated in B, C, F and G.

iii) Biomonitoring

For the ECOTONES project (Effect of Contamination on OrgaNisms of the Seine Estuary), 5 samplings of 12 to 21 fish were accomplished in the Seine estuary from June 2016 to October 2017. These samplings were all realized at the same site (Fig. 31) by a demersal (or bottom) trawler with a mesh size of 25 mm on the main part and 12 mm in the back (Fig. 32).

In addition, fish were sampled once in the channel of Tancarville (September 2015) with a fishing rod and once in the Dives and Orne estuary each (May and June 2017, respectively) with a beam trawl carried on board (1,6 m wide, 50 cm high and mesh size of 20, 16 and 10 mm). The fishing in the Dives and Orne estuary were carried on in the context of WFD biomonitoring.

The fish were generally caught during midday and kept in captivity in aerated 20 L buckets with the water from the respective sites until dissection in the morning of the following day. The dissection was carried out following the procedure described in article 2. For the isolation of cells and flow cytometric measurement, one thymus, a part of the head-kidney and the whole spleen was used. The other thymus and the other part of the head-kidney was snap frozen and kept at -80° for gene expression and other analyses. For fish with a total length superior to 15 cm, a part of the spleen was also kept for molecular biology.

Figure 31: Localisation of the different sampling of wild sea bass. Map adapted from the website: https://www.geoportail.gouv.fr/carte.

Figure 32: Demersal trawler and the boat JU-AD-LO of the society Prelev'Mar. From Patrice Cauchois and the society's website: http://www.prelevmar.fr/.

iv) Project: E2-effect on the pituitary gland proteome

(1) Animals

Fingerlings of European sea bass (*Dicentrarchus labrax*) were purchased from the hatchery "L'écloserie marine de Gravelines" (Gravelines, France). The fish were reared in the facilities of "Aquacaux" (Octeville, France) in 1,800 L tanks with continuous flow of aerated and filtered seawater at environmental temperatures and seasonally adjusted photoperiod until about three years of age. Fish were daily fed until satiety with food pellets "Torbot label rouge" (Le Gouessant, Laballe, France). Two experiments were conducted according the European Union regulations concerning the protection of the experimental animals (Dir 2010 2010/63/EU).

(2) Treatment and sampling

The experimental setup and treatment procedure is detailed in the second article (See supplementary data). Briefly, for reasons of basins availability and manpower, two successive experiments were conducted in November (Experiment 1) and December 2014 (Experiment 2). The fish were randomly distributed into four groups, *i.e.*, a control and treated group for each experimental repeat: CTR-1, E2-1, CTR-2 and E2-2. The fish injected thrice intra peritoneally over one week at day 1, day 3 and day 6. Each injection consisted of either

0.5 mg E2/kg or 0.5 mg vehicle/kg alone. On day 7, the fish were anesthetized with tricaine methanesulfonate (MS 222; Sigma) and biometric characteristics (weight and total length) were recorded. The fish were sacrificed with MS222 overdose and subsequently decapitated. The pituitary gland was dissected, snap frozen in liquid nitrogen and stored at -80 °C until the protein extraction. The fish from the two experiments were analysed in detail (biometric index and plasmatic parameters) to assure that the samples could be compared and the respective group combined. The E2-exposure was validated for both experiment using several plasmatic parameters as well as the hepatosomatic index (Article 2).

(3) Protein extraction

For the ITRAQ-based quantification, two protein extracts for each experimental group (CTR-1, E2-1, CTR-2 and E2-2) were analyzed. Total protein was extracted from pool of pituitary glands sampled on eight female fish of the same experimental group. Pools were formed based on the gonadosomatic index and the relative maturity of the gonad evaluated by histological analysis. The pituitary glands were weighted, collected in a lysis buffer (8M Urea, 50 mM of Tris-HCl; pH 7.7), sonicated on ice twice during 30 second at maximum intensity (Q500, Qsonica, LLC., USA) and incubated one hour of at room temperature. The cells debris were then removed by centrifugation at 2000 *g* for one hour. The supernatant was collected and protein concentration was determinate using the Bradford method (Bradford, 1976). The proteins were subsequently denaturated by reduction of the disulfide bond with 5mM of Tris (2-carboxyethyl) phosphine hydrochloride (TCEP, Sigma) during 1h at 37°C. The thiol group of cysteine were blocked with 55mM Iodoacetic acid (IAA) during 1 h at room temperature and obscurity. Afterwards, the samples were stored at -80°C for further analysis.

(4) Protein digestion, iTRAQ labelling and analysis

Protein digestion and Isobaric Tags for Relative and Absolute Quantification (iTRAQ) labelling and analysis was performed as described by Young *et al.* (2015). The samples were digested in 1 µg trypsin (V5111; Promega, Fitchburg, USA) per 1 µg protein at 30 °C for 15 min. Samples were then iTraq labelled from 114-117 (4374321, Sciex, Washington, USA) dissolved in EtOH for 1 h at room temperature, then subjected to isoelectric focusing in 24

well, 12.5 % w/v polyacrylamide gel strips (5188-6424, Agilent Technologies, Massy, Paris, France) using an OFFGEL fractionating system (Agilent 3100 Fractionator, Agilent Technology) for 48 h. Separated samples were analysed by ESI-LC-MS/MS (Agilent 6200 TOF, Agilent Technology) by interrogating the *D. labrax* genome database of Max Planck Institute for Molecular Genetics database (Berlin, Germany) using Spectra Mill software (Agilent Technologies). Fold changes and significance levels were determined using the iQantitator algorithm (Schwacke et al., 2009). The reporter ion peak area measurements supplied by the ABI software are used to estimate treatment-dependent peptide and protein relative expression. Estimation is accomplished using a Bayesian approach with the model given below.

B) Supplementary results

i) Thymus involution assessment

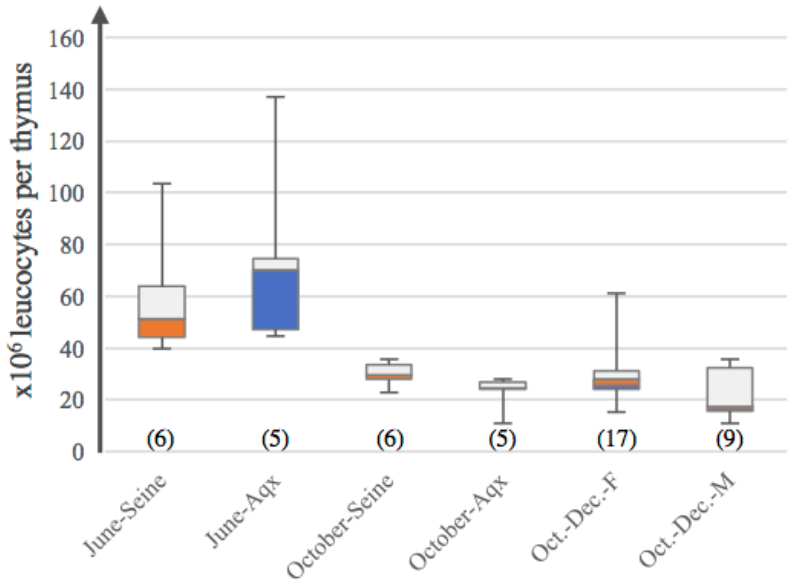


Figure 33: Thymic number of isolated leucocytes per thymus of sea bass with a total length ranging from 20-30 cm following the month of the sampling, the origin of the fish and the gender (See Table 3 for female ratio and Fulton's condition factor of the fish caught in the wild and in the hatchery). The box plots show minimum, maximum values, as well as the third and second quartile. Boxes in orange and blue indicate that the fish were sampled in the wild (Seine estuary) or taken from hatchery (Aqx, Aquacaux), respectively. The fish of 15-20 cm sampled in April-May were mainly obtained from the wild. To detect gender difference the thymic number of leucocytes of female (F) and male (M) fish of 20-30 cm caught from October to December in wild and the hatchery were compared. No significantly different at $p<0.05$ (t-test) could be detected. Numbers in parentheses correspond to biological replicates.

Table 3: Female ratio and Fulton's condition factor of fish with a total length of 20-30 cm which were used to compare the number of isolated leucocyte between the fish coming from the wild (Seine estuary) and the hatchery. ± standard error. ***, significantly different at $p<0.001$ (*t*-test).

	June-Seine	June-Aqx	October-Seine	October-Aqx
Female ratio	66.7 %	60.0%	66.7 %	80.0%
Fulton's condition factor (g/cm³)	1.01 % ± 0.08	1.11 %*** ± 0.07	1.00 % ± 0.04	1.25 %*** ± 0.06

ii) Project: E2-effect on the pituitary gland proteome

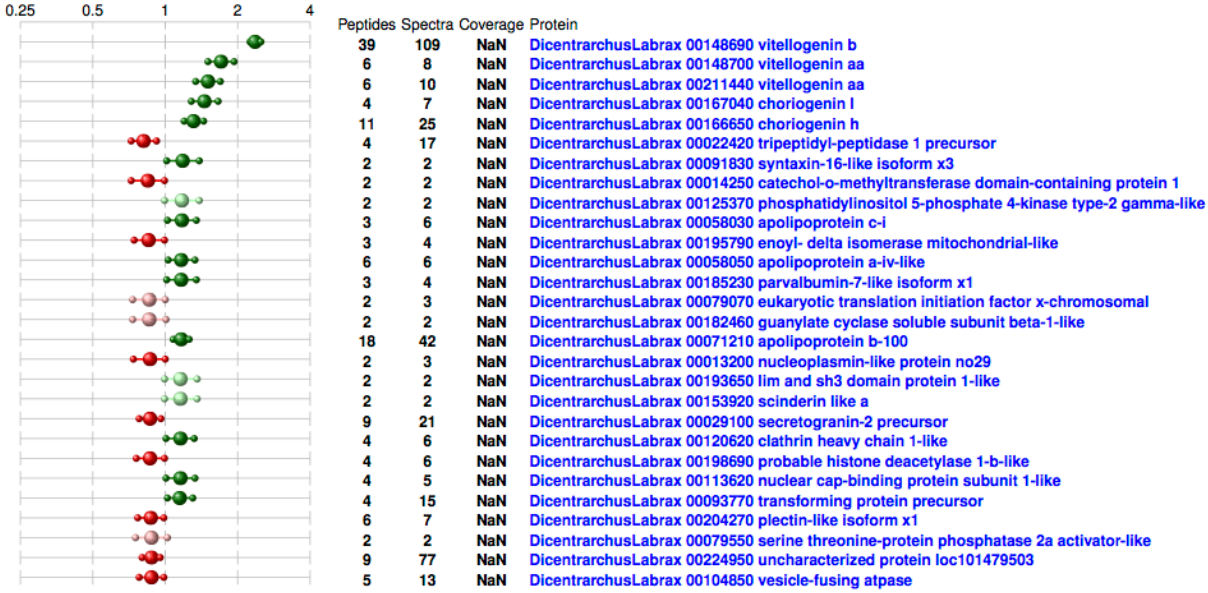
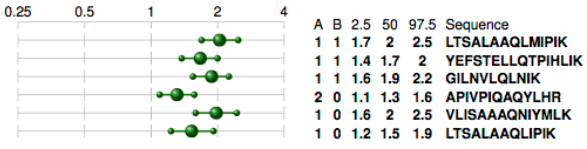


Figure 34: Example of the identified 3550 proteins listed in decreasing order of expression magnitude change

5.3 DicentrarchusLabrax 00211440 vitellogenin aa

Protein Accession	211440
Mean Expression Ratio	1.5
Median Expression Ratio	1.5
Credible Interval	(1.33, 1.69)
Associated Peptides	6
Associated Spectra	10
Coverage	NaN



5.4 DicentrarchusLabrax 00167040 choriogenin l

Protein Accession	167040
Mean Expression Ratio	1.46
Median Expression Ratio	1.46
Credible Interval	(1.28, 1.66)
Associated Peptides	4
Associated Spectra	7
Coverage	NaN

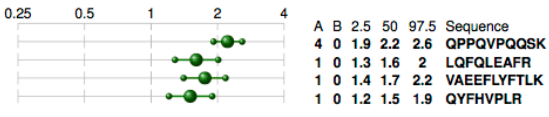


Figure 35: Examples of the detailed summary provided for each protein (Including the peptide relative expression estimates in addition to the protein-level estimates).

7) References

7) References

Abelli, L., Baldassini, M.R., Meschini, R., Mastrolia, L., 1998. Apoptosis of thymocytes in developing sea bass *Dicentrarchus labrax* (L.). Fish Shellfish Immunol. 8, 13–24.

Abi, C., 2014. Population Dynamics of the European Sea Bass (*Dicentrarchus labrax*) in Welsh Water (Msc Thesis). Bangor University.

Abo, T., 2001. Extrathymic pathways of T-cell differentiation and immunomodulation. Int. Immunopharmacol. 1, 1261–1273. https://doi.org/10.1016/S1567-5769(01)00057-1

Abramson, J., Anderson, G., 2017. Thymic Epithelial Cells. Annu. Rev. Immunol. 35, 85–118. https://doi.org/10.1146/annurev-immunol-051116-052320

Adeel, M., Song, X., Wang, Y., Francis, D., Yang, Y., 2017. Environmental impact of estrogens on human, animal and plant life: A critical review. Environ. Int. 99, 107–119. https://doi.org/10.1016/j.envint.2016.12.010

Ádori, M., Kiss, E., Barad, Z., Barabás, K., Kiszely, E., Schneider, A., Sziksz, E., Ábrahám, I.M., Matkó, J., Sármay, G., 2010. Estrogen augments the T cell-dependent but not the T-independent immune response. Cell. Mol. Life Sci. 67, 1661–1674. https://doi.org/10.1007/s00018-010-0270-5

Adurthi, S., Kumar, M.M., Vinodkumar, H.S., Mukherjee, G., Krishnamurthy, H., Acharya, K.K., Bafna, U.D., Uma, D.K., Abhishekh, B., Krishna, S., Parchure, A., Alka, M., Jayshree, R.S., 2017. Oestrogen Receptor-α binds the FOXP3 promoter and modulates regulatory T-cell function in human cervical cancer. Sci. Rep. 7, 17289. https://doi.org/10.1038/s41598-017-17102-w

Álvarez, F., Razquin, B.E., Villena, A.J., Zapata, A.G., 1998. Seasonal changes in the lymphoid organs of wild brown trout, Salmo trutta L: a morphometrical study. Vet. Immunol. Immunopathol. 64, 267–278.

