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Phylogenetic, Molecular and Functional
Characterization of Prostaglandin Receptors
in Teleost Fish

Caracterización Filogenética, Molecular Y
Funcional De Los Receptores De Prostaglandinas
De Peces Teleosteos

Dña. Fatma Hermi
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Department of Life Sciences



Escuela Internacional De
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Thesis submitted for the degree of **Ph.D.**, with International Mention, of the University of Murcia in Spain and the University of Carthage in Tunisia.

Presented by

Fatma Hermi

**PHYLOGENETIC, MOLECULAR AND FUNCTIONAL
CHARACTERIZATION OF PROSTAGLANDIN RECEPTORS IN
TELEOST FISH**

**CARACTERIZACION FILOGENETICA, MOLECULAR Y FUNCIONAL
DE LOS RECEPTORES DE PROSTAGLANDINAS DE PECES
TELEOSTEOS**

2020-2021



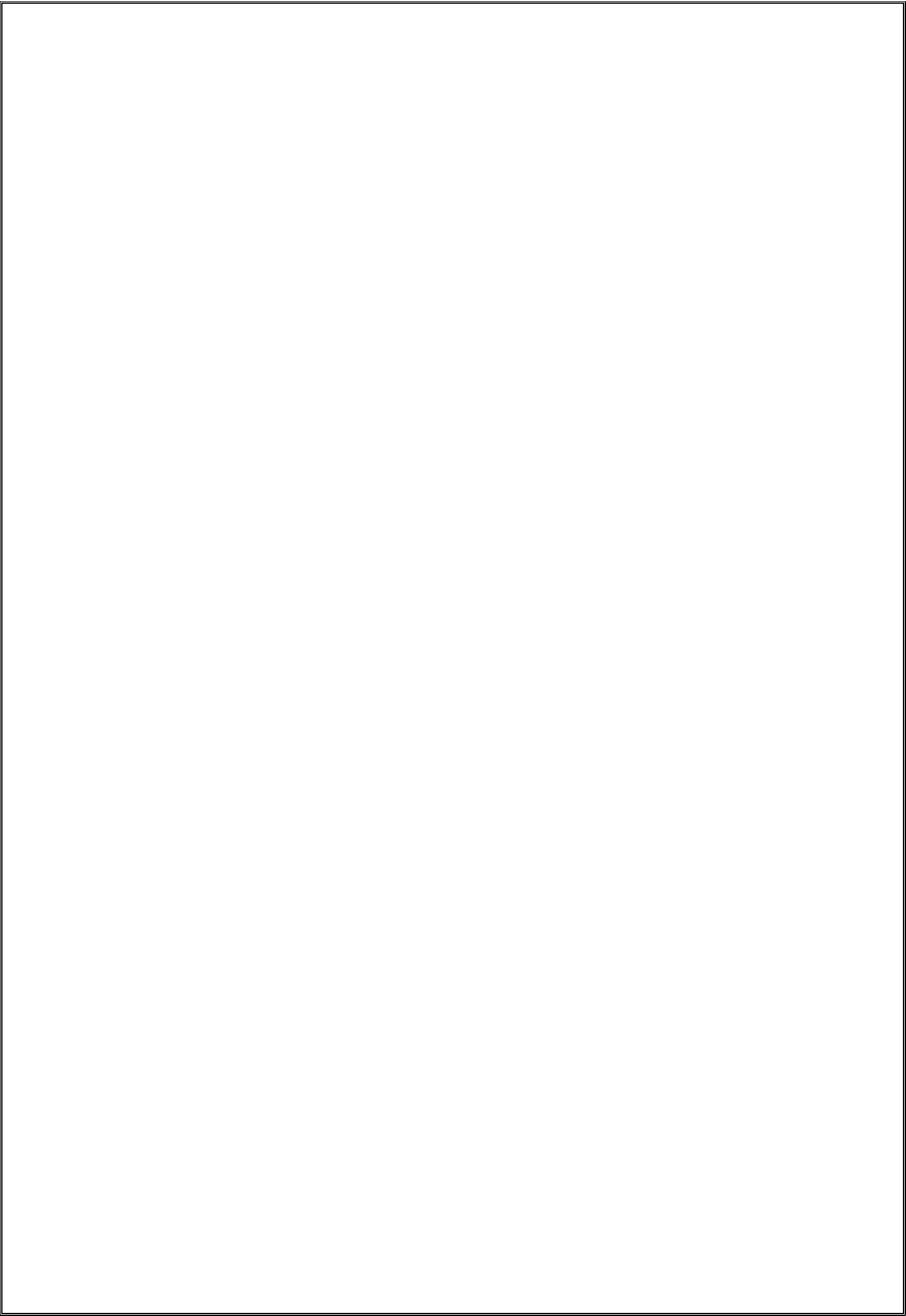
UNIVERSIDAD DE MURCIA ESCUELA INTERNACIONAL DE DOCTORADO	UNIVERSITY OF CARTHAGE DOCTORAL SCHOOL: LIFE AND MATERIAL SCIENCES
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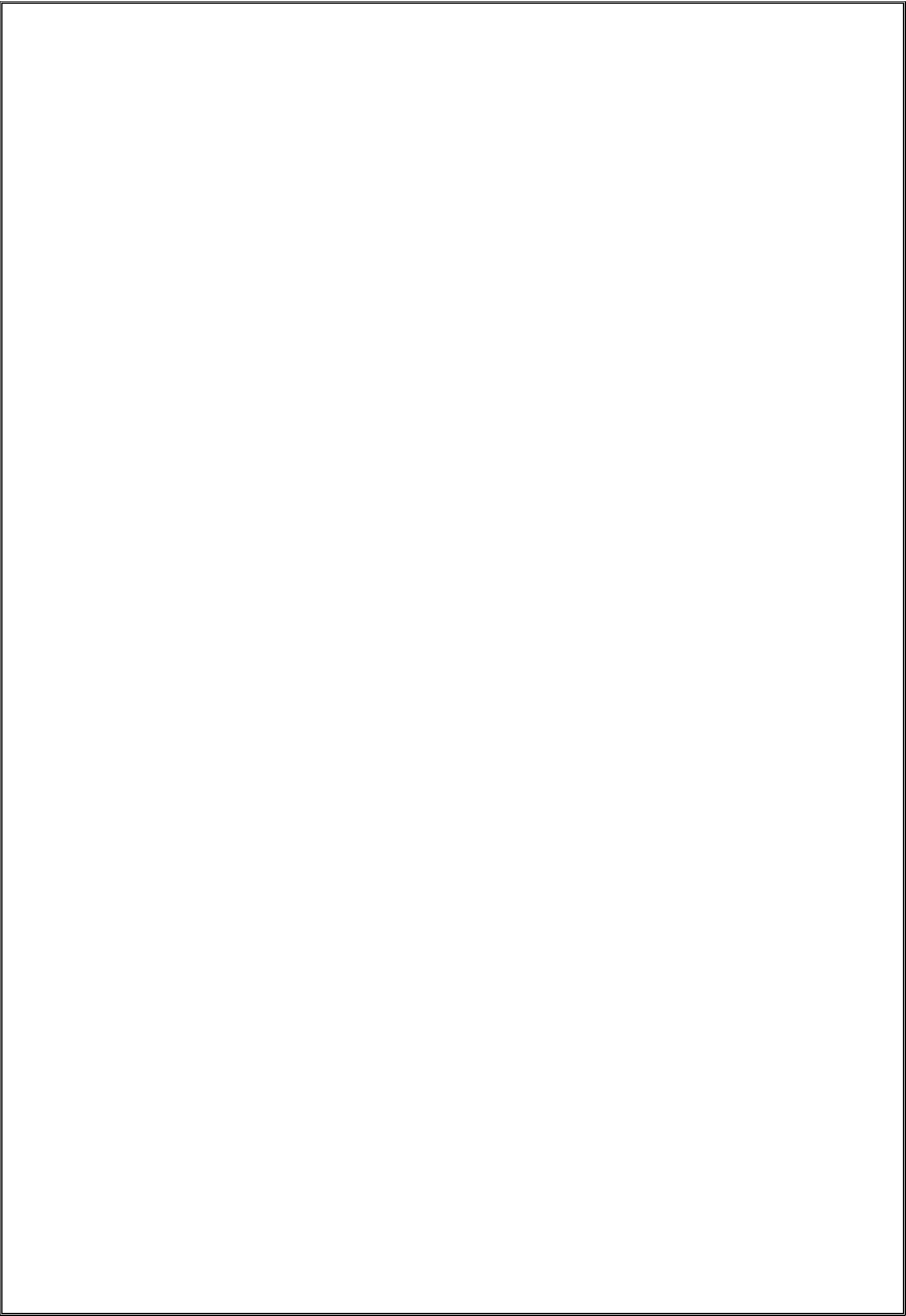


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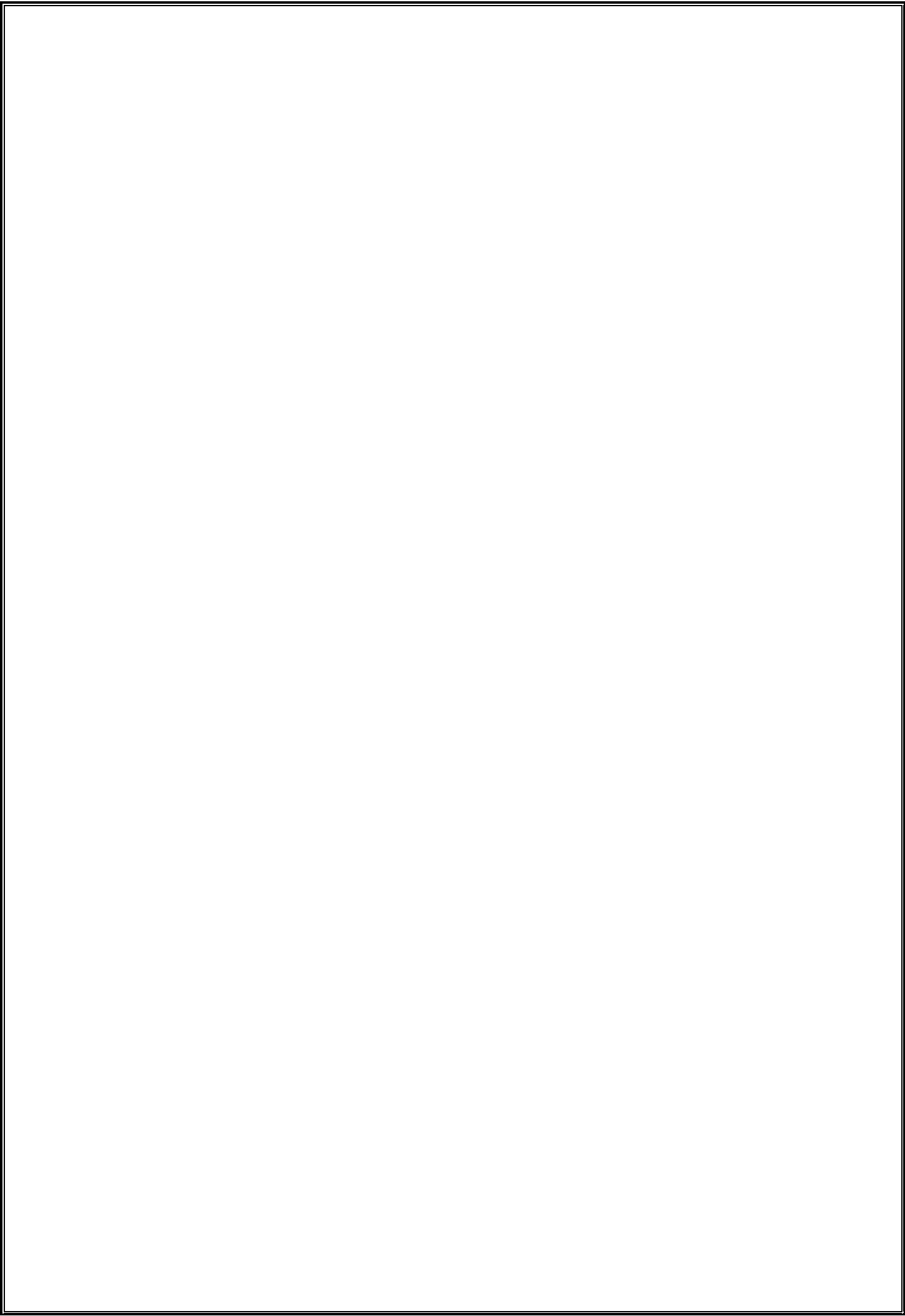
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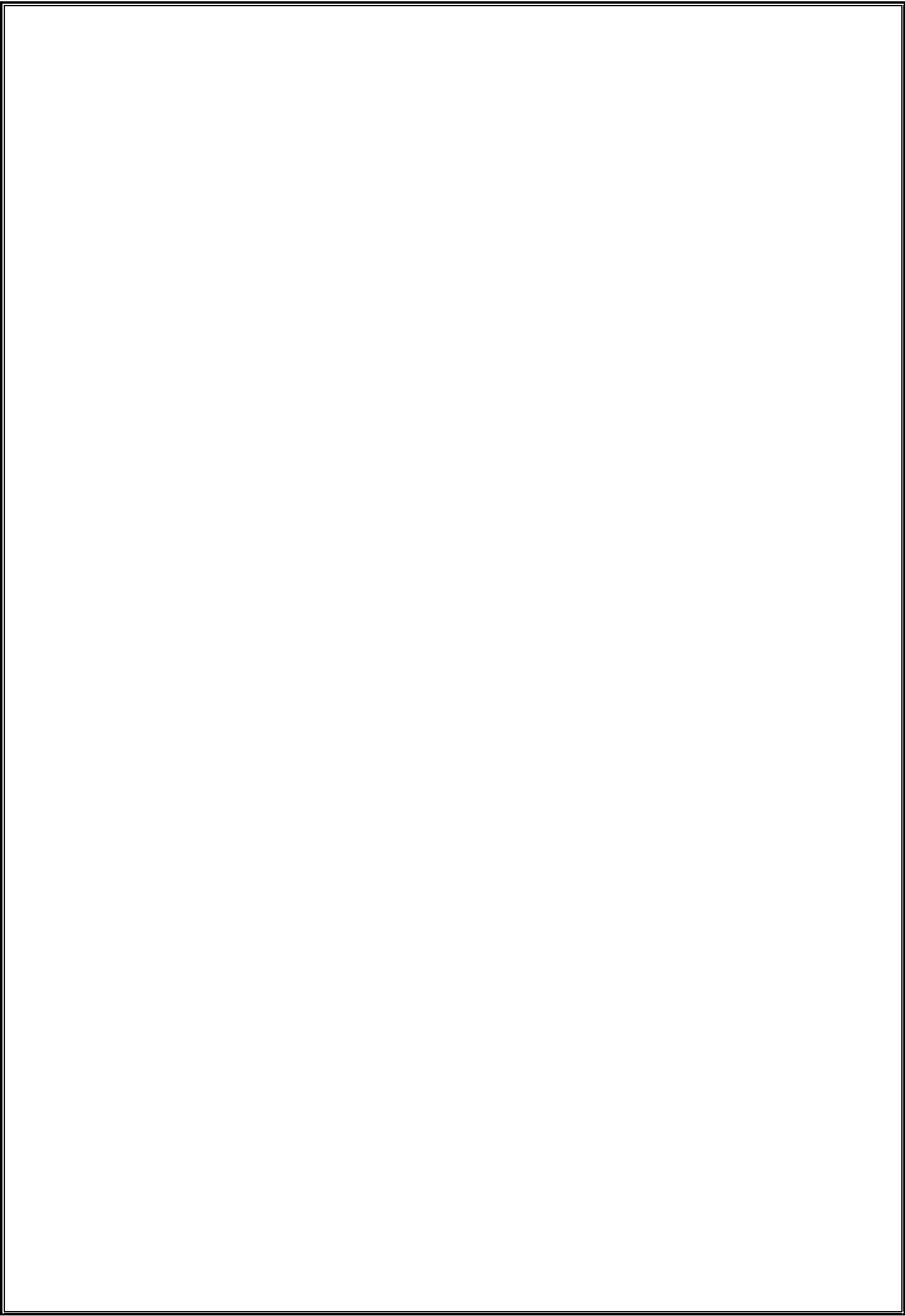
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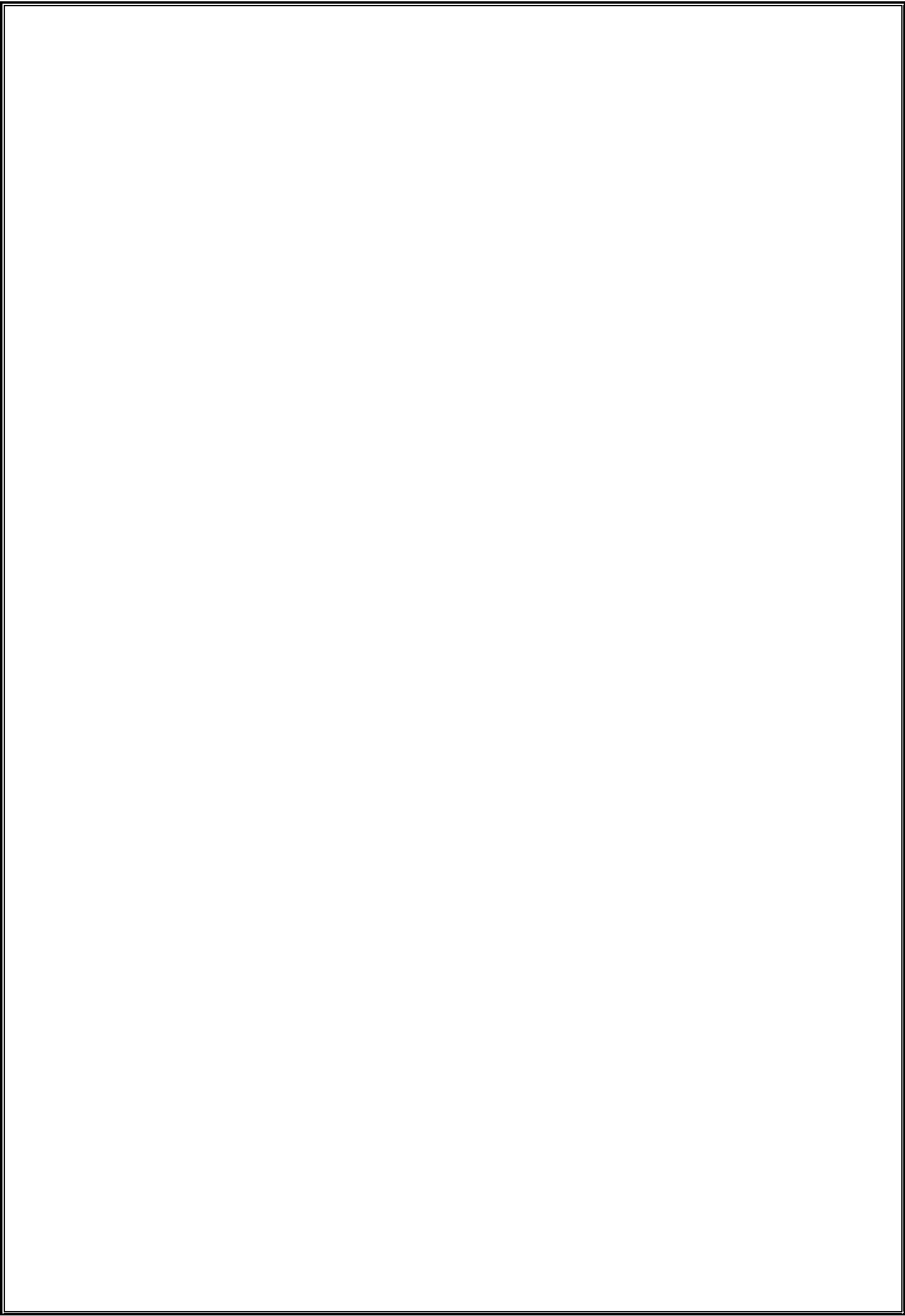
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LIST OF ABBREVIATIONS



List of abbreviations

AA	arachidonic acid
AF	activation function
AhR2	aryl hydrocarbon receptor 2
Akt	protein kinase B
APCs	antigen presenting cells
AP	AP activator protein
Ab	antibodies
AC	adenylate cyclase
AGs	acidophilic granulocytes
AR	androgen receptor
ATP	adenosine triphosphate
COX	cyclooxygenase
cAMP	cyclic adenosine monophosphate
C	cysteine
CD40L	Cluster of Differentiation 40 ligand
CCL25	Chemokine (C-C motif) ligand 25
CCR7	C-C chemokine receptor type 7
CCR9	C-C chemokine receptor type 9
CLRs	C-type lectin receptors
CRP	C-reactive protein
c-Src	Proto-oncogene tyrosine-protein kinase Src
cyPG	cyclopentenone PG
dbcAMP	dibutyryl-cAMP
D	aspartic acid
DGLA	dihomo- γ -linolenic acid DGLA
DAMPs	damage-associated molecular patterns
DNA	desoxyribonucleic acid
DBD	DNA binding domain
EP	E prostaglandin receptor
EPA	eicosapentaenoic acid EPA
EDTA	Ethylene diamine tetraacetic acid

List of abbreviations

EMBOSS	European molecular biology open software suite
EMBL	European molecular biology laboratory
EBI	European bioinformatics institute
EGFC	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EST	Expressed sequence tag
ENA	European nucleotide archive
EL	extracellular loop
ER	estrogen receptor
ERK	Extracellular signal-regulated kinases
ERR	estrogen receptor-related receptor
FCS	fetal calf serum
FOXO1	Forkhead box protein O1
GALT	gut-associated lymphoid tissue GALT
GPCR	G protein–coupled receptor
GR	glucocorticoid receptor
GRK	G protein coupled receptor kinase
GTP	guanosine triphosphate
G	arginine
HK	head kidney
HEK293	human embryonic kidney cells 293
IgG	Immunoglobulin G
IFN γ	gamma interferon
IL	intracellular loop
IL	Interleukin
IL-1RI	1 IL-1 receptor
IL-1RII	type 2 IL-1 receptor
LT- β	Lymphotoxin β
Ig	immunoglobulins
LBD	Ligand-binding domain
LPS	Lipopolysaccharide
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase

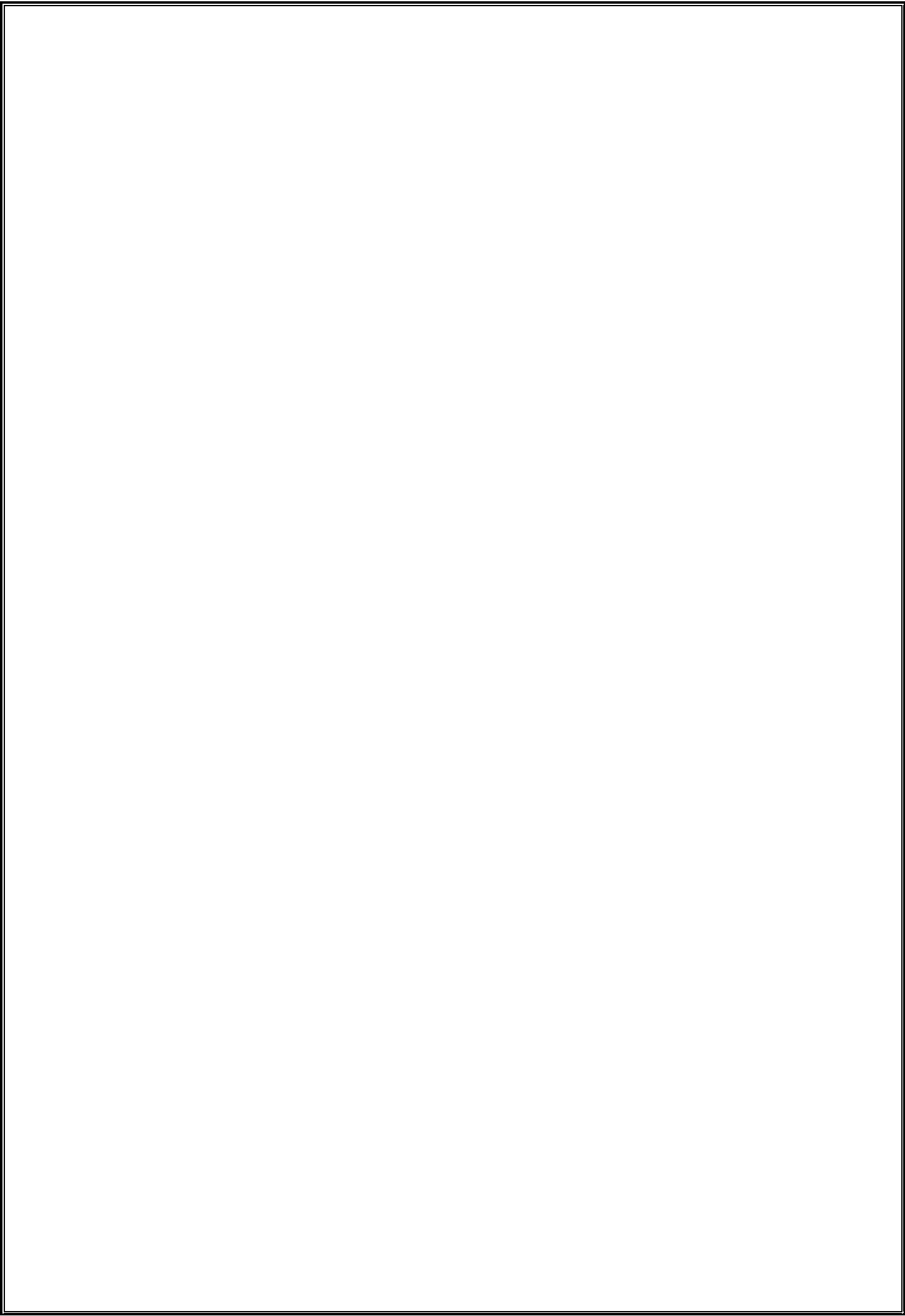
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MHCII	major histocompatibility complex II
Mrc1	mannose receptor C-type 1
MR	mineralocorticoid receptor
NBRE	NGFI-B-responsive element
NCBI	national center for biotechnology information
NF- κ B	nuclear factor kappa B
NGFI-B	nerve growth factor IB
NLRs	NOD-like receptors
NLS	nuclear localization signals
NK cells	Natural killer cell
NTD	N-terminal domain
NOR1	Neuron-Derived Orphan Receptor 1
Nr4a3	Nuclear Receptor Subfamily 4 Group A Member 3
NSAIDs	non-steroidal anti-inflammatory drugs
NR	Nuclear receptor
NRREs	NR-responsive elements
ORF	open reading frame
ONR	Orphan nuclear receptor
PGA ₂	prostaglandin A ₂
PGE ₂	prostaglandin E ₂
PE	peritoneal exudates
PAMPs	pathogenassociated molecular patterns
PG	prostaglandin
PI-3K	Phosphoinositide 3-kinase
PLA2	Phospholipase A2
PPAR	peroxisome proliferator-activated receptor
PTGER	prostaglandin E receptor
PTGS2	prostaglandin-endoperoxide synthase 2
PKA	protein kinase A
PGH2	prostaglandin H2
poly (I:C)	Polyinosinic:polycytidylic acid
PRRs	pattern recognition receptors
PR	progesterone receptor

List of abbreviations

PUFAs	polyunsaturated fatty acids
PXR	pregnane X receptor
RT-qPCR	quantitative RT-PCR
ROS	reactive oxygen species
R	arginine
RLRs	RIG-I-like receptors
S	serine
S.E	standard error
SF	steroidogenic factor
SOCS3	suppressor of cytokine signaling 3
TA	thromboxane
TMD	transmembrane domain
TNF α	Tumor necrosis factor α
T	threonine
TLRs	Toll-like receptors
TR	thyroid receptor
RIG	retinoic acid-inducible gene
RANKL	receptor activator of nuclear factor kappa-B ligand
RXR	Retinoid X Receptor
UTR	untranslated region
VaDNA	vibrio anguillarum genomic DNA
VEGF	vascular endothelial growth factor
Y	tyrosine
15-PGDH	15-hydroxy prostaglandin dehydrogenase
PGEM	13,14- dihydro-15-keto-PGE ₂

SUMMARY



Summary

The immune system of teleost fish has been studied for decades but, despite the important advances made, much is still unknown, especially concerning the presence and role of prostaglandins, such as PGE₂ and PGA₂, and their receptors. The gilthead seabream (*Sparus aurata* L.), a marine teleost fish of great commercial value in Mediterranean aquaculture, has been widely used for better understanding the fish immune response. The aim of this thesis is to advance our knowledge of the role of prostaglandins PGE₂ and PGA₂ and their receptors in the immune response of the gilthead seabream.

In the first chapter, one ortholog of mammalian PTGER1, PTGER3 and PTGER4, and two of PTGER2 (Ptger2a and Ptger2b) were identified and characterized in gilthead seabream. *In silico* analysis showed that all these receptors possessed the organization domain of G protein-coupled receptors, with the exception of Ptger2b. The corresponding *in vivo* studies revealed that they were expressed in all the tissues examined, the highest mRNA levels of *ptger1* and *ptger3* being observed in the spleen and of *ptger2a* and *ptger4* in the blood. Bacterial infection induced the highest mRNA levels of *ptger2a*, *ptger3* and *ptger4* in peritoneal exudate (the site of bacterial injection). In addition, head kidney acidophilic granulocytes and macrophages displayed different *ptger1*, *ptger2a*, *ptger3* and *ptger4* expression profiles. Furthermore, in macrophages the expression of the receptors was weakly affected by stimulation with bacterial DNA or with PGE₂, while in acidophilic granulocytes stimulation resulted in the upregulation of *ptger2a* and *ptger4*. Taken together, these results suggest different roles for seabream PGE₂ receptors in the regulation of the immune responses.

In the second chapter we have identified Nr4a3 in gilthead seabream and shown that PGA₂ regulates *in vitro* the main functions of professional phagocytes of this species. The gilthead seabream Nr4a3 revealed highly conserved motifs with all their vertebrate orthologs, particularly the DNA binding domain and sumoylation site. Expression analysis showed that *nr4a3* was constitutively expressed in macrophages and was upregulated by PGA₂ alone and combined with bacterial DNA. Importantly, PGA₂ addition weakly decreased the transcript levels of genes encoding the cytokines interleukin-1 β (Il1b), Il6 and Il10, while its combination with VaDNA promoted a synergistic induction of the mRNA levels of these three genes. However, PGA₂ failed to regulate the mRNA levels of the gene encoding the M2 polarization marker mannose receptor C-type 1 (Mrc1). In contrast, PGA₂ inhibited the

respiratory burst of acidophilic granulocytes and induced the transcript levels of the genes encoding Il1b and Il10. Finally, PGA_2 was able to signals through gilthead seabream Nr4a3 when ectopically expressed in HEK293 cells. In conclusion, we have shown for the first time that PGA_2 regulates the main functions of professional phagocytes of teleosts and provided evidences that it may signals through Nr4a3, paving the way for *in vivo* studies in order to understand the role of this prostaglandin and the Nr4a3 receptor in fish immune responses.

INTRODUCTION

INTRODUCTION

1. Immune system

The immune system is mainly defined by self-/nonself-discrimination and the action against a wide range of foreign microorganisms (viruses, bacteria, fungi, protozoa and multicellular parasites), macromolecules, (proteins and polysaccharides) and internal attacks (cancers for example). The immune response is divided into two branches, the innate or natural immunity and the adaptive, acquired or specific immunity (Parkin and Cohen 2001). Occasionally, the immune system can attack itself, resulting in autoimmune diseases.

The theory which admit that innate and the adaptative immunity differ by the presence and absences of specificity and the immune memory is being denied by the achieved recent knowledges (Muraille and Goriely 2017; Varadé et al.2020). It is more real that both systems distinguish by the speed of reactions and the component that each one dispose to eliminate or attenuate the danger (Beutler 2004; Parkin and Cohen 2001; Varadé et al.2020).

The innate immunity is the first line of defense that possesses the capability to engulfed and kills the microbe but also pave the way for the adaptive immunity to recognize the pathogen via antigen presenting cells (APCs), It disposes i) physical, chemical and microbiological barriers, ii) myeloid cells that include mononuclear phagocytes (monocytes/macrophages, dendritic cells) and the granulocytes (neutrophils, basophils, and eosinophils), and iii) humoral factors involving cytokines, complement and acute phase proteins. The microbial product, such as lipoteichoic acid, lipopolysaccharide (LPS), peptidoglycan, bacterial DNA, viral RNA, are called pathogen-associated molecular patterns (PAMPs), while endogenous molecules released from damaged cells, named damage-associated molecular patterns

(DAMPs), are recognized by a variety of germline-encoded pattern recognition receptors (PRRs) of the innate immune system. The PRRs family encompasses cell surface proteins, such as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and soluble proteins such as retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Beutler 2004; Smith et al. 2019; Takeuchi and Akira 2010).

The specific immunity disposes also humoral and cellular components that consist of i) T and B lymphocytes, ii) antibodies/immunoglobulins (Ab/Ig)(IgA, IgD, IgE, IgG and IgM) and iii) cytokines. It triggers highly specific and flexible immune responses through the ability to recognize very precise structures of the pathogen via antigen-specific receptors. Noxious agent invasion leads to T and B lymphocytes priming, activation, and differentiation in main lymphoid tissues. Then a cooperative immune response takes place through the generated immune cells and the released humoral components (**Fig. 1**). After the first pathogen attack, the adaptive immune cells develop a robust immunologic memory which permits to generate a more robust response against the second invasion (Parkin and Cohen 2001).

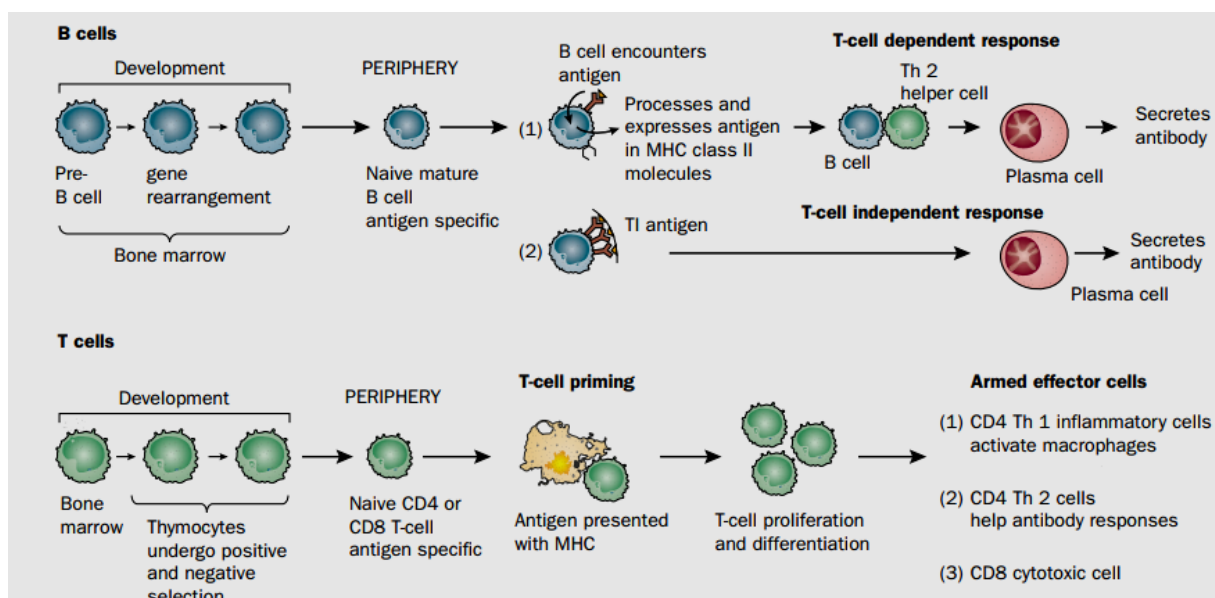


Figure 1. The role of T and B lymphocytes in specific immunity (Parkin and Cohen 2001).

2. Immune system of teleost fish

The teleost, modern branch of bony fish, are the richest vertebrate classes with more than 20,000 species (Esteban et al. 2013) whose diversity has allowed adaptations of diverse aquatic environments. The basal position of fish in the vertebrate family tree gives them a keen interest in comparative genomic and functional approaches, in particular those of the immune system (Levraud and Boudinot 2009). Fish are the first animal group that have a well-structured and differentiated innate and adaptive immune system (Rauta et al. 2012). However, unlike mammals, it is the innate immunity that has a pivotal interest in fish. Indeed, it is a set of fundamental humoral and cellular defense mechanisms (Sindermann 1980). Most species of fish hatch in the embryonic stage of life in the aquatic environment with a variety of invading microorganisms. For that innate mechanisms consist of robust system that assumes the defense against pathogens during the first weeks of their development (Scapigliati et al. 2000). The specific immune response, the last line of defense in vertebrates, also takes on two forms of immune functions, a humoral-like function with the production of antibodies and a cytotoxic cell-mediated function. Both result from the respective stimulation of B and T lymphocytes which have been shown to exist in fish (Romano et al. 2005). The response to memory is less elaborated than those of higher vertebrates.

2.1. Immune system organs

The organs and tissues of the immune system in teleosts have been classified, as in mammals, in primary and secondary lymphoid organs (Zapata et al. 2006) (**Fig.1**). In fact, the major primary organ is the kidney, which is equivalent of higher vertebrates bone marrow and lymph nodes (secondary lymphoid organ) (Kaattari and Irwin 1985). It is lodged under the ventral surface of the spine and above the swim bladder (Zapata et al. 2006). It is a single mass which presents two parts of different structures and functions, first the anterior part

(pronephros, cephalic part or head kidney, HK) with hematopoietic activity (Meseguer et al. 1991) and second, the posterior part (mesonephros) mainly ensuring the excretory function and weak immune function (Zwollo et al. 2008). The kidney produces all types of leukocytes, B lymphocytes, macrophages, granulocytes, plasma cells and T cells, with the exception of T lymphocytes. It is the primary site of antigen process and antibodies production (Dannevig et al. 1994). In addition, it is the main organ responsible for phagocytosis, also participates in the development of immunological memory with in melano macrophage centers.

The thymus is a well-developed, organ located in the branchial cavity (Kobayashi et al. 2008). This primary lymphoid organ is covered with an integument also called the cortex. It appears on histological sections as an aggregate of small thymocytes, hence the primordial role of this organ in the production of T lymphocytes. Thymus involution in fish is more dependent on hormonal cycles, seasonal variations and age (Press and Evensen 1999). The spleen is the major second immune organ located in the abdominal cavity, near the greater curvature of the stomach. Its color dark red, its edges are generally well defined in the normal state. It shows structural homology with the lymph nodes of mammals in which occur monocytopoiesis. Splenic ellipsoids (specialised terminations of arterioles) trigger the elimination of blood-borne antigens and immune complexes (Whyte 2007). It is an organ widely populated by IgM positive B cells, macrophages, erythrocytes, neutrophils and granulocytes (Rauta et al. 2012). In gilthead seabream, it was demonstrated that the pathogenic bacterium *Vibrio anguillarum* leads to the infiltration of the main innate immune cells to the spleen and was suggest that this organ is the place of the adaptative immune response initiation (Chaves-Pozo et al. 2005). As in mammals, the blood and liver are considered secondary immune tissues (Rauta et al. 2012). The liver is the metabolic center and plays an important role in the homeostasis mechanisms. Studies in fish have focused on its

immune responses to viral or toxicological infection and especially on stress responses to environmental conditions or hypoxia (Martin et al. 2010), since fish liver plays an important role in the absorption, biotransformation and excretion of pollutants (Gernhöfer et al. 2001). Other key immune secondary tissues in teleost are located in the portal entry sites; they include physical and chemical barriers which correspond to the mucus, skin epithelium, gill and intestinal tissue, namely the gut-associated lymphoid tissue (GALT) (Magnadóttir 2006).

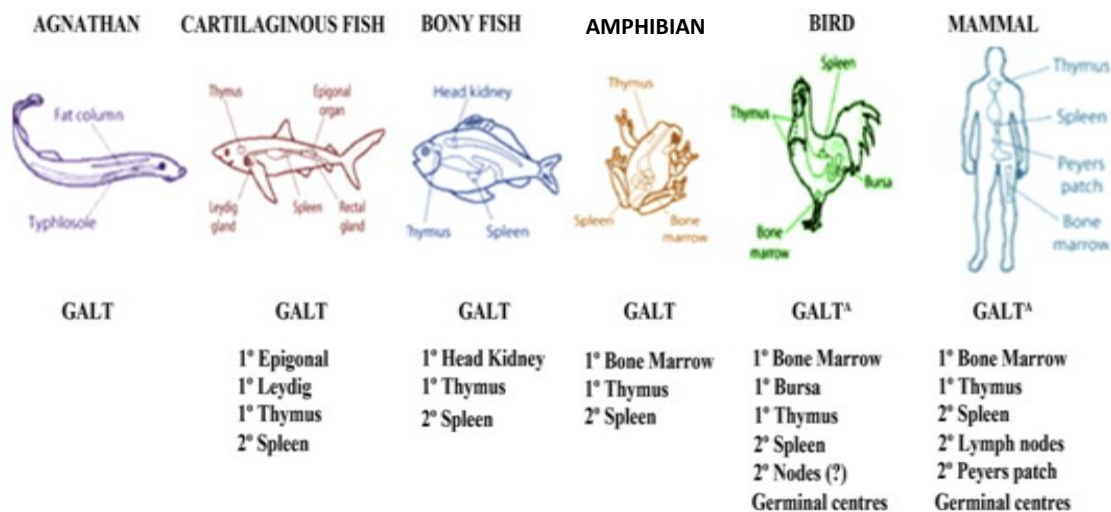


Figure.2. A comparative analysis of the immune organs in different vertebrates
(Rauta et al. 2012).

2.2. Innate immune system of teleost fish

The innate immune system of teleost fish is the first barrier of host against pathogens and it deals with any foreign material until the adaptive immune system is potent enough to take over (Dixon and Stet 2001; Fearon and Locksley 1996; Whyte 2007). The innate immune response is thought to be essential in combating diseases in fish due to the limitations of the adaptive immune response by their poikilothermic nature and their limited antibody repertoire, affinity maturation and memory, and relatively slow lymphocytes proliferation (Magnadóttir 2006; Uribe et al. 2011; Whyte 2007).

The skin and the presence of scales or the secretion of toxic substances, as well as the mucus, gills and the gastrointestinal tract represent defensive mechanisms. Moreover, there is a wide variety of humoral effectors that act in the innate of fish (Alexander and Ingram 1992). When innate immune leukocytes sense the presence of pathogens or tissue damage, inflammation appears as the innate immune response to avoid pathogen replication or infection and to initiate the process of tissue repair. Inflammation provokes redness, heat, swelling (accumulation of fluid), pain and loss of function in the inflammatory site due to the high recruitment of leukocytes and the changes in the vascular components. Although each type of leukocyte, i.e., monocytes/macrophages, neutrophils/AGs and mast cells, has a different role, they work together coordinately. The main mechanisms of the innate immunity developed in teleost fish is to eliminate harmful stimuli and include degranulation, chemotaxis, phagocytosis, oxidative burst, and opsonic and hemolytic serum activities (Secombes and Fletcher 1992; Verburg-van Kemenade et al. 1994; Whyte 2007).

2.2.1. Cellular components

Leukocytes in teleost are present in the blood and in several immune tissues and organs in addition they are endowed with morphological and functional heterogeneity which is implied by species diversity and the different environments that they inhabit (Haugland et al. 2012). In mammals, phagocytosis is mainly carried out by polymorphonuclear cells or neutrophils. In fish including, sea bass and seabream, monocytes/macrophages and granulocytes appear to be the most active phagocytic cells (Esteban and Meseguer 1997). Indeed, macrophages represent the main players in the APC family via showing the cell surface major histocompatibility complex II (MHCII) and fulfill the function of phagocytosis in response to varied immune stimuli via enhancing the expression of different inflammatory genes, namely IL-1 β , TNF α , cyclooxygenase-2 (COX-2) and TLRs (Sepulcre et al. 2007). Like in mammals,

teleost macrophages undergoes cell polarization mediated via varied mediators to leads to the appearance of M1/M2 subsets which are potent effectors in either microbial elimination or wound healing processes, respectively. However, the molecular mechanisms upstream population development of M1/M2 cells need to be investigated in teleost (Grayfer et al. 2018).