Andersen, K.G., Nissen, J.K., Betz, A.G., 2012. Comparative Genomics Reveals Key Gain-of-Function Events in Foxp3 during Regulatory T Cell Evolution. Front. Immunol. 3. https://doi.org/10.3389/fimmu.2012.00113

Anderson, M.S., Su, M.A., 2016. AIRE expands: new roles in immune tolerance and beyond. Nat. Rev. Immunol. 16, 247–258. https://doi.org/10.1038/nri.2016.9

Ankley, G.T., Feifarek, D., Blackwell, B., Cavallin, J.E., Jensen, K.M., Kahl, M.D., Poole, S., Randolph, E., Saari, T., Villeneuve, D.L., 2017. Re-evaluating the Significance of Estrone as an Environmental Estrogen. Environ. Sci. Technol. 51, 4705–4713. https://doi.org/10.1021/acs.est.7b00606

Aris, A.Z., Shamsuddin, A.S., Praveena, S.M., 2014. Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. Environ. Int. 69, 104–119. https://doi.org/10.1016/j.envint.2014.04.011

Audiger, C., Rahman, M.J., Yun, T.J., Tarbell, K.V., Lesage, S., 2017. The Importance of Dendritic Cells in Maintaining Immune Tolerance. J. Immunol. 198, 2223–2231. https://doi.org/10.4049/jimmunol.1601629

Avilés-Trigueros, M., Quesada, J.A., 1995. Presence of interdigitating cells in thymus of the sea bass, *Dicentrarchus labrax* L. (Teleost). J. Morphol. 224, 199–203. https://doi.org/10.1002/jmor.1052240208

Aw, D., Palmer, D.B., 2012. It's not all equal: a multiphasic theory of thymic involution. Biogerontology 13, 77–81. https://doi.org/10.1007/s10522-011-9349-0

Azenabor, A.A., Hoffman-Goetz, L., 2001. 17β-Estradiol Increases Ca2+ Influx and Down Regulates Interleukin-2 Receptor in Mouse Thymocytes. Biochem. Biophys. Res. Commun. 281, 277–281. https://doi.org/10.1006/bbrc.2001.4341

Bado-Nilles, A., Techer, R., Porcher, J.M., Geffard, A., Gagnaire, B., Betoulle, S., Sanchez, W., 2014. Detection of immunotoxic effects of estrogenic and androgenic endocrine disrupting compounds using splenic immune cells of the female three-spined stickleback, Gasterosteus aculeatus (L.). Environ. Toxicol. Pharmacol. 38, 672–683. https://doi.org/10.1016/j.etap.2014.08.002

Bai, M., Doukas, M., Papoudou-Bai, A., Barbouti, A., Stefanaki, K., Galani, V., Kanavaros, P., 2013. Immunohistological analysis of cell cycle and apoptosis regulators in thymus. Ann. Anat. - Anat. Anz. 195, 159–165. https://doi.org/10.1016/j.aanat.2012.07.012

Bailey, C., Segner, H., Casanova-Nakayama, A., Wahli, T., 2017. Who needs the hotspot? The effect of temperature on the fish host immune response to Tetracapsuloides bryosalmonae the causative agent of proliferative kidney disease. Fish Shellfish Immunol. 63, 424–437. https://doi.org/10.1016/j.fsi.2017.02.039

Bajoghli, B., 2013. Evolution and function of chemokine receptors in the immune system of lower vertebrates. Eur. J. Immunol. 43, 1686–1692. https://doi.org/10.1002/eji.201343557

Bajoghli, B., Aghaallaei, N., Hess, I., Rode, I., Netuschil, N., Tay, B.-H., Venkatesh, B., Yu, J.-K., Kaltenbach, S.L., Holland, N.D., Diekhoff, D., Happe, C., Schorpp, M., Boehm, T., 2009. Evolution of Genetic Networks Underlying the Emergence of Thymopoiesis in Vertebrates. Cell 138, 186–197. https://doi.org/10.1016/j.cell.2009.04.017

Bajoghli, B., Kuri, P., Inoue, D., Aghaallaei, N., Hanelt, M., Thumberger, T., Rauzi, M., Wittbrodt, J., Leptin, M., 2015. Noninvasive In Toto Imaging of the Thymus Reveals Heterogeneous Migratory Behavior of Developing T Cells. J. Immunol. 195, 2177–2186. https://doi.org/10.4049/jimmunol.1500361

Baker, M.E., 2011. Insights from the structure of estrogen receptor into the evolution of estrogens: Implications for endocrine disruption. Biochem. Pharmacol. 82, 1–8. https://doi.org/10.1016/j.bcp.2011.03.008

Baker, M.E., Nelson, D.R., Studer, R.A., 2015. Origin of the response to adrenal and sex steroids: Roles of promiscuity and co-evolution of enzymes and steroid receptors. J. Steroid Biochem. Mol. Biol. 151, 12–24. https://doi.org/10.1016/j.jsbmb.2014.10.020

Barakat, R., Oakley, O., Kim, H., Jin, J., Ko, C.J., 2016. Extra-gonadal sites of estrogen biosynthesis and function. BMB Rep. 49, 488–496. https://doi.org/10.5483/BMBRep.2016.49.9.141

Barbarossa, A., Gazzotti, T., Farabegoli, F., Mancini, F.R., Zironi, E., Badiani, A., Busani, L., Pagliuca, G., 2016. Comparison of perfluoroalkyl substances contamination in farmed and wild-caught European sea bass (*Dicentrarchus labrax*). Food Control 63, 224–229. https://doi.org/10.1016/j.foodcont.2015.12.011

Barton, M., Prossnitz, E.R., 2015. Emerging roles of GPER in diabetes and atherosclerosis. Trends Endocrinol. Metab. 26, 185–192. https://doi.org/10.1016/j.tem.2015.02.003

Bassity, E., Clark, T.G., 2012. Functional Identification of Dendritic Cells in the Teleost Model, Rainbow Trout (Oncorhynchus mykiss). PLoS ONE 7, e33196. https://doi.org/10.1371/journal.pone.0033196

Basten, A., Silveira, P.A., 2010. B-cell tolerance: mechanisms and implications. Curr. Opin. Immunol. 22, 566–574. https://doi.org/10.1016/j.coi.2010.08.001

Belikov, A.V., Schraven, B., Simeoni, L., 2015. T cells and reactive oxygen species. J. Biomed. Sci. 22. https://doi.org/10.1186/s12929-015-0194-3

Bellamri, M., Le Hegarat, L., Vernhet, L., Baffet, G., Turesky, R.J., Langouët, S., 2016. Human T lymphocytes bioactivate heterocyclic aromatic amines by forming DNA adducts. Environ. Mol. Mutagen. 57, 656–667. https://doi.org/10.1002/em.22059

Ben, W., Zhu, B., Yuan, X., Zhang, Y., Yang, M., Qiang, Z., 2017. Transformation and fate of natural estrogens and their conjugates in wastewater treatment plants: Influence of operational parameters and removal pathways. Water Res. 124, 244–250. https://doi.org/10.1016/j.watres.2017.07.065

Benten, W.P.M., Lieberherr, M., Giese, G., Wunderlich, F., 1998. Estradiol binding to cell surface raises cytosolic free calcium in T cells. FEBS Lett. 422, 349–353. https://doi.org/10.1016/S0014-5793(98)00039-8

Beraud, C., van der Molen, J., Armstrong, M., Hunter, E., Fonseca, L., Hyder, K., 2017. The influence of oceanographic conditions and larval behaviour on settlement success—the European sea bass *Dicentrarchus labrax* (L.). ICES J. Mar. Sci. https://doi.org/10.1093/icesjms/fsx195

Bergman, Å., World Health Organization, United Nations Environment Programme, 2012. State of the science of endocrine disrupting chemicals - 2012: an assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme and World Health Organization. WHO ; UNEP, Genève; Nairobi.

Bernardi, A.I., Andersson, A., Grahnemo, L., Nurkkala-Karlsson, M., Ohlsson, C., Carlsten, H., Islander, U., 2014. Effects of lasofoxifene and bazedoxifene on B cell development and function. Immun. Inflamm. Dis. 2, 214–225. https://doi.org/10.1002/iid3.37

Bernardi, A.I., Andersson, A., Stubelius, A., Grahnemo, L., Carlsten, H., Islander, U., 2015. Selective estrogen receptor modulators in T cell development and T cell dependent inflammation. Immunobiology 220, 1122–1128. https://doi.org/10.1016/j.imbio.2015.05.009

Blázquez, M., González, A., Papadaki, M., Mylonas, C., Piferrer, F., 2008. Sex-related changes in estrogen receptors and aromatase gene expression and enzymatic activity during early development and sex differentiation in the European sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 158, 95–101. https://doi.org/10.1016/j.ygcen.2008.06.001

Bly, J.E., Clem, L.W., 1992. Temperature and teleost immune functions. Fish Shellfish Immunol. 2, 159–171. https://doi.org/10.1016/S1050-4648(05)80056-7

Boehm, T., Bleul, C.C., 2007. The evolutionary history of lymphoid organs. Nat. Immunol. 8, 131–135. https://doi.org/10.1038/ni1435

Boehm, T., Hess, I., Swann, J.B., 2012. Evolution of lymphoid tissues. Trends Immunol., Special focus: Structure and function of lymphoid tissues 33, 315–321. https://doi.org/10.1016/j.it.2012.02.005

Boehm, T., Swann, J.B., 2014. Origin and Evolution of Adaptive Immunity. Annu. Rev. Anim. Biosci. 2, 259–283. https://doi.org/10.1146/annurev-animal-022513-114201

Bondesson, M., Hao, R., Lin, C.-Y., Williams, C., Gustafsson, J.-Å., 2015. Estrogen receptor signaling during vertebrate development. Biochim. Biophys. Acta BBA - Gene Regul. Mech. 1849, 142–151. https://doi.org/10.1016/j.bbagrm.2014.06.005

Boschi, I., Randelli, E., Buonocore, F., Casani, D., Bernini, C., Fausto, A.M., Scapigliati, G., 2011. Transcription of T cell-related genes in teleost fish, and the European sea bass (*Dicentrarchus labrax*) as a model. Fish Shellfish Immunol. 31, 655–662. https://doi.org/10.1016/j.fsi.2010.10.001

Bouillet, P., O'Reilly, L.A., 2009. CD95, BIM and T cell homeostasis. Nat. Rev. Immunol. 9, 514–519. https://doi.org/10.1038/nri2570

Bowden, T.J., 2008. Modulation of the immune system of fish by their environment. Fish Shellfish Immunol. 25, 373–383. https://doi.org/10.1016/j.fsi.2008.03.017

Bowden, T.J., Cook, P., Rombout, J.H.W.M., 2005. Development and function of the thymus in teleosts. Fish Shellfish Immunol., Fish Larval immunity 19, 413–427. https://doi.org/10.1016/j.fsi.2005.02.003

Bowden, T.J., Thompson, K.D., Morgan, A.L., Gratacap, R.M.L., Nikoskelainen, S., 2007. Seasonal variation and the immune response: A fish perspective. Fish Shellfish Immunol. 22, 695–706. https://doi.org/10.1016/j.fsi.2006.08.016

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Brown, N., Nagarkatti, M., Nagarkatti, P., 2006a. Induction of Apoptosis in Murine Fetal Thymocytes Following Perinatal Exposure to Diethylstilbestrol. Int. J. Toxicol. 25, 9–15. https://doi.org/10.1080/10915810500488353

Brown, N., Nagarkatti, M., Nagarkatti, P.S., 2006b. Diethylstilbestrol alters positive and negative selection of T cells in the thymus and modulates T-cell repertoire in the periphery. Toxicol. Appl. Pharmacol. 212, 119–126. https://doi.org/10.1016/j.taap.2005.07.012

Brunsing, R.L., Owens, K.S., Prossnitz, E.R., 2013. The G protein-coupled estrogen receptor (GPER) agonist G-1 expands the regulatory T cell population under T (H) 17 polarizing conditions. J. Immunother. Hagerstown Md 1997 36, 190.

Budzinski, H., Forget-Leray, J., Ait-Aissa, S., 2012. Etude de la contamination par les médicaments de l'estuaire de la Seine. Application des capteurs passifs à un meilleur diagnostic de leur présence, flux et impact toxique. (Rapport Seine-Aval 4).

Buono, M., Facchini, R., Matsuoka, S., Thongjuea, S., Waithe, D., Luis, T.C., Giustacchini, A., Besmer, P., Mead, A.J., Jacobsen, S.E.W., Nerlov, C., 2016. A dynamic niche provides Kit ligand in a stage-specific manner to the earliest thymocyte progenitors. Nat. Cell Biol. 18, 157–167. https://doi.org/10.1038/ncb3299

Buonocore, F., Stocchi, V., Nunez-Ortiz, N., Randelli, E., Gerdol, M., Pallavicini, A., Facchiano, A., Bernini, C., Guerra, L., Scapigliati, G., Picchietti, S., 2017. Immunoglobulin T from sea bass (*Dicentrarchus labrax* L.): molecular characterization, tissue localization and expression after nodavirus infection. BMC Mol. Biol. 18. https://doi.org/10.1186/s12867-017-0085-0

Burgos-Aceves, M.A., Cohen, A., Smith, Y., Faggio, C., 2016. Estrogen regulation of gene expression in the teleost fish immune system. Fish Shellfish Immunol. 58, 42–49. https://doi.org/10.1016/j.fsi.2016.09.006

Cabas, I., Rodenas, M.C., Abellan, E., Meseguer, J., Mulero, V., Garcia-Ayala, A., 2013. Estrogen Signaling through the G Protein-Coupled Estrogen Receptor Regulates Granulocyte Activation in Fish. J. Immunol. 191, 4628–4639. https://doi.org/10.4049/jimmunol.1301613

Calemine, J.B., Gogal Jr, R.M., Lengi, A., Sponenberg, P., Ahmed, S.A., 2002. Immunomodulation by diethylstilbestrol is dose and gender related: effects on thymocyte apoptosis and mitogen-induced proliferation. Toxicology 178, 101–118. https://doi.org/10.1016/S0300-483X(02)00201-9

Cardamone, C., Parente, R., Feo, G.D., Triggiani, M., 2016. Mast cells as effector cells of innate immunity and regulators of adaptive immunity. Immunol. Lett., ECI 2015 178, 10–14. https://doi.org/10.1016/j.imlet.2016.07.003

Cerdá, J., Zanuy, S., Carrillo, M., Ramos, J., Serrano, R., 1995. Short- and long-term dietary effects on female sea bass (*Dicentrarchus labrax*): seasonal changes in plasma profiles of lipids and sex steroids in relation to reproduction. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 111, 83–91. https://doi.org/10.1016/0742-8413(95)00018-3

Chakrabarti, S., Morton, J.S., Davidge, S.T., 2014. Mechanisms of Estrogen Effects on the Endothelium: An Overview. Can. J. Cardiol. 30, 705–712. https://doi.org/10.1016/j.cjca.2013.08.006

Chaouat, G., 2016. Reconsidering the Medawar paradigm placental viviparity existed for eons, even in vertebrates; without a "problem": Why are Tregs important for preeclampsia in great apes? J. Reprod. Immunol. 114, 48–57. https://doi.org/10.1016/j.jri.2015.09.002

Chapman, J.C., Chapman, F.M., Michael, S.D., 2015. The production of alpha/beta and gamma/delta double negative (DN) T-cells and their role in the maintenance of pregnancy. Reprod. Biol. Endocrinol. 13. https://doi.org/10.1186/s12958-015-0073-5

Chapman, J.C., Min, S., Freeh, S.M., Michael, S.D., 2009. The estrogen-injected female mouse: new insight into the etiology of PCOS. Reprod. Biol. Endocrinol. 7, 47. https://doi.org/10.1186/1477-7827-7-47

Chaudhry, M.S., Velardi, E., Dudakov, J.A., van den Brink, M.R.M., 2016. Thymus: the next (re)generation. Immunol. Rev. 271, 56–71. https://doi.org/10.1111/imr.12418

Cheroutre, H., Lambolez, F., Mucida, D., 2011. The light and dark sides of intestinal intraepithelial lymphocytes. Nat. Rev. Immunol. 11, 445–456. https://doi.org/10.1038/nri3007

Chien, Y., Meyer, C., Bonneville, M., 2014. γδT Cells: First Line of Defense and Beyond. Annu. Rev. Immunol. 32, 121–155. https://doi.org/10.1146/annurev-immunol-032713-120216

Chilmonczyk, S., 1992. The thymus in fish: development and possible function in the immune response. Annu. Rev. Fish Dis. 2, 181–200.

Claridge, P.N., Potter, I.C., 1983. Movements, abundance, age composition and growth of bass, *Dicentrarchus labrax*, in the Severn Estuary and inner Bristol Channel. J. Mar. Biol. Assoc. U. K. 63, 871–879.

Clark, D.A., 2016. The importance of being a regulatory T cell in pregnancy. J. Reprod. Immunol. 116, 60–69. https://doi.org/10.1016/j.jri.2016.04.288

Cockburn, A., 1992. Evolutionary Ecology of the Immune System: Why Does the Thymus Involute? Funct. Ecol. 6, 364–370.