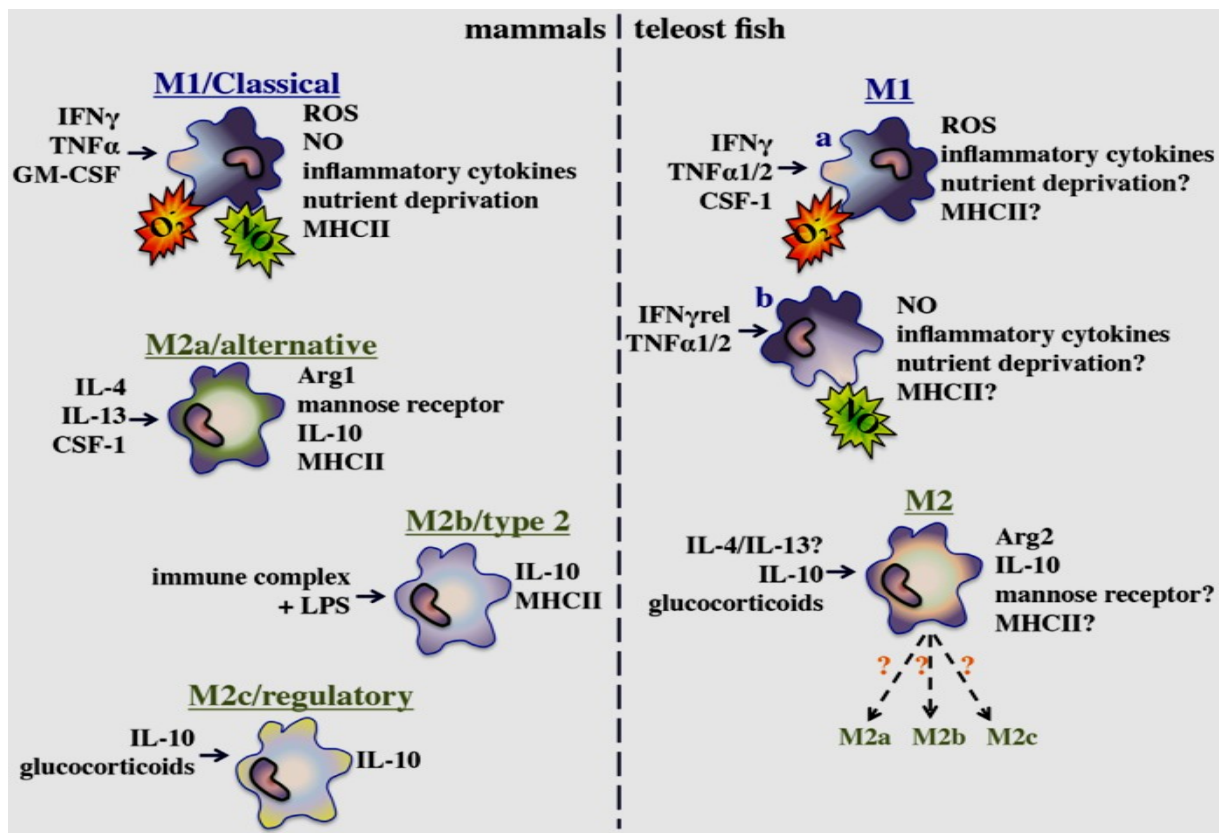


Figure 3. Functional polarization of mammalian and bony fish macrophages
(Grayfer et al. 2018).

Our group widely characterized acidophilic granulocytes in gilthead seabream (Chaves-Pozo et al. 2003; Chaves-Pozo et al. 2004; Sepulcre et al. 2002; Sepulcre et al. 2007; Sepulcre et al. 2011). They display the same functions of mammalian neutrophils (Chaves-Pozo et al.

2005; Sepulcre et al. 2002) and reside in different organs and tissues (Dezfuli et al. 2012; Lui et al. 2013; Sepulcre et al. 2002). Upon infection they are the dominant innate immune cells, since their percentage rise from 20-45% in healthy fishes to 75% of total head kidney isolated cells, being their half-life between 8 and 10 days versus 1 to 4 days for mammalian neutrophils (Sepulcre et al. 2002). Moreover, their stimulation with PAMP induces the expression of some pro-inflammatory components, for example IL-6, PTGS2 (prostaglandin-endoperoxide synthase 2 or prostaglandin H2 synthase), TLR5 in the soluble form, IL-1 β , ROS production, enhance the phagocytic activity and inhibit apoptosis (Sepulcre et al. 2011; Sepulcre et al. 2007).

2.2.2. Humoral components

The humoral components of the innate immune system are circulating proteins or glycoproteins found in the tissues, serum, mucus and eggs of fish that play pertinent roles in the immune response. On one hand, they control self-immune cells development, differentiation, maturity, mobilization survival or apoptosis, as well key immunological functions. In addition, they affect pathogens agents via its growth inhibition, neutralization and elimination (Bols et al. 2001; Grayfer et al. 2018; Zhu et al. 2013). A full list of the humoral agents has been studied in fish, namely cytokines, complement, lysozyme, lectins, antimicrobial polypeptides, eicosanoids, natural antibodies, and protease inhibitors.

Cytokines are a vast group of regulatory molecules in the innate immune response which share low similar identity with human counterparts (Whyte 2007). Teleost cytokines include interleukins (IL), tumor necrosis factor α (TNF α), interferons (IFN) and chemokines.

Interleukins (IL): Interleukin (IL) are classified into six families (1, 2, 6, 10, 12 and 17) (Savan and Sakai 2006). IL-1 family is composed of IL-1, IL-1 β and IL-18, while in contrast

to other vertebrates IL-1 α has not been identified in fish species (Lieschke and Trede 2009). They are potent early response pro-inflammatory cytokines characterized by their pleiotropic activity generally produced in inactive form and stored in the intracellular compartment (Whyte 2007). In fact, IL-1 β , is a widely distributed cytokine detected in several different teleost species (Zhu et al. 2013). In addition, two IL-1 β -like genes have been identified in channel catfish (*Ictalurus punctatus*) and IL-1 β 1 gene seems to be the main gene induced upon bacterial challenge (Wang et al. 2006). It is predominantly expressed in macrophages (Rauta et al. 2012) and neutrophilic granulocytes (Whyte 2007). In gilthead seabream (*Sparus aurata*) it was found that IL-1 β mediates its biological effects through interaction with type 1 IL-1 receptor (IL-1RI) and type 2 IL-1 receptor (IL-1RII) (López-Ruiz et al. 1992). Furthermore, IL-18 is also a constitutively widely distributed cytokine (Zou et al. 2004) which is synthesized by macrophages, dendritic cells, T and B cells (Whyte 2007). IL-18, together with IL-12, enhances IFN- γ production by Th1 T-cells and NK cells (Plouffe et al. 2005). In the IL-2 family, IL-2 has been found to modulate the production of IFN- γ (Díaz-Rosales et al. 2009) and drive lymphocyte differentiation (Corripio-Miyar et al., 2012). IL-12 is also an important inflammatory mediator as it induces IFN- γ secretion (Whyte 2007). IL-6, a member of the IL-6 family (Savan and Sakai 2006), is a multifunctional pro-inflammatory cytokine (Boltana et al. 2018) widely distributed and mainly expressed in the major immune tissues of fish, head kidney, spleen and thymus (Huang et al. 2019). It is a highly inducible cytokine upon immune challenge and leads to (TNF)- α enhancement (Zhu et al. 2016) and is involved in monocytes/macrophages functional modulation (Zhu et al. 2019). IL-10, from the IL-10 family (Savan and Sakai 2006), as described in mammals acts as an anti-inflammatory cytokine, inhibiting the production of pro-inflammatory innate immune mediators, such as IL-6, IL-1 β , IL-8 and TNF- α , while enhancing the expression of suppressor of cytokine signaling 3 (SOCS3) (Huo et al. 2019). In the IL-17 family, IL-17 has been found to regulate the

production of IL-6 and IL-8 (Monte et al. 2013) and to be regulated by the still not fully characterized regulatory T cells (Secombes et al. 2011).

Chemokines are small protein characterized by the chemoattraction of leucocytes mainly neutrophils (de Oliveira et al. 2013), monocytes, eosinophils and lymphocytes to the site of injury during the inflammatory response (Russo et al. 2014; Zlotnik and Yoshie 2000; Zlotnik and Yoshie 2012). They are further involved in homeostasis angiogenesis and angiostatic action which underline their constitutive expression (Russo et al. 2014; Zlotnik and Yoshie 2000; Zlotnik and Yoshie 2012). In teleost fish, the expression pattern of IL-8 was found inducible in response to the inflammatory stimuli (de Oliveira et al. 2013; Oehlers et al. 2010) which leads to the recruitment of leucocytes, like neutrophils and monocytes to site of injury (Harun et al. 2008; Montero et al. 2008).

Tumor necrosis factor (TNF): In mammals, TNF superfamily comprehends 19 members which interact with 29 subtypes. Fourteen distinct members of this superfamily have been identified in teleosts, including TNF- α , CD40L, RANKL (Glenney and Wiens 2007) and Lymphotoxin β (LT- β) (Kono et al. 2006). Some members of the identified TNFs exist in different isoforms in fish, such TNF- α which exist in three types in rainbow trout *Oncorhynchus mykiss* (Hong et al. 2013), while the presence of TNF- β in fish is controversial (Hong et al. 2013; Whyte 2007; Zhu et al. 2013). In teleosts, TNF- α is the most studied and the pivotal roles of TNFs as pro- and anti-inflammatory mediators against infections. In European sea bass, TNF- α play a key role in the protection against *Vibrio anguillarum* via mediating T lymphocytes infiltration in gut mucosal tissue through the activation of chemokine ligand/receptor CCL25/CCR9 pathway (Galindo-Villegas et al. 2013). In gilthead seabream, it is involved in the respiratory burst activation, rapid recruitment of phagocytic granulocytes to the site of stimulation, granulopoiesis activation in the HK in vivo, but also in the proliferation of HK cells in vitro (García-Castillo et al. 2004). In sole

(*Cynoglossus semilaevis*), it enhances IL-1, IL-6, IL-8, IL-27, TLR9 and G3BP during bacterial and viral infection (Li and Zhang 2016).

Interferon (INF): In vertebrates, IFN superfamily is divided into three groups: type I IFNs (IFN- α , IFN- β , IFN- ω , IFN- κ , IFN- ϵ), type II IFNs (IFN- γ), and type III IFNs (IFN- λ) that are structurally and functional divergent and which mediates their actions through cell surface receptors (Zhu et al. 2013). Types I and II IFNs have been characterized from several fish, such as fathead minnow (*Pimephales promelas*) and grass carp (*Ctenopharyngodon idella*). In addition, fish INF regulates macrophages activation, B and T cells proliferation, and participate in viral and bacterial infection (Zhu et al. 2013).

The complement of teleost fish, like in mammals, is activated through three different pathways: the classical pathway enhanced by the immune complex antigen-antibody (Gros et al. 2008), the alternative pathway directly induced by the pathogen, and the lectin pathway activated by the binding of the complex mannose/mannan binding lectin in bacterial cells (Uribe et al. 2011). The mechanism of action of the different pathway consist of 1) The opsonization of the foreign organisms (Zhu et al. 2013), 2) The recruitment and activation of phagocytic immune cells in the site of inflammation (Gasque 2004), and 3) Formation of membrane-attack complexes in the membrane of the pathogen causing its perforation and hence its death (Zhu et al. 2013). The key event of complement system activation is the cleavage of C3 protein into C3a and C3b (Boshra et al. 2006). Its components are largely distributed in teleost tissues, such as HK, gill, intestines, gonads and skin (Encinas et al. 2010). These mechanisms are highly conserved between fish and mammals. The characteristic of poikilothermic organism is that their complement could be activated at low temperature (0 to 4 °C) (Whyte 2007).

Lysozyme is a bacteriolytic enzyme widely distributed throughout the body (Uribe et al. 2011). It acts as a lytic enzyme of the peptidoglycan component of the bacterial cell wall

and can also have an opsonic function (Wang and Zhang 2010). The sources of lysozyme are monocytes/macrophages, neutrophils and mast cells of the intestine (Uribe et al. 2011)

Lectins are sugar-binding proteins that can specifically bind to membrane carbohydrate structures (glycoproteins and glycolipids) and agglutinates cells. Their roles consist of phagocytosis induction, platelet activation, complement system initiation, and natural killer cell activity enhancement (Osorio and Reise Sousa 2011). From the 15 member of the lectin family, the C-type lectins, galectins, F-type lectins, rhamnose-binding lectins, and intelectins have been identified in teleost (Vasta et al. 2011).

Pentraxins and C-reactive protein (CRP) are phylogenetically highly conserved (Rauta et al. 2012) markers of the acute inflammation and help to protect mammals by appearing in the serum during many acute infections (Li et al. 2013). They act in monomeric and pentameric organization in a calcium dependent manner (Falco et al. 2012). The pentameric configuration permit CRP to bind different circulating molecules, such as apoptotic bodies and intracellular components released during cell death (Du Clos 1989; Hicks et al. 1992), microbial components, such as phosphoethanolamine, lipopolysaccharide (LPS) and polysaccharides (Bottazzi et al. 2010) from gram bacteria negative (Mohamed-Jawad et al. 2012). They regulate differentially the cellular immune response through binding in innate immune cell specific receptors, such as macrophage Fc γ RI and Fc γ RIIa, which are phagocytosis stimulators and inducers of inflammatory cytokines, whereas the Fc γ RIIb is an inhibitory receptor of certain signals (Marnell et al. 2005). In fish, this protein has been isolated from a wide range of species, including dogfish (Robey et al. 1987), the rainbow trout (Murai et al. 1990; Murata, Kodama, and Onuma 1995; Jensen et al. 1997), the channel catfish (Szalai et al. 1992), common carp, (Cartwright et al. 2004) and Atlantic cod (Gísladóttir et al. 2009; Magnadóttir 2006). Furthermore, several isoforms of CRP (CRP-like) were found in certain species of

teleosts, such as common carp and zebrafish, which could increase the recognition repertoire of pathogens in animals with less developed adaptive immune systems (Sunyer et al. 2005).

Antimicrobial polypeptides have been found in several teleost species such as sole, carp, winter flounder, coho salmon, bass, tilapia and cat fish (Rauta et al. 2012). Namely histone H2B and three 60S ribosomal proteins, L40, L36A and L35, are bactericidal polypeptides found in the skin mucus of Atlantic cod (Whyte 2007). Antimicrobial polypeptides are found distributed in the mucus, liver and gill where they fight viruses and bacteria (Uribe et al. 2011).

Natural antibodies are component of the innate immune response which are found in the serum of most healthy vertebrates, they are produced by long living B1 (CD5C) lymphocytes in mammals and they offer protection from early developmental stages (Magnadóttir 2006).

Eicosanoids (see below), including prostaglandins such PGE₂ and PGD₂, and their signaling receptors play pivotal role in the inflammatory process in the most divergent group of vertebrates, teleost fish (Gómez-Abellán et al. 2015; Gómez-Abellán and Sepulcre 2016; Montero et al. 2016; Tyrkalska et al. 2016)

2.3. Adaptive immune system of teleost

2.3.1. Cellular components

Lymphocytes are the specialized immune cells of the adaptive immune response. T lymphocytes with non-specific activity, called non-specific cytotoxic cells, are equivalent to mammalian NK cells. They act against tumor cells or cells infected by intracellular organisms including viruses (Secombes, 1997). Several studies based on the differential count of leukocytes have shown that lymphocytes are the most numerous compared to monocytes, granulocytes and thrombocytes in fish while knowing that their innate immunity is more developed than acquired immunity (Rawling et al. 2014). In some species, such as salmon,

trout and cod, B lymphocytes can play the same role as phagocytes. It has been admitted that the B cells of teleosts produce only two classes of immunoglobulins (Ig), IgM and IgD, (Bado-Nilles, 2008). While another isotype, IgT, has more recently been discovered (Zhang et al. 2011). thus, it has been reported that zebrafish and rainbow trout have two different B cell lines: one expressing IgM and IgD, and the other one expressing IgT (Danilova and Steiner 2002; Hansen et al. 2005). More recently, it has been found that these B cell lines play different functional roles (Zhang et al. 2010). Indeed, studies showed in the rainbow trout IgT is an immunoglobulin specialized in immune responses in the intestinal, skin and gill mucosa, while IgM appears to be specialized in systemic immunity (Flajnik and Kasahara 2010).

2.3.2. Humoral components

Generation of the adaptive immune response starts with antigen presentation to lymphocytes via the major histocompatibility complex (MHC). It is known that CD8⁺ cytotoxic T cell enhanced cellular immune response is mediated by MHC class I, while CD4⁺ helper T cell activated humoral immunity via MHC class II. In teleost, both MHC class I and class II were characterized in teleost fish (Zhu et al. 2013).

3. Eicosanoids

3.1. Eicosanoid biosynthesis in vertebrates

Polyunsaturated fatty acids (PUFAs) including dihomo- γ -linolenic acid (DGLA), arachidonic acid (AA), and eicosapentaenoic acid (EPA) (Calder 2001) are important compounds of the phospholipid bilayer of all body cells (Park et al. 2006), including the main innate and adaptive immune cells (Calder and Grimble 2002). Prostaglandins (PGs), thromboxane and leukotrienes are collectively termed eicosanoids (Khanapure et al. 2007).

They are originated from AA (20 carbon small molecules) through cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 monooxygenase pathways (Wang and Dubois 2010).

COX-derived biologically active eicosanoids, mainly those resulting of COX-2 induction, are considerably enhanced during inflammation (Khanapure et al. 2007). Indeed, the phospholipase A2 (PLA2) hydrolyses membrane phospholipids and lead to AA release (Simmons et al. 2004; Hao and Breyer 2008; Smyth et al. 2009). Then, through their *bis*-oxygenase activity, constitutive cyclooxygenase1 (also named prostaglandin endoperoxide synthase) and inducible cyclooxygenase 2 (Cha et al. 2006), catalyze the synthesis of the unstable PGG₂, from the free AA, which is then converted to PGH₂ by their peroxidase activity (Hao and Breyer 2008). PGH₂ is used by five respective synthases: PGD synthase, PGE synthase, PGF synthase, PGI synthase and TA synthase to produce three sets of biologically active forms. The first one is the prostaglandins (PGs), including PGD₂, PGE₂ and PGF₂ α ; the second one is prostacyclin (PGI₂); and the third one is the thromboxane (TA), such as thromboxane A₂ (TA₂) (Khanapure et al. 2007). All these PGs derived from AA are designed as series-2 PGs (Calder 2001).

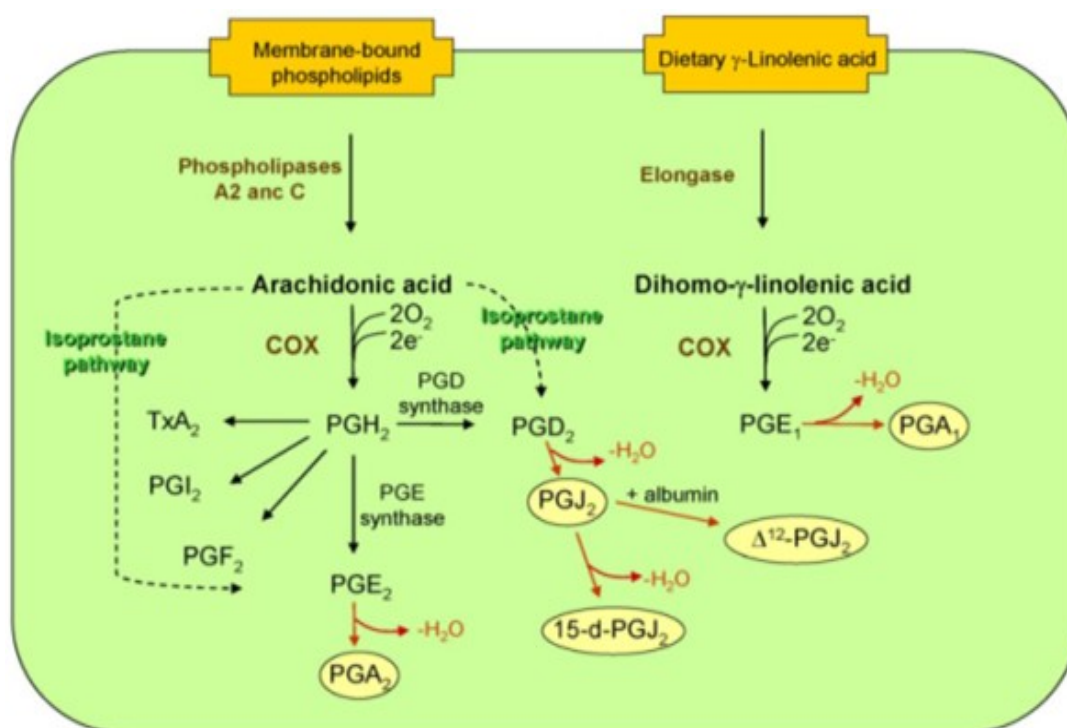


Figure4. Pathways of eicosanoids biosynthesis (Díez-Dacal and Pérez-Sala 2010).

It should be noted that in determined biological conditions, both PGE_2 and PGD_2 could be generated directly by the isoprostane pathway via AA oxidation. In addition to sequential enzymatic intracellular mobilization of AA, some PGs (PGE_1 , PGE_2 and PGD_2) are the target of non enzymatic dehydration, which produces a set of electrophilic lipids (PGA_1 , PGA_2 , and PGJ_2 , respectively) with cyclopentenone structure (cyPG), mainly characterized by a crucial anti-inflammatory effects (**Fig.4**) (Díez-Dacal and Pérez-Sala 2010).

It is important to mention that in contrast to hormones, PGs are neither circulating nor stored molecules, but they are unstable inducible mediators in vivo (**Fig.5**) (Peng and Chen 2017). Rather, its synthetic enzyme PLA2 is constitutively expressed (Park et al. 2006). Indeed, behind the cascade of synthetic reactions, PGs are transported via multidrug resistance-associated protein (MRP) into the extracellular compartment to act on cell surface

specific receptors in autocrine or paracrine manner (Reader et al. 2011). After that, for example endogenous PGE₂ suffers metabolic inactivation in two steps: the first step, via energetically active or passive influx into the intracellular compartment and the second step of oxidative inactivation by rapid degradation through the enzyme 15-hydroxy prostaglandin dehydrogenase (15-PGDH) to a stable 13,14- dihydro-15-keto-PGE₂ (PGEM) metabolite (Chi et al. 2014; Wang and DuBois 2007). PGF₂ is also a target of enzymatic inactivation through 13,14-dihydro-15-keto-PGF₂ (Wang and Dubois 2010).

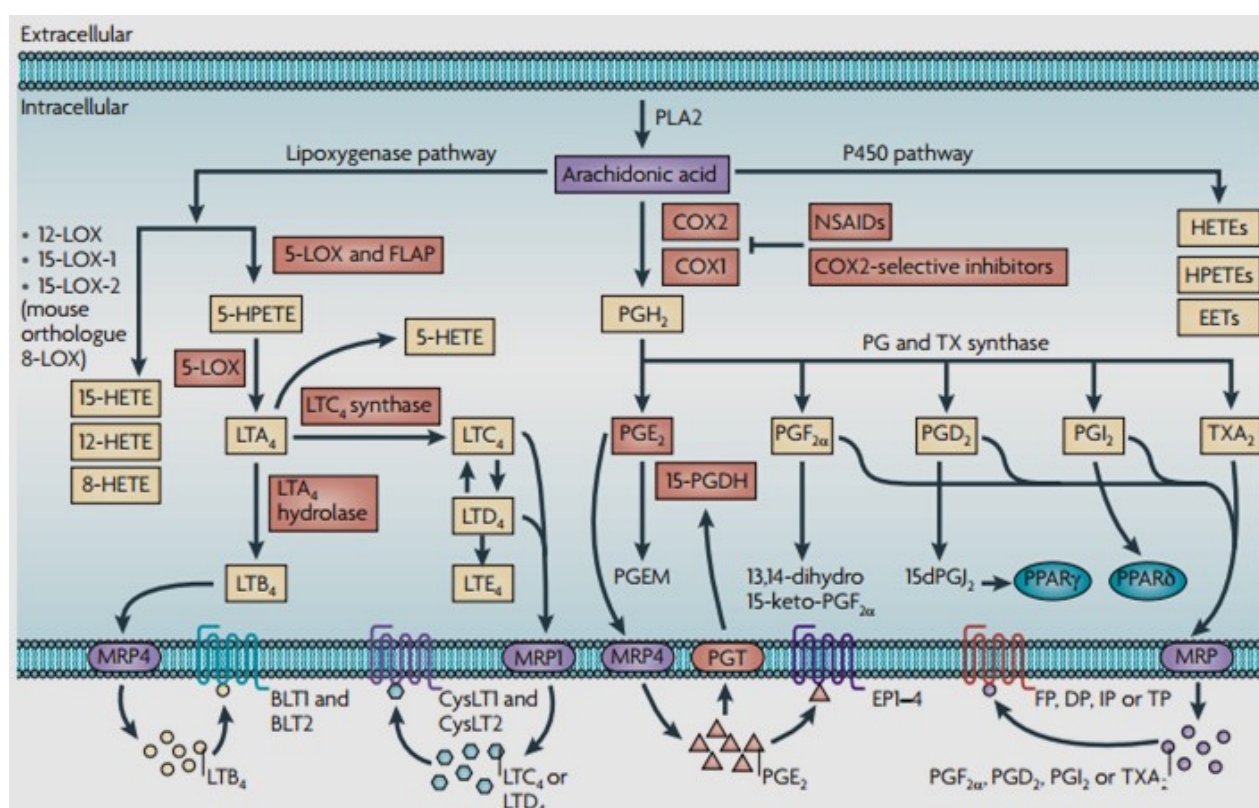


Figure5. Eicosanoid mobilization and transport (Wang and Dubois 2010).

Several pharmacological studies investigated the enzymatic transformations of PGs for the development of strong alternative therapeutic strategies against several diseases. Thus, COX inhibitors are the main target to highlight these strategies and from which started the intervention via NSAIDs (non-steroidal anti-inflammatory drugs). However, it was

interestingly proved that depending on several contexts, such as cellular, tissues environments, cognate receptors as well as prostaglandin bioavailability (Wang and Dubois 2010), PGs and their derivatives could exhibit either pro-or anti-inflammatory actions. From that, it should be taken into consideration the variable parameters that could affect the availability and the performances of these bioactive lipid in order to prevent from undesirable outcomes (Díez-Dacal and Pérez-Sala 2010). In this regard more specific target in PGs signaling has been suggested for future investigation (Wang and Dubois 2010).

3.2. The role of PGs in vertebrate inflammation and immunity

From decades many studies are oriented to explore the involvement of PGs, in several physiological and pathological mechanisms (Gamil et al. 2015; Kobayashi and Narumiya 2002). Physiologically, their potent roles were cited for example in cardiovascular homeostasis (TXA₂, PGI₂, PGE₂) (Khanapure et al. 2007), pulmonary circulation (prostaglandins A, E, F, and B) (Kadowitz et al. 1975) and reproduction (Rossitto et al. 2015). Furthermore, they are involved in several diseases like cardiovascular, gastrointestinal and renal diseases (Khanapure et al. 2007) as well as in different cancers, such as breast, colon, lung and prostate cancer (Wang and Dubois 2010). Additionally, their pivotal roles in the modulation of inflammation is attracting more attention (Khanapure et al. 2007).

3.3. Prostaglandins regulation of the cellular immune response and tissue distribution

Involvement of PGs in the immune response translates the cooperative network between cellular and humoral components. PGs are involved in the processes of cell proliferation, apoptosis and immune behavior (Wang and DuBois 2007). It is important to mention that the proportion of fatty acids could be altered for example by the diet which, in turn, affects plasma membrane fluidity, biosynthesis, metabolism and signaling pathways of phospholipid-derived molecules; key elements of the immune cell function in the immune system and

inflammation (Calder and Grimble 2002).PGs types are differentially produced by immune cells; for example, monocytes and macrophages produce large amounts of PGE₂ and PGF₂, neutrophils produce moderate amounts of PGE₂, and mast cells produce PGD₂(Calder 2001).

In vertebrates, PGE₂ is the main metabolite effectors of COX1 and COX2 (Boatman et al. 2013) and it is the major PG produced during inflammation (Khanapure et al. 2007). It is expressed by all cell types, like fibroblasts, human bone marrow stromal cells (Rodriguez et al. 2014) and innate immune cells (Kalinski 2012) such as macrophages (Harris et al. 2002). Moreover, PGE₂ is not only expressed by different cell types but it regulates the functions of macrophages, dendritic cells, granulocytes, natural killer cells, mast cells, and T and B lymphocytes acting in autocrine and paracrine manner (**Fig.6**) (Hata and Breyer 2004; Kalinski 2012). In the first stage of inflammation, PGE₂ acts locally leading to the vasodilatation and the attraction of the effective cells like neutrophils, macrophages and mast cells. In contrast, during chronic inflammation, it inverts its role to inhibit pro-inflammatory cytokines (Kalinski 2012).

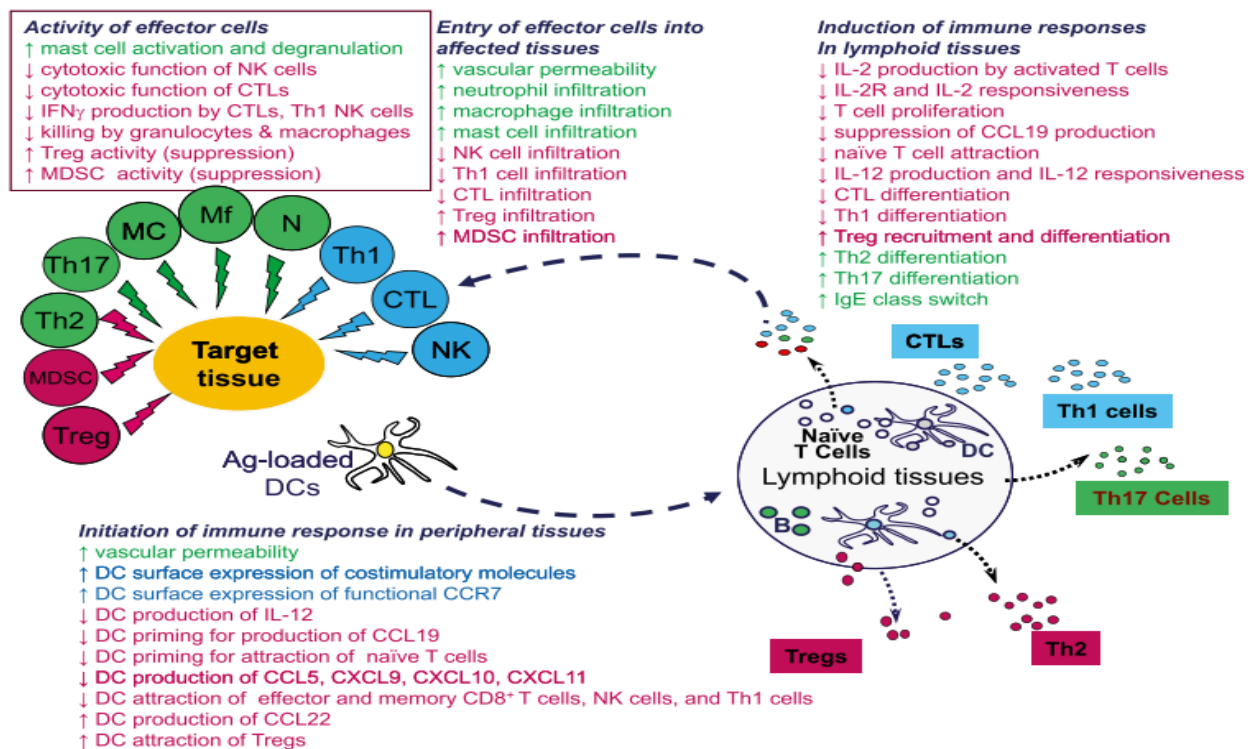


Figure6. PGE₂ regulation of the immune response (Kalinski 2012).

The PGE₂ metabolite PGA₂, has emerging roles in the development of protective and homeostatic environment, particularly in the immune system. In dose dependent manner and in response to chemical as well as biological stresses, it was demonstrated that PGA₂ controls several vital and functional process of the immune cells. Indeed, it mediates the same role of its precursor PGE₂ in TNF- α -induced maturation of human DCs and enhances pro-inflammatory IFN- γ , TNF- α and IL-5 (Thurnher et al. 2005). Furthermore, it was described as an inhibitor of immune cells recruitment through its potent effect on MCP-1 inhibition, a chemokine responsible of T cells, monocytes, microglia, DC and natural killer cells chemoattraction (Storer et al. 2005). In addition, PGA₂ exhibits antiproliferative (growth arrest) effect which is associated with the decline of c-Myc and cyclins, the rise of p21^{WAF1/CIP1} and CIP1, which induces apoptosis through the activation of caspases (Kim et al.

2002), and the induction of the heat shock protein 70 (HSP70)(Holbrook et al. 1992). These later reactions are directed tumor or virus-infected cells, which underlines that PGA_2 act as antineoplastic mediator (Holbrook et al. 1992). All together the cited findings reveal the potent immunosuppressive role of PGA_2 . However, the molecular and functional aspects of this PG are poorly investigated (Lakshmi et al. 2019).

3.4. Prostaglandins and fish immune system

In lower vertebrates, including teleost fish, the presence of PGs has been identified from 1970 (Guo et al. 2015). Indeed, PGE_2 , PGI_2 , $\text{PGF}_{2\alpha}$ and TXA_2 (Cha et al. 2006) and their rate limiting enzymes COXs was revealed in fish (Tian et al. 2017). Their multiple role in different physiological and immunological settings, including early development stages, reproduction, and olfaction system (Fujimori et al. 2012; Henrotte et al. 2011; Poureetezadi et al. 2016; Lai and Hong 2010), lipid metabolism, cell membrane fluidity (Tian et al. 2017), immune response and inflammation were figured out (Holen et al. 2018; Li et al. 2019; Zarini et al. 2014). Importantly, dietary based on AA enrichment revealed its ability to be incorporated in different organs, namely hepatopancreas, muscle, and kidney of grass carp and in other fish species (Tian et al. 2017).

Furthermore, as cited above, the involvement of prostaglandins and their derivatives in the regulation of several teleost species immune system was confirmed (Gómez-Abellán et al. 2015; Gómez-Abellán and Sepulcre 2016; Montero et al. 2016; Tyrkalska et al. 2016). Interestingly, it was proved in zebrafish that PGD_2 plays a relevant role in *Salmonella enterica* serovar Typhimurium (ST) clearance by neutrophils *in vivo* (Tyrkalska et al. 2016). In addition, in gilthead seabream acidophilic granulocytes and macrophages, all of PGE_2 , PGD_2 and its cyclopentenone derivatives (cyPGs) were found to act in the resolution of inflammation against *Vibrio anguillarum* through ROS and inflammatory cytokines modulation, and even

via M2 polarization of macrophages (Gómez-Abellán et al. 2015; Montero et al. 2016). Additionally, LPS-stimulation of Atlantic salmon macrophages with PGE₂ was found effective in dose and time depending manner (Fast et al. 2005). Importantly, it was relatively elucidated that the mediated effect by prostaglandins on the immune response control behind infections is conserved between teleost fish and mammals (Rowley et al. 1995).

To the best of our knowledge there are no available data concerning the physiological as well as immunological role of PGA₂ in teleost fish.

4. Prostaglandins receptors in vertebrates

4.1.PGE₂ receptors characterization in mammals

PGs are synthesized upon cell stimulation to act in autocrine or paracrine manner on the cells in the vicinity. These molecules act by binding to nine types and subtypes of receptors designed as Ptger1–4 for PGE₂, FP for PGF₂, IP for PGI₂, TP for TXA₂ and DP1-DP2 (CRTH2) for PGD₂ (Hata and Breyer 2004; Hirai et al. 2001). All PG receptor belong to rhodopsin-like seven transmembrane spanning G protein-coupled cell surface receptors (GPRs) subfamily (Hata and Breyer 2004). Exceptionally, some CyPGs signal through other receptor types; for example PGA₂, is actively transported in the cell (Storer et al. 2005) to bind the nuclear receptor NR4A3 and regulates target gene expression (Kagaya et al. 2005; Zhu et al. 2013; McMorro and Murphy 2011). From the known PGs, PGE₂ is the unique PG which is able to recognize four different receptors subtypes (Corry 2005; Reader et al. 2011), which reflect its potent effects in the body. In addition, its derived product PGA₂ was shown to be able to bind also EP4, in addition to NR4A3, which may result in polyvalent roles. These receptors are widely characterized in mammals (Jiang and Dingledine 2013; Kashiwagi et al. 2014; Konya et al. 2013; Poureetezadi et al. 2016). In this context, it was admitted that PTGERs, even the ones from the same group (PTGER2 and PTGER4), exhibit distinctive ligand-binding affinity, selective expression, tissue distribution as well as subcellular

localization (Sugimoto and Narumiya 2007; Yokoyama et al. 2013). That is why characterizing these receptors at molecular, structural and functional aspects in vertebrate became of great importance to understand their key roles in the health state and particularly in the immune system.

4.2.Biochemical characteristic of prostaglandin receptors

4.2.1. Structural characterization of PGs receptors

Several key functional aspects of PGs receptors are derived from their structural organization. Structurally they are organized by an N-terminal in the extracellular compartment, three interhelical loops in each side of the membrane and a cytoplasmic C-terminal (Reader et al. 2011). EP1 has also long third intracellular loop (Sugimoto and Narumiya 2007). EP2 is characterized by compact structure (Sugimoto and Narumiya 2007), a short third intracellular and C-terminal domains (Bastepe and Ashby 1999) and, exceptionally, by an extracellular region involved in structure and /or function of the receptor (Stillman et al. 1999). In the gene coding for EP2, the presence of the CAAT box in the 5'UTR (Locker and Buzard 1990) as well as polyadenylation signal in 3'UTR were noted (Smock et al. 1999). In addition, the presence of consensus binding sites for several transcription factors, including NF-KB, KLF, HMG-box binding, and GATA3 families (Smock et al. 1999). Furthermore, recently the residues (Y196 on TM5 and Y288on TM6) responsible of the interaction with PGE2 has been determined (Suganami et al. 2016). EP3 is characterized by the existence of different variants generated by alternative splicing of the C terminus region. EP4 has the longest third intracellular loop (Sugimoto and Narumiya 2007) and a long C-terminal tail which is important for short term desensitization of the receptor. The conserved aspartate in the second TMD (receptor ligand interaction) cysteine in the second and the third EL (form disulfide bond) characteristic of the GPCRs (Savarese and Fraser 1992) and the conserved arginine of the seventh TMD (Narumiya et al. 1999) are

conserved in EP4 among vertebrates (Yokoyama et al. 2013) as well as threonine and serine residues of the C-terminus responsible of agonist-induced short-term desensitization (Bastepe and Ashby 1999) and internalization (Neuschäfer-Rube et al. 2004). Moreover, pertinent binding sites of regulatory elements are scattered in the form of mosaic in EP4 gene such as the repetitive CCAAT boxes (Arakawa et al. 1996; Foord et al. 1996) as well as binding sites of proinflammatory and cancer related mediators are characterized in human and mouse EP4 gene, like NF-IL6 (nuclear factor interleukin (IL-6), AP-1 (activator protein-1), AP-2 (activator protein-2), NF- κ B (nuclear factor-kappa B), SP-1, IL6, CRE, PIT 1 and E-BOX (Arakawa et al. 1996; Foord et al. 1996; Seira et al. 2018). The amino acid identity between EP receptors is weak (28-33%) even between the one from the same group (EP2 and EP4). However, they share higher identity with other prostanoid receptors (Sugimoto and Narumiya 2007).

4.2.2. Interactive ligands with PGs receptors

Ligand concentration, conformation and binding affinity are of primordial importance in PG receptor mediated functions (Bilson et al. 2004; Markovič et al. 2017). Interestingly, it is crucial to mention that EP1-EP4 receptors are able to bind alternate PGs (PGE₂ analogs) rather than the native (cognate) ligand (PGE₂) in addition to pharmacologically developed agonists and antagonists molecules (Hata and Breyer 2004; Leduc et al. 2009; Markovič et al. 2017; Suganami et al. 2016), paving the way for receptors subtype-selective activity. Indeed, regarding endogenous PGE₂, it binds with higher affinity EP3 and EP4 than EP1 and EP2 (Ricciotti and FitzGerald 2011). Furthermore, it is crucial to mention that the characterized EP3 isoforms have the same ligand binding affinity (Bilson et al. 2004). However, from decades other agonist which are able to bind all the receptors, but with lower affinity and with not detected specificity for each receptor, except butaprost which binds only on EP2, were

defined (Narumiya et al. 1999). Interestingly, since PGE₂ is characterized by an extremely short half-life and while it could be involved in several undesirable effects, like lethargy, headache, diarrhea flushing and inflammation, highly selective receptors agonists and antagonists were recently developed as alternative EP modulators for the treatment of several diseases, such as ulcerative colitis, glaucoma, bone healing, B cell lymphoma and neurological diseases (Markovič et al. 2017).

4.3.Receptors signaling pathways

In mammals, prostanoid GPCR receptor were identified and then their genes structure, domain organization, specially the sequences coding for ligand binding site, and the signaling pathway were all determined (Narumiya et al. 1999). EPs are widely conserved between mammals species (Dey et al. 2006) while their amino acid identity between each other is weak, even between the one from the same group (EP2 and EP4) which range from 28 to 33 %. However, they share higher identity percentage with other prostanoid receptors (Sugimoto and Narumiya 2007). These receptors were divided into three groups regarding the second messengers used to transduce the intracellular signal, cyclic adenosine monophosphate (cAMP) or calcium (Ca²⁺), via receptors C terminal tail coupling to different G protein subunits (Kim et al. 2013) (**Fig. 7**)

The first group are designed stimulatory receptors, to which belong EP2 and EP4 receptors (in addition to IP and DP receptors), and are coupled to G_{as}-type G protein that stimulates adenylyl cyclase (AC) which convert ATP to cAMP and activates PKA (Fujino et al. 2002; Sheppe et al. 2018). The described pathway could be involved in gene transcription alteration by phosphorylation of cAMP-responsive binding element (CREB) (Sheppe et al. 2018). Both of EP2 and EP4 are implicated in the phosphorylation activation of Akt kinase (known as PKB) followed by phosphorylation inhibition of GSK-3 β which, in turn, inhibits the

transcriptional mediator β -catenin. (Dey et al. 2009; Fujino and Regan 2006; Kawahara et al. 2015). However, EP4 downstream signaling was found to be more complex, since it further couples to pertussis toxin-sensitive $G_{\alpha(i)}$ and involved activation of the transcription factor EGR-1 (Early growth response factor-1) in a way depending of G_{α_i} /PI3K/ERKs (extracellular signal-regulated kinases) and cAMP inhibition (Fujino and Regan 2005; Fujino et al. 2003). In addition, EP4 activation results in β -arrestin mediated signaling (Markovič et al. 2017). Interestingly, stimulatory receptors pathways were found to be involved in normal cells growth and differentiation, whereas they play antiproliferative effects in cancer cells (Dey et al. 2006; Fujino et al. 2003).

The second group coupled to $G_{\alpha q}$ -type G protein, to which belong EP1 receptor (in addition to TP and FP receptors). EP1 is implicated in the activation of phospholipase C (Dey, et al. 2006) leading to downstream phosphatidyl inositol metabolism to inositol trisphosphate and intracellular free Ca^{2+} increase (Hosono et al. 2016; Ricciotti and FitzGerald 2011). It was shown that EP1 increases intracellular Ca^{2+} through receptor-activated Ca^{2+} channels (Reader et al. 2011). It is further involved in EGFC and EGFR activation via c-Src and Akt, respectively (Dey et al. 2006)

The third group is the group of inhibitory receptors that include EP3 and is defined predominantly by decreasing cAMP production through $G_{\alpha i}$ -type G protein (Narumiya, et al. 1999) upon inhibition of AC (Kawahara et al. 2015). However, other variants generated by alternative splicing in EP3 C-terminal tail were identified in human, mice, rat, rabbit and bovine (Sugimoto and Narumiya 2007). The divergent variants are able to couple other different types of G proteins, such as $G_{\alpha s}$, $G_{\alpha i}$ and $G_{\alpha 12/13}$ (Tang et al. 2017) which leads to cAMP or Ca^{2+} enhancement, so they may play contradictory functions (Hata and Breyer 2004).

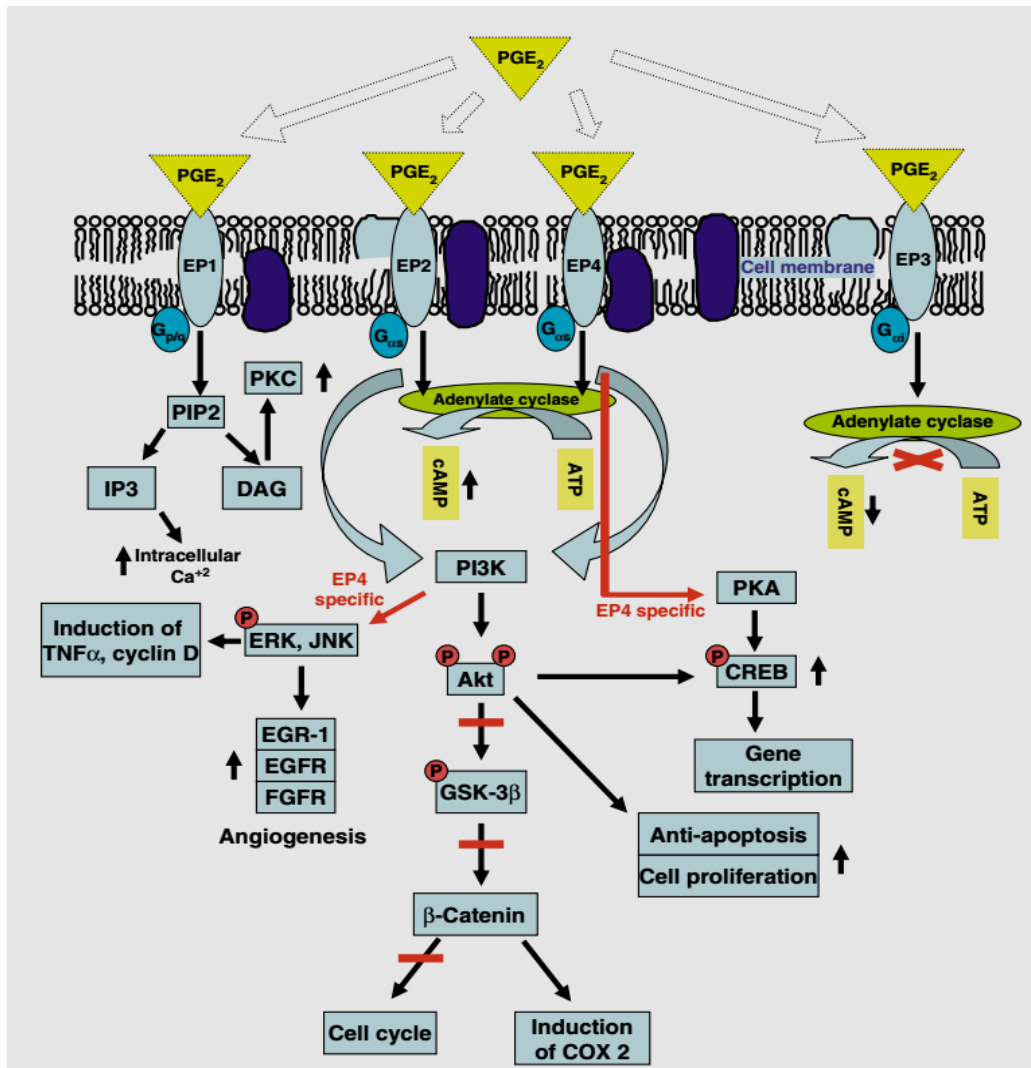


Figure 7. EP downstream signaling (Dey et al. 2006).

4.4.Receptors induced desensitization and internalization

From the main structure related modulators of GPCR receptors is their ligand induced sensitivity translated by their desensitization and internalization. The mechanism of GPCR desensitization and internalization is mediated by phosphorylation of serine and threonine residues on the third intracellular and/or C-terminal domains, via cAMP dependent protein kinase as well as G protein coupled receptor kinase (GRKs), resulting in uncoupling of the receptor from its G protein sequestration and endocytosis in intracellular compartment vesicles (Bastepe and Ashby 1999). β -arrestin and other molecules are involved in the

mechanism of receptors trafficking (Bilson et al. 2004; Desai and Ashby 2001). In human EP3 isoforms differ in PGE₂ induced desensitization (Negishi et al. 1993) and internalization through the C-terminal tail, exhibiting different subcellular localization. EP4 is also the target of PGE₂ induced rapid desensitization (10 min) (Nishigaki et al. 1996) and internalization with cooperation of β -arrestin molecule independently of MAP kinase activation pathway (Desai and Ashby 2001). It was suggested that the processes of receptors desensitization and internalization are a form of downregulation (Bilson et al. 2004; Bastepe and Ashby 1999). From these findings, it is clear that EPs present different sensitivity regarding the same primary ligand and agonist induced receptors sensitivity, widely dependent of the time which reflects a balance between their mediated effect during the course of cell signaling.

4.5.Receptors distribution and subcellular localization

Histological distribution and cytological receptors localization are important factors that give information about their related functions (Markovič et al. 2017). PGE₂ is known by its ubiquitous distribution and its mediated pleiotropic effects in different cell types. While, EPs are characterized by their functional divergence this fact was found related to specific site of expression. Several studies proved that EP3 and EP4 are ubiquitous receptors. EP1 detected in determined tissues while EP2 is the less expressed receptors (Ricciotti and FitzGerald 2011).

At cellular level, EP3 exhibits different subcellular localization and different isoforms could be found in plasma membrane, cytoplasmic compartment or perinuclear membrane (Bilson et al. 2004). Several works have shown that EPs mediated PGE₂ effects are predominantly through the four plasma membrane receptors. However, other studies have further shown that these receptors may exist in different subcellular localization. Indeed, it has been demonstrated that EP3 and EP4 are found on nuclear membranes. (Bhattacharya et al. 1999; Dey et al. 2006). It was shown that EP4 receptors translocate from Golgi apparatus to

plasma membrane during nervous system development (Davidson et al. 2016). Other evidence mentioned that the four receptors are localized in the nuclear membrane but with not defined role (Reader et al. 2011). Furthermore, intracellular localization of EP3 has been discussed (Hasegawa et al. 2000) and EP4-receptor-associated protein (EPRAP) allows the interaction with NF- κ B, leading to the suppression of pro-inflammatory proteins (Markovič et al. 2017).

4.6. Receptors modulating cellular inflammation and immune response

Table 1: Physiological and pathophysiological functions of EP receptor subtypes (Sugimoto and Narumiya 2007).

Sybttype	Function
Ptger1	Mediates stress responses (ACTH secretion and stress behavior) Promotes chemical carcinogenesis Mediates inflammatory thermal hyperalgesia
Ptger2	Facilitates ovulation and fertilization Mediates intestinal polyp formation in <i>Apc</i>^{A716} mice 4 Facilitates pain transmission by abolishing glycinergic inhibition Mediates joint inflammation in collagen-induced arthritis Suppresses dendritic cell differentiation Facilitates neutrophil recruitment by G-CSF production Promotes amyloid- formation in Alzheimer's disease Mediates COX-2-induced mammary hyperplasia
Ptger3	Mediates fever generation Suppresses type I allergy Mediates angiogenesis associated with tumor and

	chronic inflammation
	Regulates duodenal secretion
	Induces endotoxin-elicited enhanced pain perception
	Mediates pain associated with virus infection
Ptger4	Facilitates closure of ductus arteriosus
	Induces bone formation
	Protects against inflammatory bowel disease
	Facilitates Langerhans cell migration and maturation
	Mediates joint inflammation in collagen-induced arthritis

The major symptoms of inflammation are edema vascular permeability and cellular infiltration. In response to multiple stimuli PGE₂ with cooperation of its receptors are widely involved in these different steps (Kawahara et al. 2015). In this context, it was found that EP1 and EP3 are predominantly pro-inflammatory effectors, while EP2 and EP4 are mainly anti-inflammatory mediators. However, opposite actions of receptors even between the one which belong to the same group are revealed in particular physiological environments (Tilley et al. 2001). For example, vascular permeability is mediated by mast cells histamine release (Kawahara et al. 2015). In correlation with this, mast cells attraction, activation and their degranulation were found to be enhanced by EP1 and EP3, while both of EP2 and EP4 are able to oppose the cited roles (Kalinski 2012; Tilley et al. 2001). Indeed, in a specific model this phenomenon was described by the fact that in response to irritants, such as chemical irritants and PAMPs, a COX-1/PGE₂ pathway activated in keratinocytes and the released PGE₂ enhanced EP3/Gi/PI3K pathway in neighbor mast cells, which leads to PIP₃ and Ca²⁺ increase and permit to evoke histamine degranulation accompanied with vascular

permeability in one hand and, in the other part, enhances IL-6 production that promotes immune cell recruitment (**Fig. 8**) (Kawahara et al. 2015). EP1 and EP3 are involved in inflammation pain response (Hata and Breyer 2004)

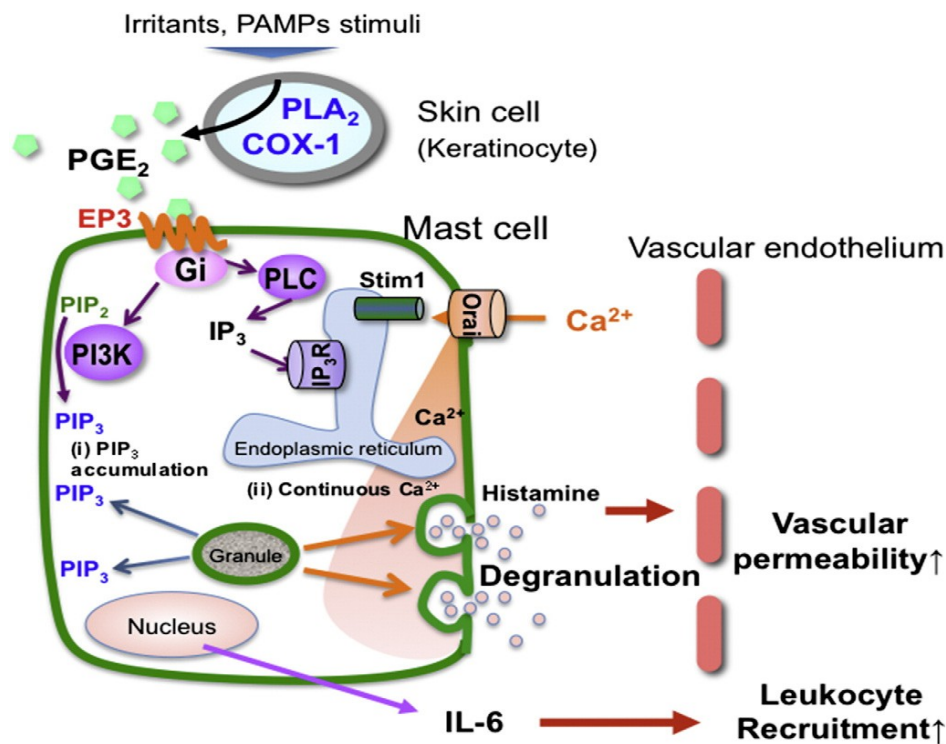


Figure 8. PGE₂ mediated EP3-mast cells activation in acute inflammation (Kawahara et al. 2015).

Depending from several conditions including cell types, infection model and virulence factors of pathogenic bacteria (Sheppe et al. 2018), EP2 and EP4 show differential modulation in some context (Akaogi et al. 2004; Bonfill-Teixidor et al. 2017; Yamane et al. 2000) but also synergic effects in others or even selective action. Indeed, it was widely proved *in vivo* and *in vitro* (Birrell et al. 2015; Sakamoto et al. 2004; St-Jacques and Ma 2011) that in response to varied stimuli (Sheppe et al. 2018) or during inflammatory diseases (Birrell et al. 2015; Sakamoto et al. 2004; Takayama et al. 2002) their immunosuppressive effects were underlined by the release inhibition of the classic innate immune cytokines cluster, such as

TNF- α , IL-12 (Ikegami et al. 2001), IL-8, MCP-1, MIP-1, MIP-1, IP-10 (Takayama et al. 2002) and MHC class II (Harizi, Grosset, and Gualde 2003) together with the enhancement of the anti-inflammatory cytokine IL-10 (Harizi et al. 2003; Németh et al. 2009; Yun et al. 2016), leading to immunosuppressive effects observed mainly on in the innate immune cells, such as dendritic cells (Harizi et al. 2003), neutrophils (Yamane et al. 2000), macrophages (Akaogi et al. 2004; Takayama et al. 2002), microglia and lymphocytes (Dutton et al. 2018; Li et al. 2017). However, in the pro-inflammatory environment EP4 was found more effective (Hata and Breyer 2003; Narumiya 2009). It was confirmed that predominantly PGE₂-EP4 upregulation and activation in human macrophages is effective against *Salmonella entericaserovar* Typhimurium by the induction of inflammasome, IL-8 and IL1- β , which was enhanced by the effect of the bacterial virulence factors (Sheppe et al. 2018). In this experimental model, eicosanoid signaling promoted M1 phenotype to stimulate bacterial clearance. However, in the same model *Yersinia enterocolitica* virulence factors have shown opposite effect by suppressing eicosanoid signaling (**Fig. 9**) (Sheppe et al. 2018). Herein, EP2 has limit effect in PGE₂ induced inflammation (Sheppe et al. 2018). In addition, EP4 via PI3K pathway enhances IL-8 in human activated T cells (Caristi et al. 2005). In injured nerve, PGE₂ upregulated IL-6 through EP4 (St-Jacques and Ma 2011). In another case, such as during embryonic implantation, EP2 and EP4 were differentially modulated in response to the same stimuli (LPS) in macrophages where EP2 was more significantly induced than EP4, suggesting that they do not play the same cellular role (Sugimoto, et al. 2000). Nevertheless, both EP2 and EP4 are enhanced during LPS-stimulated B cell switch class into IgE secreting profile (Fedyk and Phipps 1996).

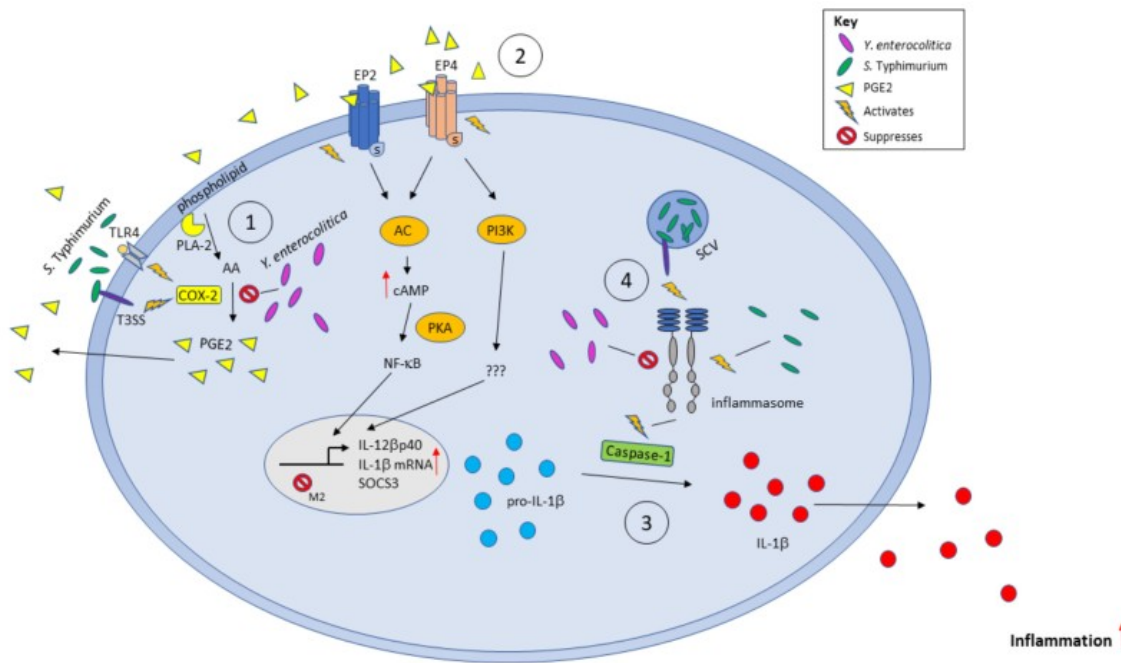


Figure 9. EP2 and EP4 mediated inflammation in response to bacterial infection (Sheppe et al. 2018).

EP4 acts on DCs and T cells leading to IL-23-dependent Th1 differentiation into Th17 profile. Similar effect registered by EP2. In early stages of atherosclerosis, EP4 was found to modulate the pro-inflammatory cytokines IL-1β, IL-6 and monocyte chemotactic protein-1. However, in response to LPS-induced neuroinflammation both of EP2 and EP4 exhibited suppressive effects in microglial cells (Ricciotti and FitzGerald 2011).

G protein-independent actions of prostanoids in inflammation are mediated by direct binding of prostanoids on nuclear receptors that control genes expression and inhibit intracellular target proteins. The main mechanism behind expression deletion is underlined by the inhibition of IκB kinase (IKK), which in response to pro-inflammatory stimuli activates NF-κB (Tilley et al. 2001).

Our group widely explored PGs (PGE₂, PGD₂, PGA₂) mediated immune and inflammatory response in zebrafish (Tyrkalska et al. 2016) and gilthead seabream (Gómez-Abellán et al.

2015; Gómez-Abellán and Sepulcre 2016; Montero et al. 2016). The collected data paved the way to deep insight into the signaling pathways of these molecules.

5. PGE₂ receptors identification in teleost fish

Researches based on the investigation of Ptger1-Ptger4 in lower vertebrate comprehending teleost fish are attracting more attention. In this context different tools involving cloning molecular phylogenetic analysis as well as pharmacological approach (Guo et al. 2015; Hagiwara et al. 2014; Henrotte et al. 2011; Rong et al. 2016; Tsuge et al. 2013) were used to explore the receptors at different levels. Indeed, Ptger1-Ptger4 nucleotide and polypeptide sequences were characterized in teleost fish (Guo et al. 2015; Iwasaki et al. 2013; Fujimori et al. 2012). To the best of our knowledge, genomic analysis based on the available *in silico* data revealed the existence of varied orthologues of Ptger1, Ptger2 and Ptger4 but not for Ptger3 in teleosts (**Table 2**). The isoforms of each ancestral receptor differ mainly in their C-terminal tail hence it is not surprising that they offer varied abilities to bind distinct G protein subunits and to evoke related downstream second messenger activation, what was seen for example in zebrafish Ptger1a and Ptger1b activating cAMP-PKA pathway (Kwok et al. 2012). The fact that constitute a common characteristic with higher vertebrates (Kim et al. 2013) or suggesting that multiple orthologs are the source of differential desensitization and internalization of the receptors, since it was proved that docosahexaenoic acid (DHA) derived from fish oil is able to raise β -arrestin (Liu et al. 2014), which is involved to receptors internalization, as defined in human (Bilson et al. 2004). Furthermore, the question if teleost receptors isoforms are the results of alternative splicing or gene duplication need to be resolved.

Table 2. Examples of PGE₂ receptors isoforms in some teleost species.

Species	Identified isoforms	Reference
Zebrafish	Ptger1a-c/ Ptger2a-b/Ptger4a-c	(Kwok et al. 2012;Tsuge et al. 2013)
Medaka	ptger1a-b / ptger4a-b	(Fujimori et al. 2011)
Atlantic salmon	Ptger4a-b	(Guo et al. 2015)

5.1. Structural feature of teleost Ptgers

Structurally as in higher vertebrate counterpart, teleost Ptgers subtypes are configured in seven transmembrane domains that signal in response to PGE₂ via coupling to GPR protein. They are arranged in an extracellular N-terminal region and cytosolic C-terminal tail. They share high percentage of similarity with their human and mice counterparts, especially at second extracellular loop, third and seventh transmembrane domain, the site of interaction with G protein (Iwasaki et al., 2013; Kwok et al.,2012; Lai and Hong 2010 ;Tsuge et al., 2013). Furthermore, conserved structural as well as functional regulatory key residues elements between teleost PGE₂ receptors, amphibian, bird and mammals were figured out (for details see chapter I). Furthermore, Ptger4b is the main conserved receptor among fish species (Guo et al. 2015; Hagiwara et al. 2014) and the closest to human homologue receptor. However, zebrafish Ptger2 appears in the same cluster as HDP receptor (Tsuge et al. 2013).

It has also been found that in teleost PGE₂ binds to cells membrane receptors with different affinity (Iwasaki et al. 2013; Kwok, et al. 2012). However, some exception exist, for example zebrafish Ptger1c respond to PGF_{2a} mediated Ca²⁺ signaling (Kwok et al. 2012).As described in mammals, in teleost Ptger2 and Ptger4 mediate cAMP increases (Tsuge et al. 2013). It was

determined that Ptger4 further induce GP/PI-3K/AKT during zebrafish gastrulation (Cha et al. 2006). Moreover, Ptger4 cAMP-independent signaling has been suggested in Atlantic Salmon (Guo et al. 2015).

5.2.Distribution of Ptger1-Ptger4 in teleost tissues

The tissue mRNA expression profile of Ptgers subtypes has been determined in a few number of teleost fish species. From the three identified zebrafish Ptger1 isoforms Ptger1a and Ptger1b are widely distributed (Kwok et al. 2012) and in contrast to mammals Ptger1 shows ubiquitous expression in *Bostrichthyssinensis*, since it was detected in all examined tissues, reflecting the polyvalent physiological roles of this receptor in teleosts and suggesting also its involvement in the immune system (Lai et al. 2014). For Ptger2 it was identified in *B.sinensis* olfactory system (Lai and Hong 2010) as well as in Eurasian perch ovary (Henrotte et al. 2011), and the two isoforms (Ptger2a and Ptger2b) in zebrafish kidney, spleen, gill, liver, intestine, but their level of expression is different (Tsuge et al. 2013). Ptger2 like receptor was identified in rockfish (*Sebastes caurinus*) hepatocytes (Busby et al. 2002). Studies investigating Ptger3 are very limited. Regarding Ptger4, from the different identified isoforms, Ptger4b was ubiquitously expressed in Atlantic salmon and in zebrafish, while the other isoforms have limited distribution, hence the authors suggested that Ptger4 isoforms are the target tissues or cells type selective expression (Tsuge et al. 2013). Furthermore, Gamil et al (2015) explored the distribution of this receptors in several localization in Atlantic salmon and, importantly, they revealed its expression in the majority of chosen tissues mainly in spleen, HK, liver, skin, gill and fin.

5.3. Ptgers physiological implications in teleost

Ptgers roles has been demonstrated in a range of physiological mechanisms, such as reproduction, metabolic regulation, fever and the immune response (Boltana et al. 2018; Fujimori et al. 2011; 2012; Henrotte et al. 2011). Ptger1 and Ptger3 are involved in teleost gill epithelium ion transport (Evans et al. 2004), Ptger3 is also involved in retinal vascular angiogenesis (Chen et al. 2017) and fever generation (Boltana et al. 2018). All Ptgers, including Ptger2, were shown to play a role in response to PGE₂, which acts as sex pheromone and hormone in the reproductive behavior and pheromonal communication (Lai and Hong 2010). Ptger4 is involved in gastrulation, ovulation and ciliogenesis (Cha et al. 2006; Fujimori et al. 2012; Hagiwara et al. 2014; Hilton and Johnson 2007; Kolmakov et al. 2008; Lai and Hong 2010; Pouretezadi et al. 2016).

Their pathophysiological roles were weakly explored in a few numbers of species (Boltana et al. 2018; Evans et al. 2004; Feng et al. 2012; Hagiwara et al. 2014) and, importantly, several of these roles were found conserved among vertebrates. Teleosts are exposed to wide range of pathogens such as parasites, bacteria and virus that threat their health, quality of meat and survival. Hence, it is important to study how their immune system could be armed against the microorganism or their derived PAMPs through PGs mediated receptors function.

6. Mammalian NR4A3

6.1. Molecular and structural characteristic of nuclear receptors

In general, nuclear receptors (NRs) have common structural organization, which is divided in 5 domains with different biological actions and differing in the degree of conservation between the different groups. Structural as well as functional investigation of these receptors was done mainly by molecular cloning and crystallographic studies (Helsen and Claessens 2014). Indeed their domains organization consist of:

1) **N-terminal domain** (NTD) comprehending ligand-independent activation function (AF-1). This domain is highly variable in size and sequence between the NRs (Brélivet et al. 2012) and, in fact, it has been considered an intrinsically disordered region. It interacts with cofactors and other proteins of the transcription machinery (Helsen and Claessens 2014).

2) **DNA binding domain** (DBD). This domain is highly conserved and contains two zinc finger motifs (Helsen and Claessens 2014). These motifs interact with NR-responsive elements (NRREs) on DNA (Gadaleta and Magnani 2014). The NRREs correspond to inverted repeats, direct repeats or no repeats within the DNA sequence (Gadaleta and Magnani 2014; Porter et al. 2019).

3) **Hinge domain** (flexible region between DBD and LBD) containing a nuclear localization signals (NLS) and target site of posttranslational phosphorylation, acetylation, methylation and sumoylation. This flexible region regulates intracellular trafficking and is responsible of the connection between DNA and ligand depending binding domain (Helsen and Claessens 2014).

4) **Ligand-binding domain** (LBD) contains hydrophobic pocket composed of 75% hydrophobic residues and also polar residues enhancing ligand binding (Weikum et al. 2018), motifs responsible for dimerization and co-regulator-binding regions, and a ligand-dependent transactivation function, termed AF-2 (Gadaleta and Magnani 2014; Germain and Bourguet 2013).

5) **C-terminal region**. This region is variable between NRs (Brélivet et al. 2012; Kagaya et al. 2005; Li et al. 2006).

6.1.1. Nuclear receptor mechanism of action

Depending on how NRs bind to DNA target regions (direct repeat or inverted repeat), dimerization (homo or hetero), ligand specificity (required or not required), the NRs were divided into four groups (Porter et al. 2019; Weikum et al. 2018; Gadaleta and Magnani 2014) (**Fig. 10**).

The first group is the group of steroid receptors that homodimerize on two DNA inverted repeats. They comprehend the estrogen receptor (ER), the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), the progestagen receptor (PR) and the androgen receptor (AR).

The second group of non-steroid receptors which heterodimerize on DNA direct repeats in complex with RXR (Retinoid X Receptor), such as PPAR (peroxisome proliferator-activated receptor), but some receptors of this group are able further to homodimerize. They are called metabolic receptors.

The third group consists of the named orphan receptors which are dimeric DNA-direct repeat sequences binders.

The fourth group are orphan receptors such as Nur77, SF1 (steroidogenic factor 1) and ERR2 (estrogen receptor-related receptor 2) receptors, which are monomeric DNA-extended half-sites binders.

6.1.2. Ligand of nuclear receptors

It was widely elucidated that most NRs, excluding orphan receptors, are ligand depending-transcription factors since they are responsiveness to hormones, vitamins, endogenous small lipophilic metabolites (Gadaleta and Magnani 2014; Zhao et al. 2015) and lipids that diffuse

or are transported across the cell membrane (Weikum et al. 2018). Importantly, it was elucidated that synthetic product like pharmaceuticals, endocrine disrupting chemicals and some industrial compounds are able to generate toxic responses via NRs LBD (Zhao et al. 2015). Nevertheless, in the literature orphan nuclear receptor (ONRs) nomenclature does not include only receptors that do not have a ligand but also receptors which have not known regulatory ligand or the called endogenous (native) bona fide ligands, although the ligands of some of them were recently identified (Gadaleta and Magnani 2014; Pearen and Muscat 2010). Structurally, the ONRs appear to have a ligand binding pocket smaller of size even than small molecules (Pei et al. 2005). From this it was considered that ONRs, mainly NR4A subfamily, induce modulation of genes expression independently from LBD activation (Nomiya et al. 2006). In contrast to these findings, it was suggested that some orphan receptors are able to bind several small molecule agonists and fatty acids (Pearen and Muscat 2010; Weikum et al. 2018). Ligand binding induce conformational change of the receptor from inactive to active form (Gadaleta and Magnani 2014). Furthermore, ligand selectivity is defined by the size of ligand binding pocket between NRs.

6.2.Subcellular localization and cytological distribution of nuclear receptors

The metabolic receptors are located exclusively in the cytoplasm in complex with chaperone protein which upon activation they translocate to the nucleus to mediate their function. Metabolic and orphan receptors are mainly located in the nucleus but also could be found in the cytoplasm (Gadaleta and Magnani 2014; Sala and Ampe 2018). Whence activated the NRs bind on DNA regulatory regions of their target genes named response element then induce transcription by recruiting specific co-regulators and components of the transcription initiation complex or RNA polymerase II. (Helsen and Claessens 2014) either for

transactivation or transrepression (Gadaleta and Magnani 2014; Weikum et al. 2018). It was further shown their involvement in the control of other cell processes, such as acetylation, phosphorylation, sumoylation and ubiquitination (Gadaleta and Magnani 2014). The cytoplasmic localization of these receptor, including Nur77 (NR4A1), Nurr1 (NR4A2) and Nor1 (NR4A3), favors their apoptotic role through mitochondrial intervention (Nomiyama et al. 2006). In the nucleus, they bind on the same DNA NGFI-B-responsive element (NBRE) sequence (AAAGGTCA) as monomers or to a palindromic NurRE sequence (TGATATTTX6AAATGCCA) as homodimers (Pei et al. 2005; Nomiyama et al. 2006).

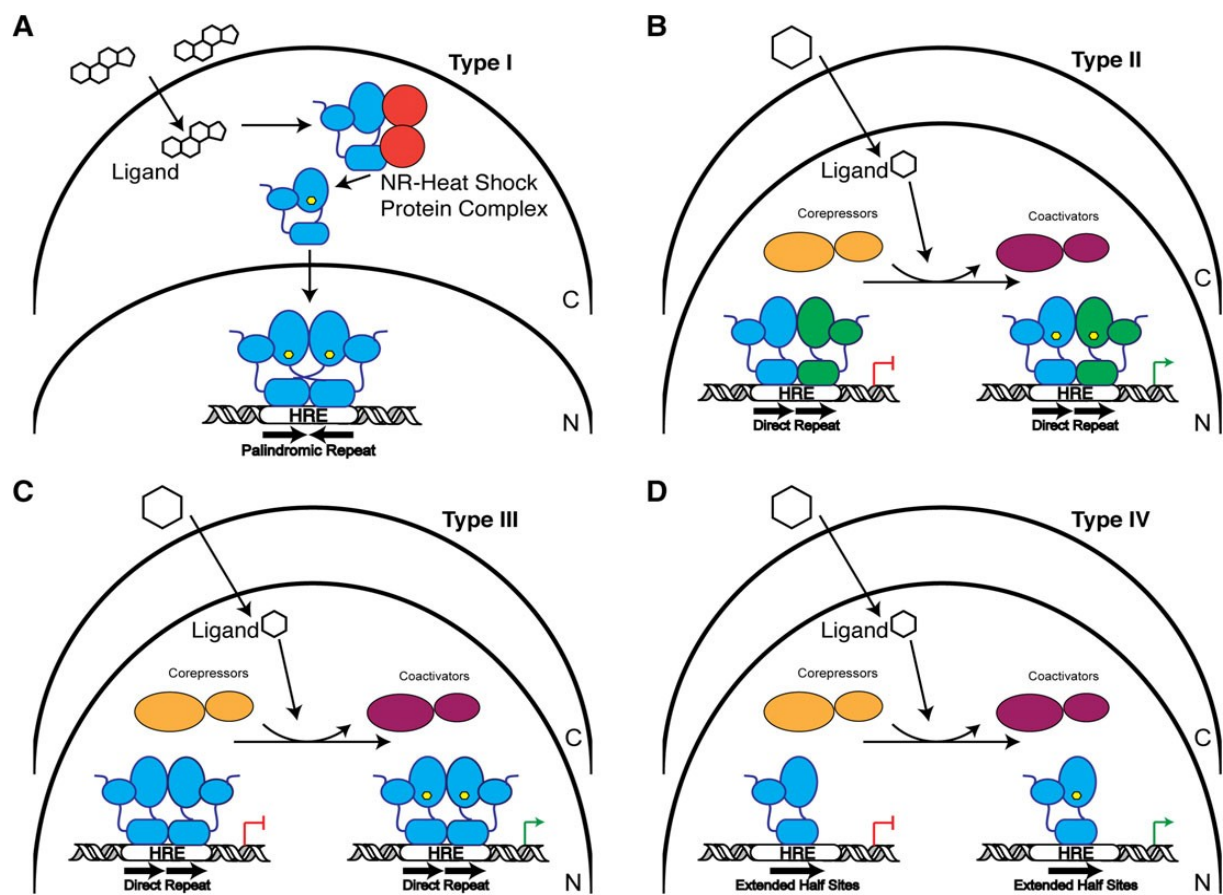


Figure 10. Schematic representation of NR signaling mechanisms (Weikum et al. 2018).

6.3. Physiological and pathological role of the NR4A receptors

In normal physiological conditions, NRs including the ONRs are constitutively active. Their multiple effects were widely highlighted in the development, reproduction metabolism, cell survival, proliferation, migration, apoptosis, angiogenesis, DNA repair, immunity, inflammation, lipid signaling and other processes (Helsen and Claessens 2014; Germain and Bourguet 2013; Nomiya et al. 2006). The type of NR4A subfamily function is highly related to their tissue as well as subcellular localization (Nomiya et al. 2006). For example, NR4A3 is implicated in mitochondrial function like energy metabolism and apoptosis via translocating to the mitochondria (Pearen and Muscat 2010). Furthermore, these receptors are gaining more and more attention in the treatment of several diseases; hence they were targeted by the development of drugs against diabetes, inflammatory diseases, myeloid leukemia, and prostate, breast, lung and colon cancer (Gadaleta and Magnani 2014). But in comparison to the other classes of NRs, the orphan receptors are still not well investigated in structural as well as functional aspects (Porter et al. 2019).

6.4.NR4A receptors in vertebrate immunology and inflammation

During the inflammatory response, particularly NR4A subfamily (NR4A1/NUR77, NR4A2/NURR1 and NR4A3/NOR1) is attracting more attention as potent regulator of several cytokines and growth factors mediated actions (McMorrow and Murphy 2011). Their expression is dramatically induced in response to various external and internal stimuli, including PAMP (LPS), TNF- α , (Bonta et al. 2006), IL-1 β , NF- κ B, vascular endothelial growth factor (VEGF), hormone, oxidized lipids (Park et al. 2016; Pei et al. 2005; Sekiya et al. 2013) and other stimuli (Pearen and Muscat 2010). NR4A receptors mRNA were analyzed in different tissues, such as thymus, heart, muscle, brain and the pituitary gland (Li et al. 2006), and in different cell types including neurons, endothelial cells, adipocytes, muscles, macrophages, and T cells *in vitro* as well as *in vivo* (Pearen and Muscat 2010; Park et al.

2019; Park et al. 2016). Indeed, in macrophages, (LPS), cytokines or oxidized lipids significantly induced all NR4A receptors, including NR4A3, suggesting their involvement in the regulation of inflammatory response (Pei et al. 2005; Pei et al. 2006). In addition, from the three members of NR4A subfamily only NR4A3 plays a crucial role in bone marrow and splenic DCs (Ag-presenting cells) development and function (Nagaoka et al. 2017). It is expressed in different populations of DC and is involved in its migration to the lymph nodes and the modulation of the expression of the cell surface chemokine CCR7 *in vivo* via NR4A3/FOXO1/CCR7 pathway (Park et al. 2016). Furthermore, Nagaoka et al. (2017) has demonstrated that NR4A3 is highly induced in response to TLR-mediated immune response in DCs and is involved in CD80, CD86, IL-10, IL-6, and IL-12 modulation in response to LPS, CpG, or poly (I:C) underlining a pro-inflammatory profile (Nagaoka et al. 2017). NR4A1 and NR4A3 trigger thymic Treg lymphocytes development, differentiation and immune homeostasis during systemic immunity in mice (Sekiya et al. 2013). Together these findings show a crosstalk between the Ag presenting cells (DCs) and the effective T cells during pathogen clearance via NR4A3. In correlation with this findings, it was demonstrated that *Citrobacter rodentium* infection is able to cause the death of mice DCs deficient in NR4A3 (Park et al. 2016). However, overexpression of NR4A3 in vascular smooth cells leads to the suppression of TNF- α in response to LPS (Calvayrac et al. 2015).

It is important to mention that in some context the exhibited immunological functions of the NR4A subfamily is redundant between the different receptors members which was contributed by their high homology between NR4A2 and NR4A3, between NR4A1 and NR4A2 as well as their ability to bind the same DNA responsive element. This fact was found in both adaptative (T cell) and innate immune cells (macrophages) (Pei et al. 2005; Pei et al. 2006; Sekiya et al. 2013).

6.5. Involvement of prostaglandins signaling in NR4A3 activation

Ranging from lower vertebrates (teleost fishes) to human the immune response orchestrates and interconnects a wide range of innate and adaptive effectors in order to favor an homeostatic microenvironment inside the body. In this context, NR4A receptors, mainly NR4A3, participate during the first stage of the immune response enhancing target genes expression, such as pro-inflammatory cytokines, but also in later stages to brake the hyperactivity of some inflammatory mediators in order to avoid the undesirable effects that could for example generate autoimmune diseases. Interestingly, it has been shown that directly and indirectly PGE₂ and its derived PGA₂, respectively, are involved in NR4A3 signaling (Prince et al. 2017; Zhu et al. 2013; Kagaya et al. 2005). Indeed, it was determined that PGE₂ and ATP are activators of NR4A3-mediated transcription as well as it was suggested that PKA induction of NR4A3 is prostaglandin-dependent during neutrophils inflammatory response (Prince et al. 2017). In addition, NR4A receptors, and particularly NR4A3, activates mitogen-activated protein kinase (MAPK) (Nagaoka et al. 2017). Furthermore, MAPK/ERK is an upstream pathway of NR4A3 activation in vascular smooth muscle cells (Nomiya et al. 2006) and other cell types (McMorrow and Murphy 2011) during cartilage degradation and inflammation. In human chondrocytes and cancer cells both COX-2, PGE₂ and NR4A₂ are concomitantly enhanced (Jennings et al. 2019). Other evidences show that NF- κ B and cAMP/PKA signaling pathway are involved in NR4A2 immune response by T_{reg} lymphocytes (Nagaoka et al. 2017). These findings show evidences that COX-2/PGE₂/cAMP pathway induces NR activation coordinating cell surface and intracellular receptors activation.

6.6. Evidences of PGA₂ activation of NR4A3

PGA₂ is responsiveness to immune stimuli such as LPS, INF- γ and TNF- α (Storer et al.

2010). During cell signaling it is actively delivered to the intracellular compartment via cell membrane specific transporter in order to act with target nuclear molecules (Holbrook et al. 1992). It was further indicated that this cyPGs could be found in the nucleus (Lakshmi et al. 2019). It has been confirmed that NR4A3 is a receptor of PGA_2 (Kagaya et al. 2005; Zhu et al. 2013). Interestingly, in a recent study Lakshmi et al (2019) clarified that through its structural composition the cyPGA_2 form a covalent adduct between its endocyclic β -carbon, C9, and Cys566 in NR4A1 LBD (Lakshmi et al. 2019) and in correlation with this finding that NR4A3 is a cognate receptor of PGA_2 (Kagaya et al. 2005). Lakshmi and his collaborators (2019) linked this characteristic with the conserved LBD between the previously designed ONRs. However, it was rarely suggested that PGA_2 binds to cell membrane receptors, enhancing release of cAMP and Ca^{2+} (Muallem et al. 1989). EP4 was also identified as a receptor for PGA_2 (Ohmura et al. 2017).

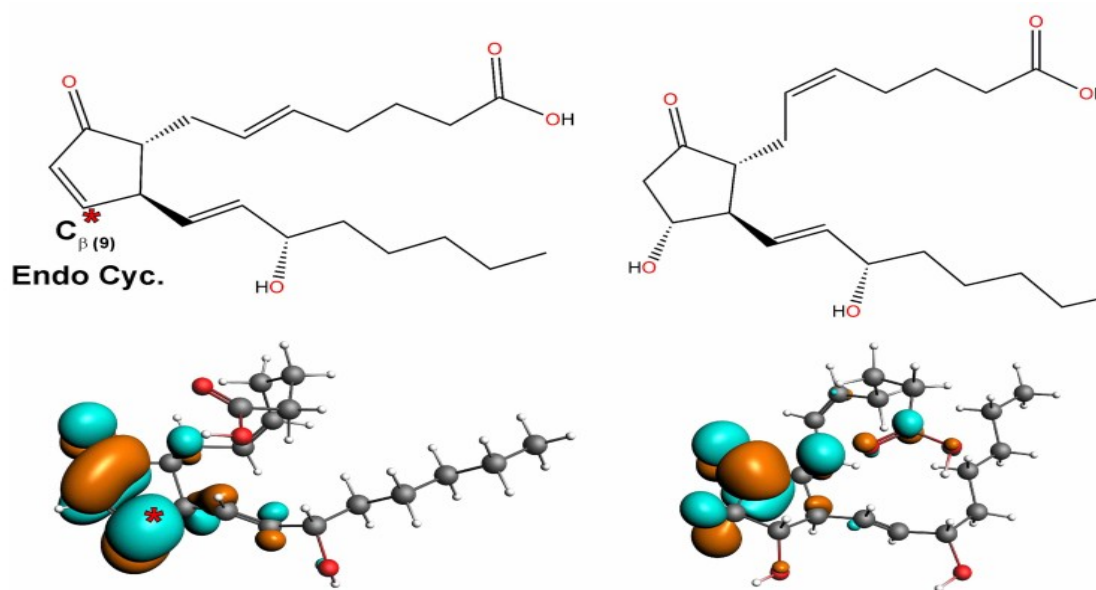


Figure11. Structural conformation of PGA_2 and its precursor PGE_2 . The electrophilic endocyclic $\text{C}\beta$ (C_9) of PGA_2 is indicated with an asterisk (Lakshmi et al. 2019).

The evidences which suggest that ONRs could act in complex with other receptors may explain the manner of ligand induced response by these receptors. Indeed, it could be logic

that for example PGA_2 binds on one of the NRs that possesses ligand binding dependent activation and hence this later forming a complex with ONRs, such as NR4A3.

In conclusion despite the limited research work based on PGA_2 and NR4A3 investigation in vertebrates, the collected information is useful at the physiological immunological and therapeutic levels. The new discovered way of interaction between the ligand and its receptors will pave the way for revolutionary functional interactions inside the family of ONRs.

7. PGA_2 receptor characterization in teleost fish

7.1. Identification of teleost fish Nr4a receptors

Regarding their strategical position in vertebrates, evolutionary phylogenetic tree as well as the specific adapted environment teleost constitute a great animal model to explore PGA_2 and NR4A3 roles. Indeed, all the subfamily of NRs have been identified in teleost fish. Their number is variable between species (between 68 and 74) (Baldwin et al. 2017) but still higher in comparison with mammals (48 in human and 49 in mouse) due to existence of several paralogue genes in teleost fish, including zebrafish medaka, tilapia and stickleback (Zhao et al. 2015). *In silico* analysis have shown that the more conserved motifs between vertebrates are mainly in the DBDs followed by the LBDs (Zhao et al. 2015). Molecular analysis based on their mammalian counterpart homology revealed the existence of the three known Nr4a receptors (Nr4a1, Nr4a2 and Nr4a3) in zebrafish which share significantly conserved genomic organization with higher vertebrates (Blin et al. 2008). The existence of two paralogs of Nr4a2 (Nr4a2a and Nr4a2b) was also shown in zebrafish and medaka (*Oryzias latipes*), and two isoforms of Nr4a3 in Fugu (*Fugu rubripes*) and tetraodon (*Tetraodon nigroviridis*) (Blin et al. 2008).

7.2. Physiological role of NRs in teleost fish

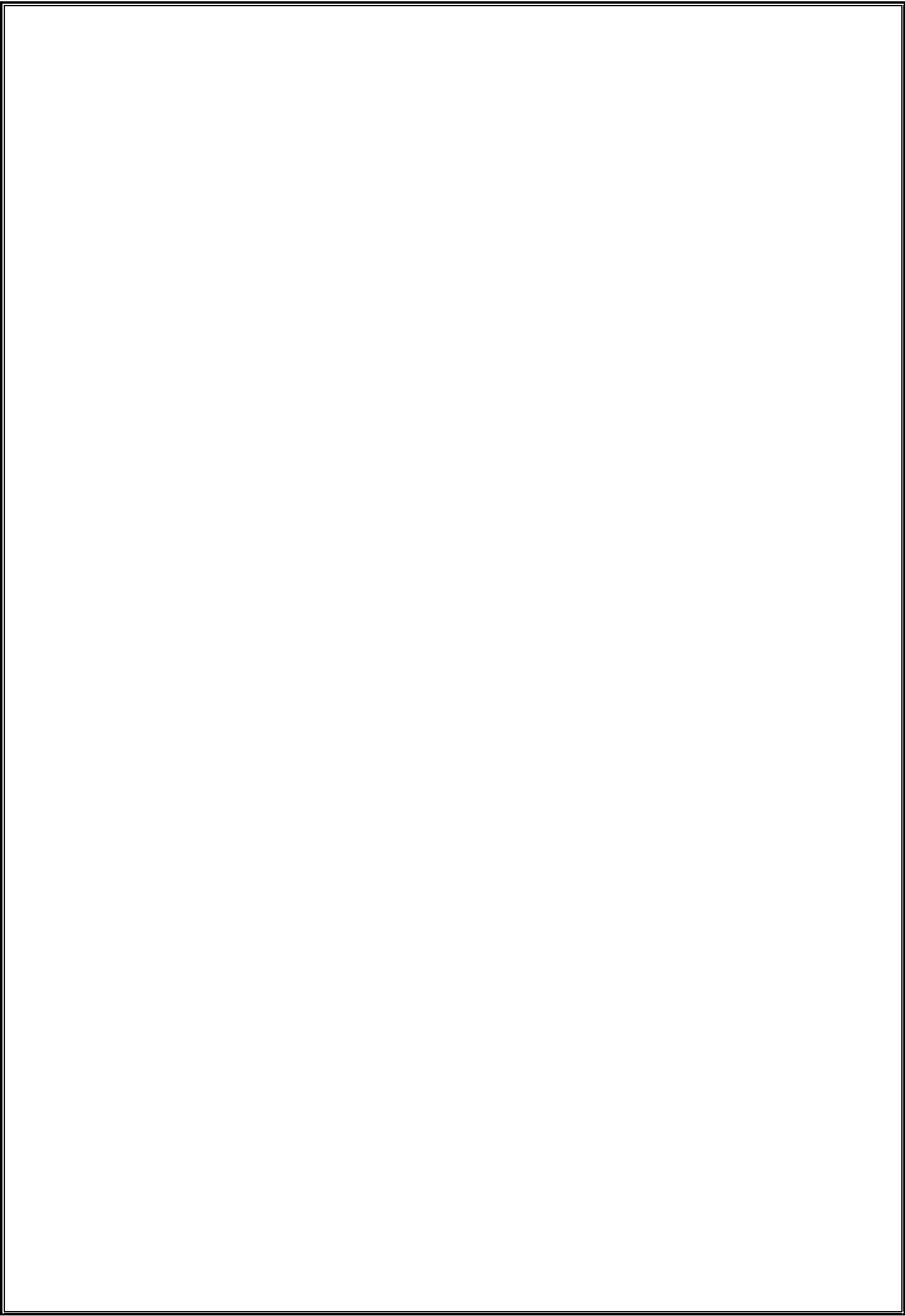
NRs involved in circadian rhythms, development, energy metabolism and resource allocation (Baldwin et al. 2017). NR4A2 participates in dopamine-synthesizing neurons differentiation and control locomotor activity in zebrafish (Blin et al. 2008). The role of some NRs receptors such as aryl hydrocarbon receptor 2(AhR2), pregnane X receptor (PXR), peroxisome proliferator activated receptor alpha (PPAR α), glucocorticoid receptor (GR) and thyroid receptor (TR) has been visualized in the toxicological field(Zhang et al. 2018; Zhao et al. 2015).