Cooke, P.S., Nanjappa, M.K., Ko, C., Prins, G.S., Hess, R.A., 2017. Estrogens in Male Physiology. Physiol. Rev. 97, 995–1043. https://doi.org/10.1152/physrev.00018.2016

Cordero, H., Brinchmann, M.F., Cuesta, A., Meseguer, J., Esteban, M.A., 2015. Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). Proteomics 15, 4007–4020. https://doi.org/10.1002/pmic.201500120

Cowan, J.E., Jenkinson, W.E., Anderson, G., 2015. Thymus medulla fosters generation of natural Treg cells, invariant γδ T cells, and invariant NKT cells: What we learn from intrathymic migration. Eur. J. Immunol. 45, 652–660. https://doi.org/10.1002/eji.201445108

Daley, S.R., Teh, C., Hu, D.Y., Strasser, A., Gray, D.H.D., 2017. Cell death and thymic tolerance. Immunol. Rev. 277, 9–20. https://doi.org/10.1111/imr.12532

Danel, L., Souweine, G., Monier, J.C., Saez, S., 1983. Specific estrogen binding sites in human lymphoid cells and thymic cells. J. Steroid Biochem. 18, 559–563. https://doi.org/10.1016/0022-4731(83)90131-0

Dee, C.T., Nagaraju, R.T., Athanasiadis, E.I., Gray, C., Fernandez del Ama, L., Johnston, S.A., Secombes, C.J., Cvejic, A., Hurlstone, A.F.L., 2016. CD4-Transgenic Zebrafish Reveal Tissue-Resident Th2- and Regulatory T Cell-like Populations and Diverse Mononuclear Phagocytes. J. Immunol. https://doi.org/10.4049/jimmunol.1600959

del Pozo, A., Falcon, J., Sanchez-Vazquez, F.J., 2014. The Biological Clock and Dualism, in: Biology of European Sea Bass. CRC Press, pp. 34–56.

Devi, K.S.P., Anandasabapathy, N., 2016. The origin of DCs and capacity for immunologic tolerance in central and peripheral tissues. Semin. Immunopathol. https://doi.org/10.1007/s00281-016-0602-0

DeWitt, J.C., Keil, D.E., 2017. Current Issues in Developmental Immunotoxicity, in: Immunopathology in Toxicology and Drug Development, Molecular and Integrative Toxicology. Humana Press, Cham, pp. 601–618. https://doi.org/10.1007/978-3-319-47377-2_13

DeWitt, J.C., Peden-Adams, M.M., Keil, D.E., Dietert, R.R., 2012. Current Status of Developmental Immunotoxicity: Early-Life Patterns and Testing. Toxicol. Pathol. 40, 230–236. https://doi.org/10.1177/0192623311427709

Do, Y., Ryu, S., Nagarkatti, M., Nagarkatti, P.S., 2002a. Role of Death Receptor Pathway in Estradiol-Induced T-Cell Apoptosis in Vivo. Toxicol. Sci. 70, 63–72. https://doi.org/10.1093/toxsci/70.1.63

Dong, M., Seemann, F., Humble, J.L., Liang, Y., Peterson, D.R., Ye, R., Ren, H., Kim, H.-S., Lee, J.-S., Au, D.W.T., Lam, Y.W., 2017. Modification of the plasma complement protein profile by exogenous estrogens is indicative of a compromised immune competence in marine medaka (Oryzias melastigma). Fish Shellfish Immunol. https://doi.org/10.1016/j.fsi.2017.09.020

Doyle, T.K., Haberlin, D., Clohessy, J., Bennison, A., Jessopp, M., 2017. Localised residency and inter-annual fidelity to coastal foraging areas may place sea bass at risk to local depletion. Sci. Rep. 8, 45841. https://doi.org/10.1038/srep45841

Dragin, N., Bismuth, J., Cizeron-Clairac, G., Biferi, M.G., Berthault, C., Serraf, A., Nottin, R., Klatzmann, D., Cumano, A., Barkats, M., Le Panse, R., Berrih-Aknin, S., 2016. Estrogen-mediated downregulation of AIRE influences sexual dimorphism in autoimmune diseases. J. Clin. Invest. 126, 1525–1537. https://doi.org/10.1172/JCI81894

Dragin, N., Nancy, P., Villegas, J., Roussin, R., Panse, R.L., Berrih-Aknin, S., 2017. Balance between Estrogens and Proinflammatory Cytokines Regulates Chemokine Production Involved in Thymic Germinal Center Formation. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-08631-5

Drayton, D.L., Liao, S., Mounzer, R.H., Ruddle, N.H., 2006. Lymphoid organ development: from ontogeny to neogenesis. Nat. Immunol. 7, ni1330. https://doi.org/10.1038/ni1330

Eick, G.N., Thornton, J.W., 2011. Evolution of steroid receptors from an estrogen-sensitive ancestral receptor. Mol. Cell. Endocrinol., Evolution of Nuclear Hormone Receptors 334, 31–38. https://doi.org/10.1016/j.mce.2010.09.003

Elnwishy, N., Sedky, N., 2016. E2, an Aquatic Hazard Worldwide. J. Agric. Sci. 8, 147. https://doi.org/10.5539/jas.v8n10p147

Erlandsson, M.C., Ohlsson, C., Gustafsson, J.-A., Carlsten, H., 2001a. Role of oestrogen receptors α and β in immune organ development and in oestrogen-mediated effects on thymus. Immunology 103, 17–25. https://doi.org/10.1046/j.1365-2567.2001.01212.x

Esteban, M.Á., Cuesta, A., Chaves-Pozo, E., Meseguer, J., 2015. Phagocytosis in Teleosts. Implications of the New Cells Involved. Biology 4, 907–922. https://doi.org/10.3390/biology4040907

Finger, J.W., Gogal, R.M., 2013. Endocrine-Disrupting Chemical Exposure and the American Alligator: A Review of the Potential Role of Environmental Estrogens on the Immune System of a Top Trophic Carnivore. Arch. Environ. Contam. Toxicol. 65, 704–714. https://doi.org/10.1007/s00244-013-9953-x

Food and Agriculture Organization of the United Nations, 2016. The State of World Fisheries and Aquaculture 2016 (SOFIA).

Forsberg, J.-G., 2000. Neonatal estrogen treatment and its consequences for thymus development, serum level of autoantibodies to cardiolipin, and the delayed-type hypersensitivity response. J. Toxicol. Environ. Health A 60, 185–213. https://doi.org/10.1080/009841000156484

Forsberg, J.-G., 1996. The Different Responses of the Female Mouse Thymus to Estrogen after Treatment of Neonatal, Prepubertal, and Adult Animals. Cells Tissues Organs 157, 275–290. https://doi.org/10.1159/000147890

Forsberg, J.-G., 1984. Short-term and long-term effects of estrogen on lymphoid tissues and lymphoid cells with some remarks on the significance for carcinogenesis. Arch. Toxicol. 55, 79–90. https://doi.org/10.1007/BF00346044

Fujikado, N., Mann, A.O., Bansal, K., Romito, K.R., Ferre, E.M.N., Rosenzweig, S.D., Lionakis, M.S., Benoist, C., Mathis, D., 2016. Aire Inhibits the Generation of a Perinatal Population of Interleukin-17A-Producing γδ T Cells to Promote Immunologic Tolerance. Immunity 45, 999–1012. https://doi.org/10.1016/j.immuni.2016.10.023

Galindo-Villegas, J., Garcia-Garcia, E., Mulero, V., 2016. Role of histamine in the regulation of intestinal immunity in fish. Dev. Comp. Immunol., Special Issue: Intestinal Immunity 64, 178–186. https://doi.org/10.1016/j.dci.2016.02.013

Galindo-Villegasa, J., Mulero, V., 2015. Current Knowledge on the Development and Functionality of Immune Responses in the European Sea Bass.

Gameiro, J., Nagib, P., Verinaud, L., 2010. The thymus microenvironment in regulating thymocyte differentiation. Cell Adhes. Migr. 4, 382–390. https://doi.org/10.4161/cam.4.3.11789

Gascoigne, N.R.J., Rybakin, V., Acuto, O., Brzostek, J., 2016. TCR Signal Strength and T Cell Development. Annu. Rev. Cell Dev. Biol. 32, 327–348. https://doi.org/10.1146/annurev-cellbio-111315-125324

Gaspéri, J., Moreau-Guigon, E., Labadie, P., Blanchard, M., Teil, M.J., Tlili, K., Dinh, T.Q., Tran, C., Alliot, F., Desportes, A., others, 2010. Contamination de la Seine par les micropolluants organiques: évolution selon les conditions hydriques et l'urbanisation. Rapp. Act. PIREN-Seine 2010.

Gerondakis, S., Fulford, T.S., Messina, N.L., Grumont, R.J., 2013. NF-κB control of T cell development. Nat. Immunol. 15, 15–25. https://doi.org/10.1038/ni.2785

Giusti, R.M., Iwamoto, K., Hatch, E.E., 1995. Diethylstilbestrol Revisited: A Review of the Long-Term Health Effects. Ann. Intern. Med. 122, 778–788. https://doi.org/10.7326/0003-4819-122-10-199505150-00008

Glucksmann, A., Cherry, C.P., 1968. The effect of castration, oestrogens, testosterone and the oestrous cycle on the cortical epithelium of the thymus in male and female rats. J. Anat. 103, 113–133.

Görlach, A., Bertram, K., Hudecova, S., Krizanova, O., 2015. Calcium and ROS: A mutual interplay. Redox Biol. 6, 260–271. https://doi.org/10.1016/j.redox.2015.08.010

Gould, K.A., Shull, J.D., Gorski, J., 2000. DES action in the thymus: inhibition of cell proliferation and genetic variation. Mol. Cell. Endocrinol. 170, 31–39. https://doi.org/10.1016/S0303-7207(00)00336-1

Granja, A.G., Leal, E., Pignatelli, J., Castro, R., Abos, B., Kato, G., Fischer, U., Tafalla, C., 2015. Identification of Teleost Skin CD8 + Dendritic-like Cells, Representing a Potential Common Ancestor for Mammalian Cross-Presenting Dendritic Cells. J. Immunol. 195, 1825–1837. https://doi.org/10.4049/jimmunol.1500322

Greene, G.L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., Shine, J., 1986. Sequence and expression of human estrogen receptor complementary DNA. Science 231, 1150–1154. https://doi.org/10.1126/science.3753802

Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.-F., 2010. Ovarian aromatase and estrogens: A pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol. 165, 352–366. https://doi.org/10.1016/j.ygcen.2009.03.002

Gulino, A., Screpanti, I., Torrisi, M.R., Frati, L., 1985. Estrogen Receptors and Estrogen Sensitivity of Fetal Thymocytes Are Restricted to Blast Lymphoid Cells*. Endocrinology 117, 47–54. https://doi.org/10.1210/endo-117-1-47

Halkias, J., Melichar, H.J., Taylor, K.T., Robey, E.A., 2014. Tracking migration during human T cell development. Cell. Mol. Life Sci. 71, 3101–3117. https://doi.org/10.1007/s00018-014-1607-2

Hareramadas, B., Rai, U., 2006. Cellular mechanism of estrogen-induced thymic involution in wall lizard: caspase-dependent action. J. Exp. Zoolog. A Comp. Exp. Biol. 305A, 396–409. https://doi.org/10.1002/jez.a.260

Havixbeck, J., Barreda, D., 2015. Neutrophil Development, Migration, and Function in Teleost Fish. Biology 4, 715–734. https://doi.org/10.3390/biology4040715

Hayday, A.C., 2000. γδ cells: a right time and a right place for a conserved third way of protection. Annu. Rev. Immunol. 18, 975–1026. https://doi.org/10.1146/annurev.immunol.18.1.975

Hedi, H., Norbert, G., 2004. 5-Lipoxygenase Pathway, Dendritic Cells, and Adaptive Immunity. J. Biomed. Biotechnol. 2004, 99–105. https://doi.org/10.1155/S1110724304310041

Hess, I., Boehm, T., 2012. Intravital Imaging of Thymopoiesis Reveals Dynamic Lympho-Epithelial Interactions. Immunity 36, 298–309. https://doi.org/10.1016/j.immuni.2011.12.016

Hildeman, D.A., Mitchell, T., Kappler, J., Marrack, P., 2003. T cell apoptosis and reactive oxygen species. J. Clin. Invest. 111, 575–581. https://doi.org/10.1172/JCI18007

Hince, M., Sakkal, S., Vlahos, K., Dudakov, J., Boyd, R., Chidgey, A., 2008. The role of sex steroids and gonadectomy in the control of thymic involution. Cell. Immunol. 252, 122–138. https://doi.org/10.1016/j.cellimm.2007.10.007

Hogquist, K.A., Jameson, S.C., 2014. The self-obsession of T cells: how TCR signaling thresholds affect fate "decisions" and effector function. Nat. Immunol. 15, ni.2938. https://doi.org/10.1038/ni.2938

Holladay, S.D., Blaylock, B.L., Comment, C.E., Heindel, J.J., Fox, W.M., Korach, K.S., Luster, M.I., 1993. Selective prothymocyte targeting by prenatal diethylstilbesterol exposure. Cell. Immunol. 152, 131–142. https://doi.org/10.1006/cimm.1993.1273

Honma, Y., Tamura, E., 1984. Histological changes in the lymphoid system of fish with respect to age, seasonal and endocrine changes. Dev Comp Immunol 3, 239–244.

Hui, S.P., Sheng, D.Z., Sugimoto, K., Gonzalez-Rajal, A., Nakagawa, S., Hesselson, D., Kikuchi, K., 2017. Zebrafish Regulatory T Cells Mediate Organ-Specific Regenerative Programs. Dev. Cell 43, 659–672.e5. https://doi.org/10.1016/j.devcel.2017.11.010

Huttenhuis, H.B.T., Romano, N., Oosterhoud, C.N.V., Taverne-Thiele, A.J., Mastrolia, L., Muiswinkel, W.B.V., Rombout, J.H.W.M., 2006. The ontogeny of mucosal immune cells in common carp (Cyprinus carpio L.). Anat. Embryol. (Berl.) 211, 19–29. https://doi.org/10.1007/s00429-005-0062-0

ICES, 2015. ICES Advice on fishing opportunities, catch, and effort Celtic Seas and Greater North Sea Ecoregions (No. 5.3.43).

ICES, 2012. Advice Report European seabass in the Northeast Atlantic (No. 9.4.23).

Isensee, J., Meoli, L., Zazzu, V., Nabzdyk, C., Witt, H., Soewarto, D., Effertz, K., Fuchs, H., Gailus-Durner, V., Busch, D., Adler, T., de Angelis, M.H., Irgang, M., Otto, C., Noppinger, P.R., 2009. Expression Pattern of G Protein-Coupled Receptor 30 in LacZ Reporter Mice. Endocrinology 150, 1722–1730. https://doi.org/10.1210/en.2008-1488

Islander, U., Erlandsson, M.C., Hasséus, B., Jonsson, C.A., Ohlsson, C., Gustafsson, J.-\AA, Dahlgren, U., Carlsten, H., 2003. Influence of oestrogen receptor α and β on the immune system in aged female mice. Immunology 110, 149–157.

Iwanowicz, L.R., Blazer, V.S., 2011. An overview of estrogen-associated endocrine disruption in fishes: evidence of effects on reproductive and immune physiology 266–275.

Iwanowicz, L.R., Stafford, J.L., Patiño, R., Bengten, E., Miller, N.W., Blazer, V.S., 2014. Channel catfish (Ictalurus punctatus) leukocytes express estrogen receptor isoforms ERα and ERβ2 and are functionally modulated by estrogens. Fish Shellfish Immunol. 40, 109–119. https://doi.org/10.1016/j.fsi.2014.06.021

Jessen, J.R., Willett, C.E., Lin, S., 1999. Artificial chromosome transgenesis reveals long-distance negative regulation of rag1 in zebrafish. Nat. Genet. 23, 15–16.

Jia, C., Zhou, Y., Huang, X., Peng, X., Liu, L., Zhou, L., Jin, L., Shi, H., Wei, J., Wang, D., 2015. The cellular protein expression of Foxp3 in lymphoid and non-lymphoid organs of Nile tilapia. Fish Shellfish Immunol. 45, 300–306. https://doi.org/10.1016/j.fsi.2015.03.021

Jochmanova, I., Lazurova, Z., Rudnay, M., Ba ova, I., Marekova, M., Lazurova, I., 2015. Environmental estrogen bisphenol A and autoimmunity. Lupus 24, 392–399. https://doi.org/10.1177/0961203314560205

Kabelitz, D., Peters, C., Wesch, D., Oberg, H.-H., 2013. Regulatory functions of γδ T cells. Int. Immunopharmacol. 16, 382–387. https://doi.org/10.1016/j.intimp.2013.01.022

Katayama, M., Fukuda, T., Narabara, K., Abe, A., Kondo, Y., 2012. Localization of Estrogen Receptor in the Central Lymphoid Organs of Chickens during the Late Stage of Embryogenesis. Biosci. Biotechnol. Biochem. 76, 2003–2007. https://doi.org/10.1271/bbb.120182

Kawashima, I., Sakabe, K., Akatsuka, A., Seiki, K., 1995. Effects of estrogen on female mouse thymus, with special reference to ER-mRNA and T cell subpopulations. Pathophysiology 2, 235–241. https://doi.org/10.1016/0928-4680(95)00047-2

Kelley, D.F., 1988. The importance of estuaries for sea-bass, *Dicentrarchus labrax* (L.). J. Fish Biol. 33, 25–33.

Khan, D., Ansar Ahmed, S., 2016. The Immune System Is a Natural Target for Estrogen Action: Opposing Effects of Estrogen in Two Prototypical Autoimmune Diseases. Front. Immunol. 6. https://doi.org/10.3389/fimmu.2015.00635

Khan, D., Cowan, C., Ahmed, S.A., 2012. Estrogen and signaling in the cells of immune system. Adv. Neuroimmune Biol. 3, 73–93.

Kimura, M., Hanawa, H., Watanabe, H., Ogawa, M., Abo, T., 1995. Synchronous Expansion of Intermediate TCR Cells in the Liver and Uterus during Pregnancy. Cell. Immunol. 162, 16–25. https://doi.org/10.1006/cimm.1995.1046

Klein, L., Kyewski, B., Allen, P.M., Hogquist, K.A., 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat. Rev. Immunol. 14, 377–391. https://doi.org/10.1038/nri3667

Klein, S.L., Flanagan, K.L., 2016. Sex differences in immune responses. Nat. Rev. Immunol. https://doi.org/10.1038/nri.2016.90

Kondo, Y., Goto, C., Abe, A., 2004. Effects of estrogen treatment during the embryogenic period on chick antibody production. J. Poult. Sci. 41, 85–93.

Kondo, Y., Oka, H., Abe, A., Tanabe, A., 1994. Dose-related Effects of. Beta-estradiol on Chicken T Cell Activity. J. pult. sci. 31, 327–334. https://doi.org/10.2141/jpsa.31.327

Kovats, S., 2015. Estrogen receptors regulate innate immune cells and signaling pathways. Cell. Immunol. 294, 63–69. https://doi.org/10.1016/j.cellimm.2015.01.018

Kuiper, G.G., Carlsson, B.O., Grandien, K.A.J., Enmark, E., Haggblad, J., Nilsson, S., Gustafsson, J., 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138, 863–870.

Laffont, S., Seillet, C., Guéry, J.-C., 2017. Estrogen Receptor-Dependent Regulation of Dendritic Cell Development and Function. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.00108

Lafont, A.-G., Rousseau, K., Tomkiewicz, J., Dufour, S., 2016. Three nuclear and two membrane estrogen receptors in basal teleosts, Anguilla sp.: Identification, evolutionary history and differential expression regulation. Gen. Comp. Endocrinol. 235, 177–191. https://doi.org/10.1016/j.ygcen.2015.11.021

Lam, S. h., Chua, H. l., Gong, Z., Wen, Z., Lam, T. j., Sin, Y. m., 2002. Morphologic transformation of the thymus in developing zebrafish. Dev. Dyn. 225, 87–94. https://doi.org/10.1002/dvdy.10127

Lam, S.H., Chua, H.L., Gong, Z., Lam, T.J., Sin, Y.M., 2004. Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. Dev. Comp. Immunol. 28, 9–28. https://doi.org/10.1016/S0145-305X(03)00103-4

Lambolez, F., Arcangeli, M.-L., Joret, A.-M., Pasqualetto, V., Cordier, C., Di Santo, J.P., Rocha, B., Ezine, S., 2006. The thymus exports long-lived fully committed T cell precursors that can colonize primary lymphoid organs. Nat. Immunol. 7, 76. https://doi.org/10.1038/ni1293

Lange, I.G., Hartel, A., Meyer, H.H., 2002. Evolution of oestrogen functions in vertebrates. J. Steroid Biochem. Mol. Biol. 83, 219–226.

Langenau, D.M., Zon, L.I., 2005. The zebrafish: a new model of T-cell and thymic development. Nat. Rev. Immunol. 5, 307–317. https://doi.org/10.1038/nri1590

Laverty, G., Skadhauge, E., 2012. Adaptation of teleosts to very high salinity. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 163, 1–6. https://doi.org/10.1016/j.cbpa.2012.05.203

Leceta, J., Fernandez-Galaz, C., Sanchez, C., Navarro, R., Gomariz, R.P., Zapata, A., Tejero, A., 1988. Effects of neonatal treatment with estrogens on the development of the thymus in rats. Dev. Comp. Immunol. 12, 375–383.

Leitman, D.C., Paruthiyil, S., Vivar, O.I., Saunier, E.F., Herber, C.B., Cohen, I., Tagliaferri, M., Speed, T.P., 2010. Regulation of specific target genes and biological responses by estrogen receptor subtype agonists. Curr. Opin. Pharmacol. 10, 629–636. https://doi.org/10.1016/j.coph.2010.09.009

Lewis, K.L., Del Cid, N., Traver, D., 2014. Perspectives on antigen presenting cells in zebrafish. Dev. Comp. Immunol., Zebrafish Immunity and Infection Models 46, 63–73. https://doi.org/10.1016/j.dci.2014.03.010

Li, C.-L., Toda, K., Saibara, T., Zhang, T., Ono, M., Iwasaki, S., Maeda, T., Okada, T., Hayashi, Y., Enzan, H., Shizuta, Y., Onishi, S., 2002. Estrogen deficiency results in enhanced expression of Smoothened of the Hedgehog signaling in the thymus and affects thymocyte development. Int. Immunopharmacol. 2, 823–833. https://doi.org/10.1016/S1567-5769(02)00023-1

Li, J., Barreda, D.R., Zhang, Y.-A., Boshra, H., Gelman, A.E., LaPatra, S., Tort, L., Sunyer, J.O., 2006. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. Nat. Immunol. 7, 1116–1124. https://doi.org/10.1038/ni1389

Li, M.O., Rudensky, A.Y., 2016. T cell receptor signalling in the control of regulatory T cell differentiation and function. Nat. Rev. Immunol. 16, 220–233. https://doi.org/10.1038/nri.2016.26

Liarte, S., Chaves-Pozo, E., Abellán, E., Meseguer, J., Mulero, V., García-Ayala, A., 2011. 17β-Estradiol regulates gilthead seabream professional phagocyte responses through macrophage activation. Dev. Comp. Immunol. 35, 19–27. https://doi.org/10.1016/j.dci.2010.07.007

Litman, G.W., Rast, J.P., Fugmann, S.D., 2010. The origins of vertebrate adaptive immunity. Nat. Rev. Immunol. 10, 543–553. https://doi.org/10.1038/nri2807

Liu, Z., Kanjo, Y., Mizutani, S., 2009. Urinary excretion rates of natural estrogens and androgens from humans, and their occurrence and fate in the environment: A review. Sci. Total Environ. 407, 4975–4985. https://doi.org/10.1016/j.scitotenv.2009.06.001

Liu, Z., Lu, G., Yin, H., Dang, Z., Rittmann, B., 2015. Removal of Natural Estrogens and Their Conjugates in Municipal Wastewater Treatment Plants: A Critical Review. Environ. Sci. Technol. 49, 5288–5300. https://doi.org/10.1021/acs.est.5b00399

Loizeau, V., Abarnou, A., Ménesguen, A., 2001. A steady-state model of PCB bioaccumulation in the sea bass (*Dicentrarchus labrax*) food web from the Seine estuary, France. Estuaries 24, 1074–1087.

Lugo-Villarino, G., Balla, K.M., Stachura, D.L., Banuelos, K., Werneck, M.B.F., Traver, D., 2010. Identification of dendritic antigen-presenting cells in the zebrafish. Proc. Natl. Acad. Sci. 107, 15850–15855. https://doi.org/10.1073/pnas.1000494107

Lutton, B., Callard, I., 2006. Evolution of reproductive–immune interactions. Integr. Comp. Biol. 46, 1060–1071. https://doi.org/10.1093/icb/icl050

Macian, F., 2005. NFAT proteins: key regulators of T-cell development and function. Nat. Rev. Immunol. 5, 472–484. https://doi.org/10.1038/nri1632

MacLatchy, D., Palace, V.P., Evans, R.E., Wautier, K.G., Mills, K.H., Blanchfield, P.J., Park, B.J., Baron, C.L., Kidd, K.A., 2009. Interspecies differences in biochemical, histopathological, and population responses in four wild fish species exposed to ethynylestradiol added to a whole lakeThis paper is part of the series "Forty Years of Aquatic Research at the Experimental Lakes Area". Can. J. Fish. Aquat. Sci. 66, 1920–1935. https://doi.org/10.1139/F09-125

Maggiano, N., Piantelli, M., Ricci, R., Larocca, L.M., Capelli, A., Ranelletti, F.O., 1994. Detection of growth hormone-producing cells in human thymus by immunohistochemistry and non-radioactive in situ hybridization. J. Histochem. Cytochem. 42, 1349–1354.

Maggioli, E., McArthur, S., Mauro, C., Kieswich, J., Kusters, D.H.M., Reutelingsperger, C.P.M., Yaqoob, M., Solito, E., 2016. Estrogen protects the blood–brain barrier from inflammation-induced disruption and increased lymphocyte trafficking. Brain. Behav. Immun. 51, 212–222. https://doi.org/10.1016/j.bbi.2015.08.020

Magnadóttir, B., 2006. Innate immunity of fish (overview). Fish Shellfish Immunol., Reviews in Fish ImmunologyReviews in Fish Immunology 20, 137–151. https://doi.org/10.1016/j.fsi.2004.09.006

Makrinos, D.L., Bowden, T.J., 2016. Natural environmental impacts on teleost immune function. Fish Shellfish Immunol., Special Issue: ISFSI 2016 53, 50–57. https://doi.org/10.1016/j.fsi.2016.03.008

Mañanós, E.L., Zanuy, S., Carrillo, M., 1997. Photoperiodic manipulations of the reproductive cycle of sea bass (*Dicentrarchus labrax*) and their effects on gonadal development, and plasma 17β-estradiol and vitellogenin levels. Fish Physiol. Biochem. 16, 211–222.

Manning, M.J., Nakanishi, T., 1996. The specific immune system: cellular defenses, in: The Fish Immune System: Organism, Pathogen, and Environment, Academic Press, Inc. pp. 159–205.

Martín, A., Alonso, L.M., Del Moral, M.G., Zapata, A.G., 1994a. Ultrastructural changes in the adult rat thymus after estradiol benzoate treatment. Tissue Cell 26, 169–179. https://doi.org/10.1016/0040-8166(94)90092-2

Martin, A., Alonso, L.M., Gomez del Moral, M., Zapata, A.G., 1994b. Morphometrical changes in the rat thymic lymphoid cells after treatment with two different doses of estradiol benzoate.

Martín, A., Casares, F., Alonso, L., Nieuwenhuis, P., Vicente, A., Zapata, A.G., 1995a. Changes in the Blood-Thymus Barrier of Adult Rats after Estradiol-Treatment. Immunobiology 192, 231–248. https://doi.org/10.1016/S0171-2985(11)80100-2

Martín, A., Vicente, A., Torroba, M., Moreno, C., Jiménez, E., Zapata, A., 1995b. Increased numbers of CD5+ B cells in the thymus of estradiol benzoate-treated rats. Thymus 24, 111–127.

Massart, S., Milla, S., Kestemont, P., 2014. Expression of gene, protein and immunohistochemical localization of the estrogen receptor isoform ERα1 in male rainbow trout lymphoid organs; indication of the role of estrogens in the regulation of immune mechanisms. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 174, 53–61. https://doi.org/10.1016/j.cbpb.2014.06.001

Matthews, J., Gustafsson, J.-A., 2003. Estrogen Signaling: A Subtle Balance Between ER and ER. Mol. Interv. 3, 281–292. https://doi.org/10.1124/mi.3.5.281

McMurray, R.W., Suwannaroj, S., Ndebele, K., Jenkins, J.K., 2001. Differential Effects of Sex Steroids on T and B Cells: Modulation of Cell Cycle Phase Distribution, Apoptosis and bcl-2 Protein Levels. Pathobiology 69, 44–58. https://doi.org/10.1159/000048757

Menuet, A., Page, Y.L., Torres, O., Kern, L., Kah, O., Pakdel, F., 2004. Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. J. Mol. Endocrinol. 32, 975–986. https://doi.org/10.1677/jme.0.0320975

Milla, S., Depiereux, S., Kestemont, P., 2011. The effects of estrogenic and androgenic endocrine disruptors on the immune system of fish: a review. Ecotoxicology 20, 305–319. https://doi.org/10.1007/s10646-010-0588-7

Minier, C., Abarnou, A., Jaouen-Madoulet, A., Le Guellec, A.-M., Tutundjian, R., Bocquené, G., Leboulenger, F., 2006. A pollution-monitoring pilot study involving contaminant and biomarker measurements in the Seine Estuary, France, using zebra mussels (Dreissena polymorpha). Environ. Toxicol. Chem. 25, 112–119. https://doi.org/10.1897/05-161R.1

Mo, R., Chen, J., Grolleau-Julius, A., Murphy, H.S., Richardson, B.C., Yung, R.L., 2005. Estrogen Regulates CCR Gene Expression and Function in T Lymphocytes. J. Immunol. 174, 6023–6029. https://doi.org/10.4049/jimmunol.174.10.6023

Mondoon, S., Shibata, K., Yoshikai, Y., 2017. In vivo blockade of T cell development reveals alternative pathways for generation of intraepithelial lymphocytes in mice. Immunol. Lett. 191, 40–46. https://doi.org/10.1016/j.imlet.2017.09.008

Mor, G., Munoz, A., Redlinger JR, R., Silva, I., Song, J., Lim, C., Kohen, F., 2001. The role of the Fas/Fas ligand system in estrogen-induced thymic alteration. Am. J. Reprod. Immunol. 46, 298–307. https://doi.org/10.1034/j.1600-0897.2001.d01-16.x

Moreno, A.M., Zapata, A.G., 1991. In situ effects of estrogens on the stroma of rat thymus. Lymphat. Tissues Vivo Immune Responses Dekker N. Y. 81–87.