7.3. Immunoregulatory aspect of nuclear receptors in teleost fish

In their aquatic environment fish, including teleosts, are exposed to several variable conditions such as seasonal changes (affect immune and endocrine system), physiological microenvironment (reproduction) (Szwejsjer et al. 2017), invading microorganisms (Luo et al. 2019) and stress (Szwejsjer et al. 2017). Recently, several studies revealed the immunomodulatory roles of NRs in order to face these constraints. For example, three ERs have been identified in several teleost fish species comprising in gilthead seabream immune tissues such as spleen, blood and head-kidney (Cabas et al. 2018) as well as in its head kidney derived macrophages challenged with genomic DNA from *Vibrio anguillarum*(VaDNA) and in lymphocytes (Liarte et al. 2011). Interestingly, their involvement in the modulation of the teleost immune response revealed mainly upon PAMP introduction (Cabas et al. 2018). Furthermore, PPAR has potent effect in Nile tilapia (*Oreochromis niloticus*) resistance against *Aeromonas hydrophila* via enhancing and inhibiting anti- and pro-inflammatory cytokines, respectively (Luo et al. 2019). Although PPAR has been shown to have similar subcellular localization as in mammals, it has been found highly induced upon viral infection in teleost reducing a set of inflammatory cytokines and exerting an anti-viral effect (Yang et al. 2020).

Concerning the subfamily of ONRs, since they are apoptotic factor regulators as described before, Prince and his collaborators (2017) explored the role of Nr4a1-3 receptors in the development and survival of zebrafish neutrophils in response to injury during the first larval stages. Indeed, they found that that knockdown of Nr4a3 (and Nr4a2) in zebrafish larvae reduced the total number of neutrophils by more than 50% *in vivo* but did not affect their percentage in the site of injury, suggesting the involvement of this Nr4a3 in the lifespan of these immune cells independently of their activity. However, to the best of our knowledge, despite the attractive attention of ONRs in higher vertebrates, studies on their role in teleost fish are absent (Zhao et al. 2015).

OBJECTIVES

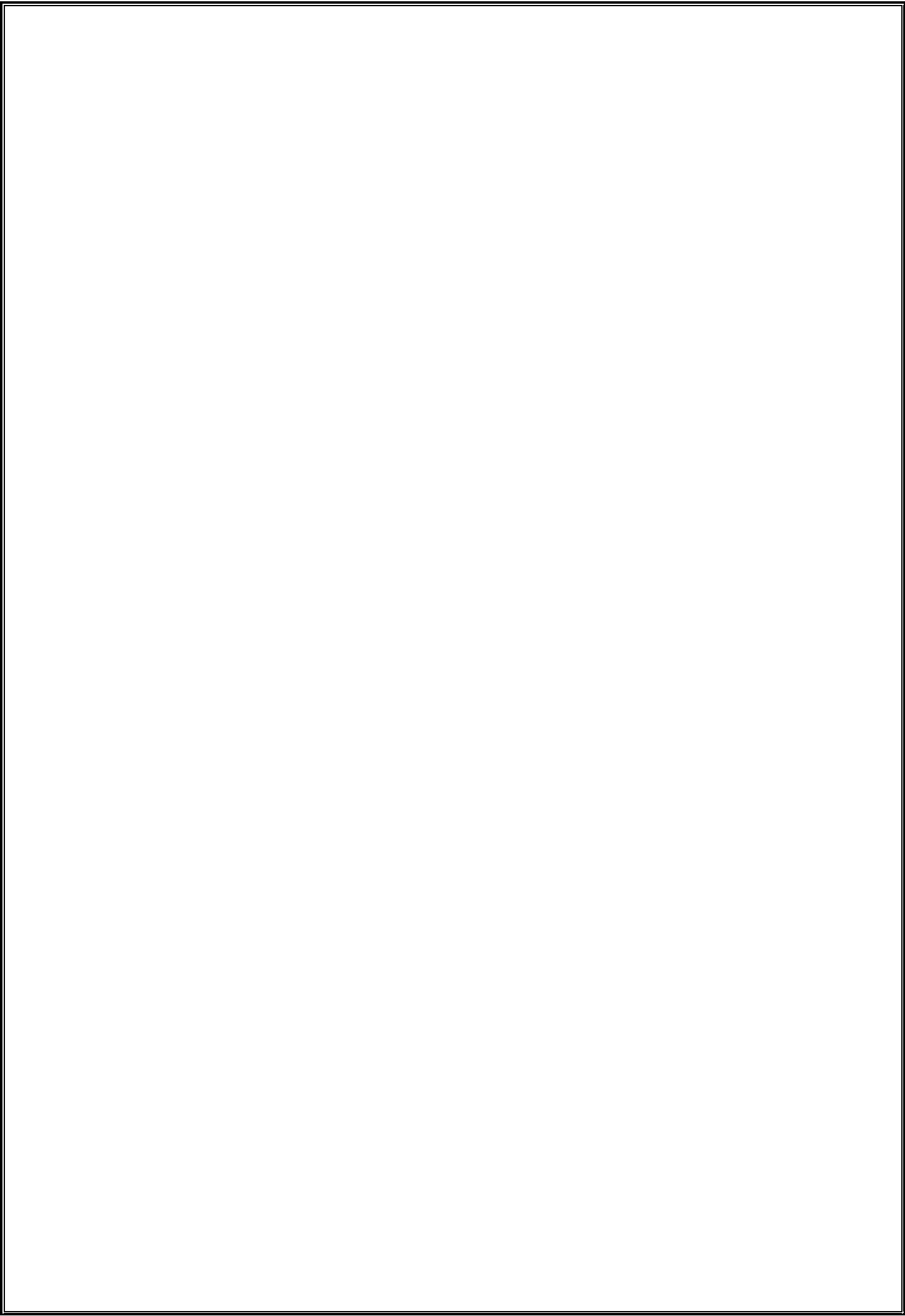


Objectives

This work has the following specific objectives:

1. Identification and phylogenetic characterization of gilthead seabream PGE₂ receptors.
2. Study the gene expression profile of gilthead seabream PGE₂ receptors *in vivo* upon bacterial challenge and *in vitro* in professional phagocytes in response to PAMPs and PGE₂.
3. Identification and phylogenetic characterization of the gilthead seabream putative PGA₂ receptor Nr4a3.
4. Study the gene expression profile of gilthead seabream Nr4a3 receptor *in vivo* upon bacterial challenge and *in vitro* in macrophages in response to PAMPs and PGA₂.
5. Study the role of exogenous addition of PGA₂ in gilthead seabream macrophages.
6. Determine whether PGA₂ signals through gilthead seabream Nr4a3 receptor.

CHAPTER I: The molecular, functional and
phylogenetic characterization of PGE₂ receptors
reveals their different roles in the immune
response of the teleost fish gilthead seabream
(*Sparus aurata* L.)



The molecular, functional and phylogenetic characterization of PGE₂ receptors reveals their different roles in the immune response of the teleost fish gilthead seabream (*Sparus aurata* L.).

Abstract

Prostaglandin E₂ (PGE₂) plays an important role in immune activities in teleost fish, including seabream. However, receptors involved in PGE₂ signaling, as well as the pathways activated downstream, are largely unknown. In this study, one ortholog of mammalian PTGER1, PTGER3 and PTGER4, and two of PTGER2 (Ptger2a and Ptger2b) were identified and characterized in gilthead seabream. *In silico*, analysis showed that all these receptors possessed the organization domain of G protein-coupled receptors, with the exception of Ptger2b. The corresponding *in vivo* studies revealed that they were expressed in all the tissues examined, the highest mRNA levels of *ptger1* and *ptger3* being observed in the spleen and of *ptger2a* and *ptger4* in the blood. Bacterial infection induced higher mRNA levels of *ptger2a*, *ptger3* and *ptger4* in peritoneal exudate (the site of bacterial injection). In addition, head kidney acidophilic granulocytes and macrophages displayed different *ptger1*, *ptger2a*, *ptger3* and *ptger4* expression profiles. Furthermore, in macrophages the expression of the receptors was weakly affected by stimulation with bacterial DNA or with PGE₂, while in acidophilic granulocytes stimulation resulted in the upregulation of *ptger2a* and *ptger4*. Functionnally, the cloned Ptgers and drastically Pger4 induced IL-8 mRNA in basal conditions while in response to PGE₂ Ptger1, Ptger2b and Ptger3 upregulated significantly this chemokine. Taken together, these results suggest different roles for seabream PGE₂ receptors in the regulation of the immune responses.

1. Introduction

Prostanoids including PGE₂ are bioactive lipid-derived autacoids (Ricciotti and FitzGerald 2011) involved in a broad array of physiological and pathological processes (Gamil et al. 2015; Kobayashi and Narumiya 2002). They are derived from the arachidonic acid released from cell membranes (Calder and Grimble 2002) in response to a variety of stimuli (Harris et al. 2002; MacKenzie et al. 2010; Ricciotti and FitzGerald 2011; Sargent et al. 1999), which is subsequently converted into PGH₂ through the action of two rate-limiting enzymes: constitutive (COX-1) and inducible (COX-2) cyclooxygenases (Wang and Dubois 2010). PGH₂ is converted into several prostanoids by the action of the respective synthases (Narumiya et al. 1999).

The divergent effects of PGE₂ have been involved in a large spectrum of biological processes (Hao and Breyer 2008; Ning et al. 2014; Miller 2006), including both pro- and anti-inflammatory mechanisms, ranging from lower vertebrates (fishes) (MacKenzie et al. 2010; Gómez-Abellán and Sepulcre 2016; Tyrkalska et al. 2016) to human (Calder and Grimble 2002; Hsu et al. 2014; Fattahi and Mirshafiey 2014; Miller 2006).

In mammals, the pleiotropic effects of PGE₂ are mediated by binding to one or to a combination of four receptors subtypes PTGER1-PTGER4 (also named EP1-EP4) (Harris et al. 2002, Qu et al. 2015), belonging to the family of rhodopsin-like seven transmembrane-spanning, G-protein-coupled receptors (Guo et al. 2015). These receptors can be grouped into three groups of prostanoid receptors according to the corresponding associated G-protein subunit, and hence to the intracellular second messengers and the effect triggered by them (Yokoyama et al. 2013). The group of relaxant receptors include EP2 and EP4, whose activation results in increased cAMP/PKA through G α_s and adenylyl cyclase (Fujino, West, and Regan 2002), leading to smooth muscle relaxation. EP1 is coupled to G α_q , which increases Ca²⁺ levels following the activation of phospholipase C (Fujino and Regan 2005) and belongs to the group of contractile receptors. EP3 is generally coupled to G α_i , leading to a decrease in cAMP via G α_i and resulting in the inhibition of muscle contraction (Narumiya et al. 1999), although different isoforms resulting from alternative splicing in the EP3 gene are coupled to different types of G protein, such as G α_s , G α_i and G $\alpha_{12/13}$ (Tang et al. 2017).

Mammalian EP1-EP4 receptors exhibit distinctive levels of ligand-binding affinity, different selective expression, tissue distribution and subcellular localization (Sugimoto and Narumiya 2007; Yokoyama et al. 2013). These receptors have been characterized at genomic (Locker and Buzard 1990; Smock et al. 1999; Suganami et al. 2016; Yokoyama et al. 2013), proteomic (Margan et al. 2012; Stillman et al. 1999; Sugimoto and Narumiya 2007; Yokoyama et al. 2013) and expression levels, both *in vitro* and *in vivo*, in response to a variety of stimuli (Birrell et al. 2015; Sakamoto et al. 2004; St-Jacques and Ma 2011, Sheppe et al. 2018). Mouse EP3 and EP4 are the most widely PGE₂ receptor distributed (Yokoyama et al. 2013) and both have a much higher affinity than EP1 and EP2 for PGE₂ (Fujino et al. 2002).

In teleost fish, the production of PGE₂ by immune cells has been described in a broad range of species, including black seabream (*Spondylusoma cantharus*), black rockfish (*Sebastes melanops*), red seabream (*Pagrus major*), Atlantic cod (*Gadus morhua*), yellow croaker (*Larimichthys polyactis*), rainbow trout (*Oncorhynchus mykiss*), goldfish (*Carassius auratus*) and gilthead seabream (*Sparus aurata* L.) (Gómez-Abellán and Sepulcre 2016). Furthermore, there is evidence concerning the immunoregulatory activity of PGE₂ in fish: for example, the modulation of cytokine expression, ROS production and phagocytic responses (Gómez-Abellán and Sepulcre 2016). More specifically, we have shown that PGE₂ leads to M2 polarization of macrophages in gilthead seabream (Montero et al. 2016), in a similar way to that seen in mammals (Harris et al. 2002). PGE₂ deactivates acidophilic granulocytes (AGs, the functionally equivalent cell to mammalian neutrophils) through the modulation of the cytokine expression profile (Montero et al. 2016). However, studies concerning the molecular characterization of PGE₂ receptors and their role in the immune response in teleost fish are scant. Mammalian homologs of EP1-4 have been identified and characterized in several fish species: zebrafish (*Danio rerio* H.) (Kwok et al. 2012; Tsuge et al. 2013), Atlantic salmon (*Salmo salar* L.) (Guo et al. 2015), *Bostrichthys sinensis* (Lai et al. 2014) and ayu (*Plecoglossus altivelis*) (Rong et al. 2016), EP4 being the most characterized (Fujimori et al. 2011; Gamil et al. 2015; Guo et al. 2015; Tsuge et al. 2013). They are involved in several functions such as reproduction (Fujimori et al. 2011, 2012), metabolic regulation (Busby et al. 2002) and the immune response (Guo et al. 2015; Rong et al. 2016). Ptger4 has also been considered as an early marker of T cells precursor in zebrafish development (Villablanca et al. 2007). However, our knowledge of the role of PGE₂ receptors in the teleost

immune response is still limited, which makes a comparative analysis with their higher vertebrate counterparts quite difficult.

In previous studies, we showed that the cell-permeable analog of cAMP (dbcAMP) mimicked the effect of PGE₂ in the regulation of cytokine mRNA levels in stimulated macrophages but not in AGs. The variety of effects that PGE₂ elicits in both phagocytic cell types could reflect the presence of specific receptors in those cells and suggest that PGE₂ signals through EP2 and/or EP4 in seabream macrophages but not in AGs. Taking into consideration all the above, the aim of this study was the identification and *in silico* characterization of gilthead seabream PGE₂ receptors (Ptger1-4) and to gain further insight into their role in the resolution of inflammation *in vivo* and *in vitro*. In particular, their distribution *in vivo* and modulation after bacterial challenge and their contribution to phagocytic cell functions were analyzed. These data pave the way for analysis of the role of PGE₂ receptors during the immune response of teleost fish.

2. Materials and Methods

2.1. Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*Sparus aurata*, Actinopterygii, Sparidae) were bred and kept at the Oceanographic Centre of Murcia (Spain) in a 14 m³ running seawater tank (dissolved oxygen 6 ppm, flow rate 20% tank volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Burgos, Spain). The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals. Seabream were killed by cervical dislocation using approved procedures following anaesthetization with clove oil. All efforts were made to minimize animal stress and to ensure that termination procedures were performed efficiently.

2.2. *In vivo* experimental infections and sampling

Five fish were injected intraperitoneally with 1 ml of phosphate-buffered saline (PBS) alone or containing a sublethal dose (10⁸) of exponentially growing *Vibrio anguillarum* R82 cells (Chaves-Pozo et al. 2004). Head kidney, spleen, thymus, liver, gills, blood and peritoneal

exudates cells were obtained 4 h after bacterial challenge, a time-point previously described as sufficient to trigger the mobilization of immune cells (Chaves-Pozo et al. 2005). All samples were processed for subsequent real-time RT-PCR (see below).

2.3. Isolation of phagocytes

AGs were obtained by Magnetic-Activated Cell Sorting (MACS), as described previously (Roca et al. 2006). Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a mAb specific to gilthead seabream AGs (G7) (Sepulcre et al. 2002), washed twice with PBS containing 2 mM EDTA (Sigma-Aldrich) and 5% FCS (Invitrogen) and then incubated with 100-200 μ l per 10⁸ cells micro-magnetic-bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec). After washing, G7⁺ (AGs) cell fractions were collected by MACS following the manufacturer's instructions and their purity was analyzed by flow cytometry (Roca et al. 2006). Head kidney macrophage monolayers were then obtained by overnight culture of G7⁻ fractions in serum-free medium as described previously (Roca et al. 2006).

2.4. Cell culture and treatments

Phagocytes (AGs and macrophages) were treated at 23 °C with 50 μ g/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (VaDNA) (Pelegrín et al. 2004) and/or PGE₂ (10 μ M, Cayman Chemical) in sRPMI (RPMI-1640) culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl, supplemented with 0.1% FCS and 100 IU/ml penicillin and 100 μ g/ml streptomycin (Biochrom). Stimulation times were 16 hours for AGs and 3 hours for macrophages. The concentrations used above have been found to be optimal for the *in vitro* activation of seabream phagocytes (Sepulcre et al. 2007 ; Montero et al. 2016).

2.5. Analysis of gene expression

Total RNA was extracted from cell pellets with TRIzol Reagent (Invitrogen) following the manufacturer's instructions, and the upper phases containing RNA were purified with RNAqueous Micro Kit (Ambion) according to the manufacturer's protocols. Final RNA concentration was measured at 260 nm by means of the nanodrop system (ND-1000), and RNA quality was

checked by electrophoresis. RNA was stored at -80 °C until use. Total RNA was treated with amplification grade DNase I (1 unit/μg RNA, Life Technologies). Then, SuperScrip III RNase H-Reverse Transcriptase (Life Technologies) was used to synthesize first strand cDNA with oligo-dT18 primer from 1 μg of total RNA for 50 min at 50°C, followed by 15 min at 70 °C. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reactions were performed in 10 μl mixtures, containing 5 μl of 2× SYBR Green PCR Core Reagents (Applied Biosystems), 0.15 μl of each primer (0.15 μM, final concentration), and 2.5 μl of cDNA. The amplification profile was: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and, finally, 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. The RNA was quantified using ribosomal protein S18 (*rps18*) transcript content as housekeeping gene using the comparative Ct method ($2^{-\Delta\Delta C_t}$ for “fold change” and $2^{-\Delta C_t}$ for constitutive expression). The primers used (**Table 1**) had an amplification efficiency of 2 and their specificity was confirmed by melting curve analysis and the sequencing of each amplified product (Genetic Analyzer ABI PRISM 3130, Applied Biosystems) (data not shown). In all cases, each PCR was performed with triplicate samples.

Table 1: Primer sequences used for cloning and qRT-PCR.

Gene	Forward/Reverse	Primer sequence (5'->3')	Technique
<i>ptger1</i>	Forward	TATCGGAGAGCACCTGTCCA	qRT-PCR
	Reverse	CTGGCACTTAGCGATGAGGT	
	Forward	AAAAAGGATCCATGTTAGCGTTGAGCCACT	cloning
	Reverse	AAAAAGCGGCCGCAACGTGGTTGACATCATTCT	
<i>ptger2a</i>	Forward	TTCTTCAGCCTTGCGACCAT	qRT-PCR
	Reverse	TGCACCAAATCCCGCAAAAG	
	Forward	AAAAAGGATCCATGGGGAATCCAGAGAAC	cloning
	Reverse	AAAAAGCGGCCGCTGGTAATAGAGTTTCTGCAG	
<i>ptger2b</i>	Forward	AAAAAGGATCCATGGGGAATCCAGAGAAC	cloning
	Reverse	AAAAAGCGGCCGCGAAGTCAACGGTCAACACTT	

<i>ptger3</i>	Forward	TCCCTCACCAGATCCTGGAC	qRT-PCR
	Reverse	AGTCTGGGTCTCCTTCCGAT	
	Forward	AAAAAGGATCCATGACTATGGACGCTTCTGA	cloning
	Reverse	AAAAAGCGGCCGCGCTACTTTGTGGTTTATGGATGT	
<i>ptger4</i>	Forward	GGGCAGAGATCCAGATGGTG	qRT-PCR
	Reverse	TGTGCAGCTGATTCGCAAAG	
	Forward	AAAAAGGATCCATGAACTCCACGGTAAAAC	cloning
	Reverse	AAAAAGCGGCCGCTATGCATTTCTCTTGTATGTTTGC	
<i>il8</i>	Forward	GTTTTTGAAGAGGGCTGAG	qRT-PCR
	Reverse	TTTGCTTGAAGTTTCACTGG	

2.6. *In silico* analysis of seabream PGE₂ receptor sequences

Nucleotide sequences of seabream *ptger1*, *ptger2a*, *ptger2b*, *ptger3* and *ptger4* (Accession numbers in Table 2), were obtained from a non-public EST data base. The deduced amino acid sequences were obtained via EMBOSS: Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). Areas of seabream protein sequences similar to those of other vertebrate sequences obtained from the NCBI data base were compared after multiple amino acid sequence alignments within the website tool Clustal Omega, (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) provided by the European Bioinformatics Institute (EMBL-EBI), in our case: fish (large yellow croaker and zebrafish), reptiles (frog), bird (chicken), mammals (mouse and human). Phylogenetic reconstructions were performed with phylogeny software (<http://www.phylogeny.fr/>) (Dereeper et al. 2008). The molecular weight of each protein was determined using the Science Gateway tool (<http://www.sciencegateway.org/tools/proteinmw.htm>). Finally the structure and domain organization of each receptor were determined using the psipred protein structure predictor, (<http://bioinf.cs.ucl.ac.uk/psipred/>), SMART program, and TMHMM Software (<http://www.cbs.dtu.dk/services/TMHMM/>) and with the HMMTOP server (<http://www.enzim.hu/hmmtop/>).

Table 2: Genbank or Ensembl accession numbers of Ptger used in this study.

Specie	Ptger1	Ptger2a	Ptger3	Ptger4
<i>Homo sapiens</i>	AAH51286	AAD44177	AAI18660	AAI13524
<i>Mus musculus</i>	NP_038669	NP_032990	NP_035326	NP_001129551
<i>Gallus gallus</i>	Unknown	ABM92941	ABF13295	NP_001074972 XP_424770
<i>Xenopustropicalis</i>	XP_002935465	XP_002936949	XP_002936132	NP_001120554
<i>Danio rerio</i>	ACX47465.1	zEP2a: NM_200635 zEP2b:NM_001030250 zEP2L: ABB88955.1	XP_009300798	NM_001039629
<i>SparusaurataL.</i>	-	-	-	-
<i>Salmo salar</i>	XP_014048970.1	XP_014000607.1	XP_014072350.1	NP_001167426.1
<i>Larimichthyscrocea</i>	XP_010732951	XP_010742830	XP_010727602	KKF22113
<i>Etheostomaspectabile</i>	XP_032394488.1	XP_032362064.1	XP_032382424.1	XP_032395955.1

2.7. Cloning the full-length cDNA of seabream Ptger1-4

Sequences of seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4 were obtained by PCR amplification with Pfu DNA polymerase (Fermentas) using cDNA from seabream head kidney as template. The specific primers used for amplification (listed in Table 1) were designed by using the Primer Designing Tool of NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). PCR were performed at low melting temperature: first 5 cycles at 58°C followed by 30 cycles at 72°C. The PCR amplified fragments were cloned between BamHI and NotI restriction sites into the plasmid pcDNA6/V5-His C (Life Technologies) in frame with the sequence coding for the V5 epitope. All constructs were sequenced by using ABI PRISM 377 (Applied Biosystems).

2.8. Functional analysis by transfection assay

Plasmid DNAs of the *Ptger1*, *Pger2a*, *Pger3* and *Pger4* were prepared using the Midi-Prep procedure (Sigma) and transfected into HEK293 cells with Lipofectamine (InvivoGen) according to the manufacturer's instructions. Briefly, forty-eight hours before transfection, HEK293 cells were harvested and seeded into 6-well plates at density of 1.2×10^5 cells/well. For transfection, each well was transfected with 1.5 μ g of the corresponding expression constructor empty vector and 1 μ l of Lipofectamine in incomplete milieu for 6h and then incubated in complete milieu. Forty-eight hours after transfection, cells were cultured with 10 μ M of PGE₂ for 3h, collected in TRIzol Reagent (Invitrogen) and processed for subsequent real-time RT-PCR.

2.9. Statistical analysis

Data were analyzed by ANOVA and Tukey's multiple range tests to determine differences between groups. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Identification and characterization of genes encoding *Ptger1*, *Ptger2a*, *Ptger2b*, *Ptger3* and *Ptger4* in gilthead seabream

Searches within a non-publicly EST database allowed us to identify two genes encoding for gilthead seabream *Ptger2* designated as *Ptger2a* and *Ptger2b* (ENA accession numbers XM_030410287.1 and XM_030410288.1, respectively) and one for *Ptger1*, *Ptger3* and *Ptger4* (ENA accession numbers XM_030412901.1, XM_030434233.1 and XM_030436477.1, respectively). The full-length open reading frame (ORF) of *ptger1* (**Fig. 1A**), *ptger2a* (**Fig. 1B**), *ptger2b* (**Fig. 1C**), *ptger3* (**Fig. 1D**) and *ptger4* (**Fig. 1E**) are 1140, 1140, 885, 1203 and 1341 base pairs (bp), respectively, which translated into the putative 379, 379, 294, 391 and 446 amino acid polypeptides with estimated molecular weights of 41.34, 42.71, 33.46, 43.46 and 49.54 kDa, respectively. The coding sequences for *Ptger2a* and *Ptger2b* showed 73% nucleotide identity and differed in the last 312 nucleotides before the stop codon (**Fig. 1B** and **C**). The 5' and 3' UTR sequences of *ptger1* had 381 and 855 bp, respectively (**Fig. 1A**). However, the 5' and 3' UTR sequences of *ptger2a* are not available in public databases. The sequences of *ptger2b* 5' and 3' UTR had 90 and 330 bp (**Fig. 1C**), while *ptger3* had 78 and 1203, respectively (**Fig. 1D**). By

contrast, *ptger4* receptor had the longest UTR sequences: 860 bp for 5'UTR and 1414bp for 3'UTR (**Fig.1E**).

The pivotal regulatory elements found in each gene are shown in **Fig.1**: i) two putative *ccaat* boxes in *ptger2b* (one in the 5'UTR and one in 3'UTR) and three in *ptger4* (two in the 5'UTR and one in 3'UTR), ii) a polyadenylation signal, an important 3' end element involved in mRNA maturation, transport and translation in eukaryotes (Sachs 1990), was found in *ptger1*, *ptger3* and *ptger4* 3'UTRs. iii) three motifs *attta*, associated with the instability of mammalian (Han et al. 1990) and fish (Roca et al. 2007) cytokine mRNAs, were found in *ptger1* 3'UTR, four in *ptger3* and six in *ptger4* (one located in the 5'UTR and five in 3'UTR). The presence of these motifs, especially the sequences with three or more ATTTA motifs, is concomitant with rapid posttranscriptional modulation, splicing, mRNA export, degradation, translation activation or inhibition, and turnover (Akashi et al. 1994; Khabar 2010; Moore, Young, and Dixon 2011). Iv) a consensus sequence (ttatttat) of mammalian inflammatory mediator mRNAs was found in *ptger3* 3'UTR) and a cytoplasmic polyadenylation element (tttttttttt) was identified in *ptger4* 3'UTR.

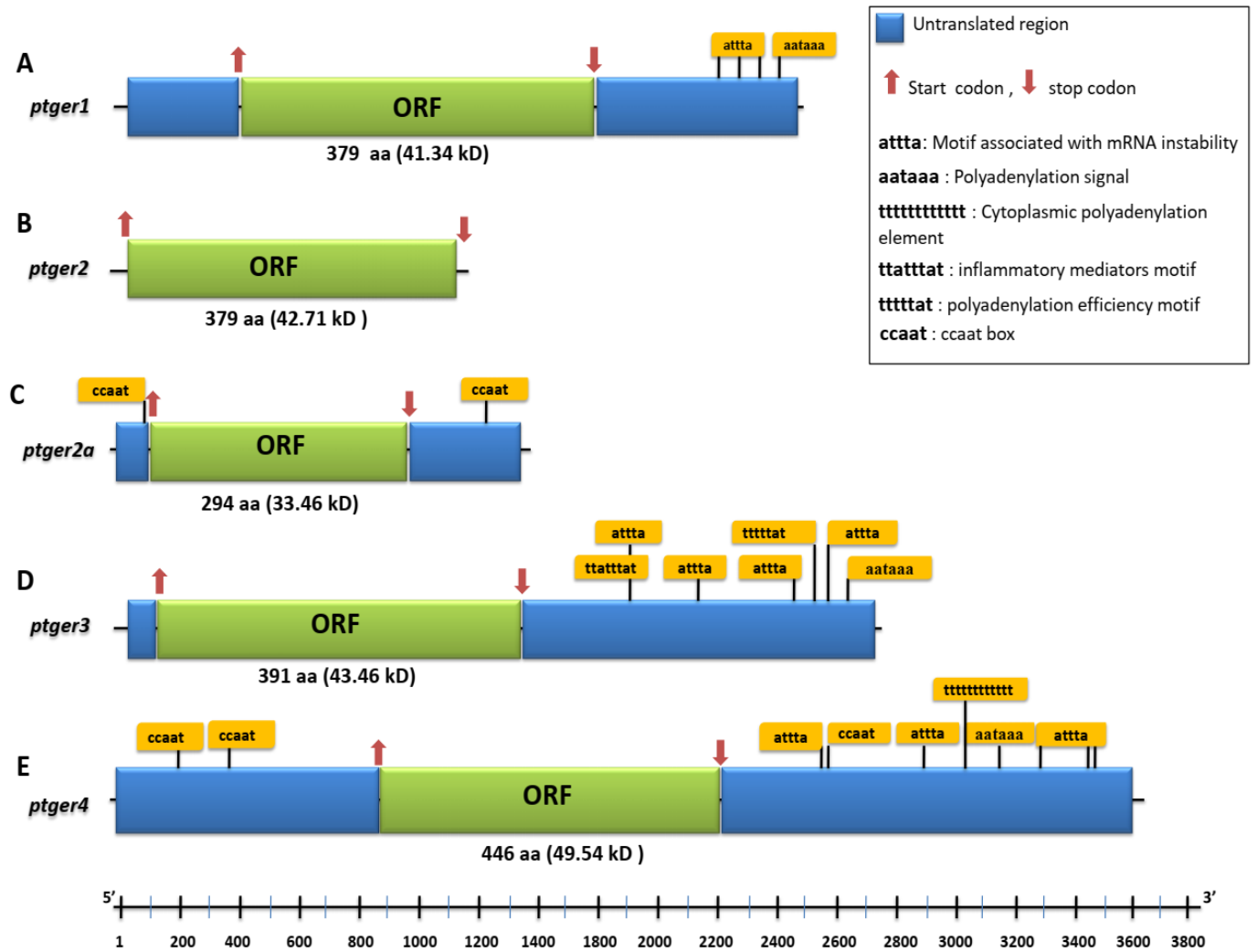


Figure 1: Schematic representation of seabream Ptger subtype sequences. Schematic representation of seabream *ptger1* (A), *ptger2a* (B), *ptger2b* (C), *ptger3*(D) and *ptger4* (E).The open reading frames are represented in green boxes and the 5`- and 3`-UTR in blue boxes. The number of amino acids, as well as the molecular weight of the deduced amino acid sequences, are shown below each ORF. Also shown are the start (ATG, upward-pointing red arrow) and stop codon (TAA or TGA, downward-pointing red arrow) and motifs: **attta** (motif associated with mRNA instability), **aataaaa** (polyadenylation signal), **ttattttat** (inflammatory mediator motif), **tttttat** (polyadenylation efficiency motif), **tttttttttt** (Cytoplasmic polyadenylation element) and **ccaat** (ccaat box).

3.2. Polypeptide sequences and topology analysis of seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4

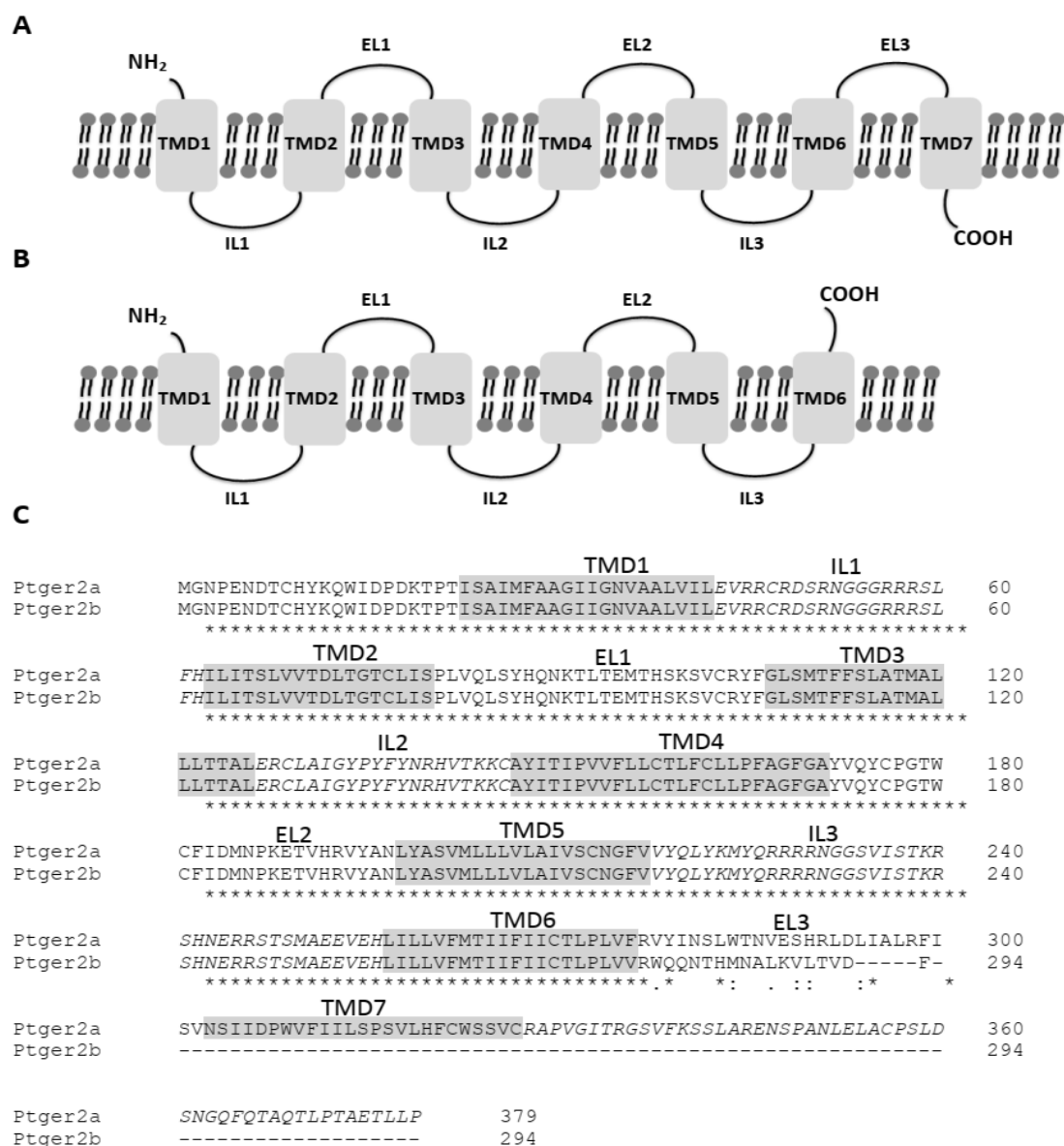


Figure 2: Schematic domain organization and sequence alignment of seabream Ptger2a and Ptger2b. Diagrams showing the domain organization of seabream Ptger2a, which is also representative of Ptger1, Ptger3 and Ptger4 (A), and Ptger2b (B). TMD: transmembrane domain, IL: intracellular loop, EL: extracellular loop. (C) Alignment of seabream Ptger2a and Ptger2b sequences. (*) Identity in one position; (:) conservative substitutions in one position; (.) semiconservative substitutions in one position.

The *in silico* analysis of the deduced amino acid sequences of *ptger1*, *ptger2a*, *ptger2b*, *ptger3* and *ptger4* and of their domain organization revealed that they are G protein-coupled receptors, as has been documented in higher vertebrates (Narumiya et al. 1999). They consist of integral membrane proteins that possess seven transmembrane domains (TMD): the extracellular N-terminal, the intracellular C-terminal, three extracellular loops (EL) and three intracellular loops (IL) (**Fig.2A**). However, Ptger2b possesses six instead of seven TMD, and hence two EL and a C-terminal on the extracellular side (**Fig.2B**).

Table 3: Amino acid sequence identity (%) between seabream Ptger1, Ptger2a, Ptger3 and Ptger4 and other vertebrates one.

	Ptger1	Ptger2a	Ptger3	Ptger4
Human	42.8	46.7	61.3	56.8
Mouse	43.8	47.1	60.4	56.2
Chicken	-	44.3	63	58.6
Frog	46.1	45.5	65	64.9
Zebrafish	zEP1a: 67.8	zEP2a: 59.8	76.1	zEP4a: 66.6
	zEP1b: 63.8	zEP2L: 58		zEP4b: 62
	zEP1c: 47	zEP2b: 49.4		zEP4c: 49
Atlantic salmon	65.8	53.3	79.5	65.2
Yellow Croaker	87.1	74.4	86.2	62
Orange throat darter	88.1	71.3	80.4	84.7

In addition, multiple sequence alignments of Ptger1, Ptger2a, Ptger3 and Ptger4 with those of other vertebrates showed long areas of conserved amino acid residues in all the sequences analyzed. The most conserved regions were located within the seven putative transmembrane domains among the identified seabream Ptger1 (**Fig. 3**), Ptger2a (**Fig. 4**), Ptger3 (**Fig. 5**) and Ptger4 (**Fig-6**) and their respective orthologs. The identities between whole-length seabream Ptger subtypes and other vertebrates Ptger ranged from 42.8 % to 88.1% (**Table 3**). The motif

TWCFI was identified in the second extracellular loop of seabream Ptger1 (**Fig. 3**), Ptger2a (**Fig. 4**), Ptger3 (**Fig. 5**) and Ptger4 (**Fig. 6**) and in those of the other sequences analyzed with the exception of zebrafish Ptger1c, frog Ptger1 and yellow croaker Ptger4, which contain leucine instead of isoleucine (**Fig 3-6**). This motif constitutes part of the 12 highly conserved amino acid residues in Ptger2a, Ptger2b (YVQYCPGTWCFI) and in Ptger3 (QWPGTWCFISTG) of most of the vertebrates mentioned in this work, except chicken Ptger3, which lacks the G (**Fig. 2C** and **Fig. 4**). These invariant residues form part of the second extracellular loop in all the mentioned vertebrate sequences, and has been suggested to act not only as a link between the transmembrane domains, but also to have a conserved structural role (allosteric conformation) and functional actions related with GTP sensitivity as well as agonist binding interaction (Stillman, Audoly, and Breyer 1998). In this context, it is important to mention that the complex G protein/GTP plays an important role in the receptor's passage from inactive "R" to the active conformation "R*" in GPCR (De Lean et al. 1980; Nataraj et al. 2001; Samama et al. 1993; Stillman et al. 1999; Venkatakrishnan et al. 2013). Furthermore, seabream Ptger4 sequence has more highly conserved regions in their TMD, EL and IL than Ptger1, Ptger2a and Ptger3 (**Fig. 6**). Notably, key residues responsible for structural as well as functional aspects of mammalian Ptger, were also found in seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4. In particular, the conserved T (in EL1) (Stillman et al. 1999), D (TMD2), C (EL1 and 2) (Savarese and Fraser 1992) and Y (TMD5) (Suganami et al. 2016), as well as the R (TMD7, a charged amino acid residue that is further conserved in the TMD7 of all PGE₂ receptors of the vertebrates mentioned in the present study except in zebrafish Ptger2b. In support of this finding, it was suggested previously that this arginine is one of the residues that may be involved in relevant functional aspects of these receptors, including conformation stability, ligand binding and signal transduction in zebrafish (Kwok et al. 2012) and in human (Audoly and Breyer 1997; Neuschäfer-Rube et al. 2004; Wilson et al. 2001). The T and S residues of the C terminus of Ptger4, responsible for agonist-induced short-term desensitization (Bastepe and Ashby 1999), were also found in seabream Ptger4 (**Fig.6**).

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		TMD1	
Seabream	-----MLALSHYNSSASPLPLLSNDSAGKVEAQVAVEELSQLGNTLTPQPTT---SGVI	52	
Y.croaker	-----MLALSHYNSSASTLLPRFTNDSTGKVEASVAVEGLLQQGNLTSVQPST---SGVI	52	
zPtgerla	-----MHHANSSGAVPLNPFSNRS--QWEGQAGSLSLADPSNVTSSRPIL---SGPI	47	
zPtgerlb	-----MLSIQQYNSTVLAEDALVNQSHHY-----VAPLRCRENLTT-----TSPT	41	
zPtgerlc	-----MDIFFTPSPFVLCQDSRGHNY----TIPCADNQTNLNSTSPSTVNPPAFG	45	
Frog	-----MFPHQRYNSSLPALSTLP-----PTSLSPSSPTPRPSS---SVPL	38	
Mouse	MSPCG-LNLSLADEAATCATPRL-----PNTSVVLPTGDNG---TSPA	39	
Human	MSPCGPLNLSLAGEATTCAAPWV-----PNTSAVPPS---G---ASPA	37	
		IL1	TMD2
Seabream	VVILSMTLGIISNIVALFILVSAYYRQ--RRRTKATFLLFATSLVVTDFIGHLIPGALVL	110	
Y.croaker	VVILSMTLGIISNIVALFILANAYSIQ--RRRTKATFLLFAASLVATDFIGHLVPGALVL	110	
zPtgerla	AAMLSMTLGILSNIVALLIPANAYARL--RRRSK-AFLLFAGSLVATDFVGHVIPGAIVM	104	
zPtgerlb	AAGLSMTLGIVSNIIAAILANAYSRR--SKTKATFLLFASSLVVTDLVGHVIPGALVL	99	
zPtgerlc	MSYFTMTLGALSNTALAILAHSYASF--RRRAKVPFLLLASTLLSDDLGHILITGALAL	103	
Frog	LPSLSMAFGVTSNALGLWILARAYTYSRRQRRSRAQFLLLASGLLTDLAGHLIAGSFVL	98	
Mouse	LPIFSMTLGAVSNVLALALLAQVAGRM--RRRSAAATFLLFVASLLAIDLGHVIPGALVL	98	
Human	LPIFSMTLGAVSNLLALALLAQAGRL--RRRSATFLLFVASLLATDLAGHVIPGALVL	96	
		::*: * * * : : : * * * : * : * : * : *	
		EL1	TMD3
Seabream	RLYLSGGVDP--ADFNSSDKMCQFLGGS		
Y.croaker	RLYLSGGVHP--EDFNSSDRMCQFLGGS		
zPtgerla	RLYLSGGVPA--EEYNRADPLCQFLGGS		
zPtgerlb	HLYLSGTGP--CLPAVPDGTQCFLLGGS		
zPtgerlc	NHLERVKHHGQMEVEPPRIYCKLFGAC		
Frog	WLYSHGGLP-----VAGCQFLGGC		
Mouse	RLYTAGRAP-----AGGACHFLGGC		
Human	RLYTAGRAP-----AGGACHFLGGC		
		* : * : * : * : * : * : * : * : * : *	
		EL2	TMD4
Seabream	VTKTRTKMCLSVIWLAAALCVALLPCFQLGSYTYQEPCT		
Y.croaker	VTKTRTKICLSVIWLAAALFVALLPCFQLGSYTYQVPGT		
zPtgerla	VTTTRTKLSLSVIWLAAALCVALLPCFQLGSYTYQYPGT		
zPtgerlb	VCTTQTQKIVMVMVWLLALSVAFLPFFRLGSYTYQYPWT		
zPtgerlc	VTMTRVRFVLLITSMALTLAGLPLLVNGNYKQFPCT		
Frog	VTQNRARLSLALAWAVALLVSIPLFLGYGAYDLQPSCT		
Mouse	VSVARARLALAVLAAMALAVALLPLVHVGRYELQYPC		
Human	VSVARARLALAABAVALAVALLPLARVGRYELQYPC		
		* : * : * : * : * : * : * : * : * : *	
		EL2	TMD5
Seabream	GLGLTSLAVAFVCNTISGLTLVLARLRKPG-----	259	
Y.croaker	GLGLTSLAIA-LCNTISGWTLVLARLRKPG-----	258	
zPtgerla	GLGLTSLAVALVCNTISGLTLVLGRIRKPC-----	253	
zPtgerlb	GLGLASLAVALVCNTISGVTLVAAARLRKQKQESIRQSKKDK-----	258	
zPtgerlc	ILGLLTLIVSLVNTLSGLKLLQARLDKGLKSSAA-----	259	
Frog	GLGLGCLAAALVCNVLVGATLLRARLRQPPEERRR-----	238	
Mouse	GLGLAALLAALVCNTLSGLALLRARWRRRSRFRKTAGPDDRRRWGSRGPRLASASSAS	270	
Human	SLGLVALLAALVCNTLSGLALLRARWRRR-SRRPPPASGPDSSRRRWGAHGPRASASSAS	267	
		*** * : : * : * : * : *	
		IL3	TMD6
Seabream	-----SHHSAKSHDIEMVVQLVGMVTSICICWSPLLIIGLMSAIRSYTG	303	
Y.croaker	-----SHHSARSHDIEMVVQLVGMVTSICICWSPLLIIFGLMSVIRSYSG	302	
zPtgerla	-----NRRSARSHDIEMVVQLVGMVTSICICWSPLLIIVGLITVKQSYEH	297	
zPtgerlb	-----QESVRLSRKDKQNKSSKTHDIEMVVQLVGMVTSICICWSPLLIIFGLMSVIRSSIS	313	
zPtgerlc	-----RRHGSFSSSSLSLDEVMTQLAVITVWSCVWSPLLIYILMSVSRYEG	309	
Frog	-----RQSRSHTHDLEMVVQLLAITFVSCVSWTPLLVSVMMLHTGI---	279	
Mouse	SITSATATLRSRGGGSARRVHAHDVEMVGQLVGMVVSICICWSPLLVLVLAIGG---	326	
Human	SIASASTFFGGSRSSGSARRARAHDEVVGQLVGMVVSICICWSPLMLVLVALAVGG---	323	
		: : * * : * * * : * * : * : *	
		EL3	TMD7
Seabream	SIGEHLS-NYTTLMVMGVRLATWNQIILDPWVYILLRRTVLRKIYLIKQAGLRG----	357	
Y.croaker	SIGEDLS-SYKTLMMVMGVRLATWNQIILDPWVYILLRRTVLRKIYLIKQAGLRG----	356	
zPtgerla	SIGDDLA-TYKTLMMGVRRIASWNQIILDPWVYILLRSVLQKIYFIFTRQTDLKE----	351	
zPtgerlb	SMGDDLEMSYRITLLVMGVRLASWNQIILDPWVYILLRAILRKIYYLTGRTGRKG----	368	
zPtgerlc	VSRPK--HQCEKLFLLALRLASWNQIILDPWVYILLRAVLRRVLQLQPDRAVYT----	362	
Frog	-----PLPRPDWLLFTVRLASLNQIILDPWVYILLRSVLFRLYTIFVRATRLRN----	328	
Mouse	----WNSNSLQRPFLFLAVRLASWNQIILDPWVYILLRQAMLRQLRLPLRVSAGKGPTL	382	

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Human	----WSSTSLQRF LFLAVRLASWNQIILDPWVY ILLRQAVLRQLRLLPFRAGAKGGPAGL	379
	::: :*: *: *****::: : :	
Seabream	-NMLGRWEPSFQSSSEKNDVNHV-----	379
Y.croaker	-NMLARWEPTSFHSSEKKQVSQV-----	378
zPtger1a	-STLRCEIHSFSPSEKNPVS RV-----	373
zPtger1b	-STFRWEASSFQNSVKKAINQT-----	390
zPtger1c	-----QSSSYRDSHRKTIS-----	376
Frog	-GKLSRWDAGEFQSSERSQGTQVLKILRALQNEGLFVKPNLSRSTQLTTNTAGAEPLPQK	387
Mouse	GLTKSAWEASSLRSSRHSGFSL-----	405
Human	GLTPSAWEASSLRSSRHSGLSHF-----	402
	: . . * : . .	
Seabream	-	379
Y.croaker	-	378
zPtger1a	-	373
zPtger1b	-	390
zPtger1c	-	376
Frog	V	388
Mouse	-	405
Human	-	402

Figure 3: Sequence alignment and domain organization of seabream Ptger1 sequence with known vertebrate Ptger1 sequences. Transmembrane domains (TMD) are shaded, extracellular loop (EL), intracellular loop (IL). (*) Identity in one position; (:) conservative substitutions in one position; (.) semiconservative substitutions in one position. The accession numbers for the sequences used are listed in **Table2**. Conserved motifs are in bold and underlined. Signal peptides are in bold. Key conserved residues are shaded in blue.