Morin, J., Brind'Amour, A., Fisson, C., Bacq, N., Boulet, M., Guillerme, D., Berthelé, O., Martin, J., 2015. Peuplements halieutiques en estuaire de Seine et baie de Seine orientale – Distribution et évolution des abondances des principales espèces capturées au chalut à perche lors des campagnes scientifiques réalisées par l'Ifremer entre 1995 et 2010. (Rapport d'étude).

Morin, J., Riou, P., Lemoine, M., Le Pape, O., Bessineton, C., Vedieu, C., Simon, S., 1999. Etude des nourriceries de la baie de Seine orientale et de l'estuaire de la Seine. Synthese des connaissances. Identification d'une nourricerie en estuaire de Seine. Analyse de la fonctionnalite de l'estuaire comme nourricerie 1–87.

Mosselman, S., Polman, J., Dijkema, R., 1996. ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett. 392, 49–53. https://doi.org/10.1016/0014-5793(96)00782-X

Mulero, I., Sepulcre, M.P., Meseguer, J., García-Ayala, A., Mulero, V., 2007. Histamine is stored in mast cells of most evolutionarily advanced fish and regulates the fish inflammatory response. Proc. Natl. Acad. Sci. 104, 19434–19439.

Muñoz-Ruiz, M., Sumaria, N., Pennington, D.J., Silva-Santos, B., 2017. Thymic Determinants of γδ T Cell Differentiation. Trends Immunol. https://doi.org/10.1016/j.it.2017.01.007

Nagler, J.J., Cavileer, T., Sullivan, J., Cyr, D.G., Rexroad III, C., 2007. The complete nuclear estrogen receptor family in the rainbow trout: Discovery of the novel ERα2 and both ERβ isoforms. Gene 392, 164–173. https://doi.org/10.1016/j.gene.2006.12.030

Nakanishi, T., 1986. Seasonal changes in the humoral immune response and the lymphoid tissues of the marine teleost, Sebastiscus marmoratus. Vet. Immunol. Immunopathol., Fish Immunology 12, 213–221. https://doi.org/10.1016/0165-2427(86)90125-X

Nakanishi, T., Shibasaki, Y., Matsuura, Y., 2015. T Cells in Fish. Biology 4, 640–663. https://doi.org/10.3390/biology4040640

Nancy, P., Berrih-Aknin, S., 2005. Differential estrogen receptor expression in autoimmune myasthenia gravis. Endocrinology 146, 2345–2353.

Narita, J., Miyaji, C., Watanabe, H., Honda, S., Koya, T., Umezu, H., Ushiki, T., Sugahara, S., Kawamura, T., Arakawa, M., others, 1998. Differentiation of forbidden T cell clones and granulocytes in the parenchymal space of the liver in mice treated with estrogen. Cell. Immunol. 185, 1–13.

Narita, S., Goldblum, R.M., Watson, C.S., Brooks, E.G., Estes, D.M., Curran, E.M., Midoro-Horiuti, T., 2006. Environmental Estrogens Induce Mast Cell Degranulation and Enhance IgE-Mediated Release of Allergic Mediators. Environ. Health Perspect. 115, 48–52. https://doi.org/10.1289/ehp.9378

Navas, J.M., Mañanós, E., Thrush, M., Ramos, J., Zanuy, S., Carrillo, M., Zohar, Y., Bromage, N., 1998. Effect of dietary lipid composition on vitellogenin, 17β-estradiol and gonadotropin plasma levels and spawning performance in captive sea bass (*Dicentrarchus labrax* L.). Aquaculture 165, 65–79. https://doi.org/10.1016/S0044-8486(98)00246-4

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol., 7th International Symposium on Fish Endocrinology 192, 15–24. https://doi.org/10.1016/j.ygcen.2013.03.032

Nelson, L.R., Bulun, S.E., 2001. Estrogen production and action. J. Am. Acad. Dermatol. 45, S116–S124. https://doi.org/10.1067/mjd.2001.117432

Noppe, H., Le Bizec, B., Verheyden, K., De Brabander, H.F., 2008. Novel analytical methods for the determination of steroid hormones in edible matrices. Anal. Chim. Acta 611, 1–16. https://doi.org/10.1016/j.aca.2008.01.066

Nuñez Ortiz, N., Gerdol, M., Stocchi, V., Marozzi, C., Randelli, E., Bernini, C., Buonocore, F., Picchietti, S., Papeschi, C., Sood, N., Pallavicini, A., Scapigliati, G., 2014. T cell transcripts and T cell activities in the gills of the teleost fish sea bass (*Dicentrarchus labrax*). Dev. Comp. Immunol. 47, 309–318. https://doi.org/10.1016/j.dci.2014.07.015

Offner, H., Adlard, K., Zamora, A., Vandenbark, A.A., 2000. Estrogen potentiates treatment with T-cell receptor protein of female mice with experimental encephalomyelitis. J. Clin. Invest. 105, 1465–1472.

Oh-hora, M., Rao, A., 2008. Calcium signaling in lymphocytes. Curr. Opin. Immunol. 20, 250–258. https://doi.org/10.1016/j.coi.2008.04.004

Okasha, S.A., Ryu, S., Do, Y., McKallip, R.J., Nagarkatti, M., Nagarkatti, P.S., 2001. Evidence for estradiol-induced apoptosis and dysregulated T cell maturation in the thymus. Toxicology 163, 49–62. https://doi.org/10.1016/S0300-483X(01)00374-2

Okuyama, R., Abo, T., Seki, S., Ohteki, T., Sugiura, K., Kusumi, A., Kumagai, K., 1992. Estrogen administration activates extrathymic T cell differentiation in the liver. J. Exp. Med. 175, 661–669.

Olde, B., Leeb-Lundberg, L.M.F., 2009. GPR30/GPER1: searching for a role in estrogen physiology. Trends Endocrinol. Metab. 20, 409–416. https://doi.org/10.1016/j.tem.2009.04.006

Öner, H., Ozan, E., 2002. Effects of gonadal hormones on thymus gland after bilateral ovariectomy and orchidectomy in rats. Arch. Androl. 48, 115–126. https://doi.org/10.1080/014850102317267427

Ottaviani, E., Malagoli, D., Capri, M., Franceschi, C., 2008. Ecoimmunology: is there any room for the neuroendocrine system? BioEssays 30, 868–874. https://doi.org/10.1002/bies.20801

Ouyang, W., Kolls, J.K., Zheng, Y., 2008. The Biological Functions of T Helper 17 Cell Effector Cytokines in Inflammation. Immunity 28, 454–467. https://doi.org/10.1016/j.immuni.2008.03.004

Parra, D., Korytar, T., Takizawa, F., Sunyer, J.O., 2016. B cells and their role in the teleost gut. Dev. Comp. Immunol. 64, 150–166. https://doi.org/10.1016/j.dci.2016.03.013

Parra, D., Takizawa, F., Sunyer, J.O., 2013. Evolution of B Cell Immunity. Annu. Rev. Anim. Biosci. 1, 65–97. https://doi.org/10.1146/annurev-animal-031412-103651

Pascoli, F., Lanzano, G.S., Negrato, E., Poltronieri, C., Trocino, A., Radaelli, G., Bertotto, D., 2011. Seasonal effects on hematological and innate immune parameters in sea bass *Dicentrarchus labrax*. Fish Shellfish Immunol. 31, 1081–1087. https://doi.org/10.1016/j.fsi.2011.09.014

Pasman, L., Kasper, D.L., 2017. Building conventions for unconventional lymphocytes. Immunol. Rev. 279, 52–62. https://doi.org/10.1111/imr.12576

Paulsen, M., Janssen, O., 2011. Pro- and anti-apoptotic CD95 signaling in T cells. Cell Commun. Signal. 9, 7. https://doi.org/10.1186/1478-811X-9-7

Pawson, M.G., Pickett, G.D., 1996. The annual pattern of condition and maturity in bass, *Dicentrarchus labrax*, in waters around England and Wales. J. Mar. Biol. Assoc. U. K. 76, 107–125.

Pawson, M.G., Pickett, G.D., Kelley, D.F., 1987. The distribution and migrations of bass, *Dicentrarchus labrax* L., in waters around England and Wales as shown by tagging. J. Mar. Biol. Assoc. U. K. 67, 183–217.

Pawson, M.G., Pickett, G.D., Leballeur, J., Brown, M., Fritsch, M., 2007. Migrations, fishery interactions, and management units of sea bass (*Dicentrarchus labrax*) in Northwest Europe. ICES J. Mar. Sci. 64, 332–345.

Peel, E., Belov, K., 2017. Immune-endocrine interactions in marsupials and monotremes. Gen. Comp. Endocrinol. 244, 178–185. https://doi.org/10.1016/j.ygcen.2017.01.026

Peluso, I., Morabito, G., Riondino, S., La Farina, F., Serafini, M., 2012. Lymphocytes as internal standard in oxidative burst analysis by cytometry: A new data analysis approach. J. Immunol. Methods 379, 61–65. https://doi.org/10.1016/j.jim.2012.02.015

Pérez-Ruzafa, A., Concepción, M., 2015. Ecology and Distribution of *Dicentrarchus labrax* (Linnaeus 1758), in: Biology of European Sea Bass. pp. 3–33.

Phalen, L.J., Köllner, B., Hogan, N.S., van den Heuvel, M.R., 2017. Transcriptional response in rainbow trout (Oncorhynchus mykiss) B cells and thrombocytes following in vivo exposure to benzo[a]pyrene. Environ. Toxicol. Pharmacol. 53, 212–218. https://doi.org/10.1016/j.etap.2017.06.002

Picchietti, S., Abelli, L., Guerra, L., Randelli, E., Proietti Serafini, F., Belardinelli, M.C., Buonocore, F., Bernini, C., Fausto, A.M., Scapigliati, G., 2015. MHC II-β chain gene expression studies define the regional organization of the thymus in the developing bony fish *Dicentrarchus labrax* (L.). Fish Shellfish Immunol. 42, 483–493. https://doi.org/10.1016/j.fsi.2014.11.012

Picchietti, S., Guerra, L., Bertoni, F., Randelli, E., Belardinelli, M.C., Buonocore, F., Fausto, A.M., Rombout, J.H., Scapigliati, G., Abelli, L., 2011. Intestinal T cells of *Dicentrarchus labrax* (L.): Gene expression and functional studies. Fish Shellfish Immunol. 30, 609–617. https://doi.org/10.1016/j.fsi.2010.12.006

Picchietti, S., Guerra, L., Buonocore, F., Randelli, E., Fausto, A.M., Abelli, L., 2009. Lymphocyte differentiation in sea bass thymus: CD4 and CD8-α gene expression studies. Fish Shellfish Immunol. 27, 50–56. https://doi.org/10.1016/j.fsi.2009.04.003

Picchietti, S., Guerra, L., Selleri, L., Buonocore, F., Abelli, L., Scapigliati, G., Mazzini, M., Fausto, A.M., 2008. Compartmentalisation of T cells expressing CD8α and TCRβ in developing thymus of sea bass *Dicentrarchus labrax* (L.). Dev. Comp. Immunol. 32, 92–99. https://doi.org/10.1016/j.dci.2007.04.002

Picchietti, S., Nuñez-Ortiz, N., Stocchi, V., Randelli, E., Buonocore, F., Guerra, L., Scapigliati, G., 2017. Evolution of lymphocytes. Immunoglobulin T of the teleost sea bass (*Dicentrarchus labrax*): Quantitation of gene expressing and immunoreactive cells. Fish Shellfish Immunol. 63, 40–52. https://doi.org/10.1016/j.fsi.2017.02.002

Piferrer, F., Blázquez, M., 2005. Aromatase distribution and regulation in fish. Fish Physiol. Biochem. 31, 215–226. https://doi.org/10.1007/s10695-006-0027-0

Pinto, C., Grimaldi, M., Boulahtouf, A., Pakdel, F., Brion, F., Aït-Aïssa, S., Cavaillès, V., Bourguet, W., Gustafsson, J.-A., Bondesson, M., Balaguer, P., 2014. Selectivity of natural, synthetic and environmental estrogens for zebrafish estrogen receptors. Toxicol. Appl. Pharmacol. 280, 60–69. https://doi.org/10.1016/j.taap.2014.07.020

Pinto, P., Estêvão, M., Power, D., 2014. Effects of Estrogens and Estrogenic Disrupting Compounds on Fish Mineralized Tissues. Mar. Drugs 12, 4474–4494. https://doi.org/10.3390/md12084474

Plitas, G., Rudensky, A.Y., 2016. Regulatory T Cells: Differentiation and Function. Cancer Immunol. Res. 4, 721–725. https://doi.org/10.1158/2326-6066.CIR-16-0193

Polanczyk, M.J., Carson, B.D., Subramanian, S., Afentoulis, M., Vandenbark, A.A., Ziegler, S.F., Offner, H., 2004. Cutting Edge: Estrogen Drives Expansion of the CD4+CD25+ Regulatory T Cell Compartment. J. Immunol. 173, 2227–2230. https://doi.org/10.4049/jimmunol.173.4.2227

Polanczyk, M.J., Hopke, C., Huan, J., Vandenbark, A.A., Offner, H., 2005. Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. J. Neuroimmunol. 170, 85–92. https://doi.org/10.1016/j.jneuroim.2005.08.023

Prieto, G.A., Rosenstein, Y., 2006. Oestradiol potentiates the suppressive function of human CD4+ CD25+ regulatory T cells by promoting their proliferation. Immunology 118, 58–65. https://doi.org/10.1111/j.1365-2567.2006.02339.x

Priyanka, H.P., Krishnan, H.C., Singh, R.V., Hima, L., ThyagaRajan, S., 2013. Estrogen modulates in vitro T cell responses in a concentration- and receptor-dependent manner: Effects on intracellular molecular targets and antioxidant enzymes. Mol. Immunol. 56, 328–339. https://doi.org/10.1016/j.molimm.2013.05.226

Prossnitz, E.R., Barton, M., 2014. Estrogen biology: New insights into GPER function and clinical opportunities. Mol. Cell. Endocrinol. 389, 71–83. https://doi.org/10.1016/j.mce.2014.02.002

Prykhozhij, S.V., Berman, J.N., 2014. The progress and promise of zebrafish as a model to study mast cells. Dev. Comp. Immunol. 46, 74–83. https://doi.org/10.1016/j.dci.2014.01.023

Qin, B., Dong, L., Guo, X., Jiang, J., He, Y., Wang, X., Li, L., Zhao, J., 2014. Expression of G protein-coupled estrogen receptor in irritable bowel syndrome and its clinical significance. Int. J. Clin. Exp. Pathol. 7, 2238.

Quintana, F.J., Iglesias, A.H., Farez, M.F., Caccamo, M., Burns, E.J., Kassam, N., Oukka, M., Weiner, H.L., 2010. Adaptive Autoimmunity and Foxp3-Based Immunoregulation in Zebrafish. PLoS ONE 5, e9478. https://doi.org/10.1371/journal.pone.0009478

Raica, M., Encică, S., Motoc, A., Cîmpean, A.M., Scridon, T., Bârsan, M., 2006. Structural heterogeneity and immunohistochemical profile of Hassall corpuscles in normal human thymus. Ann. Anat. - Anat. Anz. 188, 345–352. https://doi.org/10.1016/j.aanat.2006.01.012

Rathore, J.S., Wang, Y., 2016. Protective role of Th17 cells in pulmonary infection. Vaccine 34, 1504–1514. https://doi.org/10.1016/j.vaccine.2016.02.021

Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms. Immunol. Lett. 148, 23–33. https://doi.org/10.1016/j.imlet.2012.08.003

Ren, W., Liu, G., Chen, S., Yin, J., Wang, J., Tan, B., Wu, G., Bazer, F.W., Peng, Y., Li, T., Reiter, R.J., Yin, Y., 2017. Melatonin signaling in T cells: Functions and applications. J. Pineal Res. 62, n/a-n/a. https://doi.org/10.1111/jpi.12394

Rezzani, R., Nardo, L., Favero, G., Peroni, M., Rodella, L.F., 2013. Thymus and aging: morphological, radiological, and functional overview. AGE 36, 313–351. https://doi.org/10.1007/s11357-013-9564-5

Riera Romo, M., Pérez-Martínez, D., Castillo Ferrer, C., 2016. Innate immunity in vertebrates: an overview. Immunology 148, 125–139. https://doi.org/10.1111/imm.12597

Rijhsinghani, A.G., Bhatia, S.K., Tygrett, L.T., Waldschmidt, T.J., 1996a. Effect of pregnancy on thymic T cell development. Am. J. Reprod. Immunol. 35, 523–528.

Rijhsinghani, A.G., Thompson, K., Bhatia, S.K., Waldschmidt, T.J., 1996b. Estrogen blocks early T cell development in the thymus. Am. J. Reprod. Immunol. N. Y. N 1989 36, 269–277. https://doi.org/10.1111/j.1600-0897.1996.tb00176.x

Roberts, N.A., White, A.J., Jenkinson, W.E., Turchinovich, G., Nakamura, K., Withers, D.R., McConnell, F.M., Desanti, G.E., Benezech, C., Parnell, S.M., Cunningham, A.F., Paolino, M., Penninger, J.M., Simon, A.K., Nitta, T., Ohigashi, I., Takahama, Y., Caamano, J.H., Hayday, A.C., Lane, P.J.L., Jenkinson, E.J., Anderson, G., 2012. Rank Signaling Links the Development of Invariant γδ T Cell Progenitors and Aire+ Medullary Epithelium. Immunity 36, 427–437. https://doi.org/10.1016/j.immuni.2012.01.016

Rodenas, M.C., Cabas, I., Abellán, E., Meseguer, J., Mulero, V., García-Ayala, A., 2015. Tamoxifen persistently disrupts the humoral adaptive immune response of gilthead seabream (Sparus aurata L.). Dev. Comp. Immunol. 53, 283–292. https://doi.org/10.1016/j.dci.2015.06.014

Rodenas, M.C., Cabas, I., García-Alcázar, A., Meseguer, J., Mulero, V., García-Ayala, A., 2016. Selective estrogen receptor modulators differentially alter the immune response of gilthead seabream juveniles. Fish Shellfish Immunol. 52, 189–197. https://doi.org/10.1016/j.fsi.2016.03.041

Rodenas, M.C., Cabas, I., Gómez-González, N.E., Arizcun, M., Meseguer, J., Mulero, V., García-Ayala, A., 2017a. Estrogens Promote the Production of Natural Neutralizing Antibodies in Fish through G Protein-Coupled Estrogen Receptor 1. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.00736

Rodenas, M.C., Tamassia, N., Cabas, I., Calzetti, F., Meseguer, J., Cassatella, M.A., García-Ayala, A., Mulero, V., 2017b. G Protein-Coupled Estrogen Receptor 1 Regulates Human Neutrophil Functions. Biomed. Hub 2, 2–2. https://doi.org/10.1159/000454981

Romano, N., Caccia, E., Piergentili, R., Rossi, F., Ficca, A.G., Ceccariglia, S., Mastrolia, L., 2011. Antigen-dependent T lymphocytes (TcRβ+) are primarily differentiated in the thymus rather than in other lymphoid tissues in sea bass (*Dicentrarchus labrax*, L.). Fish Shellfish Immunol. 30, 773–782. https://doi.org/10.1016/j.fsi.2010.12.032

Romano, N., Fanelli, M., Del Papa, G., Scapigliati, G., Mastrolia, L., 1999a. Histological and cytological studies on the developing thymus of sharpsnout seabream, Diplodus puntazzo. J. Anat. 194, 39–50.

Romano, N., Rossi, F., Abelli, L., Caccia, E., Piergentili, R., Mastrolia, L., Randelli, E., Buonocore, F., 2007. Majority of TcRβ+ T-lymphocytes located in thymus and midgut of the bony fish, *Dicentrarchus labrax* (L.). Cell Tissue Res. 329, 479–489. https://doi.org/10.1007/s00441-007-0429-z

Romano, N., Scapigliati, G., Abelli, L., 2017. Water Oxygen Content Affects Distribution of T and B Lymphocytes in Lymphoid Tissues of Farmed Sea Bass (*Dicentrarchus Labrax*). Fishes 2, 16. https://doi.org/10.3390/fishes2030016

Romano, N., Taverne-Thiele, A.J., Fanelli, M., Baldassini, M.R., Abelli, L., Mastrolia, L., Van Muiswinkel, W.B., Rombout, J.H.M.W., 1999b. Ontogeny of the thymus in a teleost fish, Cyprinus carpio L.: developing thymocytes in the epithelial microenvironment. Dev. Comp. Immunol. 23, 123–137. https://doi.org/10.1016/S0145-305X(98)00053-6

Romano, R., Palamaro, L., Fusco, A., Giardino, G., Gallo, V., Del Vecchio, L., Pignata, C., 2013. FOXN1: A Master Regulator Gene of Thymic Epithelial Development Program. Front. Immunol. 4. https://doi.org/10.3389/fimmu.2013.00187

Romano, S.N., Gorelick, D.A., 2017. Crosstalk between nuclear and G protein-coupled estrogen receptors. Gen. Comp. Endocrinol. https://doi.org/10.1016/j.ygcen.2017.04.013

Ross, M.A., Korenchevsky, V., 1941. The thymus of the rat and sex hormones. J. Pathol. Bacteriol. 52, 349–360.

Roved, J., Westerdahl, H., Hasselquist, D., 2017. Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. Horm. Behav. 88, 95–105. https://doi.org/10.1016/j.yhbeh.2016.11.017

Ruscher, R., Kummer, R.L., Lee, Y.J., Jameson, S.C., Hogquist, K.A., 2017. CD8αα intraepithelial lymphocytes arise from two main thymic precursors. Nat. Immunol. advance online publication. https://doi.org/10.1038/ni.3751

Russler-Germain, E.V., Rengarajan, S., Hsieh, C.-S., 2017. Antigen-specific regulatory T-cell responses to intestinal microbiota. Mucosal Immunol. https://doi.org/10.1038/mi.2017.65

Ryan, M.R., Shepherd, R., Leavey, J.K., Gao, Y., Grassi, F., Schnell, F.J., Qian, W.-P., Kersh, G.J., Weitzmann, M.N., Pacifici, R., 2005. An IL-7-dependent rebound in thymic T cell output contributes to the bone loss induced by estrogen deficiency. Proc. Natl. Acad. Sci. U. S. A. 102, 16735–16740.

Salinas, I., 2015. The Mucosal Immune System of Teleost Fish. Biology 4, 525–539. https://doi.org/10.3390/biology4030525

Saltis, M., Criscitiello, M.F., Ohta, Y., Keefe, M., Trede, N.S., Goitsuka, R., Flajnik, M.F., 2008. Evolutionarily conserved and divergent regions of the Autoimmune Regulator (Aire) gene: a comparative analysis. Immunogenetics 60, 105–114. https://doi.org/10.1007/s00251-007-0268-9

Samavat, H., Kurzer, M.S., 2015. Estrogen metabolism and breast cancer. Cancer Lett. 356, 231–243. https://doi.org/10.1016/j.canlet.2014.04.018

Samstein, R.M., Josefowicz, S.Z., Arvey, A., Treuting, P.M., Rudensky, A.Y., 2012. Extrathymic Generation of Regulatory T Cells in Placental Mammals Mitigates Maternal-Fetal Conflict. Cell 150, 29–38. https://doi.org/10.1016/j.cell.2012.05.031

Samy, T.A., Knöferl, M.W., Zheng, R., Schwacha, M.G., Bland, K.I., Chaudry, I.H., 2001. Divergent immune responses in male and female mice after trauma-hemorrhage: dimorphic alterations in T lymphocyte steroidogenic enzyme activities. Endocrinology 142, 3519–3529.

Savino, W., Dardenne, M., 2000. Neuroendocrine Control of Thymus Physiology. Endocr. Rev. 21, 412–443. https://doi.org/10.1210/edrv.21.4.0402

Savino, W., Dardenne, M., 1984. Thymic hormone-containing cells. VI. Immunohistologic evidence for the simultaneous presence of thymulin, thymopoietin and thymosin α1 in normal and pathological human thymuses. Eur. J. Immunol. 14, 987–991.

Savino, W., Mendes-da-Cruz, D.A., Lepletier, A., Dardenne, M., 2015. Hormonal control of T-cell development in health and disease. Nat. Rev. Endocrinol. https://doi.org/10.1038/nrendo.2015.168

Savino, W., Smaniotto, S., Mendes-da-Cruz, D.A., Dardenne, M., 2012. Growth hormone modulates migration of thymocytes and peripheral T cells: Growth hormone modulates T cell migration. Ann. N. Y. Acad. Sci. 1261, 49–54. https://doi.org/10.1111/j.1749-6632.2012.06637.x

Schmidt, M., Kreutz, M., Loffler, G., Scholmerich, J., Straub, R.H., 2000. Conversion of dehydroepiandrosterone to downstream steroid hormones in macrophages. J. Endocrinol. 164, 161–169. https://doi.org/10.1677/joe.0.1640161

Schneider, A.E., Kárpáti, É., Schuszter, K., Tóth, E.A., Kiss, E., Kulcsár, M., László, G., Matko, J., 2014. A dynamic network of estrogen receptors in murine lymphocytes: fine-tuning the immune response. J. Leukoc. Biol. 96, 857–872. https://doi.org/10.1189/jlb.2A0214-080RR

Schnitzler, J.G., Thomé, J.P., Lepage, M., Das, K., 2011. Organochlorine pesticides, polychlorinated biphenyls and trace elements in wild European sea bass (*Dicentrarchus labrax*) off European estuaries. Sci. Total Environ. 409, 3680–3686. https://doi.org/10.1016/j.scitotenv.2011.06.018

Schwacke, J.H., Hill, E.G., Krug, E.L., Comte-Walters S. and Schey S. K., 2009. iQuantitator: A tool for protein expression inference using iTRAQ . BMC Bioinformatics. 10:342; PMID:19835628. doi:10.1186/1471-2105-10-342

Screpanti, I., Gulino, A., Pasqualini, J.R., 1982. The Fetal Thymus of Guinea Pig as an Estrogen Target Organ*. Endocrinology 111, 1552–1561.

Screpanti, I., Meco, D., Morrone, S., Gulino, A., Mathieson, B.J., Frati, L., 1991. In Vivo modulation of the distribution of thymocyte subsets: Effects of estrogen on the expression of different T cell receptor Vβ gene families in CD4−, CD8− thymocytes. Cell. Immunol. 134, 414–426. https://doi.org/10.1016/0008-8749(91)90314-2

Seemann, F., Knigge, T., Duflot, A., Marie, S., Olivier, S., Minier, C., Monsinjon, T., 2016. Sensitive periods for 17β-estradiol exposure during immune system development in sea bass head kidney: Sensitivity of sea bass head kidney development to E2. J. Appl. Toxicol. 36, 815–826. https://doi.org/10.1002/jat.3215

Seemann, F., Knigge, T., Olivier, S., Monsinjon, T., 2015. Exogenous 17-β-oestradiol (E2) modifies thymus growth and regionalization in European sea bass *Dicentrarchus labrax*. J. Fish Biol. 86, 1186–1198. https://doi.org/10.1111/jfb.12626

Seemann, F., Peterson, D.R., Chiang, M.W.L., Au, D.W.T., 2017. The development of cellular immune defence in marine medaka Oryzias melastigma. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. https://doi.org/10.1016/j.cbpc.2017.03.010

Segner, H., Casanova-Nakayama, A., Kase, R., Tyler, C.R., 2013. Impact of environmental estrogens on fish considering the diversity of estrogen signaling. Gen. Comp. Endocrinol. 191, 190–201. https://doi.org/10.1016/j.ygcen.2013.05.015

Segner, H., Verburg-van Kemenade, B.M.L., Chadzinska, M., 2017. The immunomodulatory role of the hypothalamus-pituitary-gonad axis: Proximate mechanism for reproduction-immune trade offs? Dev. Comp. Immunol. 66, 43–60. https://doi.org/10.1016/j.dci.2016.07.004

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

Selvaraj, P., Pitchappan, R.M., 1985. Effect of oestradiol dipropionate on the immune system of the pigeon, Columba livia. Dev. Comp. Immunol. 9, 669–677. https://doi.org/10.1016/0145-305X(85)90031-X

Selvaraj, V., Bunick, D., Finnigan-Bunick, C., Johnson, R.W., Wang, H., Liu, L., Cooke, P.S., 2005. Gene Expression Profiling of 17β-Estradiol and Genistein Effects on Mouse Thymus. Toxicol. Sci. 87, 97–112. https://doi.org/10.1093/toxsci/kfi219

Seto, K., Hoang, M., Santos, T., Bandyopadhyay, M., Kindy, M.S., Dasgupta, S., 2016. Non-genomic oestrogen receptor signal in B lymphocytes: An approach towards therapeutic interventions for infection,

autoimmunity and cancer. Int. J. Biochem. Cell Biol. 76, 115–118. https://doi.org/10.1016/j.biocel.2016.04.018

Sfacteria, A., Brines, M., Blank, U., 2015. The mast cell plays a central role in the immune system of teleost fish. Mol. Immunol. 63, 3–8. https://doi.org/10.1016/j.molimm.2014.02.007

Shang, N., Sun, X.-F., Hu, W., Wang, Y.-P., Guo, Q.-L., 2008. Molecular cloning and characterization of common carp (*Cyprinus carpio* L.) TCRγ and CD3γ/δ chains. Fish Shellfish Immunol. 24, 412–425. https://doi.org/10.1016/j.fsi.2007.12.007

Shao, T., Zhu, L.-Y., Nie, L., Shi, W., Dong, W.-R., Xiang, L.-X., Shao, J.-Z., 2015. Characterization of surface phenotypic molecules of teleost dendritic cells. Dev. Comp. Immunol. 49, 38–43. https://doi.org/10.1016/j.dci.2014.11.010

Shelley, L.K., Osachoff, H.L., van Aggelen, G.C., Ross, P.S., Kennedy, C.J., 2013. Alteration of immune function endpoints and differential expression of estrogen receptor isoforms in leukocytes from 17β-estradiol exposed rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 180, 24–32. https://doi.org/10.1016/j.ygcen.2012.09.014

Shelley, L.K., Ross, P.S., Kennedy, C.J., 2012. The effects of an in vitro exposure to 17β-estradiol and nonylphenol on rainbow trout (*Oncorhynchus mykiss*) peripheral blood leukocytes. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 155, 440–446. https://doi.org/10.1016/j.cbpc.2011.11.006

Smith, C., Wootton, R.J., 2016. The remarkable reproductive diversity of teleost fishes. Fish Fish. 17, 1208–1215. https://doi.org/10.1111/faf.12116

Spanier, J.A., Nashold, F.E., Mayne, C.G., Nelson, C.D., Hayes, C.E., 2015. Vitamin D and estrogen synergy in Vdr-expressing CD4+ T cells is essential to induce Helios+FoxP3+ T cells and prevent autoimmune demyelinating disease. J. Neuroimmunol. 286, 48–58. https://doi.org/10.1016/j.jneuroim.2015.06.015

Staples, J.E., Fiore, N.C., Frazier, D.E., Gasiewicz, T.A., Silverstone, A.E., 1998. Overexpression of the anti-apoptotic oncogene, bcl-2, in the thymus does not prevent thymic atrophy induced by estradiol or 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmacol. 151, 200–210.