		TMD1	IL1	
sbPtger2a	-----MGNPENDTCHYKQWIDPKTPTISAIMFAAGTIIGNVAALVILEVRRCRDSR--NG	53		
Y.croaker	-----MSAMSDDPCHTDLHISPNTSPVTSAIMFAAGVVGNNMAALLILEVRRCRDAR--TG	53		
zPtger2L	-----MGTENGTCIDSVNVEQNGGPAISALMFAAGAI GNVLALVLEFRRRKE-----K	49		
zPtger2a	-----MGTENGTCIDSVNVEQNGGPAISALMFAAGAI GNVLALVLEFRRRKE-----K	49		
zPtger2b	-----MASQNVSCREQISV-SGGSP TIPAFMFSTGVLGNVVALILLEMRRRKQ-----	47		
Frog	-----MRGNSSDLCSQLHLNPGVSPHSAAMFSLGVLGNLIALVLEHRRRGI-----	49		
Chicken	-----MNGTGREPCGHGEALAAGARPLVSALMFSAGLLGNLALGLLRCRRPP-----	49		
Mouse	MDNFLNDSKLMEDCKSRQWLLSGESPAISSVMFSAGVLGNLIALALLARRWRGDTGCSAG	60		
Human	MGNASN-DSQSEDCETRQWLPPGESPAISSVMFSAGVLGNLIALALLARRWRGDVGCSAG	59		
	* : . * : . * : . * : . * : . * : . * : . * : . * : . * : . * : . * : .			
	TMD2	EL1		
sbPtger2a	GGRRRSLFHILITSLVVTDLTGTCCLISPLVQLSYHQNKTLTEMTH--SKSVCRYFGLSMT	111		
Y.croaker	DRRCRSLFHILITSLVVTDLAGTCCLISPLVQLSYSRNDTLVGMSSS-NDNVCKYFGCTMT	112		
zPtger2L	NRQRQSLFHLLVTTLVITDLTGTCCLISPVVQTAYLTNTTLVGMMSK--THAVCEYFGFAMT	107		
zPtger2a	NRQRQSLFHLLVTTLVITDLTGTCCLISPVVQTAYLTNTTLVGMMSK--THAVCEYFGFAMT	107		
zPtger2b	---PASLFHVLVSSLVITDLLGTFSLSPLVLTAYFRNASLLGMNE--NRLACGHFAYAMT	102		
Frog	-RGKISLFHILATGLVITDLMGTCMLSPVVL SAYSSNLTKALGGSDNFIVCHYFAMT	108		
Chicken	RARPPSLFHVLVLAALVITDLLGTCSVSPFVLASYHRNLTALGRGG--HICLYFGFAMS	107		
Mouse	SRTSISLFHVLVTELVTDLTGTCCLISPVVLTASYSRNQTALVALAPES--HACTYFAFTMT	118		
Human	RRSSLSLFHVLVTELVTDLTGTCCLISPVVLTASYARNQTALVALAPES--HACTYFAFTMT	117		
	****: * ** . * * * * * * * : * * * : * : * : * : * : * : * : *			
	TMD3	IL2	TMD4	
sbPtger2a	FFSLATMALLLTALERC LAIGYPYFYNRHVTKKAYITIPVVFLCTLFLCFLPFAGFGA	171		
Y.croaker	FFSLATMSLLFTALERCFAIGYPYLYNRVYTKKAYITIPLVFVLCILFCLLPFVGFGA	172		
zPtger2L	FLSLATLSILLAMALERCISIGYPYHYGRHITKRCGYITIPCIYLACFLFCIMPFGFGK	167		
zPtger2a	FLSLATLSILLAMALERCISIGYPYHYGRHITKRCGYITIPCIYLACFLFCIMPFGFGK	167		
zPtger2b	FFSVVTLAILLSMAVERWLSIGHPPFYEKYSSKRCGYITIALIYLLCALFCVTPFLGFGK	162		
Frog	FFSLATMLVLFAMALERAMAIGHYPYEFKFSKRCGLVMFPVIYSFCIFFCLFPAMGAGE	168		
Chicken	FFGLATMLVLFAMALERCLALGRPYFYERFLSPRAGLVALPAIYTFSAAFCSLPLLGFGR	167		
Mouse	FFSLATMLMLFAMALERYLSIGYPYFYRRHLSRRGLAVLPVIYGASLLFCSLPLLNYGE	178		

Figure 5: Sequence alignment and domain organization of seabream Ptger3 sequence with known vertebrate Ptger3 sequences. Transmembrane domains (TMD) are shaded, extracellular loop (EL), intracellular loop (IL). (*) Identity in one position; (:) conservative substitutions in one position; (.) semiconservative substitutions in one position. The accession numbers for the sequences used are listed in **Table2**. Conserved motifs are in bold and underlined. Key conserved residues are shaded in blue.

		TMD1	IL1	
Seabream	-----MNSTVKPHTRPPTIPAIMFIFGVGNVIAIVVLRISRKEQKET			44
A.salmon	-----MSGMNGTMTSGPREPTIPVIMFIFGVGNVIAIVVLRKSRKEQKET			47
Y.croaker	-----MVPTIPSIMFIFGVGNVIAIVVLRKSRKEQKET			34
Zebrafish	-----MEPTVPTIPAIMFIFGVISNLIIVVLRKSRKEQKET			37
Frog	MSYEQN-FSVP-NATSIQNLVAPRGSTPPIIPAVMFLGVLGNLIAIVVLRKSRKEQKET			58
Chicken	MSFNSTTILQPANGSANGTASAAEGGKPAITPTVMFIFGVGNLIAIVVLRKSRKEQKET			60
Mouse	-----MSIP-GVNASFSTPERLNSPVTIPAVMFI FGVGNLVAIVVLRKSRKEQKET			52
Human	-----MSTP-GVNSSASLSPDRLNSPVTIPAVMFI FGVGNLVAIVVLRKSRKEQKET			52
		** :*:~::~~		

TMD7		
Seabream	WRIFANQLHINQSGK-----RIEDL AIRMASVNPILD PWYIYILLRKTVVLKLM MEKIKC	314
A. salmon	LRIFVNLRSRVEFDARSTPIEKNPDL AIRIASVNPILD PWYIYILLRKTVLKL VEKIKY	324
Y. croaker	AQVFLNQLYKTPVEL---RLEKNPDL AIRFASNPILD PWYIYILLRKAVLLKL IENIKC	308
Zebrafish	VQVFLNQLYKTPVEK---RLDKNPDL AIRFASTNPILD PWYIYILLRKAVLSKV VENIKC	310
Frog	VRIFINQLYQPAVVE---DISKNPDL AIRMASVNPILD PWYIYILLRKTVLSKL IEKIKC	330
Chicken	VRVFNQLYRPPFVK---DLRQNPDL AIRIASVNPILD PWYIYILLRKTVLRKA IEKIKC	335
Mouse	VRVFINQLYQPNVVK---DISRNPDL AIRIASVNPILD PWYIYILLRKTVLSKA IEKIKC	349
Human	VRVFNQLYQPSLER---EVSKNPDL AIRIASVNPILD PWYIYILLRKTVLSKA IEKIKC	346
::* *** : ** ***.* *****.*****:* : * :*.***		
Seabream	LFC KMGQGGR--NGGQFRCADGHLNS-SIVSRDSPSLVSRELRELVS STSQT FL---YPS	368
A. salmon	LFC RLGSHGRSGVSGQFCCGGESHQNSSIVSLDSPSLVSRELKDV STSQT FL---YLP	381
Y. croaker	LFC KIGARGQ--QRQGNFHCIDAHQLSSVISKRDSQSLVSHNFRDVT STSQT FL---YLP	363
Zebrafish	LFC RSDSRGQ--QTQNSLLCINGQQFSS---RDSPSGVSRQLQDV STSQT SL---NLA	361
Frog	LFC RIGARRRH-SAGSFNCTEGRRASSA-LSSHSPSFISRELREVS STSQT LL---YPL	385
Chicken	LFC QIGGARRQH-SGSNFNCVDGRRTSSA-MSSQSPSFISRELREIS STSQT LL---YPP	390
Mouse	LFC RIGGSGRDS-SA--QHCSERRTSSA-MSGHSRSLFLARELKEIS STSQT LL---YLP	402
Human	LFC RIGGSRRE-SG--QHCSDSQRTSSA-MSGHSRSLFLARELKEIS STSQT LLPDL	402
***: .. : *		
Seabream	EGNPRRSFQ----AGAQYG----SSPPSAQTLQGP---QAGEGPQRE-----LSQSDLED	412
A. salmon	EGTKGGLGGF---RGTGGEGRPIL-PAVERSLLQDQQTQAGKGMQKDPKKGPRISMSEG	437
Y. croaker	EGCET-----Y---PGSCHTAQRLS-GSQHLSVRNSQASYSE-----QDSVSGSHRE	406
Zebrafish	KAPAGGQNSS---SESCRCSSHD-----G	382
Frog	ELSDGSLKGSVAVLPGTTIAIN--LPGSTDRTLGRSDQSVSSQGPES-----EQVSLVN	436
Chicken	ELSESVGGVLLPGP-SASLAQSDTTSVRTLRSETSDSSQGQDS-----ESVLLVN	442
Mouse	DLTESSLGGRNLLPGSHGMGLTQADTSLRLTRLISSETSDSSQGQDS-----ESVLLVD	455
Human	DLSENGLGRNLLPGVPGMLAQEDTSLRLTRLISSETSDSSQGQDS-----ESVLLVD	455
.		
Seabream	DAPGRPLNELPVC-PKDSLHVTFTDETANIEKCI	446
A. salmon	TVDPSRLNRVQLDRKDKTLHVTFTDETNLNLEKCI	472
Y. croaker	TNVSKDLSALP-CPKDTALQVLSLNE--TVEEKCI	438
Zebrafish	-----Q-C-KKQSLQMSITND--SVQEKS	403
Frog	ETKPTRVTQNGSLSKGNPLRVTFPNETLHLSGKSI	471
Chicken	DIRSG--GGATSMKSGSPLQVTFPTETLNLEKCI	475
Mouse	EVSQS--HREEPASKGNSLQVTFPSETLKLSEKCI	488
Human	EAGGS--GRAGPAPKGSSLQVTFPSETLNLEKCI	488
*::: : .. *.*		

Figure 6: Sequence alignment and domain organization of seabream Ptger4 sequence with known vertebrate Ptger4 sequences. Transmembrane domains (TMD) are shaded, extracellular loop (EL), intracellular loop (IL). (*) Identity in one position; (:) conservative substitutions in one position; (.) semiconservative substitutions in one position. The accession numbers for the sequences used are listed in **Table2**. Conserved motifs are in bold and underlined. Signal peptides are in bold. Key conserved residues are shaded in blue.

3.3. Phylogenetic analysis of seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4

The phylogenetic tree constructed with the Neighbor-Joining method, based on full amino acid sequences of seabream Ptger1-4, revealed two branches. The first branch comprised Ptger2 and Ptger4 and the second branch comprised Ptger1 and Ptger3 (**Fig. 7A**). Furthermore, the phylogenetic tree constructed with mammalian and teleost Ptger sequences formed two clusters (**Fig. 7B-E**). Furthermore, gilthead seabream Ptger1-4 have the closest phylogenetic relationship

with their respective receptors of other fish species belonging to the Perciformes order, followed by Salmoniformes (Fig. 7B-E).

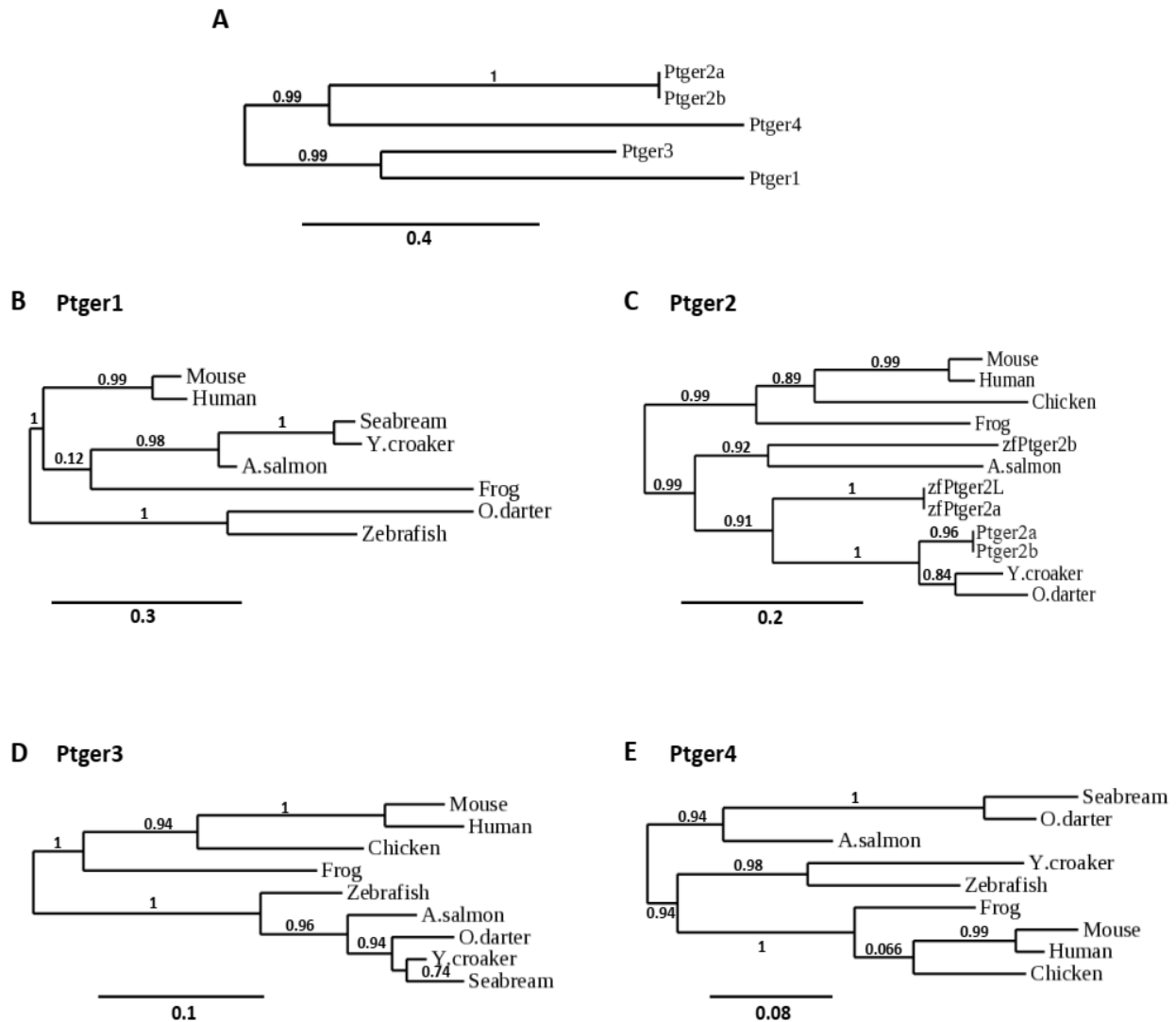


Figure 7: Phylogenetic relationships seabream Ptger subtypes sequences. Phylogenetic tree of seabream Ptger1-4 (A) and vertebrate Ptger subtypes and seabream Ptger1 (B), Ptger2a and Ptger2b (C), Ptger3 (D) and Ptger4(E) polypeptides. The tree was generated by phylogeny.fr software (Dereeper et al. 2008) using amino acid sequences. Numbers shown are percentages of 100 bootstrap replicates in which the same internal branch was observed. The horizontal lines are drawn proportional to the inferred phylogenetic distances, while the vertical lines have no significance. ENA accession numbers are listed in **Table 2**.

3.4. Gilthead seabream *Ptger1-4* are differentially distributed and modulated upon bacterial infection

To gain further insight into the role of the *Ptger1-4* immune response in gilthead seabream, RT-qPCR was used to analyze the expression pattern of their corresponding genes as well as their modulation upon bacterial challenge. Seabream *ptger1*, *ptger2a*, *ptger3* and *ptger4* were expressed in all the tissues examined, including such key immune organs as head kidney (HK), spleen, thymus and gills (**Fig. 8**). In particular, the highest mRNA levels of *ptger1* were found in spleen, followed by HK, thymus, gill and peritoneal exudate (PE, the site of bacterial injection) (**Fig. 8B**). In a similar way, the most abundant transcript levels of *ptger3* were also found in spleen and gill, followed by thymus, HK, and liver (**Fig. 8C**). Interestingly, the weakest mRNA levels of *ptger1* and *ptger3* were found in blood (**Fig. 8C**). By contrast, the highest mRNA levels of *ptger2a* and *ptger4* were found in blood (**Fig. 8B** and **8D**), followed by spleen and thymus for *ptger2a* (**Fig. 8B**) and by spleen, thymus, PE and gill for *ptger4* (**Fig. 8D**). However, it is important to note that the magnitude order of the mRNA levels of *ptger2a* is higher compared to those of *ptger4* and much higher compared to *ptger1* and *ptger3* ones (**Fig. 8A-D**).

Furthermore, infection of fish with the pathogenic bacterium *V. anguillarum* resulted in increased mRNA levels of *ptger1*, mainly in blood followed by HK, thymus and liver (**Fig. 8A**). However, bacterial challenge led to decreased mRNA levels of *ptger1* in PE (**Fig. 8A**). By contrast, the mRNA levels of *ptger3* did not significantly change, or slightly decreased, in all the tissues examined, with the exception of PE, in which increased four-fold. Similarly, bacterial challenge resulted in increased mRNA levels of *ptger2a* and *ptger4*, mainly in PE (**Fig. 8B** and **4D**) followed by gill in the case of *ptger2a* (**Fig. 8B**), and HK, spleen and gill in the case of *ptger4* (**Fig. 8D**). However, the mRNA levels of *ptger2a* did not significantly change in HK, and even decreased dramatically in blood (the highest basal expression site), spleen and thymus after bacterial challenge (**Fig. 8B**). However, the mRNA levels of *ptger4* increased in all organ tissues analyzed, mainly in PE, but not in blood, where they did not change (**Fig. 8D**). Taken together, these results showed a different distribution and modulation pattern of the four PGE₂ receptors in the main immune organs of gilthead seabream.

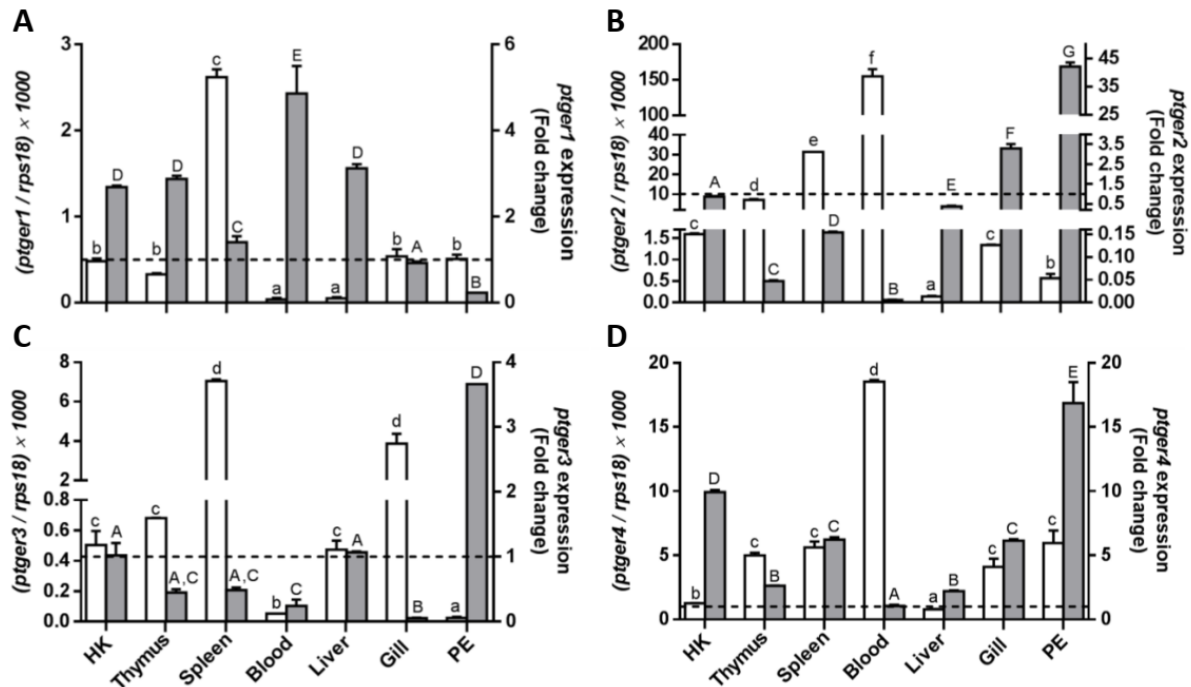


Figure 8: Tissue expression pattern of seabream *Ptger* subtypes. Basal mRNA levels (left y axis, white bars) and those following bacterial challenge (right y axis, gray bars) of *ptger1* (A), *ptger2a* (B), *ptger3* (C) and *ptger4* (D) were determined by real-time RT-PCR in the indicated tissues of control adult specimens or following a 4 h challenge with 10^8 cells of *Vibrio anguillarum*. The results are representative of five fishes. The constitutive expression is shown relative to *rps18* and the regulation of the expression following infection is shown as the mean $\pm$ S.E. of them RNA fold change in infected fish relative to control fish (indicated with horizontal dashed lines). Different letters denote statistically significant differences among the groups according to a Tukey test ($p < 0.05$). The groups marked “A” did not show statistically significant differences from non-infected fish.

3.5. Expression of gilthead seabream *Ptger1-4* in professional phagocytes

We next analyzed the constitutive expression of *ptger1*, *ptger2a*, *ptger3* and *ptger4* in seabream professional phagocytes, macrophages and AGs, as well as the effect of their activation with VaDNA or/and PGE₂ (the receptor ligand) in the mRNA levels of those receptors. It is important to note that although the methods used for phagocyte isolation could induce some level of activation, previous studies showed that this induction was minimal compared to those of

VaDNA for other activation markers, like *il1b* and *tlr5* (Sepulcre et al., 2007) or *pges* (Montero et al., 2016). The results showed that the four receptors were constitutively expressed in both cell types, more strongly in macrophages than in AGs, with the exception of *ptger1* (**Fig.9A**). Interestingly, while AGs showed similar mRNA levels of the four receptors, the transcription levels of the genes encoding for Ptger2a and Ptger3 in macrophages were much higher than those of Ptger1 or Ptger4 (**Fig.9A**). Furthermore, the results revealed that, in macrophages, the mRNA levels of *ptger1*, *ptger2a*, *ptger3* and *ptger4* decreased upon stimulation with VaDNA (**Fig. 9B-E**). However, treatment of macrophages with PGE₂, resulted in a similar expression pattern of *ptger1* and *ptger3*, whose mRNA level decreased (**Fig.9B and 9D**), and of *ptger2a* and *ptger4*, whose mRNA levels did not significantly change (**Fig.9C and 9E**). In addition, the joint treatment of macrophages with PGE₂ and VaDNA had a synergistic effect in the reduction of the mRNA levels of *ptger1*, but led to a less pronounced decrease in the transcription levels of *ptger3*. Furthermore, the joint treatment of macrophages with PGE₂ and VaDNA abolished the effect that VaDNA alone had on the reduction of *ptger2a* and *ptger4* transcript levels.

In the case of AGs, stimulation by VaDNA or PGE₂ resulted in increased mRNA levels of *ptger2a* and *ptger4*, especially in the case of PGE₂ (**Fig. 9C and 9E**). In addition, treatment of AGs with PGE₂ and VaDNA together abolished the effect of PGE₂ in the induction of *ptger2a* and *ptger4* transcript levels (**Fig. 9C and 9E**). In the case of *ptger1* mRNA levels, the individual treatment of AGs with VaDNA or PGE₂ had a much weaker effect, and their combined use resulted in a dramatic decrease (**Fig. 9B**). However, the results differed in the case of *ptger3*, when VaDNA increased *ptger3* mRNA levels and PGE₂ had no effect on its own. However, the combination of both stimuli resulted in the lower induction of *ptger3* transcript levels compared with VaDNA alone (**Fig.9D**). All these results further demonstrate that seabream phagocytic cells are armed with Ptger1, Ptger2a, Ptger3 and Ptger4, which allows them to respond to their ligand, PGE₂, which interfere in the regulation of the mRNA levels of each receptor by PAMPs. Furthermore, in the light of our data, it is tempting to speculate that each phagocyte population would play different roles in the innate immune responses through these receptors, however further functional studies are required.

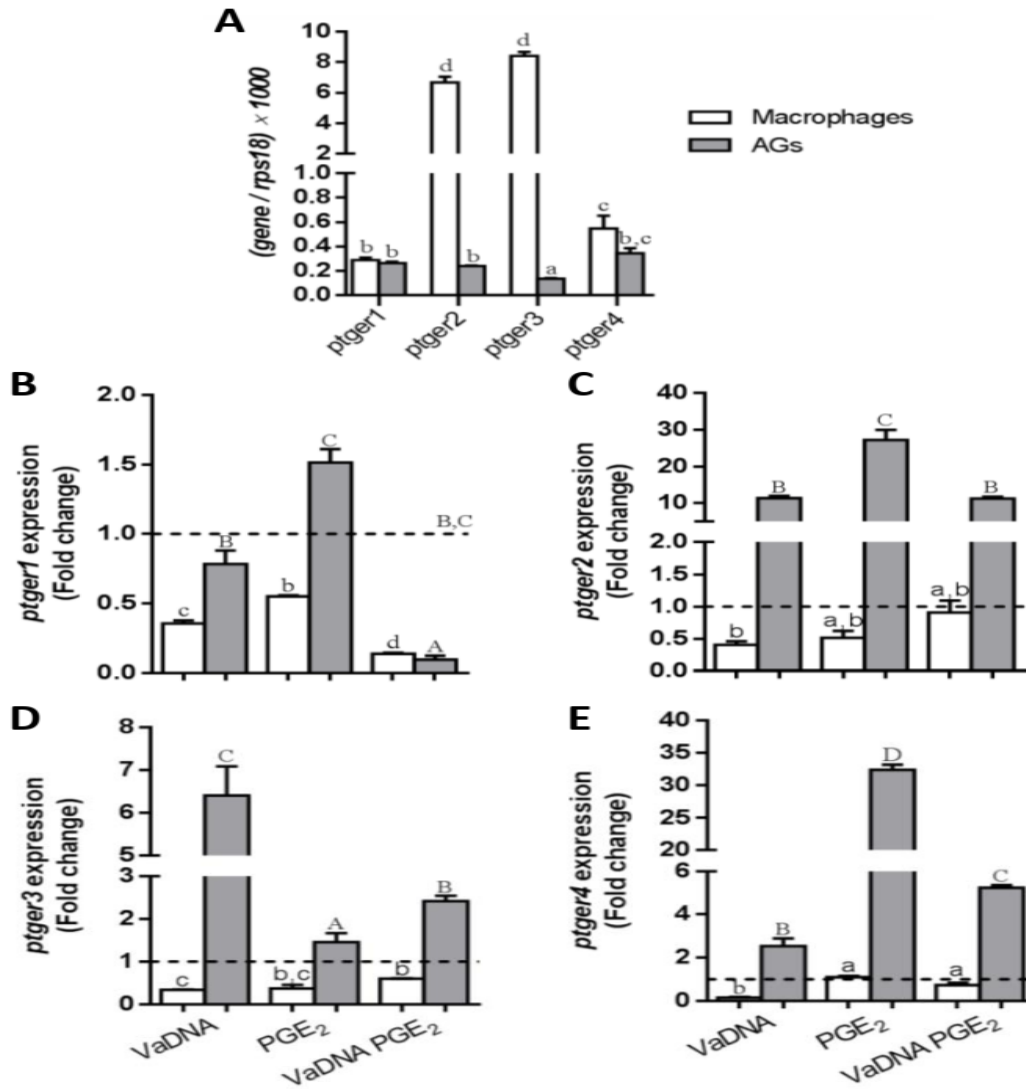


Figure 9: Expression pattern of seabream Ptgers subtypes in professional phagocytes. Basal mRNA levels of seabream Ptger subtypes in macrophages (white bars) and GAs (grey bars) (A) and following stimulation with VaDNA and/or PGE₂. Levels of *ptger1* (B), *ptger2a*(C), *ptger3*(D) and *ptger4* (E) were determined by real-time RT-PCR. The results are representative of six independent experiments. The constitutive expression is shown as relative to *rps18* and the regulation of expression following stimulation is shown as the mean ± S.E. of them. RNA fold change in non-stimulated cells. Different letters denote statistically significant differences among the groups. The groups marked “a” for macrophages or “A” for AGs did not show statistically significant differences from non-stimulated cells with the exception of Ptger1 where this group is marked “B,C”.

3.6. Ectopic expression of Ptgers resulted in increased mRNA levels of *il8*

In order to determine the role of the cloned seabream *ptger1*, *ptger2a*, *ptger2b*, *ptger3* and *ptger4* in the immune response, human *il8* expression pattern was performed by RT-qPCR in HEK cells transfected or no with the target genes in milieu with or without PGE₂ as determined in higher vertebrate (Brant and Fabisiak 2013; Neuschäfer-Rube et al. 2013). Importantly, from the collected results we realized that PGE₂ induced significantly *il8* expression pattern in HEK cells containing either *ptger1* or with *ptger3* constructions in comparison with both non transfected and non stimulated cells (**Figure 10A**). However, for Ptger2a and Ptger4, no remarkable effect observed after adding PGE₂ in the time when this ligand enhanced the target chemokine in Ptger2b expressing cells (**Figure 10B**). These data let us to suggest that PGE₂ trigger important role in the regulation of the inflammatory response or in the maintain of homeostasis via Ptger1, Ptger2b and Ptger3 in lower vertebrates but more functional analysis are needed to understand the specific signaling pathway of each receptors in response to their ligand in teleost.

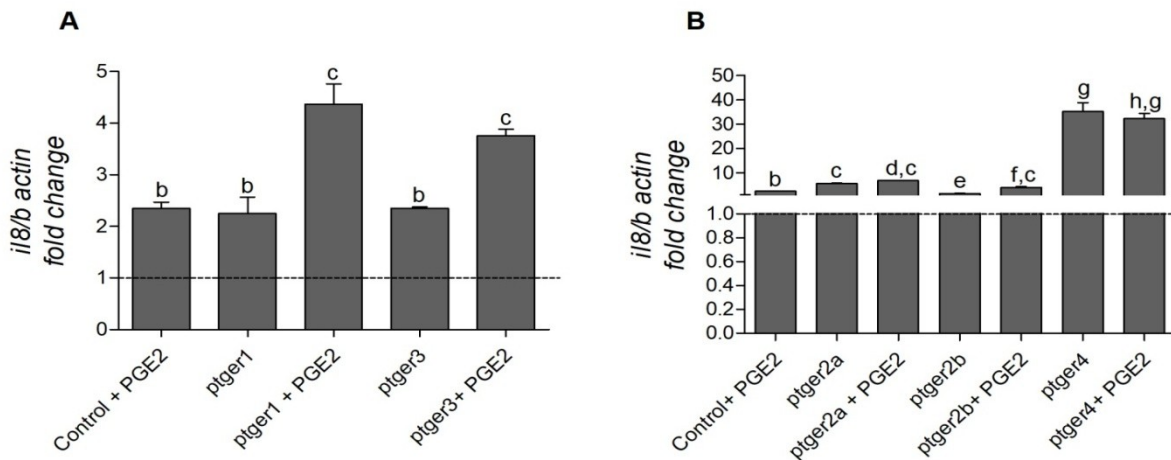


Figure 10: *ptger1*, *ptger2a*, *ptger2b*, *ptger3*, and *ptger4* promote *in vitro* *il8* modulation in both non stimulated and PGE₂ stimulated HEK293 cells. Basal expression data were normalized to the housekeeping gene β actin. IL-8 mRNA modulation in HEK 293 cells transfected with *ptger1* and with *ptger3*(A) and in HEK 293 cells transfected with *ptger2a*, *ptger2b* and *ptger4* (B) data are shown as the mean $\pm$ SEM of the gene mRNA fold change in stimulated milieu relative to non-stimulated milieu (indicated with horizontal dashed lines). Different letters denote significant differences ($p < 0.05$) among groups and within each group, using one-way analysis of variance (ANOVA) and Tukey's multiple range test.

4. Discussion

Recent studies from our group have shown that PGs, including PGE₂, play a key role in the inflammatory process in the most divergent group of vertebrates, teleost fish (Gómez-Abellán et al. 2015; Gómez-Abellán and Sepulcre 2016; Montero et al. 2016; Tyrkalska et al. 2016). However, studies concerning the PGE₂ signaling pathways are scarce. In the present study, seabream PGE₂ receptors, Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4, were cloned and characterized. Multiple alignment, structure domain and phylogenetic tree analysis revealed that the characterized receptors are the bonafide orthologs of mammalian PTGERs, with the typical domain organization of G protein-coupled receptors. Furthermore, a variant of seabream Ptger2a, called Ptger2ab, was also identified and found to be structurally composed of six TMD rather than the seven present in all PGE₂ receptor subtypes of the vertebrates mentioned in this study, similar to zebrafish Ptger2a and mouse Ptger3, which have six TMD. It is possible that these two isoforms exhibit different degrees of sensitivity with regard to PGE₂ binding, resulting in different levels of action. Two isoforms, Ptger2a and Ptger2b, have been identified in zebrafish, in which both enhance cAMP synthesis (Tsuge et al. 2013). Interestingly, putative ccaat boxes and polyadenylation signals were found in the UTRs of *ptger2a* and *ptger4*, as they have been in higher vertebrates (Arakawa et al. 1996; Foord et al. 1996; Locker and Buzard 1990; Smock et al. 1999). Furthermore, motifs associated with mRNA instability were identified in *ptger1*, *ptger3* and *ptger4*; however, Cytoplasmic polyadenylation elements were identified only in *ptger4* UTRs. In addition, seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4 share with their vertebrate orthologs the key residues responsible for structural as well as functional aspects, namely the conserved threonine (Stillman et al. 1999), aspartic acid, cysteine (Savarese and Fraser 1992) and tyrosine (Suganami et al. 2016) residues. Conserved arginine of the seventh TMD (Narumiya et al. 1999), which is preserved among vertebrates (Yokoyama et al. 2013) and targeted by N/O glycosylation, as well as the threonine and the serine residues of the C terminus responsible for agonist-induced short-term desensitization (Bastepe and Ashby 1999) were only found in Ptger4. The motifs of the extracellular and intracellular loops involved in G protein coupling and/or ligand binding are conserved among fish and their orthologs (Kwok et al. 2012), which was further confirmed by the phylogenetic analysis which showed that seabream PGE₂ receptors clustered into two groups: one involving higher vertebrates and the other encompassing all fish species. These new findings, together with previous results (Margan et al. 2012; Stillman

et al. 1999; Hagiwara et al. 2014; Guo et al. 2015), support the idea that PGE₂ signaling through Ptger1-4 subtypes predated the split of fish and tetrapods more than 450 million years ago.

Several reports exist about the effect of PGs on immune-related activities in fish, most of them focused on the biological activity of PGE₂ (Gómez-Abellán and Sepulcre 2016). The variety of effects that PGE₂ can elicit, may reflect the presence of specific Ptger subtypes in different tissues and cell types. We have shown herein that gilthead seabream Ptger subtypes were expressed in all the immune tissues analyzed, including HK, spleen, blood, gill, thymus and PE, but with different distributions. In this respect, the results pointed to two distinct expression patterns: one for *ptger1* and *ptger3* and another for *ptger2* and *ptger4*. The highest mRNA levels of the genes coding for Ptger1 and Ptger3 were found in spleen, while those coding for Ptger2a and Ptger4 were also observed in blood. However, *ptger2a* and *ptger4* were the most abundant subtypes in most of the tissues analyzed, unlike in mouse, where *Ptger3* and *Ptger4* are the most robustly distributed. It is important to note that the basal expression profile of the four receptors differs in the main phagocytic cell types of seabream, being *ptger2a* and *ptger3* more strongly expressed in macrophages than in AGs and showing AGs similar basal mRNA levels of *ptger1-4*. Hence, the basal expression profile of *ptger1-4* in the different organs reflects the contribution of the breadth of cell types present in the specific organ, not only of macrophages and AGs.

Furthermore, seabream challenged with *V. anguillarum* modulated the expression of genes encoding the different Ptger subtypes, *ptger2a* and *ptger4*, showed similar expression profiles, PE (the site of bacterial injection) being the tissue with the highest induction rate of the mRNA levels of both receptors, and blood with lowest rate. In contrast, *ptger1* showed a modulation profile that was the opposite of that shown by the rest of the *ptger* subtypes. Taken together, these results suggest not only that mRNA levels in the specific tissues are modulated in response to bacterial challenge but also could reflect the mobilization of different immune cells from one organ to another (Chaves-Pozo et al. 2005). To get further into this point, we also analyzed the constitutive expression of the genes encoding Ptger1-4 and their modulation by PAMP and ligand stimulation in the main innate immune cells of seabream. The results showed that stimulation of phagocytic cells with VaDNA and PGE₂, alone or together, up-regulated the mRNA levels of *ptger2a*, *ptger3* and *ptger4* in AGs, but not in macrophages, where the levels decreased very slightly or did not change. This data, together with those previously reported showing that

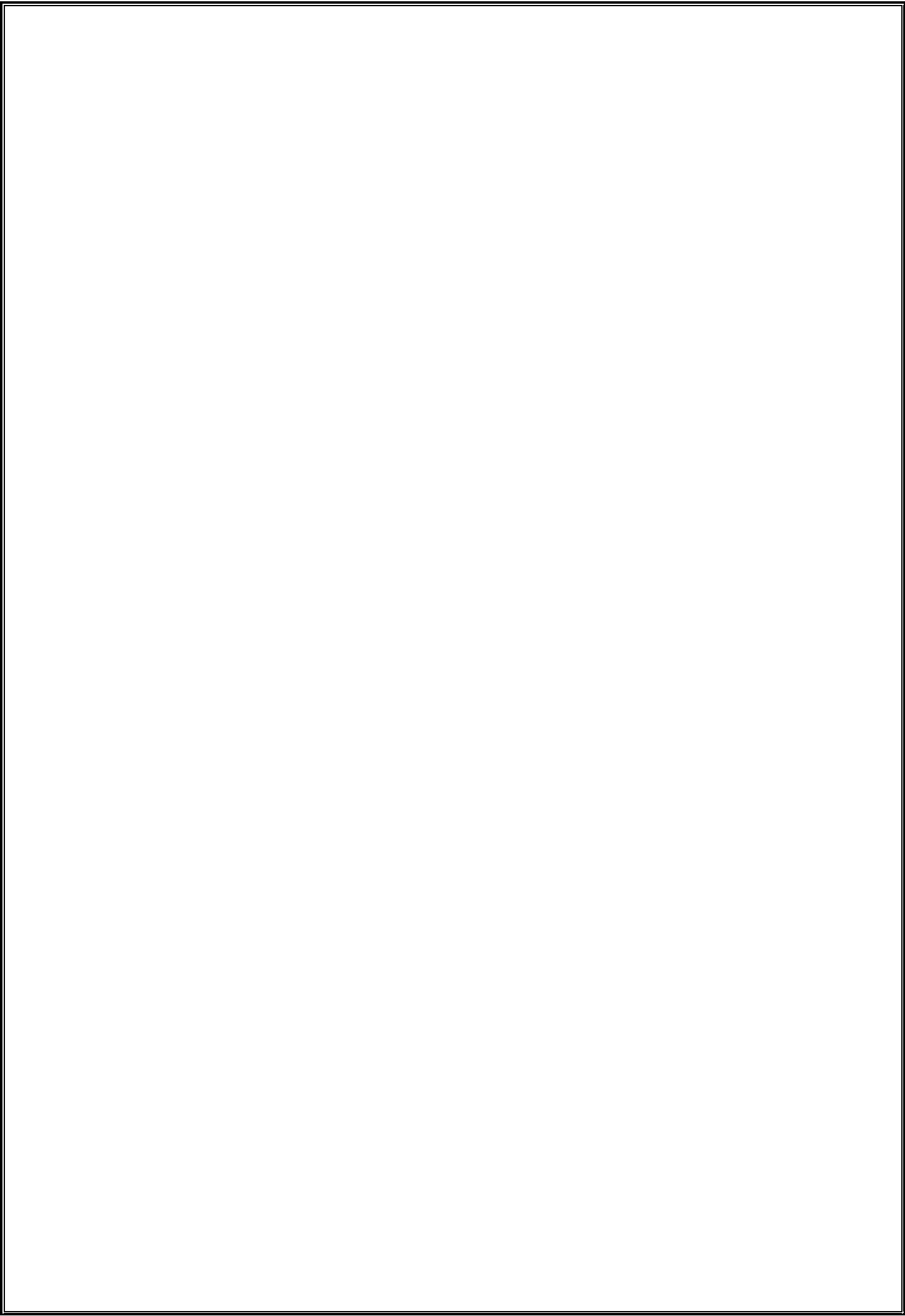
seabream infection with *V. anguillarum* results in AGs recruitment and activation at the injection site at the time tested herein (Chaves-Pozo et al. 2005), support the hypothesis that mobilization of AGs from different organs contribute to the mRNA levels of *ptger1-4* in the specific tissues after bacterial challenge. Furthermore, the results confirmed the *in vivo* results by demonstrating the similar regulation of *ptger2a* and *ptger4* expression by VaDNA or/and PGE₂ in both AGs and macrophages. Nevertheless, *ptger1* and *ptger3* showed a similar degree of modulation in macrophages, but behaved in a totally different way in AGs. This differential regulation of *ptger1-4* in macrophages and AGs is in agreement with the fact that PGE₂ promotes M2 polarization of macrophages but deactivates AGs.

As defined chemokines including IL-8 are small protein characterized by the chemoattraction of leucocytes such as neutrophils monocytes, eosinophils and lymphocytes to the site of injury during the inflammatory response, in mammals (Russo et al. 2014; Zlotnik and Yoshie 2000) and in teleost (Harun et al. 2008; Montero et al. 2008 de Oliveira et al. 2013; Oehlers et al. 2010). Its homeostatic and angiostatic action that underline their constitutive expression were further revealed (Russo et al. 2014; Zlotnik and Yoshie 2000). For that, in the current study we expected a role of at least one of the investigated Ptgers receptors in the modulation of IL-8 as determined in human (Brant and Fabisiak 2013; Neuschäfer-Rube et al. 2013). Interestingly, our results showed that in all the transfected HEK cells and mainly Ptger4 expressing cells IL-8 mRNA was enhanced significantly in comparison with control condition. However, in response to PGE₂ stimulation we have found that Ptger1, Ptger2b and Ptger3 remarkably upregulated this chemokine while Ptger2a and Ptger4 did not. This finding supports our previous suggesting that Ptger2a and Ptger2b may exhibit different sensitivity regarding PGE₂ stimulation. In other hand, in accordance, with our finding several studies in mammals have shown the implication of PGE₂ receptors in the induction of IL-8. Indeed, *in vitro* HEK293 transfection study has shown that costimulation of Ptger1 and Ptger4 activate the transcriptional factor NF- κ B that bind to IL-8 promoter to enhance the mRNA and the protein rate of this molecule (Neuschäfer-Rube et al. 2013). In gastrointestinal inflammation PGE₂, through Ptger2 and Ptger4, modulated IL-8 (Srivastava et al. 2012). Furthermore, during colonic inflammation in PGE₂/EP4/cAMP dependent mechanism (Dey et al. 2009). However, the involvement of Ptger3 in IL-8 induction still largely unknown. The obtained data reflect a conserved PGE₂-mediated Ptgers enhancement

of IL-8 in vertebrates, but, further functional investigations are needed to determine the pathway upstream this phenomenon.