Staples, J.E., Gasiewicz, T.A., Fiore, N.C., Lubahn, D.B., Korach, K.S., Silverstone, A.E., 1999. Estrogen receptor alpha is necessary in thymic development and estradiol-induced thymic alterations. J. Immunol. Baltim. Md 1950 163, 4168–4174.

Steinman, R.M., Banchereau, J., 2007. Taking dendritic cells into medicine. Nature 449, 419–426. https://doi.org/10.1038/nature06175

Straub, R.H., 2007. The Complex Role of Estrogens in Inflammation. Endocr. Rev. 28, 521–574. https://doi.org/10.1210/er.2007-0001

Sufi, B.G., Mori, K., Nomura, I., 1980. Involution of Thymus in Relation to Sexual and Steroid Hormone Treatments in Salmonid Fish. Tohoku J. of Agri. Res. 31, 97–105.

Sugimoto, K., Hui, S.P., Sheng, D.Z., Nakayama, M., Kikuchi, K., 2017. Zebrafish FOXP3 is required for the maintenance of immune tolerance. Dev. Comp. Immunol. 73, 156–162. https://doi.org/10.1016/j.dci.2017.03.023

Suzuki, G., Sawa, H., Kobayashi, Y., Nakata, Y., Nakagawa, K., Uzawa, A., Sakiyama, H., Kakinuma, S., Iwabuchi, K., Nagashima, K., 1999. Pertussis Toxin-Sensitive Signal Controls the Trafficking of Thymocytes Across the Corticomedullary Junction in the Thymus. J. Immunol. 162, 5981–5985.

Szwejser, E., Maciuszek, M., Casanova-Nakayama, A., Segner, H., Verburg-van Kemenade, B.M.L., Chadzinska, M., 2017. A role for multiple estrogen receptors in immune regulation of common carp. Dev. Comp. Immunol. 66, 61–72. https://doi.org/10.1016/j.dci.2016.04.003

Szwejser, E., Pijanowski, L., Maciuszek, M., Ptak, A., Wartalski, K., Duda, M., Segner, H., Verburg-van Kemenade, B.M.L., Chadzinska, M., 2017. Stress differentially affects the systemic and leukocyte estrogen network in common carp. Fish Shellfish Immunol. 68, 190–201. https://doi.org/10.1016/j.fsi.2017.07.011

Szwejser, E., Verburg-van Kemenade, B.M.L., Maciuszek, M., Chadzinska, M., 2016b. Estrogen-dependent seasonal adaptations in the immune response of fish. Horm. Behav. https://doi.org/10.1016/j.yhbeh.2016.10.007

Tafalla, C., Leal, E., Yamaguchi, T., Fischer, U., 2016. T cell immunity in the teleost digestive tract. Dev. Comp. Immunol., Special Issue: Intestinal Immunity 64, 167–177. https://doi.org/10.1016/j.dci.2016.02.019

Taghon, T., Yui, M.A., Pant, R., Diamond, R.A., Rothenberg, E.V., 2006. Developmental and Molecular Characterization of Emerging β- and γδ-Selected Pre-T Cells in the Adult Mouse Thymus. Immunity 24, 53–64. https://doi.org/10.1016/j.immuni.2005.11.012

Tai, P., Wang, J., Jin, H., Song, X., Yan, J., Kang, Y., Zhao, L., An, X., Du, X., Chen, X., Wang, S., Xia, G., Wang, B., 2008. Induction of regulatory T cells by physiological level estrogen. J. Cell. Physiol. 214, 456–464. https://doi.org/10.1002/jcp.21221

Takano, K., Takemura, A., Furihata, M., Nakanishi, T., Hara, A., 1991. Annual reproductive and spawning cycles of female Sebastiscus marmoratus. Environ. Biol. Fishes 30, 39–48. https://doi.org/10.1007/BF02296875

Takao, T., 2005. Effect of 17 -estradiol on tumor necrosis factor- -induced cytotoxicity in the human peripheral T lymphocytes. J. Endocrinol. 184, 191–197. https://doi.org/10.1677/joe.1.05914

Takizawa, F., Dijkstra, J.M., Kotterba, P., Korytář, T., Kock, H., Köllner, B., Jaureguiberry, B., Nakanishi, T., Fischer, U., 2011. The expression of CD8α discriminates distinct T cell subsets in teleost fish. Dev. Comp. Immunol. 35, 752–763. https://doi.org/10.1016/j.dci.2011.02.008

Takizawa, F., Magadan, S., Parra, D., Xu, Z., Koryta, T., Boudinot, P., Sunyer, J.O., 2016. Novel Teleost CD4-Bearing Cell Populations Provide Insights into the Evolutionary Origins and Primordial Roles of CD4+ Lymphocytes and CD4+ Macrophages. J. Immunol. 196, 4522–4535. https://doi.org/10.4049/jimmunol.1600222

Tamaki, M., Konno, Y., Kobayashi, Y., Takeda, M., Itoga, M., Moritoki, Y., Oyamada, H., Kayaba, H., Chihara, J., Ueki, S., 2014. Expression and functional roles of G-protein-coupled estrogen receptor (GPER) in human eosinophils. Immunol. Lett. 160, 72–78. https://doi.org/10.1016/j.imlet.2014.03.012

Tamura, E., Honma, Y., Kitamura, Y., 1981. Seasonal changes in the thymus of the viviparous surfperch, Ditrema temmincki, with special reference to its maturity and gestation. Jpn. J. Ichthyol. Jpn.

Tappin, A.D., Millward, G.E., 2015. The English Channel: Contamination status of its transitional and coastal waters. Mar. Pollut. Bull. 95, 529–550. https://doi.org/10.1016/j.marpolbul.2014.12.012

Taranger, G.L., Carrillo, M., Schulz, R.W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F.-A., Dufour, S., Karlsen, Ø., Norberg, B., Andersson, E., Hansen, T., 2010. Control of puberty in farmed fish. Gen. Comp. Endocrinol. 165, 483–515. https://doi.org/10.1016/j.ygcen.2009.05.004

Tatner, M.F., 1996. Natural changes in the immune system of fish, in: The Fish Immune System: Organism, Pathogen, and Environment. pp. 255–287.

Teles, A., Schumacher, A., Kühnle, M.-C., Linzke, N., Thuere, C., Reichardt, P., Tadokoro, C.E., Hämmerling, G.J., Zenclussen, A.C., 2013a. Control of Uterine Microenvironment by Foxp3+ Cells Facilitates Embryo Implantation. Front. Immunol. 4. https://doi.org/10.3389/fimmu.2013.00158

Teles, A., Thuere, C., Wafula, P.O., El-Mousleh, T., Zenclussen, M.L., Zenclussen, A.C., 2013b. Origin of Foxp3+ cells during pregnancy. Am. J. Clin. Exp. Immunol. 2, 222–233.

Tetel, M.J., Pfaff, D.W., 2010. Contributions of estrogen receptor-α and estrogen receptor-β to the regulation of behavior. Biochim. Biophys. Acta BBA - Gen. Subj., Estrogen Actions in the Brain 1800, 1084–1089. https://doi.org/10.1016/j.bbagen.2010.01.008

Thomas, P., 2012. Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. Gen. Comp. Endocrinol. 175, 367–383. https://doi.org/10.1016/j.ygcen.2011.11.032

Ting, Y.F., Praveena, S.M., 2017. Sources, mechanisms, and fate of steroid estrogens in wastewater treatment plants: a mini review. Environ. Monit. Assess. 189. https://doi.org/10.1007/s10661-017-5890-x

Toda, H., Saito, Y., Koike, T., Takizawa, F., Araki, K., Yabu, T., Somamoto, T., Suetake, H., Suzuki, Y., Ototake, M., Moritomo, T., Nakanishi, T., 2011. Conservation of characteristics and functions of CD4 positive lymphocytes in a teleost fish. Dev. Comp. Immunol. 35, 650–660. https://doi.org/10.1016/j.dci.2011.01.013

Tokarz, J., Möller, G., Hrabě de Angelis, M., Adamski, J., 2015. Steroids in teleost fishes: A functional point of view. Steroids 103, 123–144. https://doi.org/10.1016/j.steroids.2015.06.011

Torroba, M., Zapata, A.G., 2003. Aging of the vertebrate immune system. Microsc. Res. Tech. 62, 477–481. https://doi.org/10.1002/jemt.10409

Trampont, P.C., Tosello-Trampont, A.-C., Shen, Y., Duley, A.K., Sutherland, A.E., Bender, T.P., Littman, D.R., Ravichandran, K.S., 2010. CXCR4 acts as a costimulator during thymic β-selection. Nat. Immunol. 11, 162–170. https://doi.org/10.1038/ni.1830

Turchinovich, G., Pennington, D.J., 2011. T cell receptor signalling in γδ cell development: strength isn't everything. Trends Immunol. 32, 567–573. https://doi.org/10.1016/j.it.2011.09.005

Ulges, A., Schmitt, E., Becker, C., Bopp, T., 2016. Context- and Tissue-Specific Regulation of Immunity and Tolerance by Regulatory T Cells. Adv. Immunol. 132, 1–46. https://doi.org/10.1016/bs.ai.2016.08.002

van de Wijngaert, F.P., Kendall, M.D., Schuurman, H.-J., Rademakers, L.H., Kater, L., 1984. Heterogeneity of epithelial cells in the human thymus. Cell Tissue Res. 237, 227–237. https://doi.org/10.1007/BF00217140

Vantourout, P., Hayday, A., 2013. Six-of-the-best: unique contributions of γδ T cells to immunology. Nat. Rev. Immunol. 13, 88–100. https://doi.org/10.1038/nri3384

Vera, L.M., Migaud, H., 2014. Melatonin Rhythms, in: Biology of European Sea Bass. CRC Press, pp. 90–115.

Wan, F., Hu, C., Ma, J., Gao, K., Xiang, L., Shao, J., 2017. Characterization of γδ T Cells from Zebrafish Provides Insights into Their Important Role in Adaptive Humoral Immunity. Front. Immunol. 7. https://doi.org/10.3389/fimmu.2016.00675

Wang, C., Dehghani, B., Li, Y., Kaler, L.J., Proctor, T., Vandenbark, A.A., Offner, H., 2009. Membrane Estrogen Receptor Regulates Experimental Autoimmune Encephalomyelitis through Up-regulation of Programmed Death 1. J. Immunol. 182, 3294–3303. https://doi.org/10.4049/jimmunol.0803205

Wang, C., Dehghani, B., Magrisso, I.J., Rick, E.A., Bonhomme, E., Cody, D.B., Elenich, L.A., Subramanian, S., Murphy, S.J., Kelly, M.J., Rosenbaum, J.S., Vandenbark, A.A., Offner, H., 2008. GPR30 Contributes to Estrogen-Induced Thymic Atrophy. Mol. Endocrinol. 22, 636–648. https://doi.org/10.1210/me.2007-0359

Wang, T., Monte, M.M., Huang, W., Boudinot, P., Martin, S.A.M., Secombes, C.J., 2010. Identification of two FoxP3 genes in rainbow trout (Oncorhynchus mykiss) with differential induction patterns. Mol. Immunol. 47, 2563–2574. https://doi.org/10.1016/j.molimm.2010.06.015

Watanabe, N., Wang, Y.-H., Lee, H.K., Ito, T., Wang, Y.-H., Cao, W., Liu, Y.-J., 2005. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. Nature 436, 1181–1185. https://doi.org/10.1038/nature03886

Wee, S.Y., Aris, A.Z., 2017. Endocrine disrupting compounds in drinking water supply system and human health risk implication. Environ. Int. 106, 207–233. https://doi.org/10.1016/j.envint.2017.05.004

Wei, C., Mei, J., Tang, L., Liu, Y., Li, D., Li, M., Zhu, X., 2016. 1-Methyl-tryptophan attenuates regulatory T cells differentiation due to the inhibition of estrogen-IDO1-MRC2 axis in endometriosis. Cell Death Dis. 7, e2489. https://doi.org/10.1038/cddis.2016.375

Weist, B.M., Kurd, N., Boussier, J., Chan, S.W., Robey, E.A., 2015. Thymic regulatory T cell niche size is dictated by limiting IL-2 from antigen-bearing dendritic cells and feedback competition. Nat. Immunol. 16, 635–641. https://doi.org/10.1038/ni.3171

Wen, Y., Fang, W., Xiang, L.-X., Pan, R.-L., Shao, J.-Z., 2011. Identification of Treg-like cells in Tetraodon: insight into the origin of regulatory T subsets during early vertebrate evolution. Cell. Mol. Life Sci. 68, 2615–2626. https://doi.org/10.1007/s00018-010-0574-5

Wiegertjes, G.F., Wentzel, A.S., Spaink, H.P., Elks, P.M., Fink, I.R., 2016. Polarization of immune responses in fish: The 'macrophages first' point of view. Mol. Immunol. 69, 146–156. https://doi.org/10.1016/j.molimm.2015.09.026

Winans, B., Humble, M.C., Lawrence, B.P., 2011. Environmental toxicants and the developing immune system: A missing link in the global battle against infectious disease? Reprod. Toxicol., Prenatal Programming and Toxicity II (PPTOX II): Role of Environmental Stressors in the Developmental Origins of Disease 31, 327–336. https://doi.org/10.1016/j.reprotox.2010.09.004

Wise, A., O'Brien, K., Woodruff, T., 2011. Are Oral Contraceptives a Significant Contributor to the Estrogenicity of Drinking Water? Environ. Sci. Technol. 45, 51–60. https://doi.org/10.1021/es1014482

Xu, Z., Liu, J., Wu, X., Huang, B., Pan, X., 2017. Nonmonotonic responses to low doses of xenoestrogens: A review. Environ. Res. 155, 199–207. https://doi.org/10.1016/j.envres.2017.02.018

Yamano, T., Steinert, M., Klein, L., 2015. Thymic B Cells and Central T Cell Tolerance. Front. Immunol. 6. https://doi.org/10.3389/fimmu.2015.00376

Yang, M., Wei, H., Zhao, T., Wang, X., Zhang, A., Zhou, H., 2012. Characterization of Foxp3 gene from grass carp (*Ctenopharyngodon idellus*): A rapamycin-inducible transcription factor in teleost immune system. Dev. Comp. Immunol. 38, 98–107. https://doi.org/10.1016/j.dci.2012.04.010

Yao, G., Hou, Y., 2004. Nonylphenol induces thymocyte apoptosis through Fas/FasL pathway by mimicking estrogen in vivo. Environ. Toxicol. Pharmacol. 17, 19–27. https://doi.org/10.1016/j.etap.2004.01.006

Yellayi, S., Naaz, A., Szewczykowski, M.A., Sato, T., Woods, J.A., Chang, J., Segre, M., Allred, C.D., Helferich, W.G., Cooke, P.S., 2002. The phytoestrogen genistein induces thymic and immune changes: a human health concern? Proc. Natl. Acad. Sci. 99, 7616–7621.

Yellayi, S., Teuscher, C., Woods, J.A., Welsh Jr, T.H., Tung, K.S., Nakai, M., Rosenfeld, C.S., Lubann, D.B., Cooke, P.S., 2000. Normal development of thymus in male and female mice requires estrogen/estrogen receptor-α signaling pathway. Endocrine 12, 207–213.