In conclusion, the above results, taken together, suggest a role for G protein-coupled PGE₂ receptors, that is conserved from fish to mammals, thus adding for our understanding of their roles in the modulation of the teleost immune response.

CHAPTER II: P G A2 regulates macrophages
and acidophilic granulocytes functions in the
teleost fish gilthead seabream (*Sparus aurata L.*)



PGA₂ regulates macrophages and acidophilic granulocytes functions in the teleost fish gilthead seabream (*Sparus aurata* L.)

Abstract

Prostaglandin A₂ (PGA₂) acting through the nuclear receptor Nr4a3 regulates the immune response of mammals. However, the relevance of PGA₂ and Nr4a3 in teleosts is scant. In the current study, we have identified Nr4a3 in gilthead seabream and shown that PGA₂ regulates *in vitro* the main functions of professional phagocytes of this species. The gilthead seabream Nr4a3 revealed highly conserved motifs with all their vertebrate orthologs, particularly the DNA binding domain and sumoylation site. Expression analysis showed that *nr4a3* was constitutively expressed in macrophages and was upregulated by PGA₂ alone and combined with bacterial DNA. Importantly, PGA₂ addition weakly decreased the transcript levels of genes encoding the cytokines interleukin-1 β (Il1b), Il6 and Il10, while its combination with VaDNA promoted a synergistic induction of the mRNA levels of the 3 genes. However, PGA₂ failed to regulate the mRNA levels of the gene encoding the M2 marker mannose receptor C-type 1 (Mrc1). In contrast, PGA₂ inhibited the respiratory burst of acidophilic granulocytes and induced the transcript levels of the genes encoding Il1b and Il10. Finally, PGA₂ was able to signals through gilthead seabream Nr4a3 when ectopically expressed in HEK293 cells. In conclusion, we have shown for the first time that PGA₂ regulates the main functions of professional phagocytes of teleosts and provided evidences that it may signals through Nr4a3, paving the way for *in vivo* studies in order to understand the role of this prostaglandin and the Nr4a3 receptor in fish immune responses.

1. Introduction

The cyclopentenone prostanoids (cPGs or cyPGs) are potent reactive electrophilic lipid which have been found to exert protective effects during pathophysiological inflammation and oxidative stress in mammals (Ohmura et al. 2017; Stamatakis and Pérez-Sala 2006; Storer et al. 2005) as well as in invertebrates (Stanley et al. 2012). PGA_1 , PGA_2 and PGJ_2 are CyPGs, derived from conventional oxygenated PGs, PGE_1 , PGE_2 and PGD_2 , respectively, by nonenzymatic dehydration (Holbrook et al. 1992; Lakshmi et al. 2019). They are characterized by α , β -unsaturated carbonyl group located in the cyclopentenone ring which allow them to react with cysteine residues of target cellular proteins, such as NF- κ B and AP-1 via Michael addition reactions (Bui and Straus, 1998; Straus et al., 2000; Straus and Glass, 2001). This property confers the CyPGs the capacity to regulate protein activities (Stamatakis and Pérez-Sala 2006) which enable them to display polyvalent and controversial roles in order to buffer the dangerous effects that could arise after overactivation of the immune system (Díez-Dacal and Pérez-Sala 2010).

In this context, PGA_2 has recently been described as a rapid metabolic product of PGE_2 dehydration, which shows potent anti-inflammatory effects *in vitro* and *in vivo* (Ohmura et al. 2017). It acts mainly in the late stage of inflammation (Díez-Dacal and Pérez-Sala 2010; Lakshmi et al. 2019), suppressing large spectrum of proinflammatory mediators, such as cytokines, chemokines and nitric oxide (NO). Furthermore, it inhibits cell mobilization and clearance by phagocytosis (Stamatakis and Pérez-Sala 2006).

In response to different stimuli (Lakshmi et al. 2019), such as lipopolysaccharide (LPS), interferon γ (INF- γ) and tumor necrosis factor α (TNF- α), and in contrast to its precursors (PGs) that act via binding on G protein-coupled cell surface receptors, CyPGs including PGA_2 , are transported into the intracellular compartment (Storer et al. 2010) where they exert their functions by directly modifying proteins, as indicated above, or acting through specific receptors. Hence, the nuclear receptor subfamily 4 group A member 3 (NR4A3) (named also NOR-1, TEC, CHN or MINOR), which belongs to the subfamily of nuclear receptors 4 (NR4A) together with NR4A1 [Nur77 or nerve growth factor IB (NGFIB)], NR4A2 (Nurr1)

(Jiang et al. 2019; Ma et al. 2020), was identified as a receptor of PGA_2 (Kagaya et al. 2005; Zhu et al. 2013). NR4A3 was initially described as an orphan nuclear receptor which acts as a transcription factor upon PGA_2 binding (Kagaya et al. 2005; Zhu et al. 2013). However, it was also found to be able to enhance transcriptional machinery independently of ligand binding, reflecting its constitutive activity (McMorrow and Murphy 2011). It can bind its DNA target sites as a monomer to NGFI-B response element (NBRE), or as homodimer or heterodimer with other family members to Nur-responsive element (NurRE) (Jiang et al. 2020; Jiang et al. 2019; Zhao and Bruemmer 2010). Nevertheless, its mechanism of action is controversial, as it was early reported that PGA_2 is able to bind to unidentified cell membrane receptors enhancing release of cAMP and Ca^{2+} (Muallem et al. 1989) and, more recently, its ability to signals through the PGE_2 receptor EP4 (Ohmura et al. 2017).

Several studies have shown a role for NR4A3 in cell survival, proliferation, mobilization and apoptosis (Fedorova et al. 2019; Prince et al. 2017). In this context various immune cells are the target of NR4A3 these actions for the resolution of the inflammation. Indeed, it is a potent regulator of inflammatory genes in mammalian macrophages (Bonta et al. 2006; Pei et al. 2005; Pei et al. 2006), dendritic cells (Nagaoka et al. 2017) and B cells (Close et al. 2019), acts as a regulator of monocyte-derived dendritic cells differentiation (Boulet et al. 2019). In addition, at high level of NR4A3 expression is involved in the resolution of inflammation by inhibiting neutrophil mobilization and NF- κ B activation through JAK2-STAT3 pathway (Jiang et al. 2019).

To the best of our knowledge, the immune role of PGA_2 and its receptors is largely unknown in non-mammalian species. Prince et al. (2017) showed the pivotal roles of Nr4a3 in neutrophil survival, development and function during inflammation in both mammalian and zebrafish. Therefore, the available information is still very limited to understand the mechanism of PGA_2 and Nr4a3 functions in teleosts.

In this study, we identified and phylogenetically characterized Nr4a3 in the teleost fish gilthead seabream and analyzed the *in vitro* effects of PGA_2 in acidophilic granulocytes, functionally equivalent to mammalian neutrophils, and macrophages of this species. We also demonstrate that PGA_2 signals through Nr4a3 in gilthead seabream.

2. Materials and Methods

2.1. Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*Sparus aurata*, Actinopterygii, Sparidae) were bred and kept at the Oceanographic Center of Murcia (Spain) in a 14 m³ running seawater tank (dissolved oxygen 6 ppm, flow rate 20% tank volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Burgos, Spain). The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals. Seabream were killed by cervical dislocation using approved procedures following anaesthetization with clove oil. All efforts were made to minimize animal stress and to ensure that termination procedures were performed efficiently.

2.2. Isolation of phagocytes

AGs were obtained by Magnetic-Activated Cell Sorting (MACS), as described previously (Roca et al., 2006). Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a monoclonal antibody (mAb) specific to gilthead seabream AGs (G7) (Sepulcre et al., 2002), washed twice with PBS containing 2 mM EDTA (Sigma-Aldrich) and 5% FCS (Invitrogen), and then incubated with 100-200 µl per 10⁸ cells micro-magnetic-bead-conjugated anti-mouse IgG antibody (MiltenyiBiotec). After washing, G7+ (AGs) cell fractions were collected by MACS following the manufacturer's instructions and their purity was analyzed by flow cytometry (Roca et al., 2006). Head kidney macrophage monolayers were then obtained by overnight culture of G7-fractions in serum-free medium as described previously (Roca et al., 2006).

2.3. Cell culture and treatments

Phagocytes (AGs and macrophages) were treated at 23 °C with 50 µg/ml phenol extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (VaDNA) (Pelegrin et al., 2004) and/or were exposed *in vitro* to different concentrations of PGA₂ at two times (1.5 and 16 h) PGA₂ in sRPMI (RPMI 1640) culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl, supplemented with 0.1% FCS and 100 IU/ml

penicillin and 100 µg/ml streptomycin (Biochrom). Stimulation concentrations were 0.1, 1 and 10 µM for AGs and 1 µM for macrophages.

2.4. Analysis of gene expression

Total RNA was extracted from cell pellets with TRIzol Reagent (Invitrogen) following the manufacturer's instructions, and the up perphases containing RNA were purified with RNAquous Micro Kit (Ambion) according to the manufacturer's protocols. Final RNA concentration was measured at 260 nm by means of the nanodrop system (ND-1000), and RNA quality was checked by electrophoresis. RNA was stored at -80 °C until use. Total RNA was treated with amplification grade DNase I (1 unit/µg RNA, Life Technologies). Then, SuperScrip III RNase H- Reverse Transcriptase (Life Technologies) was used to synthesize first strand cDNA with oligo-dT18 primer from 1 µg of total RNA for 50 min at 50 °C, followed by 15 min at 70 °C. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reactions were performed in 10 µl mixtures, containing 5 µl of 2 × SYBR Green PCR Core Reagents (Applied Biosystems), 0.15 µl of each primer (0.15µM, final concentration), and 2.5 µl of cDNA. The amplification profile was: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60°C, and, finally, 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. The RNA was quantified using ribosomal protein S18 (*rps18*) transcript content as housekeeping gene using the comparative Ct method (either $2^{-\Delta\Delta C_t}$ or $2^{-\Delta C_t}$ for normalization or not with the unstimulated sample). The primers used (**Table 1**) had an amplification efficiency of 2 and their specificity was confirmed by melting curve analysis and the sequencing of each amplified product (Genetic Analyzer ABI PRISM 3130, Applied Biosystems) (data not shown). In all cases, each PCR was performed with triplicate samples.

Table 1: Primer sequences used for cloning and qRT-PCR.

Gene	Forward/Reverse	Primer sequence (5'→3')	Technique
<i>il1b</i>	Forward	GGGCTGAACAACAGCACTCTC	qRT-PCR
	Reverse	TTAACTACTCTCCACCCTCCA	
<i>il10</i>	Forward	TGGAGGGCTTTCCTGTCAGA	qRT-PCR
	Reverse	TGCTTCGTAGAAGTCTCGGATGT	
<i>il6</i>	Forward	ATGCTGAAGTTGGTGGAAGG	qRT-PCR
	Reverse	GGGTCTGAACAACAGCACTCTC	
<i>mrc1</i>	Forward	GTTTTGACAGTTGGGCTGGT	qRT-PCR
	Reverse	CCTGAATCGCTCATTGTTGA	
<i>nr4a3</i>	Forward	CCCTAAAGTCACCCCGCTTT	qRT-PCR
	Reverse	CTGGTAGAGGCAGGAGGACT	
<i>nr4a3</i>	Forward	aaaaaggatccATGCCCTGTGTGCAGGCTCAGTACGG	cloning
	Reverse	aaaaagcggccgcGTAGGGCAGAGTGTCCAGGAACCT	
<i>ENO1</i>	Forward	TTACCACAACCTGAAGAATG	qRT-PCR
	Reverse	TCCAGGCCTTCTTTATTCTC	
<i>ENO3</i>	Forward	CAACATCCTGGAGAACAATG	qRT-PCR
	Reverse	ATTGCGATAGAACTCAGATG	

2.5. ROS production

ROS production was measured as the chemiluminescence of the cells, following the methodology of Bayne and Levy (1991). Fractions enriched in G7 +, treated *in vitro* with PGA_2 , were incubated with 100 μl of a solution of luminol (100 μM) and myristate and phorbol acetate (PMA) (1 μg / ml) in HBSS (Hanks buffer). Chemiluminescence was recorded every 117 seconds for 1 hour on a FLUO start plate reader (BGM LabTechnologies). The data were obtained as the mean of three measurements and expressed as the slope of the reaction curve from 585 to 1170 seconds, to which the nonspecific background emission was subtracted.

2.6. In silico analysis of seabream PGA_2 receptor sequences

Nucleotide sequences of seabream *nr4a3* (Accession numbers in **Table 2**) were obtained from a public EST data base. The deduced amino acid sequences was obtained via EMBOSS: Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). Areas of seabream protein sequences similar to those of other vertebrate sequences obtained from the NCBI data base were compared after multiple amino acid sequence alignments within the website tool Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), provided by the European Bioinformatics Institute (EMBL-EBI). Phylogenetic reconstructions were performed with phylogeny software (<http://www.phylogeny.fr>) (Dereeper et al.,2008). The molecular weight of each protein was determined using the Science Gateway tool (http://www.sciencegateway.org/tools/prot_einmw.htm). Finally the structure and domain organization were determined using SMART program, and with JASSA Joined advanced sumoylation site and SIM analyzer <http://www.jassa.fr/index.php?m=jassa> .

Table 2: Genbank or Ensembl accession numbers of Nr4a3 protein sequences used in this study.

Specie	Nr4a3
<i>Homo sapiens</i>	XP_016870651.1
<i>Mus musculus</i>	NP_056558.1
<i>Gallus gallus</i>	XP_015137891.2
<i>Xenopus tropicalis</i>	XP_031760505.1
<i>Danio rerio</i>	NP_001166100.1
<i>Sparus aurata</i>	Nr4a3X1: XP_030250338.1 Nr4a3X2 : XP_030250339.1
<i>Salmo salar</i>	XP_014005832.1
<i>Larimichthys crocea</i>	XP_010734298.1
<i>Etheostoma spectabile</i>	XP_032374777.1

2.7. Cloning the full-length cDNA of seabream *nr4a3*

The seabream *nr4a3* open reading frame (ORF) of variant X2 (XM_030394479.1) was obtained by PCR amplification with Pfu DNA polymerase (Fermentas) using cDNA from seabream head kidney as template. The specific primers used for amplification (listed in

Table 1) were designed by using the snapgene viewer software..The PCR amplified fragments were cloned between BamHI and NotI restriction sites into the plasmid pcDNA6/V5-His C(Life Technologies) in frame with the sequence coding for the V5 epitope. All constructs were sequenced by using ABI PRIS 377 (Applied Biosystems).

2.8. Functional analysis by transfection assay

Plasmid DNAs of the *nr4a3* was prepared using the Midi-Prep procedure (Sigma) and transfected into HEK293 cells with Lipofectamine (InvivoGen) according to the manufacturer's instructions. Briefly, forty-eight hours before transfection, HEK293 cells were harvested and seeded into 6-well plates at density of 1.2×10^5 cells/well. For transfection, each well was transfected with 1.5 µg of the corresponding expression constructor empty vector and 1 µl of Lipofectamine in FCS-free DMEM culture medium for 6h and then incubated in complete culture medium (with 10% FCS). Forty-eight hours after transfection, cells were stimulated with 10 µM of PGA_2 for 3h, collected in TRIzol Reagent (Invitrogen) and processed as described above for subsequent real-time RT-PCR analysis. The transcript levels of the NR4A3 target genes *ENO1* and *ENO3* genes were used as readout of NR4A3 activation. *ACTB* was used as the housekeeping gene for normalization.

2.9. Statistical analysis

Data were analyzed by ANOVA and Tukey's multiple range tests to determine differences among groups. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Identification and characterization of genes encoding Nr4a3 in gilthead seabream

Coding sequence for seabream *nr4a3* gene was obtained from public EST database. Two predicted transcript variants for *nr4a3* were found with ENA accession numbers XM_030394478.1 (variant X1) and XM_030394479.1 (variant X2). The full length ORF of variant X2 was 1833 base pairs (bp) while that of variant X1 was 2046 bp which corresponds to predicted proteins of 611 and 681 amino acids, respectively, with expected molecular weight of 66.97 kDa and 74.2 kDa (**Fig. 1**). The 5' UTR and 3'UTR of variant X1 was 130 and

1,571bp and the ones of variant X2 was 572 bp and 1,571bp respectively. No obvious polyadenylation signals were found in both variants.

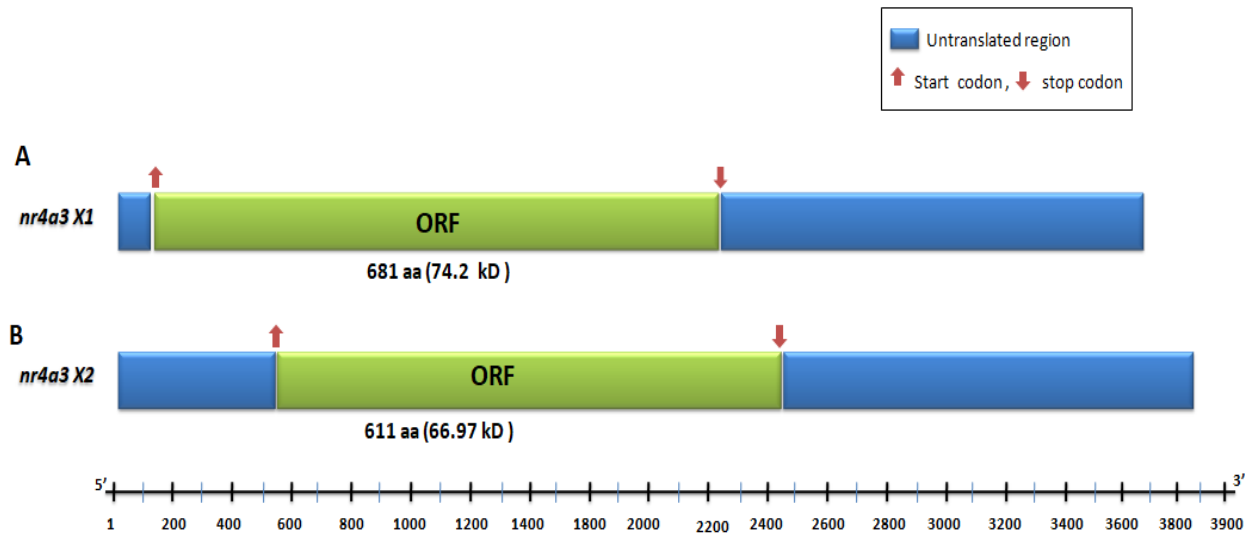


Figure 1. Schematic representation of seabream *nr4a3* transcript variants. Schematic representation of seabream *nr4a3* variant X1(A) and *nr4a3* variant X2(B). The open reading frames are represented in green boxes and the 5'- and 3'-UTR in blue boxes. The number of residues and the molecular weight of the deduced amino acid sequences are shown below each ORF. The start (ATG, upward-pointing red arrow) and stop codon (TAA or TGA, downward-pointing red arrow) were also shown.

3.2. Amino acid sequences characterization and domain prediction of seabream *Nr4a3* variants

Both seabream *Nr4a3* variants showed the following predicted domains (**Fig.2**): azF-C4 (zinc finger, C4 type), which is a DNA binding domain (DBD) (Zhao and Bruemmer 2010) and a ligand binding domain (LBD) of nuclear hormone receptor. The corresponding positions of the two predicted domains (DBD and LBD) are indicated in bold italic and between parenthesis for the variant X1 and the variant X2 respectively, thus they are not in the same position. However, the coding amino acid sequences of both domains are entirely the same between the two variants (data not shown).

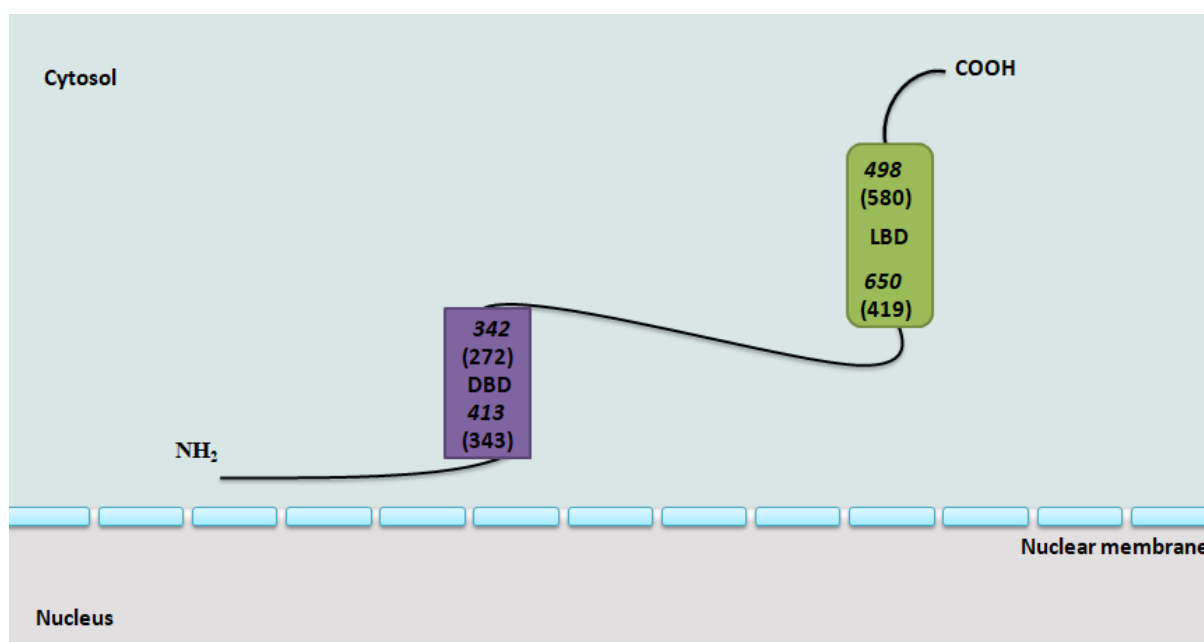


Figure 2. Domain prediction of seabream Nr4a3. DBD : DNA binding domain and LBD : ligand binding domain.

Through multiple sequences alignment identical motifs were identified. Importantly, we found that DBD was highly conserved among all species analyzed (Jiang et al. 2020; Maira et al. 2003; Zhao and Bruemmer 2010). Furthermore, 9 serine residues are maintained in all fish sequence investigated in the current study which may reflect a conserved structural or functional aspect in lower vertebrates. They may be target of phosphorylation, as it has been shown for the NR4A receptors (Galleguillos et al. 2004; Zhao and Bruemmer 2010). In fact, numerous consensus phosphorylation sites appeared in serine, threonine and tyrosine residues of seabream Nr4a3 protein sequence. Finally, putative sumoylation sites (IKKEEES, EDKL and LKLE) and SUMO-interaction motifs (LVLL) were highly conserved among fish Nr4a3.

sbNr4a3X1	SLNLDSAFACLAAL LVL LTQVPGPKDSCRK-----EELQNK-----VICCLRDLHLG	622
sbNr4a3X2	SLNLDSAFACLAAL LVL LTQVPGPKDSCRK-----EELQNK-----VICCLRDLHLG	552
Y.croaker	SLNLDSAFACLAAL LVL LTQVPGPKDSCRK-----EELQNK-----VICCLRDLHLG	553
A.salmon	SLNLDTSAFSCLAAL LVL LTGNTPTTHAHTHTHTHTHRYTQIHTQVIHTQSFVCRL-----	550
O.darter	SLNLDTSAFACLAAL LVL LTQVPGPKDSCRK-----EELQNK-----VICCLRDLHLG	551
Zebrafish	ELNLDPAPFSCLCAL LVL LTQCPGLKPEPKV-----EELQSK-----LICCLRDLHLS	533
Frog	SLNLDVSTVACLSAACLFTGR-QGLKDPKKV-----EELHNK-----IVGCLKEHLAV	538

Chicken	SLNLDIPALASLSALTMITER-HGLKEPKKV-----EELCNK-----ITSSLKDHLTF	630
Mouse	SLNLDIQALACLSALSMITER-HGLKEPKRV-----EELCTK-----ITSSLKDH---	570
Human	SLNLDIQALACLSALSMITER-HGLKEPKRV-----EELCNK-----ITSSLKDH---	569
	.**** :.:*. * :.* . : : : . *	
sbNr4a3X1	--CGPSSSSSKAAPPLSRILGLRAELRSQRTQGLQRIFY <u>LKLE</u> DLVPPPPLIDR-FLDTL	679
sbNr4a3X2	--CGPSSSSSKAAPPLSRILGLRAELRSQRTQGLQRIFY <u>LKLE</u> DLVPPPPLIDR-FLDTL	609
Y.croaker	--CGPSSSSSKAAPPLSRVLGLRAELRSQRTQGLQRIFY <u>LKLE</u> DLVPPPPLIDR-FLDTL	610
A.salmon	-----	550
O.darter	--CGPSSSSSKATPPLSRILGLRAELRSQRTQGLQRIFY <u>LKLE</u> DLVQPPPLIDR-FLDTL	608
Zebrafish	INAGGVASTGKAPPPVGAVLGLRAELRSQRTQGLQRIFY <u>LKLE</u> DLVPPPPLIDR-FLDTL	592
Frog	RGQ---NRPNQLDS--TVVMCILTSLRSLCTLGLQRIFY <u>LKLE</u> DLVPPPAVIDKLFDDL	593
Chicken	SCQ---NKGQPLESAEPKVLGLADLRSICTLGLQRIFY <u>LKLE</u> DLVPAPSIIDRLFLDTL	687
Mouse	--Q---RKQALEPSEPKVLRALVELRKICTQGLQRIFY <u>LKLE</u> DLVPPPSVIDKLFDDL	625
Human	--Q---SKGQALEPTESKVLGALVELRKICTLGLQRIFY <u>LKLE</u> DLVSPPSIIDKLFDDL	624
sbNr4a3X1	PY 681	
sbNr4a3X2	PY 611	
Y.croaker	PY 612	
A.salmon	-- 550	
O.darter	PY 610	
Zebrafish	PY 594	
Frog	PY 595	
Chicken	PF 689	
Mouse	PF 627	
Human	PF 626	

Figure 3: Sequence alignment of vertebrate Nr4a3 proteins. Putative DBD and LBD are shaded in light gray and pink respectively, putative sumoylation site IKKEEES, EDKL and LKLE motifs, SUMO-interaction motifs (SIMs) LVLL are in bold and underlined. (*) Identity in one position; () conservative substitutions in one position; (.) semiconservative substitutions in one position.

3.3. Phylogenetic analysis of seabream Nr4a3

The phylogenetic tree constructed with the Neighbor-Joining method, based on full amino acid sequences of vertebrate Nr4a3, revealed two major clusters. The first joined together fish sequences with high similarity and the second cluster comprised higher vertebrates sequences (Fig. 4).

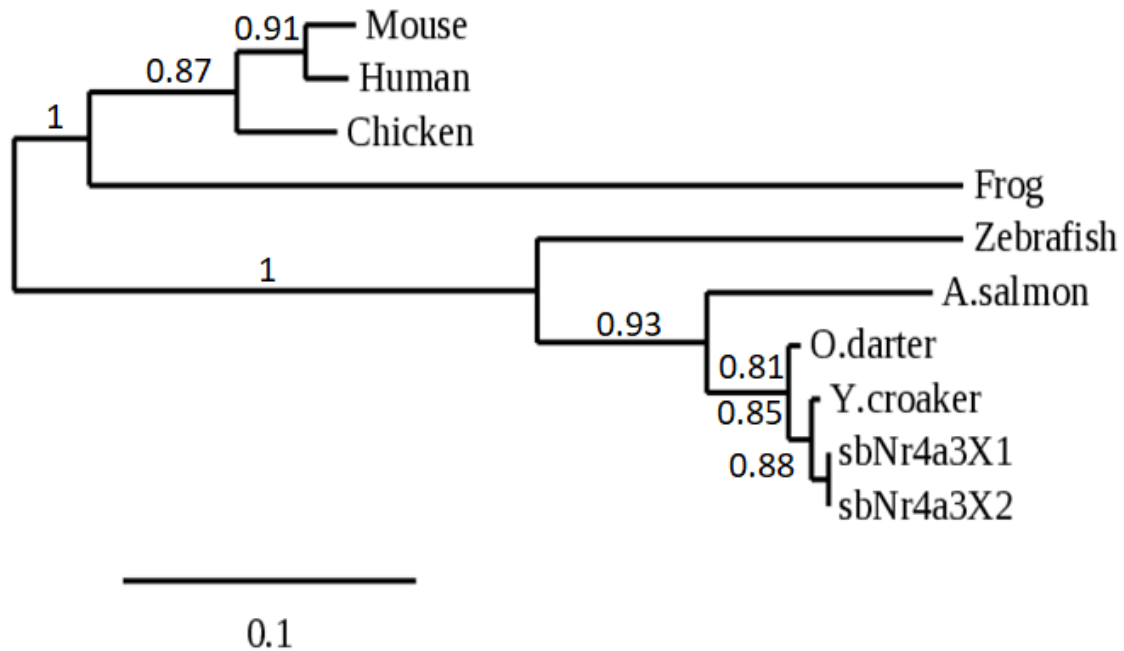


Figure 4. Phylogenetic relationship of vertebrate Nr4a3 polypeptides. The tree was generated by phylogeny.fr software (Dereeper et al. 2008) using amino acid sequences. Numbers shown are percentages of 100 bootstrap replicates in which the same internal branch was observed. The horizontal lines are drawn proportional to the inferred phylogenetic distances, while the vertical lines have no significance. ENA accession numbers are listed in **Table 2**.

3.4. Effects of PGA_2 in the viability and respiratory burst of acidophilic granulocytes

To gain further insight into the immunological role of PGA_2 in seabream, its effect on ROS production was measured *in vitro* in the main seabream professional phagocytes, namely acidophilic granulocytes. The results showed a robust dose-dependent decreased ROS production (**Fig.5**). In all cases, the viability remained above 89%, indicating that the decrease in ROS production was a direct effect of PGA_2 on this activity.

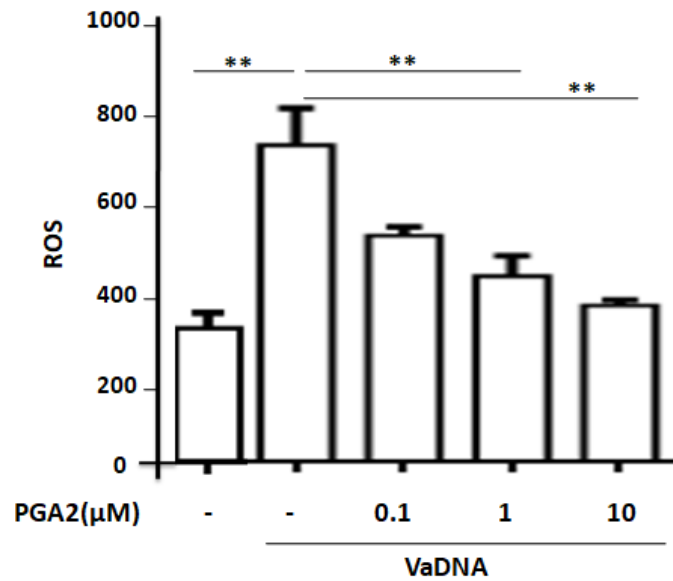


Figure 5. Production of ROS in seabream acidophilic granulocytes treated with PGA₂. Head kidney AGs were isolated by MACS and incubated for 16h with different concentrations of PGA₂ (0, 0.1, 1 and 10μM) in the presence or absence of 50 μg / ml of VaDNA. The asterisks indicate significant differences among groups according to the Tukey test: ** p <0.01; *** p <0.001; **** p <0.0001.

3.5. PGA₂ decreases *il1b* transcript levels but not those of *il10*

We next studied the role of PGA₂ in the regulation of the genes encoding the major pro- and anti-inflammatory cytokines *Il1b* and *Il10*, respectively. PGA₂ was used at 1μM, since this concentration significantly decreased the respiratory burst without affecting cells viability. The results showed that VaDNA stimulation caused a drastic increase of *il1b* (**Fig. 6**) and *il10* (**Fig. 7**) mRNA levels at both 1.5 and 16h. Notably, PGA₂ alone did not exert a significant effect at any of the times in the transcript levels of the two genes. However, it strongly decreased them RNA levels of *il1b* at 1.5 h and, to some extent, at 16 h in cells stimulated with VaDNA (**Fig. 6**). Surprisingly, PGA₂ failed to regulate *il10* mRNA levels in VaDNA-stimulated acidophilic granulocytes (**Figure 7**).

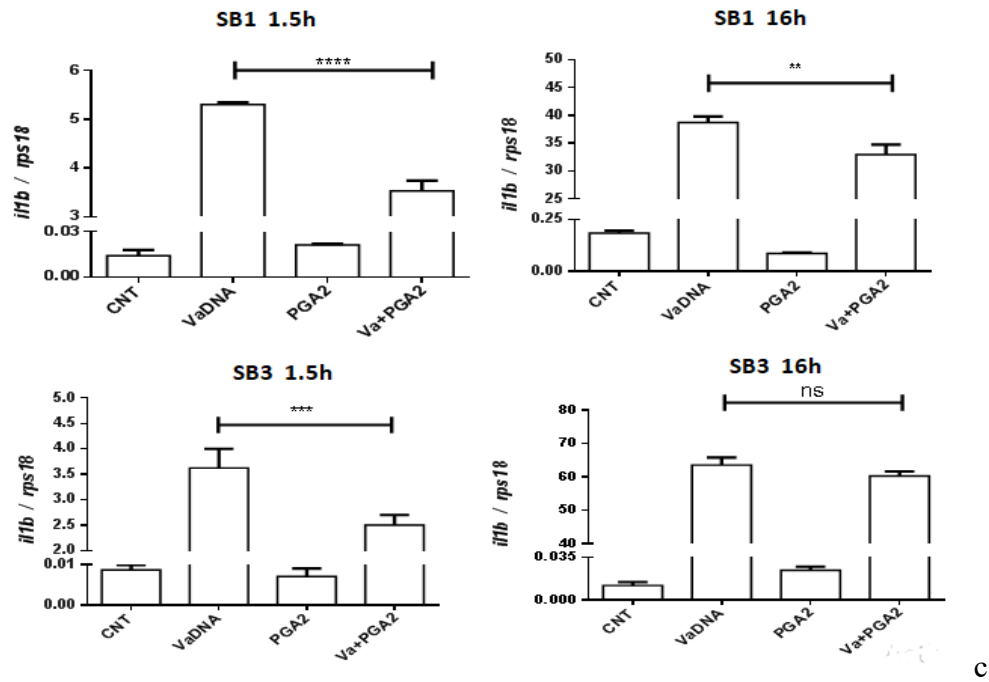


Figure 6. Transcript levels of *il1b* determined by RT-qPCR in seabream acidophilic granulocytes. Acidophilic granulocytes were stimulated *in vitro* with 1 μ M of PGA_2 for 1.5 and 16 h in the presence or absence of 50 μ g/ml of VaDNA. The asterisks indicate significant differences among groups according to ANOVA and Tukey test: ** p < 0.01; *** p < 0.001; Ns, non-significant.

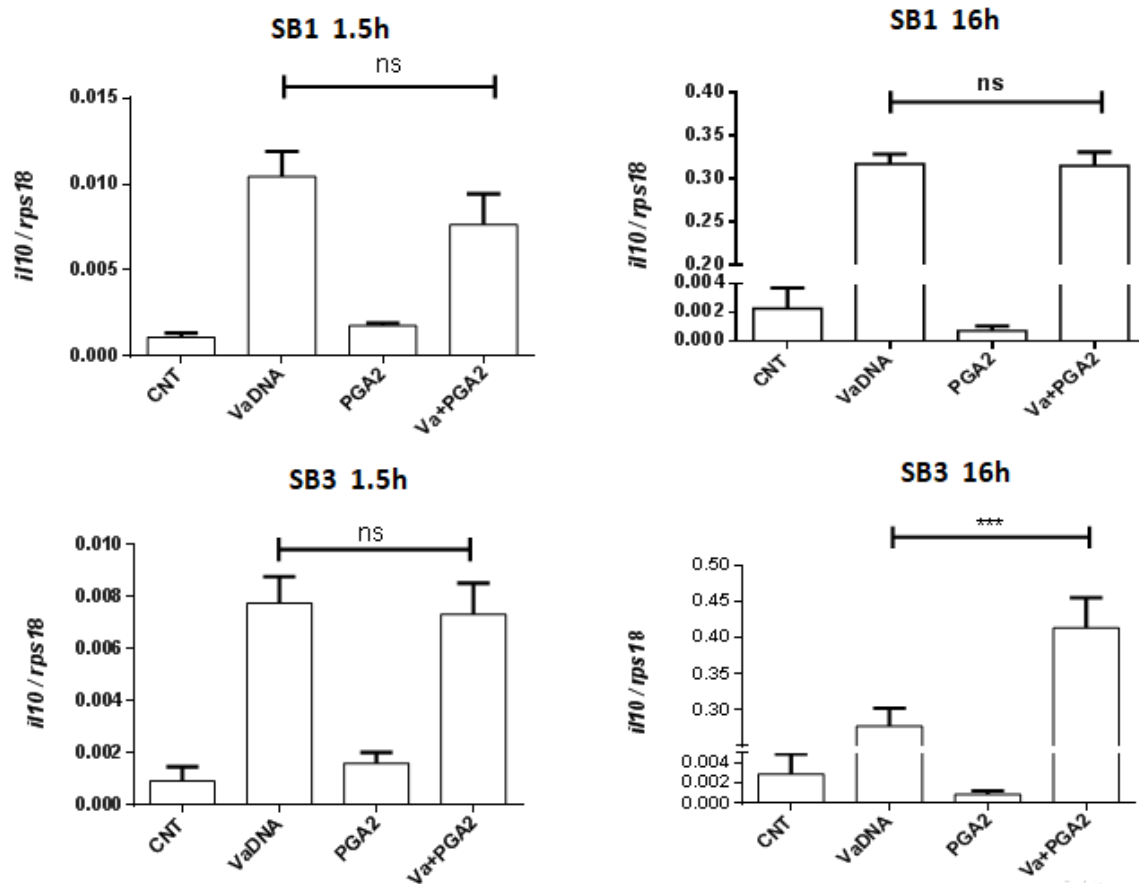


Figure 7. Transcript levels of *il10* determined by RT-qPCR in seabream acidophilic granulocytes. Acidophilic granulocytes were stimulated *in vitro* with 1 μ M of PGA₂ for 1.5 and 16 h in the presence or absence of 50 μ g/ml of VaDNA. The asterisks indicate significant differences among groups according to ANOVA and Tukey test: ** $p < 0.01$; *** $p < 0.001$; Ns, non-significant.

3.6. Dual effects of PGA₂ in seabream macrophages

The effects of PGA₂ in the gene expression profiles of immune-related genes was different to those found in acidophilic granulocytes. Thus, exogenous addition of PGA₂ alone resulted in weakly decreased transcript levels of *il1b*, *il6* and *il10* at both times, while its combination with VaDNA promoted a synergistic induction of the mRNA levels of the 3 genes at the shorter time of incubation (**Fig. 8**). However, PGA₂ addition failed to regulate the mRNA levels of the gene encoding the M2 marker Mrc1 (**Fig. 8**).

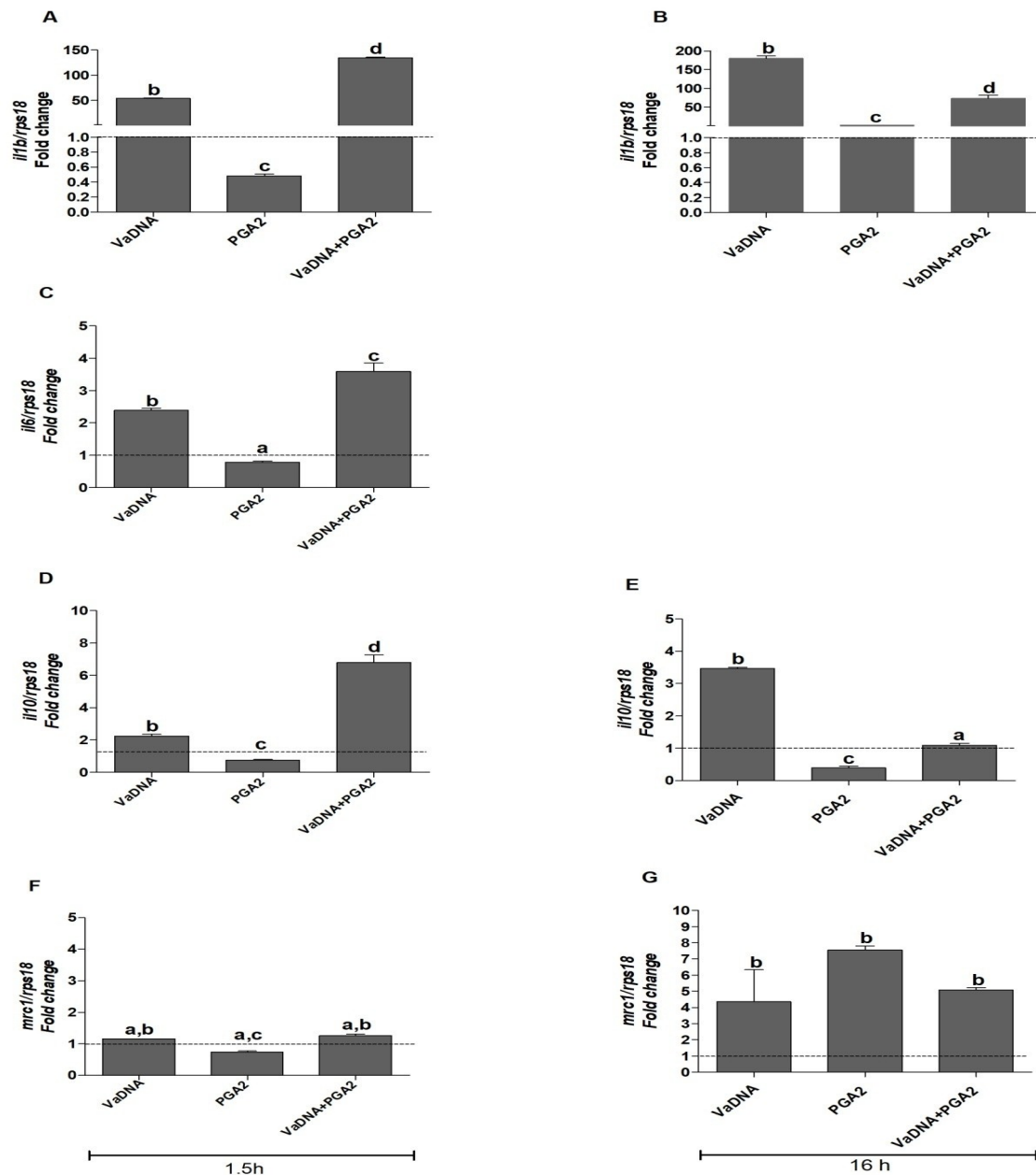


Figure 8. Transcript levels of *il1b*, *il6*, *il10* and *mrc1* determined by RT-qPCR in seabream macrophages. Macrophages were stimulated *in vitro* with 1 μ M of PGA₂ for 1.5 and 16 h in the presence or absence of 50 μ g/ml of VaDNA. Different letters denote statistically significant differences among groups according to ANOVA and Tukey test. The groups marked with “a” did not show statistically significant differences from non-stimulated fish.

3.7. PGA₂ regulates *nr4a3* transcript levels in macrophages

The dual effects of PGA₂ in macrophages led us to investigate if it was able to regulate the transcript levels of the gene encoding its putative receptor Nr4a3. The results showed that this was the case and stimulation of macrophages with PGA₂ for 1.5 h resulted in increased *nr4a3* mRNA levels, while it was able to decrease them after 16 h of stimulation (Fig. 9).

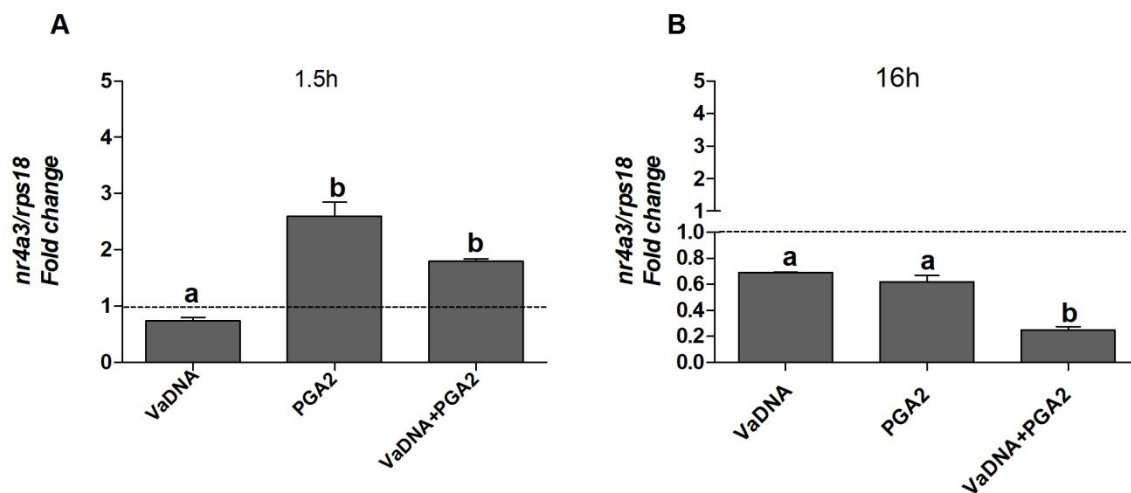
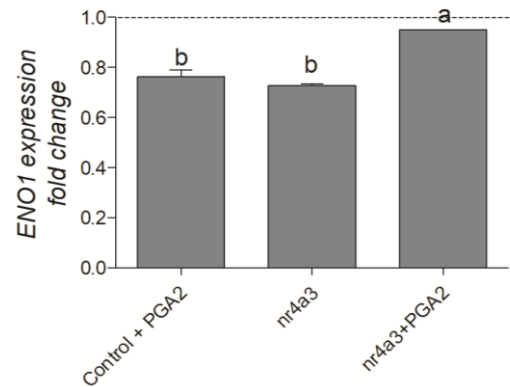


Figure 9. Transcript levels of *nr4a3* determined by RT-qPCR in seabream macrophages. Macrophages were stimulated *in vitro* with 1 μ M of PGA₂ for 1.5 and 16 h in the presence or absence of 50 μ g/ml of VaDNA. Different letters denote statistically significant differences among groups according to ANOVA and Tukey test. The groups marked with “a” did not show statistically significant differences from non-stimulated fish.

3.8. PGA₂ signals through gilthead seabream Nr4a3

We next examined whether gilthead seabream Nr4a3 is a bonafide receptor for PGA₂. To achieve this goal, gilthead seabream Nr4a3 variant X2 was ectopically expressed in human HEK293 cells before being stimulated with PGA₂. As a read out of receptor activation, the transcript levels of the NR4A3 target genes *ENO1* and *ENO3* were evaluated (PMID 17550977, 27771116). PGA₂ was able to weakly increase *ENO1* transcript levels in cells expressing seabream Nr4a3 variant X2 (Fig.10A), while those of *ENO3* were unaffected compared with unstimulated cells (Fig.10B).

A



B

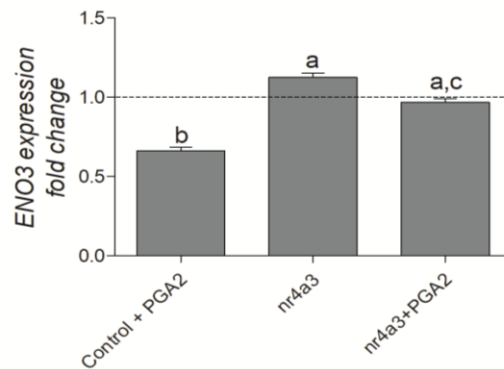


Figure 10. PGA₂ signaling through gilthead seabream Nr4a3. HEK293 cells ectopically expressing gilthead seabream Nr4a3 variant X2 were stimulated with 10 μ M PGA₂ for 3 h and then the transcript levels of *ENO1* and *ENO3* were determined by RT-qPCR. The data are shown as the mean $\pm$ S.E. of the mRNA fold change in stimulated cells relative to non-transfected, unstimulated cells (indicated with horizontal dashed lines). Different letters denote statistically significant differences among the groups according to a Tukey test ($p < 0.05$). The groups marked “a” did not show statistically significant differences from non-stimulated fish.

4. Discussion

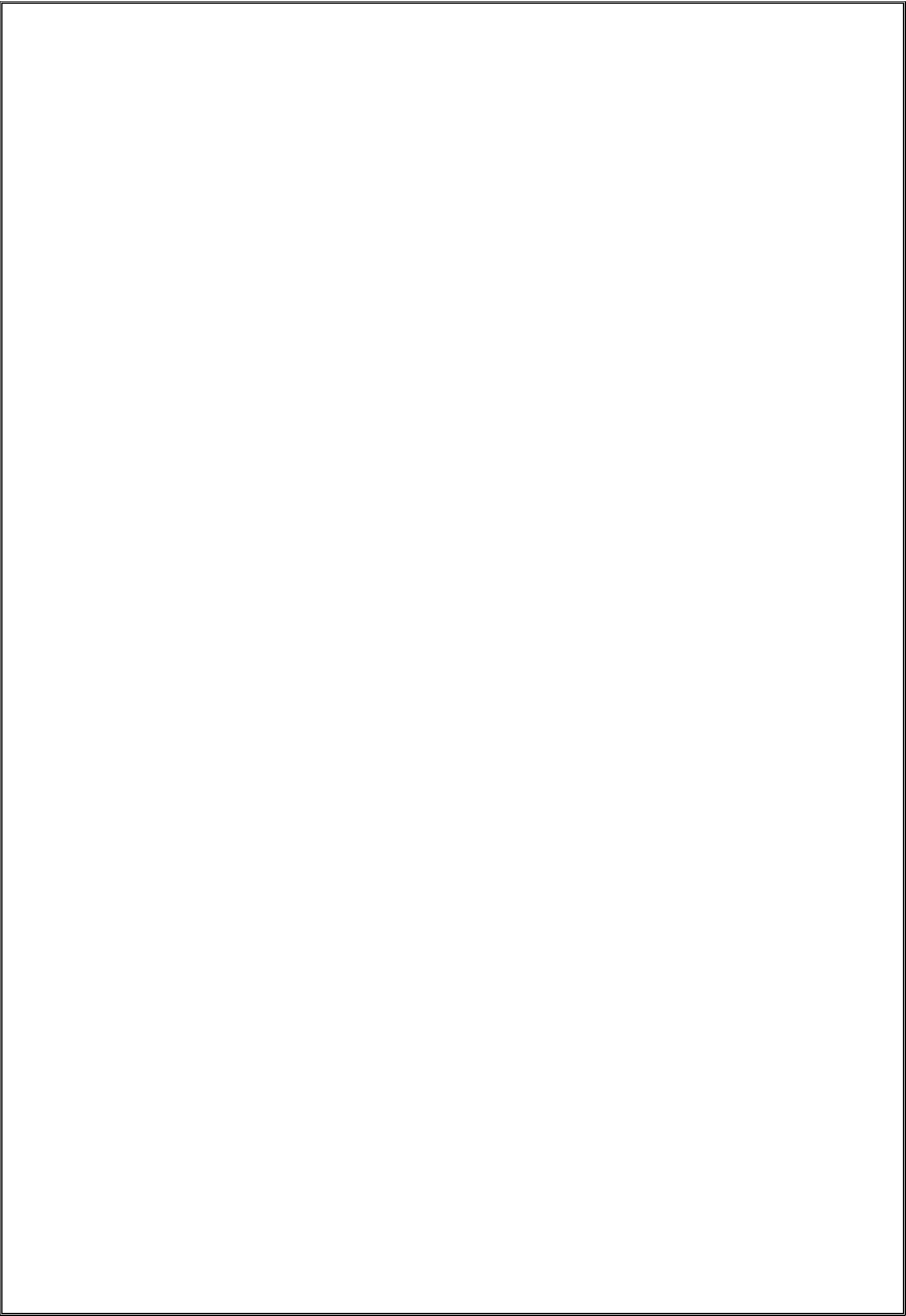
This is the first study which explores the role of orphan nuclear receptor Nr4a3 and its putative ligand PGA₂ in teleost. *In silico* analysis of seabream *nr4a3* mRNA and polypeptide sequence revealed key conserved feature between fish species and their higher vertebrate counterparts, particularly the DBD. This domain was further shown to be conserved in the family of nuclear receptor subfamily (Jiang et al. 2020; Maira et al. 2003; Zhao and Bruemmer 2010), confirming that gilthead seabream Nr4a3 receptor also belong to this family. Furthermore, the conserved DBD may reflect conserved transcriptional signaling pathway in vertebrates, as we have confirmed by functional analysis in HEK293 cells, where the gilthead seabream receptor was able to regulate the expression of *ENO1* and to be engaged by PGA₂. In addition, the sumoylation site IKKEES and SUMO-interaction motifs (SIMs) were also conserved, further indicating conserved signaling among vertebrates.

To deep insight into the immunological role of Nr4a3 and PGA₂ in teleosts, their RNA levels of Nr4a3 and the impact of PGA₂ were analyzed in the two professional phagocytic cell types of the gilthead seabream, namely macrophages and acidophilic granulocytes. We showed that PGA₂ exerted an anti-inflammatory effect in acidophilic granulocytes. Thus, PGA₂ inhibited the respiratory burst and the mRNA levels *il1b* triggered by stimulation with bacterial DNA in these cells, while concomitantly increased those of *il10*. In accordance with our study, in higher vertebrates the effects of PGA₂ on the resolution of inflammation have been shown in several cell types, such as human endothelial cells (Ke et al. 2017), astrocytes (Chistyakov et al. 2018), lymphocytes (D'Onofrio et al. 1990) and microglia (Storer et al. 2005). In addition, in murine macrophages, PGA₂ is also able to inhibit IκBα degradation, which results in enhanced NF-κB nuclear translocation and transcriptional activation, and COX-2 and iNOS induction (Musiek et al. 2005). Paradoxically, we found, that in contrast to acidophilic granulocytes, PGA₂ alone resulted in weakly decreased transcript levels of *il1b*, *il6* and *il10* at both times, while its combination with VaDNA promoted a synergistic induction of these 3 genes at the shorter time of incubation. Therefore, these results, together with inability of PGA₂ to regulate the mRNA levels of the gene encoding the M2 marker *Mrc1*, suggest a pro-inflammatory effect of PGA₂ in macrophages. Therefore, our data suggest a dual function of PGA₂ in the regulation of innate cellular immunity in gilthead seabream. This is not surprising, since NR4A3 has been shown to play cell-specific function in mammals (Ma et al. 2020).

Another interesting observation of this study is that the PGA_2 receptor *nr4a3* is expressed in resting gilthead seabream macrophages. However, PGA_2 alone or combined with bacterial DNA upregulated *nr4a3* transcript levels in these cells. These data are in agreement with several studies in mammals that have shown that PGA_2 signaling plays a key role in the late stage of inflammation (Díez-Dacal and Pérez-Sala 2010; Lakshmi et al. 2019).

In conclusion, we have shown for the first time that PGA_2 regulates the main functions of professional phagocytes of teleosts and provided evidences that it may signals through Nr4a3. *In vivo* studies are required to understand the role of this prostaglandin and the Nr4a3 receptor in fish immune responses.

CONCLUSIONS



Conclusions

1. One ortholog of mammalian PTGER1, PTGER3 and PTGER4, and two of PTGER2 (Ptger2a and Ptger2b) were identified and characterized in gilthead seabream. All receptors possessed the organization domain of G protein-coupled receptors, with the exception of Ptger2b.
2. Gilthead seabream *ptger1-4* were expressed in all the tissues examined, the highest mRNA levels of *ptger1* and *ptger3* being observed in the spleen and of *ptger2a* and *ptger4* in the blood. Bacterial infection induced higher mRNA levels of *ptger2a*, *ptger3* and *ptger4* in the site of bacterial injection.
3. Head kidney acidophilic granulocytes and macrophages displayed different *ptger1*, *ptger2a*, *ptger3* and *ptger4* expression profiles. In macrophages the expression of the receptors was weakly affected by stimulation with bacterial DNA or with PGE₂, while in acidophilic granulocytes stimulation resulted in the upregulation of *ptger2a* and *ptger4*, suggesting different roles for seabream PGE₂ receptors in the regulation of the immune responses.
4. An ortholog of mammalian NR4A3 was identified in gilthead seabream. Phylogenetic analysis revealed highly conserved motifs with all their vertebrate orthologs, particularly the DNA binding domain and sumoylation site.
5. *nr4a3* was constitutively expressed in macrophages and was upregulated by PGA₂ alone and combined with bacterial DNA. In addition, PGA₂ weakly decreased the transcript levels of genes encoding the cytokines Il1b, Il6 and Il10, while its combination with VaDNA promoted a synergistic induction of the mRNA levels of

these 3 genes. However, PGA_2 failed to regulate the mRNA levels of the gene encoding the M2 marker *Mrc1*.

6. Exogenous addition of PGA_2 inhibited the respiratory burst of acidophilic granulocytes and induced the transcript levels of the genes encoding *Il1b* and *Il10*.
7. PGA_2 was able to signals through gilthead seabream *Nr4a3* when ectopically expressed in HEK293 cells, which suggests an important role for PGA_2 and *Nr4a3* in fish immune responses.

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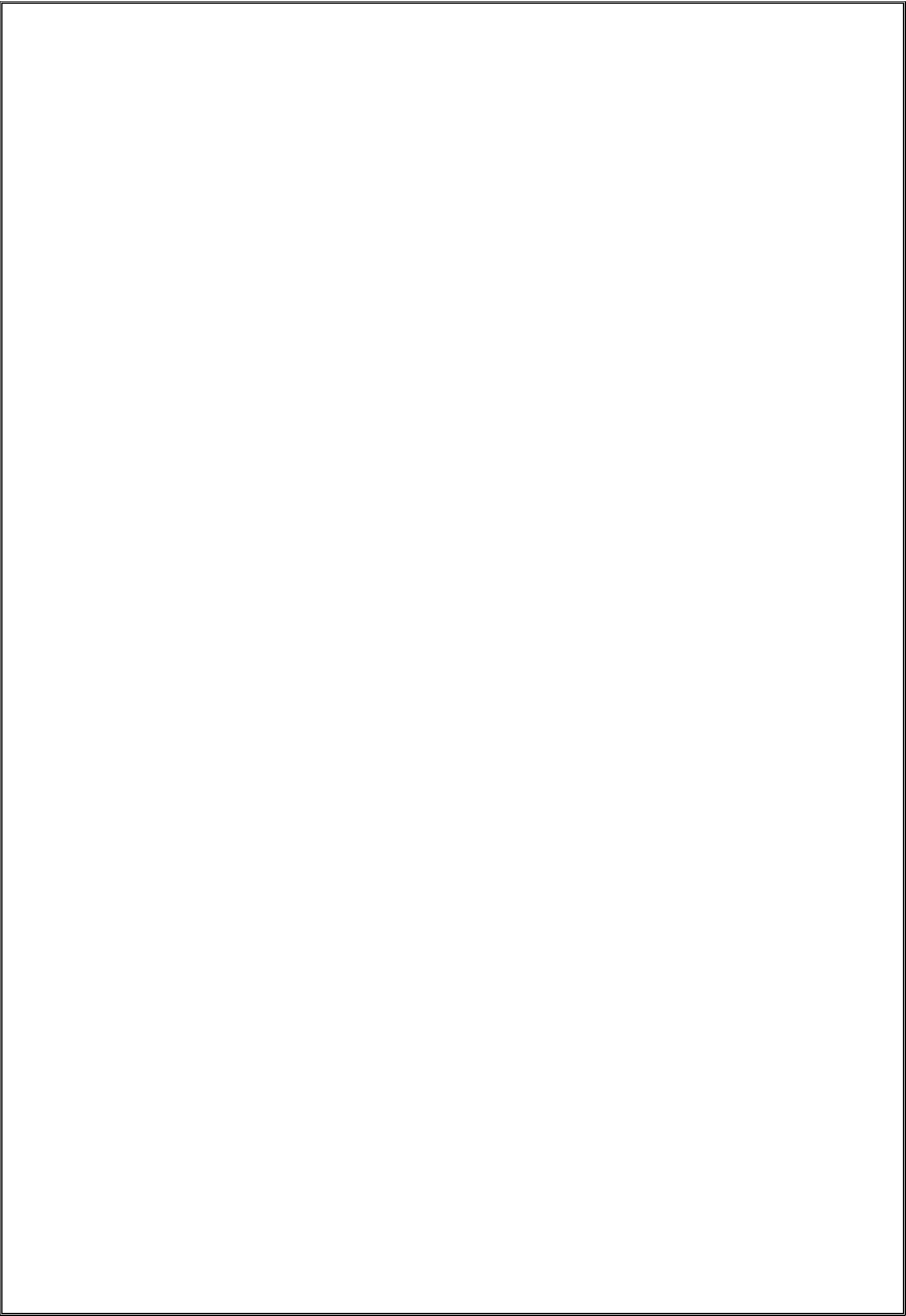
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RESUMEN EN CASTELLANO



Resumen

Los teleósteos, rama moderna de los peces óseos, son las clases de vertebrados más ricas con más de 20.000 especies cuya diversidad ha permitido adaptaciones de diversos ambientes acuáticos. La posición basal de los peces en el árbol filogenético de los vertebrados les da un gran interés en enfoques genómicos y funcionales comparativos, en particular los del sistema inmunitario. Los peces son el primer grupo de animales que tiene un sistema inmunitario innato y adaptativo bien estructurado y diferenciado. Sin embargo, a diferencia de los mamíferos, es la inmunidad innata la que tiene un interés fundamental en los peces. La mayoría de las especies de peces eclosionan en la etapa embrionaria de la vida en el medio acuático con una variedad de microorganismos invasores. Por eso los mecanismos innatos consisten en un sistema robusto que asume la defensa contra patógenos durante las primeras semanas de su desarrollo. La respuesta inmune adaptativa, la última línea de defensa en los vertebrados, también tiene dos ramas: una función de tipo humoral con la producción de anticuerpos y una función mediada por células citotóxicas. Ambos son el resultado de la estimulación respectiva de los linfocitos B y T que se ha demostrado que existen en los peces. Sin embargo, la respuesta a la memoria es menos elaborada que la de los vertebrados superiores.

La acuicultura consiste en la producción acuática de animales y plantas utilizando diferentes métodos y técnicas para mejorar su eficiencia. Esto significa que los individuos están expuestos a una manipulación en un espacio limitado que desencadena un estrés en los animales que provoca el desarrollo de enfermedades infecciosas que son responsables de una alta mortalidad y pérdidas económicas sustanciales. Por tanto, el estudio de la respuesta inmune de los peces es esencial para disminuir las pérdidas económicas en la acuicultura. Además, los peces ocupan una posición clave desde el punto de vista evolutivo debido a que son el primer grupo de animales donde está presente la respuesta inmune innata y las adaptativas basadas en el sistema Rag1/2. En consecuencia, el conocimiento del sistema inmunitario de los peces se ha convertido en uno de los principales objetivos de la investigación en acuicultura.

La dorada (*Sparus aurata* L.) es un pez teleósteo marino de reproducción estacional. Aunque esta especie de pez se ha utilizado como modelo animal de investigación para estudiar el sistema inmunitario de los peces, aún se desconoce mucho. En este contexto,

las prostaglandinas (PG) son mediadores lipídicos con un papel clave en la modulación de la función inmunológica, desencadenando una respuesta proinflamatoria o antiinflamatoria durante el proceso de inflamación.

Las prostaglandinas tipo ciclopentenona (cPG o cyPG) son potentes lípidos electrofílicos reactivos que ejercen efectos protectores durante la inflamación fisiopatológica y el estrés oxidativo en mamíferos y en invertebrados. PGA1, PGA2 y PGJ2 son CyPG, derivados de PG oxigenadas convencionales PGE1, PGE2 y PGD2, respectivamente, por deshidratación no enzimática. Se caracterizan por un grupo carbonilo α , β -insaturado ubicado en el anillo de ciclopentenona que les permite reaccionar con residuos de cisteína de proteínas celulares diana, como NF- κ B y AP-1 a través de reacciones de adición de Michael. Esta propiedad confiere a los CyPG la capacidad de regular las actividades proteicas que les permiten desplegar funciones polivalentes y controvertidas para amortiguar los peligrosos efectos que podrían surgir tras la sobreactivación del sistema inmunológico.

Los efectos divergentes de la PGE2 han estado involucrados en un amplio espectro de procesos biológicos, incluidos mecanismos proinflamatorios y antiinflamatorios, que van desde vertebrados inferiores hasta peces y humanos. En los mamíferos, los efectos pleiotrópicos de PGE2 están mediados por la unión a uno o una combinación de cuatro subtipos de receptores PTGER1-PTGER4 (también denominados EP1-EP4), pertenecientes a la familia de receptores acoplados a proteína G que poseen 7 regiones transmembrana similares a la rodopsina. Estos receptores se pueden agrupar en tres grupos de receptores prostanoides de acuerdo con la subunidad de proteína G asociada correspondiente y, por lo tanto, con los segundos mensajeros intracelulares y el efecto desencadenado por ellos. El grupo de receptores relajantes incluye EP2 y EP4, cuya activación da como resultado un aumento de cAMP / PKA a través de G α s y adenilato ciclasa, lo que conduce a la relajación del músculo liso. EP1 está acoplado a G α q, que aumenta los niveles de Ca²⁺ tras la activación de la fosfolipasa C y pertenece al grupo de receptores contráctiles. EP3 generalmente está acoplado a Gi, lo que lleva a una disminución del cAMP a través de G α i y da como resultado la inhibición de la contracción muscular, aunque diferentes isoformas resultantes del procesamiento alternativo en el gen EP3 se acoplan a diferentes tipos de G proteína, como G α s, G α i y G α 12 / 13.

En los peces teleósteos, la producción de PGE2 por células inmunes se ha descrito en una amplia gama de especies. Además, existe evidencia sobre la actividad inmunorreguladora de PGE2 en peces: por ejemplo, la modulación de la expresión de citocinas, producción de ROS y respuestas fagocíticas. Más específicamente, se ha demostrado que PGE2 conduce a la polarización M2 de los macrófagos en la dorada, de manera similar a la observada en los mamíferos. La PGE2 desactiva los granulocitos acidófilos, la célula funcionalmente equivalente a los neutrófilos de mamíferos, mediante la modulación del perfil de expresión de citocinas. Sin embargo, los estudios sobre la caracterización molecular de los receptores de PGE2 y su papel en la respuesta inmune en los peces teleósteos son escasos.

En estudios anteriores, mostramos que el análogo permeable a las células de cAMP (dbcAMP) imitaba el efecto de PGE2 en la regulación de los niveles de ARNm de citocinas en macrófagos estimulados, pero no en AG. La variedad de efectos que provoca la PGE2 en ambos tipos de células fagocíticas podría reflejar la presencia de receptores específicos en esas células y sugerir que la PGE2 envía señales a través de EP2 y / o EP4 en macrófagos de dorada pero no en granulocitos acidófilos. Teniendo en cuenta todo lo anterior, el objetivo del primer capítulo de la presente tesis doctoral es la identificación y caracterización *in silico* de los receptores de PGE2 de la dorada (Ptger1-4) y profundizar en su papel en la resolución de la inflamación *in vivo* e *in vitro*. Así, un ortólogo de los receptores de PGE2 de mamíferos PTGER1, PTGER3 y PTGER4, y dos de PTGER2 (Ptger2a y Ptger2b), fueron identificados y caracterizados en dorada. El análisis *in silico* mostró que todos estos receptores poseían el dominio de organización de los receptores acoplados a proteína G, con la excepción de Ptger2b. Los correspondientes estudios *in vivo* revelaron que se expresaban en todos los tejidos examinados, observándose los niveles más altos de ARNm de *ptger1* y *ptger3* en el bazo, y de *ptger2a* y *ptger4* en la sangre. Una infección experimental con la bacteria *Vibrio anguillarum* indujo los niveles más altos de ARNm de *ptger2a*, *ptger3* y *ptger4* en el exudado peritoneal (el lugar de la inyección bacteriana). Además, los fagocitos profesionales de esta especie, granulocitos acidófilos y macrófagos, del riñón cefálico mostraron diferentes perfiles de expresión de *ptger1*, *ptger2a*, *ptger3* y *ptger4*. Además, en los macrófagos, la expresión de los receptores se vio débilmente afectada por la estimulación con ADN bacteriano, ligando del receptor similar a toll 9 (TLR9) o con PGE2, mientras que en los granulocitos acidófilos la estimulación resultó en la

regulación positiva de *ptger2a* y *ptger4*. En conjunto, estos resultados sugieren diferentes papeles desempeñados por los receptores de PGE2 de la dorada en la regulación de la respuesta inmunitaria. No obstante, se necesitan más estudios sobre la señalización de cada uno de ellos y su función *in vivo*.

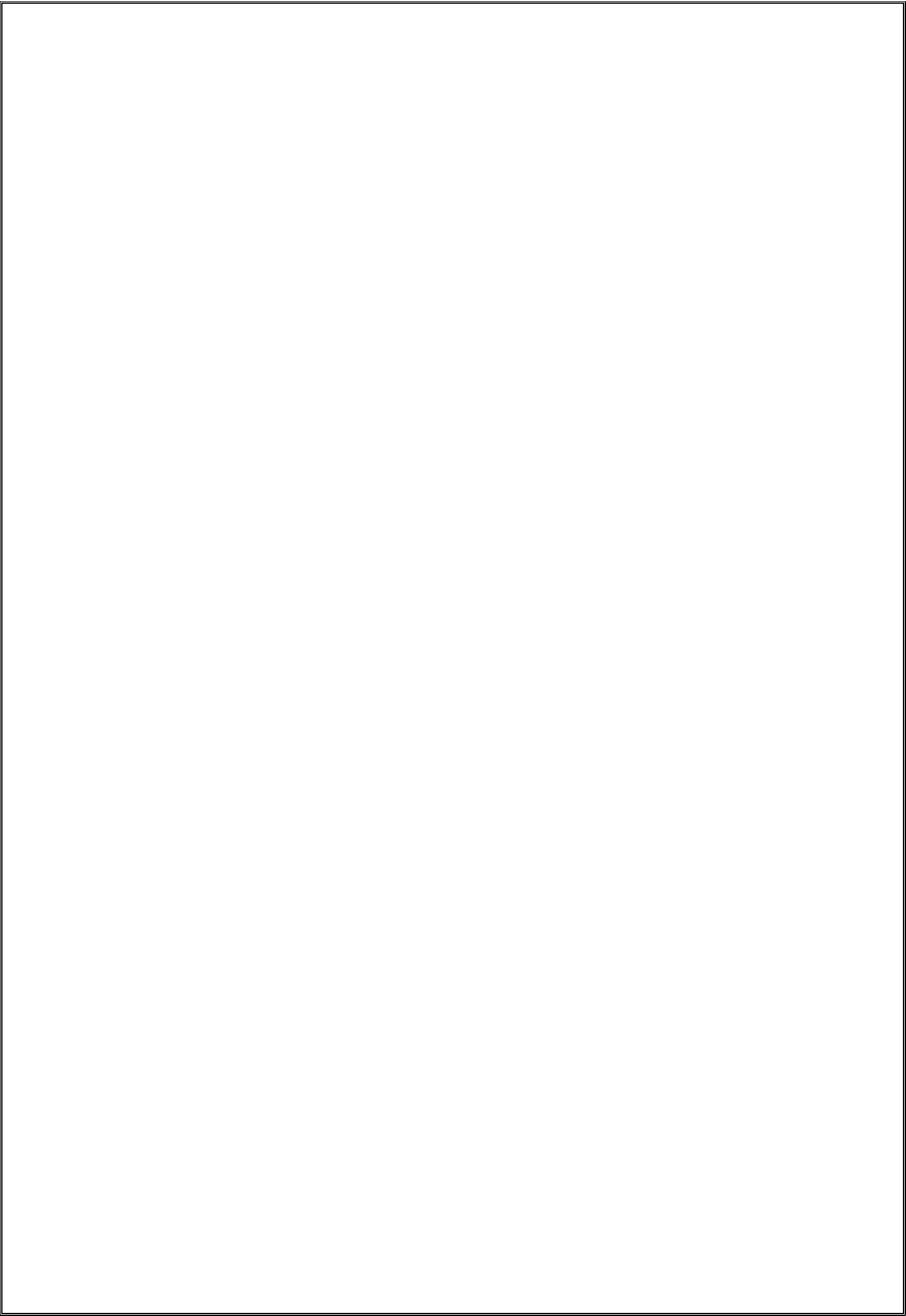
Por otro lado, la PGA2 ha sido descrita recientemente como un producto metabólico rápido de la deshidratación de la PGE2, que muestra potentes efectos antiinflamatorios *in vitro* e *in vivo*, que actúa principalmente en la etapa tardía de la inflamación, suprimiendo un amplio espectro de mediadores proinflamatorios, como citocinas, quimiocinas y óxido nítrico (NO). Además, inhibe la movilización celular y la fagocitosis. En respuesta a diferentes estímulos, como el LPS, INF- γ y TNF- α , y a diferencia de sus precursores (PGs) que actúan mediante unión en los receptores de superficie celular acoplados a proteína G, las CyPG, incluida la PGA2, se transportan al compartimento intracelular donde ejercen sus funciones modificando directamente proteínas o actuando a través de receptores específicos. Así, la subfamilia de receptores nucleares NR4A3 fue identificado como un receptor de PGA2. NR4A3 fue inicialmente descrito como receptor nuclear huérfano que actúa como factor de transcripción tras la unión de PGA2. Sin embargo, también se encontró que podía mejorar la maquinaria transcripcional independientemente de la unión del ligando, lo que refleja su actividad constitutiva. Puede unir sus sitios diana de ADN como un monómero al elemento de respuesta NGFI-B (NBRE) o como homodímero o heterodímero con otros miembros de la familia al elemento que responde a Nur (NurRE). Sin embargo, su mecanismo de acción es controvertido, ya que PGA2 es capaz de unirse a receptores de membrana celular no identificados mejorando la liberación de cAMP y Ca²⁺ y señalizar el receptor EP4 de PGE2.

Varios estudios han demostrado un papel de NR4A3 en la supervivencia celular, proliferación, movilización y apoptosis. En este contexto, es un potente regulador de genes inflamatorios en macrófagos de mamíferos, células dendríticas y células B, y actúa como regulador de la diferenciación de células dendríticas derivadas de monocitos. Además, a alto nivel de expresión de NR4A3 interviene en la resolución de la inflamación al inhibir la movilización de neutrófilos y la activación de NF- κ B a través de JAK2- Vía STAT3.

Hasta donde sabemos, el papel inmunológico de PGA2 y sus receptores se desconoce en gran medida en especies que no son mamíferos. Las funciones fundamentales de Nr4a3

en la supervivencia, el desarrollo y la función de los neutrófilos durante la inflamación tanto en mamíferos como en peces cebra ha sido demostrada recientemente. Por lo tanto, la información disponible aún es muy limitada para comprender el mecanismo de las funciones PGA2 y Nr4a3 en teleósteos. En el segundo capítulo hemos identificado el receptor Nr4a3 en dorada y demostrado que PGA2 regula *in vitro* las principales funciones de los granulocitos acidófilos y los macrófagos de esta especie. La dorada Nr4a3 reveló motivos altamente conservados con todos sus ortólogos de vertebrados, particularmente el dominio de unión al ADN y el sitio de sumoilación. El análisis de expresión mostró que nr4a3 se expresaba constitutivamente en macrófagos y se regulaba positivamente con PGA2 solo y combinado con ADN bacteriano. Es importante destacar que la adición de PGA2 disminuyó débilmente los niveles de transcripción de los genes que codifican las citocinas interleucina-1b (Il1b), Il6 e Il10, mientras que su combinación con VaDNA promovió una inducción sinérgica de los niveles de mRNA de estos tres genes. Sin embargo, PGA2 no pudo regular los niveles de ARNm del gen que codifica el marcador de polarización M2 del receptor de manosa C-tipo 1 (Mrc1). Por el contrario, PGA2 inhibió el estallido respiratorio de granulocitos acidófilos e indujo los niveles de transcripción de los genes que codifican Il1b e Il10. Finalmente, PGA2 pudo enviar señales a través de la dorada Nr4a3 cuando se expresó ectópicamente en células HEK293. En conclusión, hemos demostrado por primera vez que PGA2 regula las principales funciones de los fagocitos profesionales de teleósteos y aportado evidencias de que puede señalizar a través de Nr4a3, allanando el camino para estudios *in vivo* con el fin de comprender el papel de esta prostaglandina y la Nr4a3. receptor en las respuestas inmunitarias de los peces.

RESUME EN FRANÇAIS



Résumé

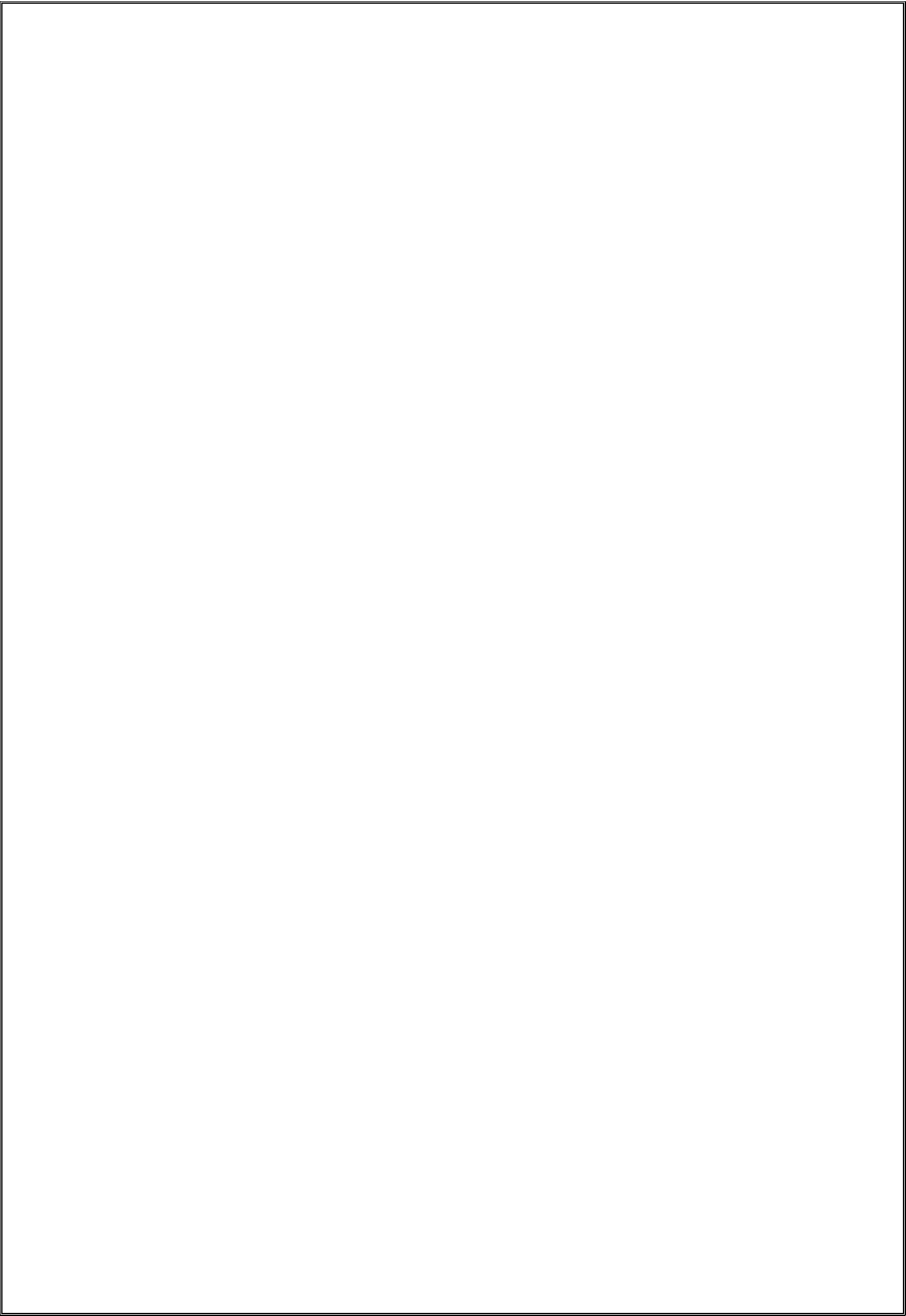
Le système immunitaire des poissons téléostéens est étudié depuis des décennies mais, malgré les progrès importants réalisés, beaucoup d'investigations sont recommandés, notamment en ce qui concerne la présence et le rôle des prostaglandines, telles que PGE₂ et PGA₂, et leurs récepteurs. La dorade royale (*Sparus aurata* L.), est un poisson téléostéen marin de grande valeur commerciale en aquaculture méditerranéenne, il a été largement utilisée pour mieux comprendre la réponse immunitaire des poissons. L'objectif de cette thèse est de faire progresser nos connaissances sur le rôle des prostaglandines PGE₂ et PGA₂ et de leurs récepteurs dans la réponse immunitaire de la dorade royale.

Dans le premier chapitre, un orthologue de PTGER1, PTGER3 et PTGER4, et deux orthologues de PTGER2 (Ptger2a et Ptger2b) mammalien ont été identifiés et caractérisés chez la dorade royale. L'analyse *in silico* a montré que tous ces récepteurs possédaient une organisation des récepteurs couplés aux protéines G, à l'exception de Ptger2b. Les études *in vivo* correspondantes ont montré que ces récepteurs ont été exprimés dans tous les tissus examinés, les taux d'ARNm les plus élevés de *ptger1* et *ptger3* étant observés dans la rate et de *ptger2a* et *ptger4* dans le sang. L'infection bactérienne a induit les niveaux d'ARNm les plus élevés de *ptger2a*, *ptger3* et *ptger4* dans l'exsudat péritonéal (le site d'injection bactérienne). De plus, au niveau des granulocytes acidophiles et des macrophages isolés du rein antérieur présentaient des profils d'expression de *ptger1*, *ptger2a*, *ptger3* et *ptger4* différents. En outre, au niveau des macrophages, l'expression de ces récepteurs était faiblement affectée par la stimulation avec l'ADN bactérien ou avec PGE₂, tandis que dans les granulocytes acidophiles, la stimulation entraînait une régulation positive de *ptger2a* et *ptger4*. Pris ensemble, ces résultats suggèrent des rôles différents pour les récepteurs PGE₂ chez la dorade dans la régulation des réponses immunitaires.

Dans le deuxième chapitre, nous avons identifié le récepteur Nr4a3 chez la dorade royale et montré que PGA₂ régule les principales fonctions des cellules phagocytaires de cette espèce *in vitro*. Nr4a3 de la dorade royale présente des motifs hautement conservés avec tous leurs orthologues des vertébrés sélectionnés dans la présente étude, en particulier le domaine de liaison à l'ADN et le site de sumoylation. L'analyse d'expression a montré que nr4a3 était exprimé de manière constitutive dans les macrophages et il a été induit par PGA₂ seul et combiné avec de l'ADN bactérien. Surtout, l'ajout de PGA₂ a diminué faiblement les niveaux de transcription des gènes codant pour l'interleukine-1b (Il1b), Il6 et Il10, tandis que sa

combinaison avec l'ADN bactérien a favorisé une induction synergique des niveaux d'ARNm de ces trois gènes. Cependant, PGA_2 n'a pas réussi à réguler le niveaux d'ARNm du gène codant pour le récepteur C-type 1 du mannose, marqueur de polarisation M2 (Mrc1). En revanche, PGA_2 a inhibé la chaîne respiratoire des granulocytes acidophiles et a induit les taux de transcription des gènes codant pour Il1b et Il10. Enfin, PGA_2 était capable de transmettre des signaux à travers Nr4a3 chez la dorade royale lorsqu'elle était ectopiquement exprimée dans des cellules HEK293. En conclusion, nous avons montré pour la première fois que la PGA_2 régule les principales fonctions des cellules phagocytaires des téléostéens et fourni des preuves qu'elle peut signaler via Nr4a3, ouvrant la voie à des études *in vivo* afin de comprendre le rôle de cette prostaglandine et du récepteur Nr4a3 dans les réponses immunitaires des poissons.

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❖ From Tunisia

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- ❖ From Spain: The department of cell biology and histology, Faculty of biology, University of Murcia.

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To my mother's soul Fathia who passed away years ago. You are always present in my heart. All the words can't express your value to us. You are the mother I dreamed of having.

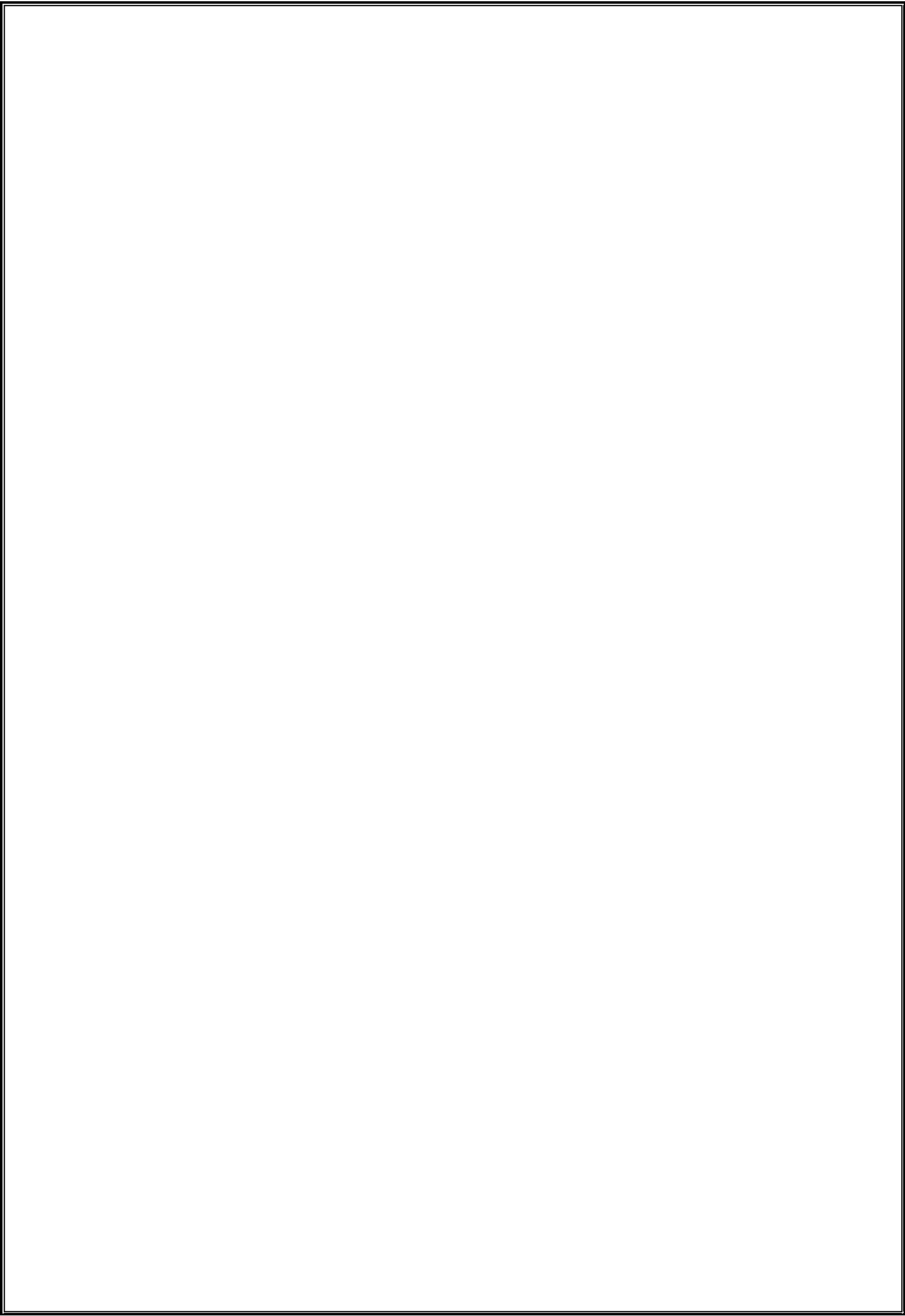
To my very dear father Belgacem, my sister Faten, brother my Hafedh, my sons Yaakoub, and Youssef, my daughter Alaa, and my husband Walid; your support, help and encouragement made me successful in my studies. Words are hardly enough to express my love for you. I dedicate this work to you with all my best wishes for happiness, health and success.