Yonezawa, Y., Shin, Y., Abe, A., Kondo, Y., 2008. Estrogen receptor alpha expression in the thymus of chick embryos during late stages of embryogenesis. Anim. Sci. J. 79, 270–273. https://doi.org/10.1111/j.1740-0929.2008.00526.x

Young, C.N.J., Sinadinos, A., Lefebvre, A., Chan, P., Arkle, S., Vaudry, D. & Gorecki, D. C., 2015. A novel mechanism of autophagic cell death in dystrophic muscle regulated by P2RX7 receptor large-pore formation and HSP90. Autophagy 11:1, 113-130, DOI: 10.4161/15548627.2014.994402

Yui, M.A., Rothenberg, E.V., 2014. Developmental gene networks: a triathlon on the course to T cell identity. Nat. Rev. Immunol. 14, 529–545. https://doi.org/10.1038/nri3702

Zaitsu, M., Narita, S.-I., Lambert, K.C., Grady, J.J., Estes, D.M., Curran, E.M., Brooks, E.G., Watson, C.S., Goldblum, R.M., Midoro-Horiuti, T., 2007. Estradiol activates mast cells via a non-genomic estrogen receptor-α and calcium influx. Mol. Immunol. 44, 1977–1985. https://doi.org/10.1016/j.molimm.2006.09.030

Zapata, A.G., Chiba, A., Varas, A., 1996. Cell and tissues of the immune system of fish, in: The Fish Immune System: Organism, Pathogen, and Environment. pp. 255–287.

Zhang, X.-J., Wang, P., Zhang, N., Chen, D.-D., Nie, P., Li, J.-L., Zhang, Y.-A., 2017. B Cell Functions Can Be Modulated by Antimicrobial Peptides in Rainbow Trout *Oncorhynchus mykiss*: Novel Insights into the Innate Nature of B Cells in Fish. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.00388

Zheng, W., Yoshimura, Y., 2001. Organ-specificity of estrogen effects on the induction of immunocompetent cells in the chicken. J. Poult. Sci. 38, 41–49.

Zhu, L. -y., Lin, A. -f., Shao, T., Nie, L., Dong, W. -r., Xiang, L. -x., Shao, J. -z., 2014. B Cells in Teleost Fish Act as Pivotal Initiating APCs in Priming Adaptive Immunity: An Evolutionary Perspective on the Origin of the B-1 Cell Subset and B7 Molecules. J. Immunol. 192, 2699–2714. https://doi.org/10.4049/jimmunol.1301312

Zoller, A.L., Kersh, G.J., 2006. Estrogen Induces Thymic Atrophy by Eliminating Early Thymic Progenitors and Inhibiting Proliferation of -Selected Thymocytes. J. Immunol. 176, 7371–7378. https://doi.org/10.4049/jimmunol.176.12.7371

Zoller, A.L., Schnell, F.J., Kersh, G.J., 2007. Murine pregnancy leads to reduced proliferation of maternal thymocytes and decreased thymic emigration. Immunology 121, 207–215. https://doi.org/10.1111/j.1365-2567.2006.02559.x

Zwollo, P., 2016. The humoral immune system of anadromous fish. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2016.12.008

Matthieu Paiola
3 rue Joseph Clerc, 76600 Le Havre
Cell phone: +33 6.58.90.77.41
Email: mat.paiola@gmail.com
Age: 26
Nationality: French

PhD
UMR-I 02 Sebio – Environmental stress and biomonitoring of aquatic environment
Normandy University – University of Le Havre Normandy

Education

2012-2014 International Master in Biology of Marine Organism program within the Master of Integrative Biology and Physiology, majoring in Marine Organism Physiology, with honours (*grade good: 14/20*)

-University of Pierre and Marie Curie (UPMC) (Sorbonne University), Paris (France) and Faculty of Agriculture and Engineering of the Pontifical Catholic University of Santiago (PUC, Chile)

-Ranks Semester 4 and Semester 3: 7/18; Semester 1: 36/148; Semester 2: 11/137

2009-2012 Bachelor of Science in Biology, majoring in Animal Physiology, with honours (*grade good: 14.3/20*)

-University of Caen (France).

-Ranks: L3: 1/32; L2: 2/152; L1: 13/100

Research experiences

Since October 2014 **Immunomodulation of thymic function and T cell differentiation by oestrogens in the European sea bass, *Dicentrarchus labrax*. An evolutionary and ecotoxicological perspective.**

-UMR-I 02 INERIS-URCA-ULH SEBIO, Normandy University – University of Le Havre Normandy

-Supervisors: Thomas Knigge and Tiphaine Monsinjon

Scientific exchanges:

-January 2016 for 1 month, research team of Pr. G. Scapigliati, Department for Innovation in Biological, Agro-food and Forest Systems, Tuscia University, 01100 Viterbo, Italy

-January 2017 for 10 days, UMR 9190 MARBEC research team AEO (Ecophysiological Adaptation and Ontogenesis), University of Montpellier 2, France

January-June 2014 Internship **Short term acclimation to hypotonic environment of the sea bass *Dicentrarchus labrax*, with a cellular approach**

-UMR 5119 ECOSYM, research team AEO (Ecophysiological Adaptation and Ontogenesis), University of Montpellier 2 (France)

-Supervisors: Catherine Lorin-Nebel and Emilie Farcy

January-April 2013 Internship **Effect of alternative protein sources on plasmatic parameter and the digestibility of the teleost *Eleginops maclovinus***

-Department of Animal Production of the University of Chile (Santiago, Chile) and at the Faculty of Agriculture and Engineering of the PUC (Santiago, Chile)

-Supervisors: Rui Sá and Jurij Wacyk

June-August 2012 facultative internship **Effect of ocean acidification on the marine worm behaviour *Nereis succinea***

-Department of Biological Sciences at the University of Hull (UK)

-Supervisor: Jörg D. Hardege

Publication list

Paiola M., Knigge T., Picchietti S., Duflot A., Guerra L., Scapigliati G., Monsinjon T., Oestrogen receptor distribution related to functional thymus anatomy of the European sea bass, *Dicentrarchus labrax,* (2017)

Paiola M., Knigge T., Duflot A., Pinto I.S.P., Farcy E., Monsinjon T., Oestrogen, an evolutionary conserved regulator of T cell differentiation and immune tolerance in jawed vertebrates? (2018)

Paiola M., Moreira C., Duflot A., Knigge T., Monsinjon T., Oestrogen differentially modulates oxidative burst capacity and ROS-signaling in lymphoid and myeloid cells of three major lymphoid organs in European sea bass, *Dicentrarchus labrax* (L.), *(in preparation)*

Communication list

International communications (4)

Paiola M., Knigge T., Picchietti S., Duflot A., Guerra L., Pinto I.S.P., Scapigliati G., Monsinjon T., Oestrogen receptor distribution on a primary immune organ, the thymus of European Sea Bass, *Dicentrarchus labrax*, 30th ESCPB congress (New European Society for Comparative and Biochemistry) (Barcelona, Spain). 4-7th of September 2016, *Oral Communication*

Paiola M., Monsinjon T., Rocher B., Chan P., Vaudry D., Knigge T., Oestrogenic effect of 17β-estradiol on pituitary gland proteome of European Sea Bass, *Dicentrarchus labrax*, 30th ESCPB congress, Barcelona (Spain), 4-7th of September 2016, *Poster*

Paiola M., Knigge T., Picchietti S., Guerra L., Pinto P., Monsinjon T. Oestrogen receptor distribution on a primary immune organ, the thymus of European Sea Bass, *Dicentrarchus labrax*, 12th annual EcoBIM conference (international network in aquatic ecotoxicology), Le Havre (France). 30th of May to the 1st of June 2016, *Oral Communication*

Sá R., Wacyk J., **Paiola M.**, Loentgen G., Bas F., Effect of dietary protein source on plasmatic parameters of Patagonian blennie (*Eleginops maclovinus*) juveniles, The XXXIX Congress of the Chilean Society of Animal Production (SOCHIPA), Buenos Aires (Argentina), 20-22th October 2014, *Poster*

National communications (3)

Paiola M., Knigge T., Duflot A., Pinto I.S.P., Farcy E., Monsinjon T., Oestrogen, an evolutionarily conserved regulator of T cell differentiation and immune tolerance in jawed vertebrates? Seminary *Sciences Appliquées à l'Environnement* (SCALE), Le Havre (France), 27th of October 2017, *Oral Communication*

Paiola M., Knigge T., Duflot A., Pinto I.S.P., Farcy E., Monsinjon T., Oestrogen modulates thymic function and T cell differentiation in European sea bass, *Dicentrachus labrax*, Seminary of the *groupement de recherche ecotoxicologie Aquatique (GDR EA)*, Lyon (France), 22th of June 2017, *Oral Communication*

Paiola M., Knigge T., Picchietti S., Guerra L., Pinto P., Monsinjon T., Oestrogen receptor distribution on a primary immune organ, the thymus of European Sea Bass, *Dicentrarchus labrax*, Doctoral school seminary (ENBISE), Caen (France), 11th of March 2016, *Oral Communication*

Paiola M., Knigge T., Picchietti S., Guerra L., Pinto P., Monsinjon T., Oestrogen receptor expression on the thymus of the European Sea Bass, *Dicentrarchus labrax*, Seminary *Sciences Appliquées à l'Environnement* (SCALE), Rouen (France), 10th of March 2016, *Poster*

Paiola M., Knigge T., Monsinjon T., Oestrogen receptor expression on the thymus of the European Sea Bass, *Dicentrarchus labrax*, with focus in GPER. Seminary *Sciences Appliquées à l'Environnement* (SCALE), Le Havre (France), 23th of June 2015, *Poster*

Teaching activity

2015-2016 **Teaching assistant (64h)**

-Plant Biology (Practical work with 2nd year of Bachelor in Biology and Chemistry, University of Le Havre), Comparative Histology between monocotyledon and dicotyledon

-Membrane Biology (Practical work with 2nd year of Bachelor in Biology and Chemistry, University of Le Havre), Study of the frog's permeability skin, osmotic phenomena and diffusion

2017-2018 **Contracted lecturer (88h)**

-Plant Biology (Practical work with 2nd year of Bachelor in Biology and Chemistry, University of Le Havre), Comparative Histology between monocotyledon and dicotyledon

-Biochemistry (Practical work with 1st year of Bachelor in Biology and Chemistry, University of Le Havre), Sugar and protein quantification (Bertrand, Bradford and Biuret methods)

-Animal Biology (Practical work with 1st year of Bachelor in Biology and Chemistry, University of Le Havre), Comparative Anatomy of the digestive, reproductive and excretory systems between teleost fish, insect and mammal

-Animal Physiology (Practical work with 2nd year of Bachelor in Biology and Chemistry, University of Le Havre), Spirometry and Electrocardiogram

Competences

Technical competences

Animal experimentation (teleost fish): dissection, blood sampling, intra-peritoneal injection

Cell Biology: Cell isolation (on Ficoll and Percoll gradient), flow cytometry (phagocytosis, oxidative burst, viability and immunostaining), cell culture

Histology: immunohistochemistry (peroxydase et fluorochrome staining), photonic and epifluorescent microscopy, Pappenheim, HES and PAS coloration

Molecular Biology: RNA and DNA purification, quantitative RT-PCR (relative to multiple reference genes), pipetting robot computer programming and use

Bioinformatic: Multalin, Primer-BLAST, FlowJo, NovoExpress, imagej, ReFinder, SigmaPlot, Zotero

Transversal competences

Languages : French (Native), English (B2), Spanish (B1) *(European levels – self assessment grid)*

Co-supervision of master degree internship: Oestrogen receptor transcripts expression on isolated leucocytes from primary and secondary lymphoid organs of the European sea bass, *Dicentrarchus labrax,* (March to August 2017), Aurélie Duflot (Master 2, University of Le Havre Normandy)

Lab and budget management

Head of the organisation comity of the 20th seminary of the doctoral school (EdNBISE) (the 16th and 17th of March 2017): Search for financial supports, budget, communication, logistic, safety management. Chairman during oral sessions (In English)

Elective office

2015-2017 **Representative PhD student at doctoral school EDNBISE**

Formations

February 2016 **Professional communication towards socio-economical actors,** University of Le Havre Normandy

December 2015 **Benefits and limits of empathy in education,** University of Rouen (ESPE, Mont-Saint-Aignan)

May 2015 **University degree, design and conduction of animal experimentation applied to biomedical research, level C,** University of Caen, *Institut de Biologie Fondamentale et Appliquée* (IBFA), France

Personal interests

Cycling, hiking, travelling, judo (14 years of practice, black belt, Dan 1)
Clarinet (7 years of practice)

References

Thomas Knigge (PhD supervisor),
UMR-I 02 Sebio (Environmental stress and biomonitoring of aquatic environment)
Le Havre Normandy University
knigget@univ-lehavre.fr

Helmut Segner,
Center of Fish and Wildlife Health
University Bern
helmut.segner@vetsuisse.unibe.ch

Abstract:

Jawed vertebrates have developed an efficient adaptive immune system partly based on T lymphocytes. They develop in an evolutionary conserved organ, the thymus. In mammals, endogenous oestrogens are well known to regulate thymus function and plasticity. The question is, therefore, whether this is also the case in lower vertebrates, such as teleosts.

To achieve these aims, firstly the distribution of oestrogen receptor subtypes was investigated on the background of a detailed description of the functional anatomy of the thymic microenvironment. Secondly, thymic function- and T cell-related gene expression was analysed in the thymus, the head-kidney and the spleen of sea bass exposed to 17β-oestradiol. Moreover, the oxidative burst capacity in the two latter organs was evaluated *in vivo* and *in vitro* in leucocytes of the head-kidney and spleen following exposure to oestrogen. Eventually, age- and size-dependent variations in thymocyte number were examined in sea bass caught at various time points over three years.

The thesis provides new insights into the evolution of the immunomodulatory function of oestrogen with respect to the thymic and peripheral T cell differentiation in vertebrates. As a matter of fact, in addition to a highly conserved morpho-functional organisation, the distribution of oestrogen receptor subtypes as well as the oestrogenic effects appear to be evolutionarily conserved. Our results suggest that in sea bass, similar to mammals, oestrogen (1) stimulates a thymic alternative pathway of T cell maturation with innate-like properties, (2) enhances immune tolerance by promoting Treg differentiation, and (3) actively regulate thymic plasticity.

Keywords: thymus, T lymphocyte, teleost fish, comparative immunology, endocrinology, regulatory T lymphocyte, gamma-delta T lymphocyte,

Résumé :

Chez les vertébrés gnathostomes, le système immunitaire repose en grande partie sur les lymphocytes T qui se développent dans un organe conservé évolutivement : le thymus. Chez les mammifères, cet organe constitue une cible privilégiée pour les œstrogènes. La question soulevée ici est donc de savoir si c'est également le cas chez les poissons téléostéens.

Dans ce but, la distribution des différents sous-types de récepteurs aux œstrogènes a d'abord été étudiée dans le contexte d'une description de l'anatomie fonctionnelle du microenvironnement thymique. Par la suite, l'expression de gènes relatifs à la fonction thymique et aux différents sous-types de lymphocytes T a été analysée dans le thymus, le rein-antérieur et la rate de bars exposés au 17β-œstradiol. De plus, la capacité de flambée oxydative a été évaluée sur des leucocytes du rein-antérieur et de rate à la suite d'expositions *in vivo* et *in vitro*. Finalement, la variation du nombre de thymocytes a été examinée sur des bars capturés durant trois ans.

La thèse fournit de nouvelles connaissances concernant l'évolution des fonctions immunomodulatrices des œstrogènes sur la différenciation des cellules T. En effet, en plus d'une organisation morpho-fonctionnelle fortement conservée, la distribution des sous-types de récepteurs aux œstrogènes ainsi que les effets œstrogéniques apparaissent conservés au cours de l'évolution. Nos résultats suggèrent que, chez le bar comme chez les mammifères, les œstrogènes (1) stimulent une voie alternative de maturation des lymphocytes T ayant des propriétés similaires aux cellules immunitaires innées, (2) augmentent la tolérance immunitaire et (3) régulent la plasticité du thymus.

Mots clefs : thymus, lymphocyte T, poisson téléostéen, immunologie comparative, endocrinologie, lymphocyte T régulateur, lymphocyte T gamma-delta