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ANNEXE I: Publications derived from the thesis

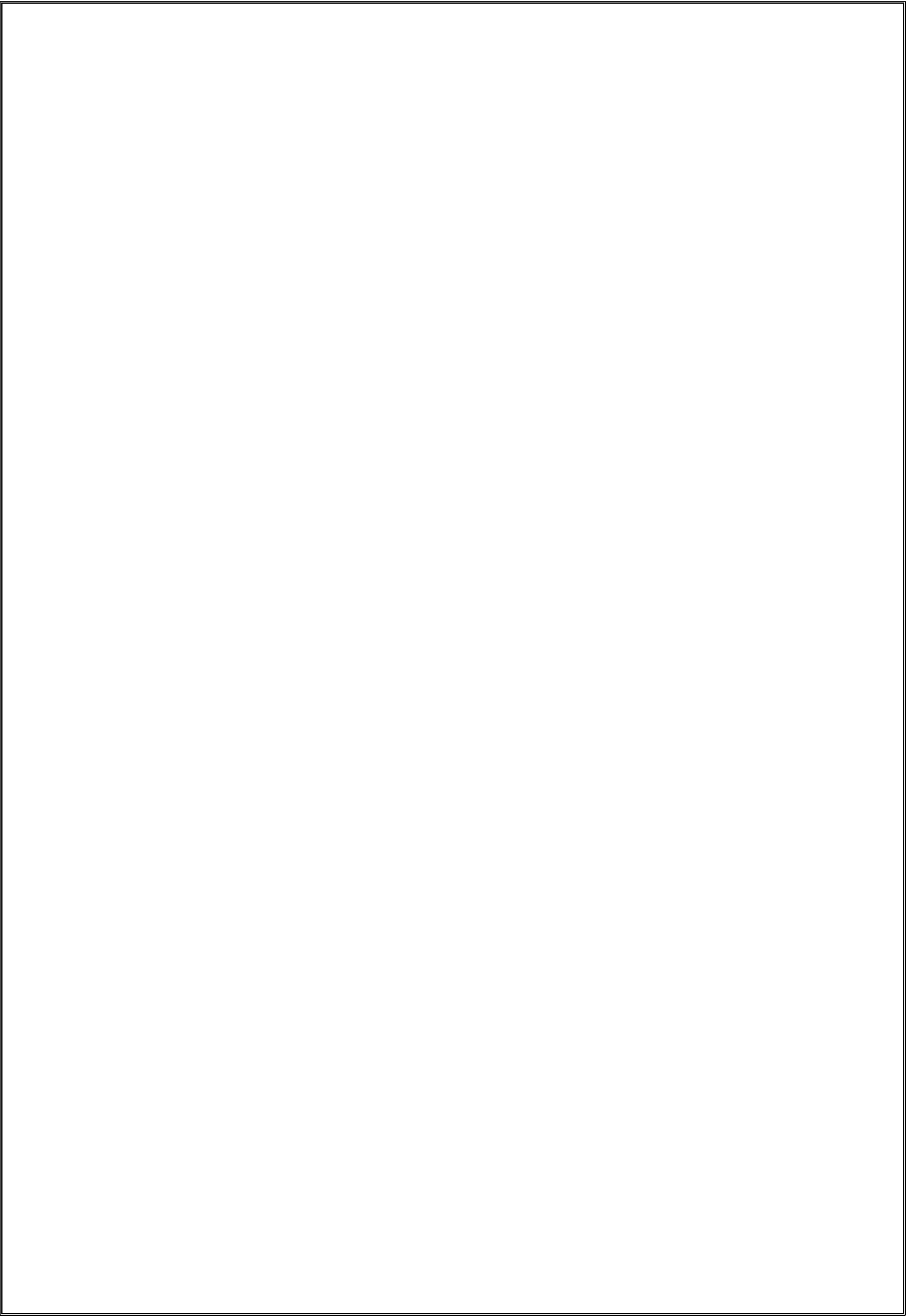


Annexe I

- 1) **Fatma Hermi**, Victoria Gómez-Abellán, Ana B. Pérez-Oliva, Diana García-Moreno, Azucena López-Muñoz, Elena Sarropoulou, Marta Arizcun, Oueslati Ridha, Victoriano Mulero, María P. Sepulcre. The molecular, functional and phylogenetic characterization of PGE₂ receptors reveals their different roles in the immune response of the teleost fish gilthead seabream (*Sparus aurata* L.). *Developmental & Comparative Immunology* 114 (2021): 103803.

- 2) **Fatma Hermi**, Victoria Gómez-Abellán, Ana B. Pérez-Oliva, Diana García-Moreno, Azucena López-Muñoz, Elena Sarropoulou, Marta Arizcun, Oueslati Ridha, María P. Sepulcre, Victoriano Mulero. PGA2 regulates macrophages and acidophilic granulocytes functions in the teleost fish gilthead seabream (*Sparus aurata* L.) (*in preparation*).

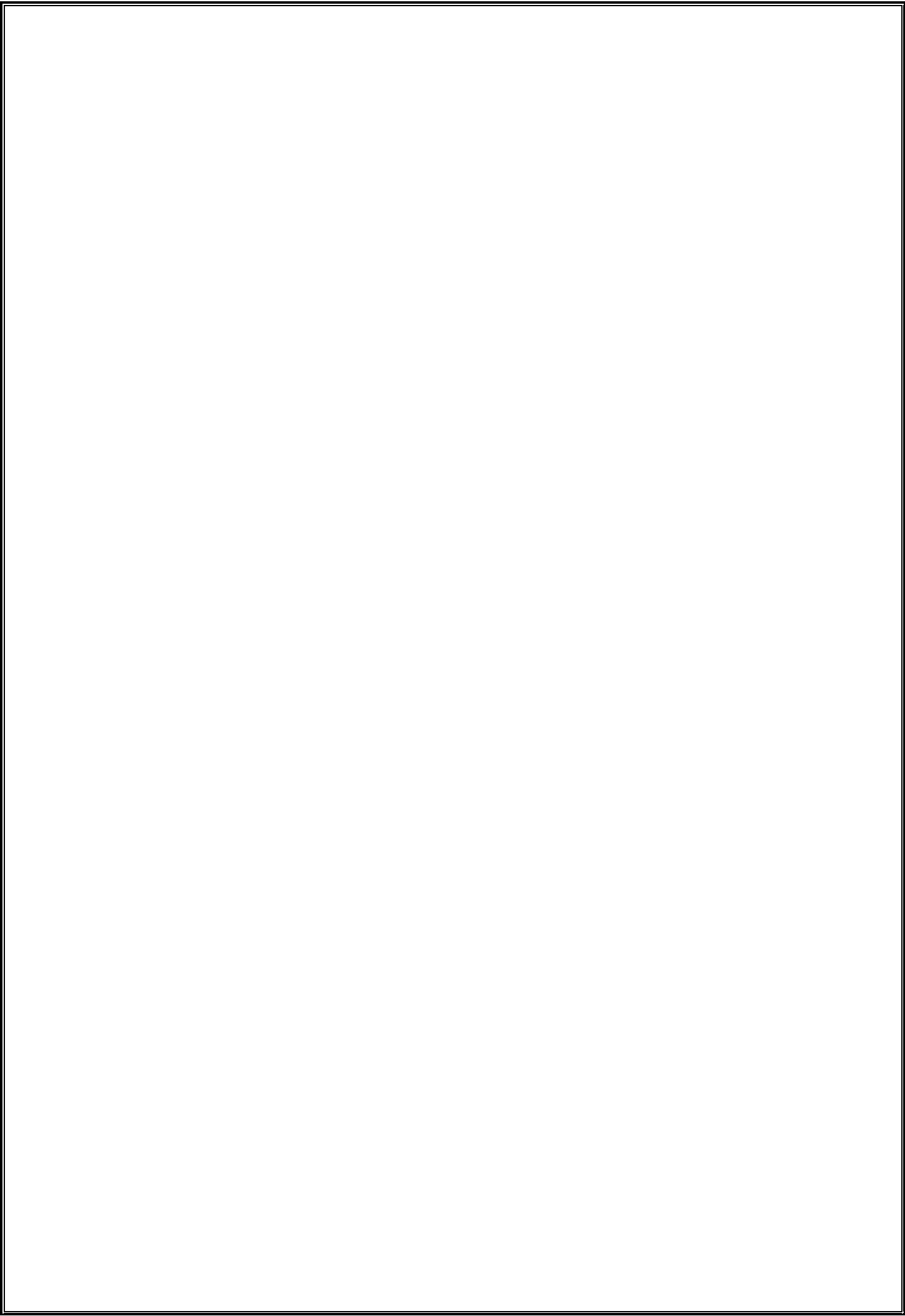
ANNEXE II: Participation in publications during the PhD



Annexe II

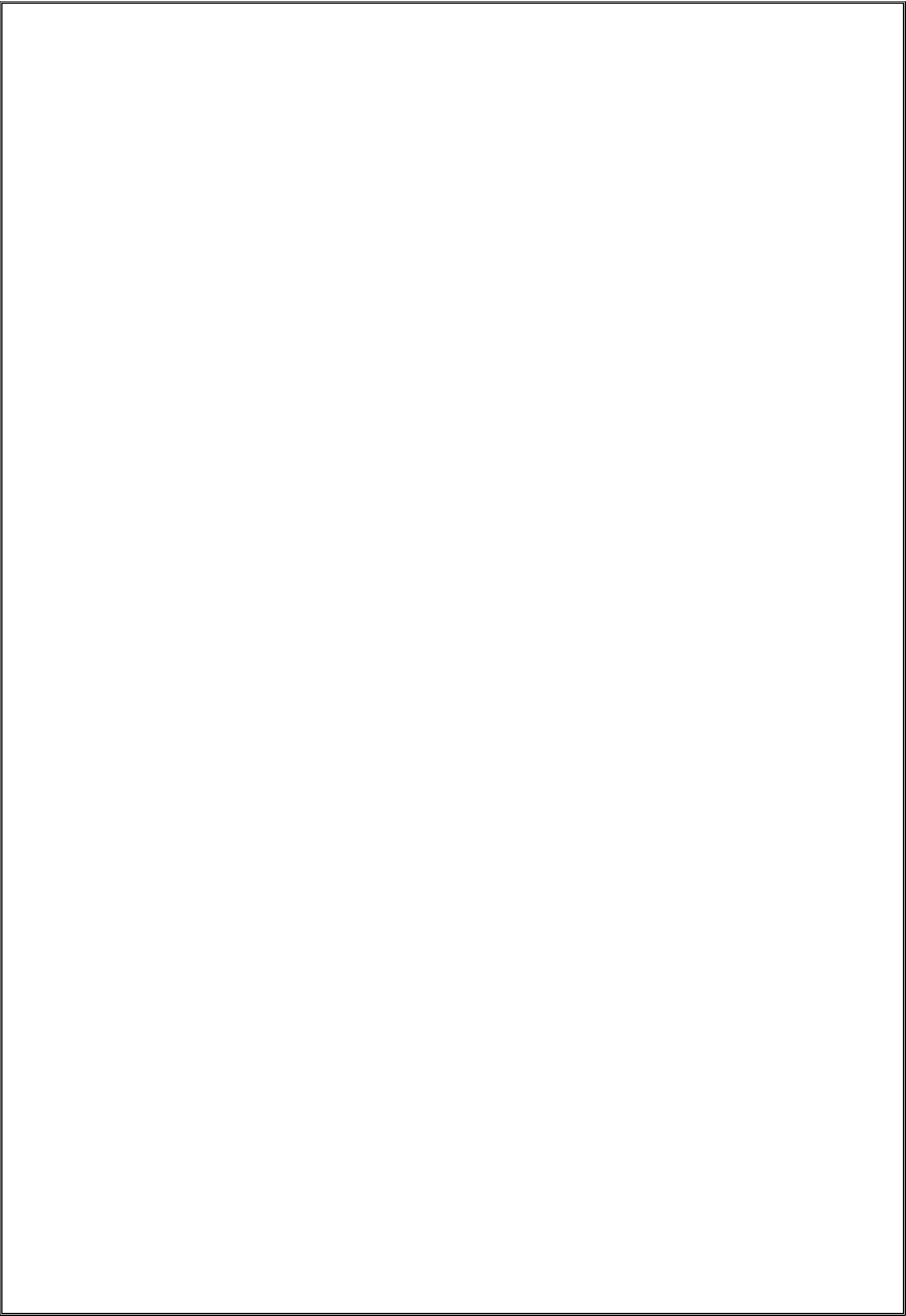
Victoria Gómez-Abellán, Ana Belén Pérez-Oliva, **Fatma Hermi**, Marta Arizcun, Victoriano Mulero, María P. Sepulcre. A role for PPAR β in the anti-inflammatory effect of the cyclopentenone prostaglandin 15-deoxy- Δ 12,14-PGJ2 in professional phagocytes of the teleost gilthead seabream (*in preparation*).

ANNEXE III: Participation in national and international conferences



Annexe III

- 1) **Hermi F**, Gómez-Abellán V, Montero J, Sarropoulou E, Oueslati R, Mulero V, Sepulcre M P. Immunomodulatory role of PGE2 through Ptger2 and Ptger4 in the teleost fish gilthead seabream (*Sparus aurata* L.). 2nd International Symposium for Young Researchers in Biology, (Hammamet, Tunisia), 05/2018.
- 2) **Hermi F**, Gómez-Abellán V, Montero J, Sarropoulou E, Oueslati R, Mulero V, Sepulcre MP. Molecular characterization, phylogenetic and expression analysis of PGE2 receptor subtypes in gilthead seabream (*Sparus aurata* L.). 39 Congreso de la Sociedad Española de Inmunología (Alicante, Spain), 05/2016.
- 3) **Hermi F**, Gómez-González NE, Gómez-Abellán V, García-Ayala A, Oueslati R, Mulero V, Sepulcre MP. Identification and expression analysis of PGE2 receptor subtypes in gilthead seabream (*Sparus aurata* L.). ISDCI (Murcia, Spain), 07/2015.





The molecular, functional and phylogenetic characterization of PGE₂ receptors reveals their different roles in the immune response of the teleost fish gilthead seabream (*Sparus aurata* L.)

Fatma Hermi^{a,b}, Victoria Gómez-Abellán^b, Ana B. Pérez-Oliva^b, Diana García-Moreno^b, Azucena López-Muñoz^b, Elena Sarropoulou^c, Marta Arizcun^d, Oueslati Ridha^a, Victoriano Mulero^b, María P. Sepulcre^{b,*}

^a Unit of Immunology, Environmental Microbiology and Cancerology, Faculty of Sciences of Bizerte, Jarzouna - Bizerte, 7021, University of Carthage, Tunis, Tunisia

^b Departamento de Biología Celular e Histología, Facultad de Biología, Universidad de Murcia, IMIB-Arrixaca, 30100, Murcia, Spain

^c Institute for Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, 71003, Heraklion, Crete, Greece

^d Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO), 30860, Murcia, Spain

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ABSTRACT

Prostaglandin E₂ (PGE₂) plays an important role in immune activities in teleost fish, including seabream. However, receptors involved in PGE₂ signaling, as well as the pathways activated downstream, are largely unknown. In this study, one ortholog of mammalian PTGER1, PTGER3 and PTGER4, and two of PTGER2 (Ptger2a and Ptger2b) were identified and characterized in gilthead seabream. *In silico* analysis showed that all these receptors possessed the organization domain of G protein-coupled receptors, with the exception of Ptger2b. The corresponding *in vivo* studies revealed that they were expressed in all the tissues examined, the highest mRNA levels of *ptger1* and *ptger3* being observed in the spleen and of *ptger2a* and *ptger4* in the blood. Bacterial infection induced higher mRNA levels of *ptger2a*, *ptger3* and *ptger4* in peritoneal exudate (the site of bacterial injection). In addition, head kidney acidophilic granulocytes and macrophages displayed different *ptger1*, *ptger2a*, *ptger3* and *ptger4* expression profiles. Furthermore, in macrophages the expression of the receptors was weakly affected by stimulation with bacterial DNA or with PGE₂, while in acidophilic granulocytes stimulation resulted in the upregulation of *ptger2a* and *ptger4*. Taken together, these results suggest different roles for seabream PGE₂ receptors in the regulation of the immune responses.

1. Introduction

Prostanoids including PGE₂ are bioactive lipid-derived autacoids (Ricciotti and FitzGerald, 2011) involved in a broad array of physiological and pathological processes (Gamil et al., 2015; Kobayashi and Narumiya, 2002). They are derived from the arachidonic acid released from cell membranes (Calder and Grimbale, 2002) in response to a variety of stimuli (Harris et al., 2002; MacKenzie et al., 2010; Ricciotti and FitzGerald, 2011; Sargent et al., 1999), which is subsequently converted into PGH₂ through the action of two rate-limiting enzymes: constitutive (COX-1) and inducible (COX-2) cyclooxygenases (Wang and Dubois, 2010). PGH₂ is converted into several prostanoids by the action of the respective synthases (Narumiya et al., 1999).

The divergent effects of PGE₂ have been involved in a large spectrum

of biological processes (Hao and Breyer, 2008; Ning et al., 2014; Miller, 2006), including both pro- and anti-inflammatory mechanisms, ranging from lower vertebrates (fishes) (MacKenzie et al., 2010; Gómez-Abellán and Sepulcre, 2016; Tyrkalska et al., 2016) to human (Calder and Grimbale, 2002; Hsu et al., 2014; Fattahi and Mirshafiey, 2014; Miller, 2006).

In mammals, the pleiotropic effects of PGE₂ are mediated by binding to one or to a combination of four receptors subtypes PTGER1-PTGER4 (also named EP1-EP4) (Harris et al., 2002; Qu et al., 2015), belonging to the family of rhodopsin-like seven transmembrane-spanning, G-protein-coupled receptors (Guo et al., 2015). These receptors can be grouped into three groups of prostanoid receptors according to the corresponding associated G-protein subunit, and hence to the intracellular second messengers and the effect triggered by them (Yokoyama et al., 2013). The group of relaxant receptors include EP2 and EP4,

* Corresponding author. Departamento de Biología Celular e Histología, Facultad de Biología, Universidad de Murcia, Murcia, Spain.

E-mail address: mpsepul@um.es (M.P. Sepulcre).

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Abbreviations			
AGs	acidophilic granulocytes	IL	intracellular loop
COX	cyclooxygenase	LPS	Lipopolysaccharide
cAMP	cyclic adenosine monophosphate	MACS	magnetic-activated cell sorting
C	cysteine	NCBI	national center for biotechnology information
dbcAMP	dibutyl- γ -cAMP	NF- κ B	nuclear factor kappa B
D	aspartic acid	ORF	open reading frame
EP	E prostaglandin receptor	PGE ₂	prostaglandin E2
EDTA	ethylenediamine tetraacetic acid	PE	peritoneal exudates
EMBOSS	European molecular biology open software suite	PAMPs	pathogen associated molecular patterns
EMBL	European molecular biology laboratory	PG	prostaglandin
EBI	European bioinformatics institute	PTGER	prostaglandin E receptor
EST	Expressed sequence tag	PGH ₂	prostaglandin H ₂
ENA	European nucleotide archive	PKA	protein kinase A
EL	extracellular loop	RT-qPCR	quantitative RT-PCR
FCS	fetal calf serum	ROS	reactive oxygen species
GPCR	G protein-coupled receptor	R	arginine
GTP	guanosine triphosphate	S	serine
G	arginine	S.E.	standard error
HK	head kidney	TMD	transmembrane domain
HEK	human embryonic kidney cells	T	threonine
IgG	Immunoglobulin G	UTR	untranslated region
		VaDNA	<i>Vibrio anguillarum</i> genomic DNA

Table 1

Primer sequences used for cloning and qRT-PCR.

Gene	Forward/Reverse	Primer sequence (5'→3')	Technique
<i>ptger1</i>	Forward	TATCGGAGAGCACCTGTCCA	qRT-PCR
	Reverse	CTGGCACTTAGCGATGAGGT	
	Forward	AAAAAGGATCCATGTTAGCGTTGAGCCACT	cloning
	Reverse	AAAAAGCGGCCGCAACGTGGTTGACATCATTCT	
<i>ptger2a</i>	Forward	TTCTTCAGCCTTGCGACCAT	qRT-PCR
	Reverse	TGCACCAAATCCCGCAAAAG	
	Forward	AAAAAGGATCCATGGGGAATCCAGAGAAC	cloning
	Reverse	AAAAAGCGGCCGCTGGTAATAGAGTTTCTGCAG	
<i>ptger2b</i>	Forward	AAAAAGGATCCATGGGGAATCCAGAGAAC	cloning
	Reverse	AAAAAGCGGCCGCGAAGTCAACGGTCAACACTT	
<i>ptger3</i>	Forward	TCCCTCACCAGATCCTGGAC	qRT-PCR
	Reverse	AGTCTGGGTCTCCTTCCGAT	
	Forward	AAAAAGGATCCATGACTATGGACGCTTCTGA	cloning
	Reverse	AAAAAGCGGCCGCGTACTTTGTGGTTTATGGATGT	
<i>ptger4</i>	Forward	GGGCAGAGATCCAGATGGTG	qRT-PCR
	Reverse	TGTGCAGCTGATTGCGAAAG	
	Forward	AAAAAGGATCCATGAACGCCGTTAAAC	cloning
	Reverse	AAAAAGCGGCCGCTATGCATTCTCTTGTATGTTGC	
<i>il8</i>	Forward	GTTTTTGAAGAGGGCTGAG	qRT-PCR
	Reverse	TTTGCTTGAAGTTTCACTGG	

Table 2

Genbank or Ensembl accession numbers of Ptger used in this study.

Specie	Ptger1	Ptger2a	Ptger3	Ptger4
<i>Homo sapiens</i>	AAH51286	AAD44177	AAI18660	AAI13524
<i>Mus musculus</i>	NP_038669	NP_032990	NP_035326	NP_001129551
<i>Gallus gallus</i>	Unknown	ABM92941	ABF13295	NP_001074972 XP_424770
<i>Xenopus tropicalis</i>	XP_002935465	XP_002936949	XP_002936132	NP_001120554
<i>Danio rerio</i> H.	ACX47465.1	zEP2a: NM_200635 zEP2b: NM_001030250 zEP2L: ABB88955.1	XP_009300798	NM_001039629
<i>Sparus aurata</i> L.	XM_030412901.1	Ptger2a: XM_030410287.1 Ptger2b: XM_030410288.1	XM_030434233.1	XM_030436477.1
<i>Salmo salar</i> L.	XP_014048970.1	XP_014000607.1	XP_014072350.1	NP_001167426.1
<i>Larimichthys crocea</i>	XP_010732951	XP_010742830	XP_010727602	KKF22113
<i>Etheostoma spectabile</i>	XP_032394488.1	XP_032362064.1	XP_032382424.1	XP_032395955.1

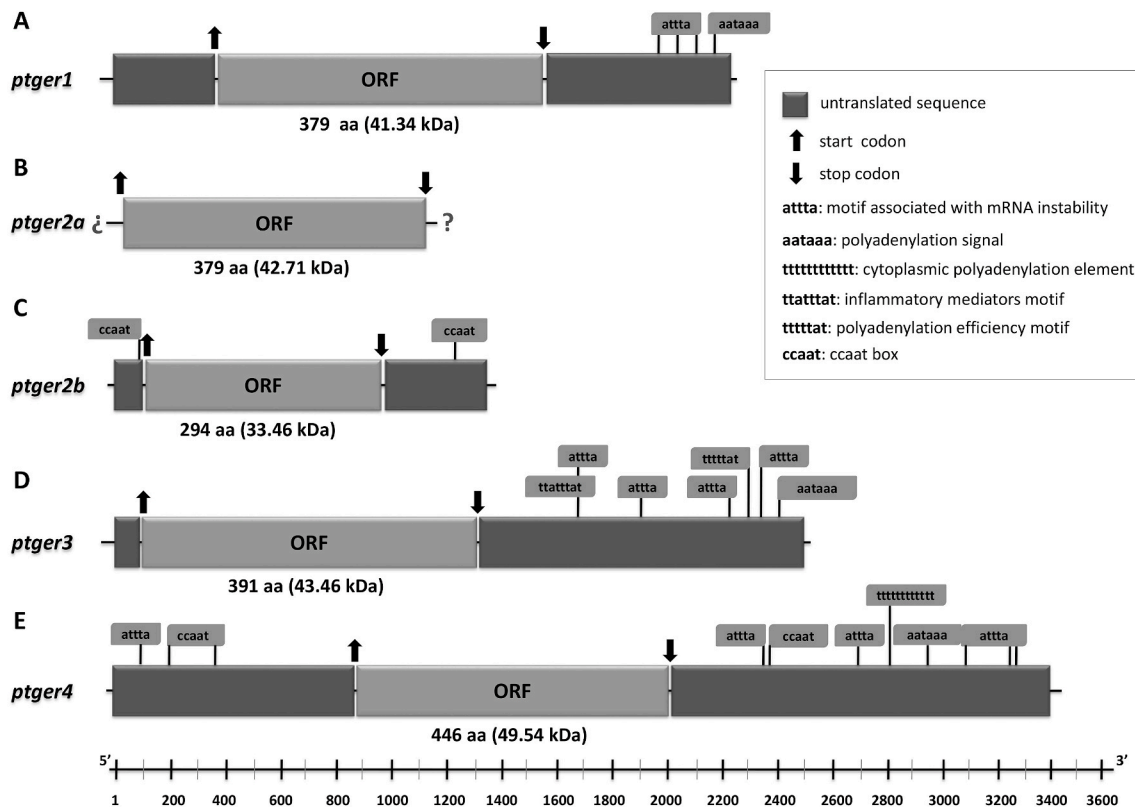


Fig. 1. Schematic representation of seabream *Ptger* subtype sequences. Schematic representation of seabream *ptger1* (A), *ptger2a* (B), *ptger2b* (C), *ptger3* (D) and *ptger4* (E). The open reading frames are represented in light gray boxes and the 5'- and 3'-UTR in dark gray boxes. The number of amino acids, as well as the molecular weight of the deduced amino acid sequences, are shown below each ORF. Also shown are the start (ATG, upward-pointing black arrow) and stop codon (TAA or TGA, downward-pointing black arrow) and motifs: **attta** (motif associated with mRNA instability), **aataaa** (polyadenylation signal), **ttatttat** (inflammatory mediator motif), **tttttttttt** (cytoplasmic polyadenylation element) and **ccaat** (ccaat box).

whose activation results in increased cAMP/PKA through G_{α_s} and adenylyl cyclase (Fujino et al., 2002), leading to smooth muscle relaxation. EP1 is coupled to G_{α_q} , which increases Ca^{2+} levels following the activation of phospholipase C (Fujino and Regan, 2005) and belongs to the group of contractile receptors. EP3 is generally coupled to G_i , leading to a decrease in cAMP via G_{α_i} and resulting in the inhibition of muscle contraction (Narumiya et al., 1999), although different isoforms resulting from alternative splicing in the EP3 gene are coupled to different types of G protein, such as G_{α_s} , G_{α_i} and $G_{\alpha_{12/13}}$ (Tang et al., 2017).

Mammalian EP1-EP4 receptors exhibit distinctive levels of ligand-binding affinity, different selective expression, tissue distribution and subcellular localization (Sugimoto and Narumiya, 2007; Yokoyama et al., 2013). These receptors have been characterized at genomic (Locker and Buzard, 1990; Smock et al., 1999; Suganami et al., 2016; Yokoyama et al., 2013), proteomic (Margan et al., 2012; Stillman et al., 1999; Sugimoto and Narumiya, 2007; Yokoyama et al., 2013) and expression levels, both *in vitro* and *in vivo*, in response to a variety of stimuli (Birrell et al., 2015; Sakamoto et al., 2004; St-Jacques and Ma, 2011; Sheppe et al., 2018). Mouse EP3 and EP4 are the most widely PGE₂ receptor distributed (Yokoyama et al., 2013) and both have a much higher affinity than EP1 and EP2 for PGE₂ (Fujino et al., 2002).

In teleost fish, the production of PGE₂ by immune cells has been described in a broad range of species, including black seabream (*Spondylosoma cantharus*), black rockfish (*Sebastes melanops*), red seabream (*Pagrus major*), Atlantic cod (*Gadus morhua*), yellow croaker (*Larimichthys polyactis*), rainbow trout (*Oncorhynchus mykiss*), goldfish (*Carassius auratus*) and gilthead seabream (*Sparus aurata* L.) (Gómez-Abellán and Sepulcre, 2016). Furthermore, there is evidence concerning the immunoregulatory activity of PGE₂ in fish: for example,

the modulation of cytokine expression, ROS production and phagocytic responses (Gómez-Abellán and Sepulcre, 2016). More specifically, we have shown that PGE₂ leads to M2 polarization of macrophages in gilthead seabream (Montero et al., 2016), in a similar way to that seen in mammals (Harris et al., 2002). PGE₂ deactivates acidophilic granulocytes (AGs), the functionally equivalent cell to mammalian neutrophils) through the modulation of the cytokine expression profile (Montero et al., 2016). However, studies concerning the molecular characterization of PGE₂ receptors and their role in the immune response in teleost fish are scant. Mammalian homologs of EP1-4 have been identified and characterized in several fish species: zebrafish (*Danio rerio* H.) (Kwok et al., 2012; Tsuge et al., 2013), Atlantic salmon (*Salmo salar* L.) (Guo et al., 2015), *Bostrichthys sinensis* (Lai et al., 2014) and ayu (*Plecoglossus altivelis*) (Rong et al., 2016), EP4 being the most characterized (Fujimori et al., 2011; Gamil et al., 2015; Guo et al., 2015; Tsuge et al., 2013). They are involved in several functions such as reproduction (Fujimori et al., 2011, 2012), metabolic regulation (Busby et al., 2002) and the immune response (Guo et al., 2015; Rong et al., 2016). *Ptger4* has also been considered as an early marker of T cells precursor in zebrafish development (Villablanca et al., 2007). However, our knowledge of the role of PGE₂ receptors in the teleost immune response is still limited, which makes a comparative analysis with their higher vertebrate counterparts quite difficult.

In previous studies, we showed that the cell-permeable analog of cAMP (dbcAMP) mimicked the effect of PGE₂ in the regulation of cytokine mRNA levels in stimulated macrophages but not in AGs. The variety of effects that PGE₂ elicits in both phagocytic cell types could reflect the presence of specific receptors in those cells and suggest that PGE₂ signals through EP2 and/or EP4 in seabream macrophages but not in AGs. Taking into consideration all the above, the aim of this study was

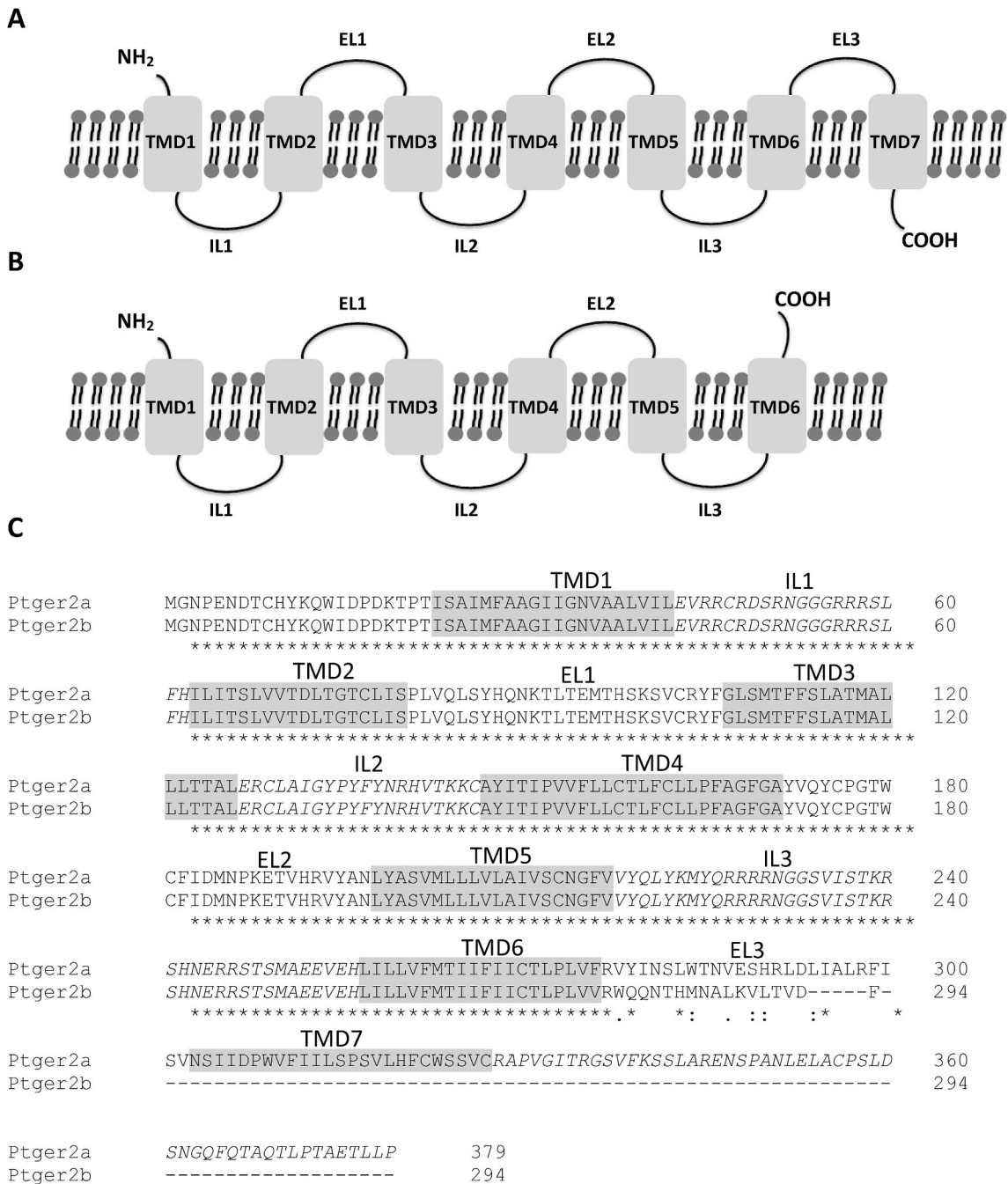


Fig. 2. Schematic domain organization and sequence alignment of seabream Ptger2a and Ptger2b. Diagrams showing the domain organization of seabream Ptger2a, which is also representative of Ptger1, Ptger3 and Ptger4 (A), and Ptger2b (B). TMD: transmembrane domain, IL: intracellular loop, EL: extracellular loop. (C) Alignment of seabream Ptger2a and Ptger2b sequences. (*) Identity in one position; (.) conservative substitutions in one position; (.) semiconservative substitutions in one position.

the identification and in silico characterization of gilthead seabream PGE₂ receptors (Ptger1-4) and to gain further insight into their role in the resolution of inflammation *in vivo* and *in vitro*. In particular, their distribution *in vivo* and modulation after bacterial challenge and their contribution to phagocytic cell functions were analyzed. These data pave the way for analysis of the role of PGE₂ receptors during the immune response of teleost fish.

2. Materials and methods

2.1. Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*Sparus aurata*, Actinopterygii, Sparidae) were bred and kept at the Oceanographic Center of Murcia (Spain) in a 14 m³ running seawater tank (dissolved oxygen 6 ppm, flow rate 20% tank volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Burgos, Spain). The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the

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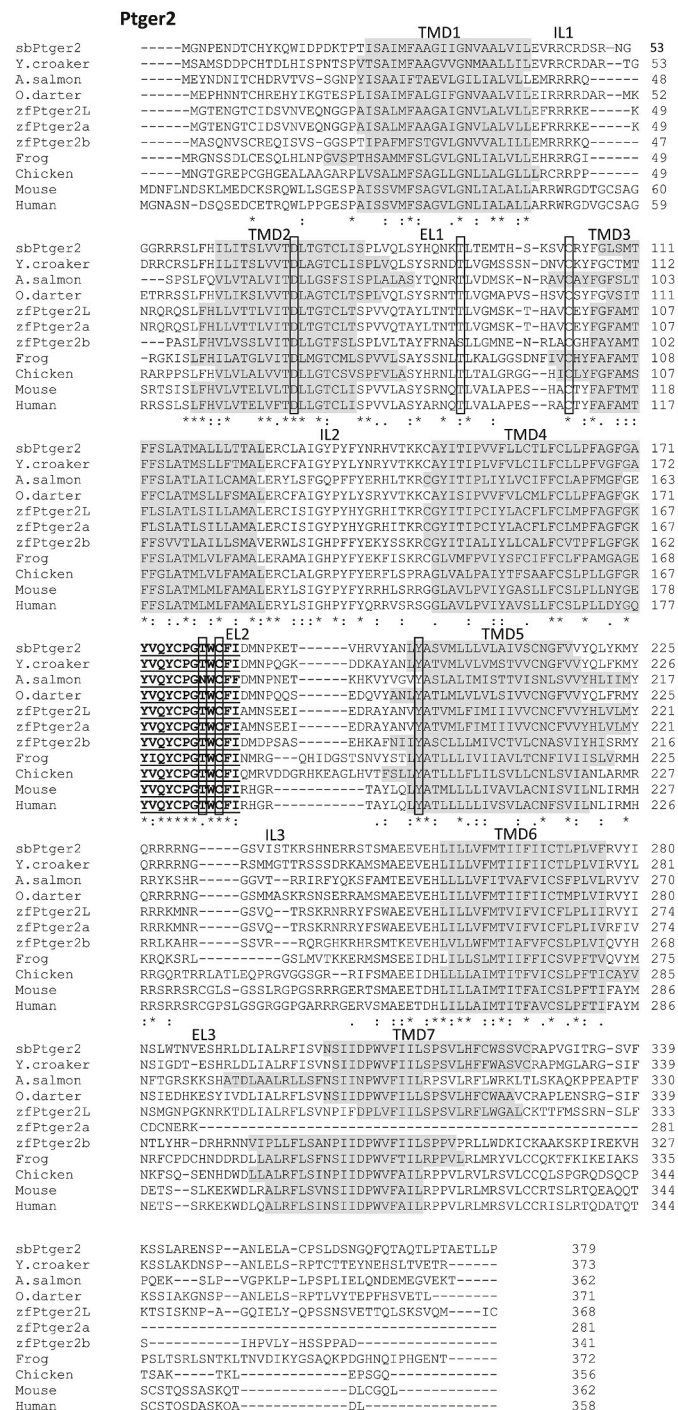


Fig. 4. Sequence alignment and domain organization of seabream *Ptger2a* sequence with known vertebrate *Ptger2* sequences. Transmembrane domains (TMD) are shaded, extracellular loop (EL), intracellular loop (IL). (*) Identity in one position; () conservative substitutions in one position; (:) semiconservative substitutions in one position. The accession numbers for the sequences used are listed in Table 2. Conserved motifs are in bold and underlined.

Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals. Seabream were killed by cervical dislocation using approved procedures following anaesthetization with clove oil. All efforts were made to minimize animal stress and to ensure that termination procedures were performed efficiently.

2.2. In vivo experimental infections and sampling

Five fish were injected intraperitoneally with 1 ml of phosphate-buffered saline (PBS) alone or containing a sublethal dose (10^8) of exponentially growing *Vibrio anguillarum* R82 cells (Chaves-Pozo et al., 2004). Head kidney, spleen, thymus, liver, gills, blood and peritoneal exudates cells were obtained 4 h after bacterial challenge, a time-point previously described as sufficient to trigger the mobilization of immune cells (Chaves-Pozo et al., 2005). All samples were processed for subsequent real-time RT-PCR (see below).

2.3. Isolation of phagocytes

AGs were obtained by Magnetic-Activated Cell Sorting (MACS), as described previously (Roca et al., 2006). Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a mAb specific to gill-head seabream AGs (G7) (Sepulcre et al., 2002), washed twice with PBS containing 2 mM EDTA (Sigma-Aldrich) and 5% FCS (Invitrogen) and then incubated with 100–200 μ l per 108 cells micro-magnetic-bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec). After washing, G7+ (AGs) cell fractions were collected by MACS following the manufacturer's instructions and their purity was analyzed by flow cytometry (Roca et al., 2006). Head kidney macrophage monolayers were then obtained by overnight culture of G7-fractions in serum-free medium as described previously (Roca et al., 2006).

2.4. Cell culture and treatments

Phagocytes (AGs and macrophages) were treated at 23 °C with 50 μ g/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (VaDNA) (Pelegri et al., 2004) and/or PGE₂ (10 μ M, Cayman Chemical) in sRPMI [RPMI-1640 culture medium (Gibco) adjusted to gilthead seabream serum osmolality (353.33 mOs) with 0.35% NaCl, supplemented with 0.1% FCS and 100 IU/ml penicillin and 100 μ g/ml streptomycin (Biochrom). Stimulation times were 16 h for AGs and 3 h for macrophages. The concentrations used above have been found to be optimal for the *in vitro* activation of seabream phagocytes (Sepulcre et al., 2007; Montero et al., 2016).

2.5. Analysis of gene expression

Total RNA was extracted from cell pellets with TRIzol Reagent (Invitrogen) following the manufacturer's instructions, and the upper phases containing RNA were purified with RNAqueous Micro Kit (Ambion) according to the manufacturer's protocols. Final RNA concentration was measured at 260 nm by means of the nanodrop system (ND-1000), and RNA quality was checked by electrophoresis. RNA was stored at –80 °C until use. Total RNA was treated with amplification grade DNase I (1 unit/ μ g RNA, Life Technologies). Then, SuperScript III RNase H- Reverse Transcriptase (Life Technologies) was used to synthesize first strand cDNA with oligo-dT18 primer from 1 μ g of total RNA for 50 min at 50 °C, followed by 15 min at 70 °C. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reactions were performed in 10 μ l mixtures, containing 5 μ l of 2 $\times$ SYBR Green PCR Core Reagents (Applied Biosystems), 0.15 μ l of each primer (0.15 μ M, final concentration), and 2.5 μ l of cDNA. The amplification profile was: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and, finally, 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. The RNA was quantified using ribosomal protein S18 (*rps18*) transcript content as housekeeping gene using the comparative Ct method ($2^{-\Delta\Delta C_t}$ for “fold change” and $2^{-\Delta C_t}$ for constitutive expression). The primers used (Table 1) had an amplification efficiency of 2 and their specificity was confirmed by melting curve analysis and the sequencing of each amplified product (Genetic Analyzer ABI PRISM 3130, Applied Biosystems) (data not shown). In all cases, each PCR was performed with

	-MTM-----DASDHLRGFNKTTIGEMPGAN-----	-VSKSLRGCNVNTENSCGRSGSVGFP	
Seabream	-MTP-----DECIDLKGLQTTTGEMLSAN-----	-VSKSLRGCNVNTENSCGRSGSVGFP	27
Y.croaker	-----ML-----	-SNKSLRERNVTKSGNCGSVSVLFP	27
A.salmon	MMTA-----ESCDFLEGLQTNIGKMLDFN-----	-VSKSLREDNATKNSCGSVSVGFP	28
O.darter	-----MNTLP-----	-EKGWHQNDSPGRSAGCGSVSVFPF	49
Zebrafish	-MK--RGA---LP-SADLNSTGRRLDPIASTAMKEDRSDNMTA--R-AEAGNTVSVAF		50
Frog	-MS-----RRPLCPQPGNGTEPMRPRNGNTG--RPAEGCGAVSVAF		39
Chicken	-----MASMWAPHE-----SAEHSNLSS---TTDDCGSVSVAF		32
Mouse	-MKETRGYGGDAPFCFTRLNHSYTG MWAPER-----SAEARGNLTRPPSGEDCGSVSVAF		55
Human			
	TMD1	IL1	TMD2
Seabream	ITMMITGMVGNLTALILVYISYRKKENK	RKKSFFLLCIGSLALTDLFGQLLTSP	107
Y.croaker	ITMMITGMVGNLTALILVYISYRKKENK	RKKSFFLLCIGSLALTDLFGQLLTSP	107
A.salmon	ITMMVITGMVGNLTALILVYSSYRKKENN	RKKSFFLLCIGSLALTDLFGQLLTSP	87
O.darter	ITSMITITGIVGNLTALILVYISYRKKENK	RKKSFFLLCIGSLAFTDLFGQLLTSP	107
Zebrafish	ITMMVITGMVGNLTALILVYISYRKKENK	RKKSFFLLCIGSLALTDLFGQLLTSP	89
Frog	LSMMITITGLVGNALAMLVYKSYRNKES	RKKSFFLLCIGSLALTDLFGQLLTSP	110
Chicken	ITMMITITGIVGNALAMLVYRSRYRAREN	RKKSFFLLCIGSLALTDLFGQLLTSP	92
Mouse	ITMMVITGFVGNALAMLVYRSRYRRES	RKKSFFLLCIGSLALTDLFGQLLTSP	99
Human	ITMLITGFVGNALAMLVYRSRYRRES	RKKSFFLLCIGSLALTDLFGQLLTSP	115
	EL1	TMD3	IL2
Seabream	AGMEWKHIDPSGNLCGFFGVCMTTFLGCLFLASAMA	TERAMAITNPHWYSNHMKTSVTK	167
Y.croaker	ADLKWERIDSSGNLCAGFGVCMTTFLGCLFLASAMA	TERAMAITNPHWYSNHMKTSVTK	167
A.salmon	ADLKWERIDSSGNLCAGFGVCMTTFLGCLFLASAMA	TERALAITSPHWYSSHMKTSTVTK	147
O.darter	ADLKWEHIDSSGNLCAGFGVCMTTFLGCLFLASAMA	TERAMAITNPHWYSSHMKTSTVTK	168
Zebrafish	ADMKWDRVDPSTLCAFFGVCMTTFLGCLFLASAMA	TERALAIAPHWYSHYMKTSVTK	149
Frog	SNRQWARVDPSTLCAFFGVCMTTFLGCLFLASAMA	TERLAIPTPHWYSSHMKTSTVTK	170
Chicken	SDRVNTWVDPSTLCAFFGVCMTTFLGCLFLASAMA	TERLAIPTPHWYSSHMKTSTVTK	159
Mouse	SQRWEQLDPSTLCAFFGVCMTTFLGCLFLASAMA	TERALAIAPHWYSHYMKTSVTK	152
Human	SKQREWHIDPSGRLCTFFGLTMTVFLGSSLFASAMA	VERALAIAPHWYSHYMKTSVTK	175
	TMD4	EL2	
Seabream	QTLAVIWCVLFPALLPIAGVGVEYKQ	OWPGTACFISTGDR-----KVPGNMFFAITFA	220
Y.croaker	QTLAVIWCVLFPALLPIAGVGVEYKQ	OWPGTACFISTGDR-----EVPGNMFFAITFA	220
A.salmon	QILAVIWCVLFPALLPIAGVGVEYKQ	OWPGTACFISTGDR-----EVPGNMFFAITFA	220
O.darter	QTLVVVWCVLFPALLPIAGVGVEYKQ	OWPGTACFISTGDR-----EVPGNMFFAITFA	201
Zebrafish	QVLVIWCVLFPALLPIAGVGVEYKQ	OWPGTACFISTGDK-----DVTGNFTFAITFA	202
Frog	MVLLGIVLGLFPALMPIIGVGQYTL	OWPGTACFISTGDK-----EAVGNIFFASTYA	223
Chicken	AVLLGIWLAVPAPALLPLAGLGRYAL	OWPGTACFIST-EG-----QRPGSVLFASAF	211
Mouse	AVLLGVWLAVPAPALLPLVGLVGQYTL	OWPGTACFISTGDRGNETDPAREPGSVAFASAF	212
Human	AVLLGVWLAVPAPALLPLVGLVGQYTL	OWPGTACFISTGRGNGTSSSHNNGVLFASAF	235
	TMD5	IL3	TMD6
Seabream	GLGIFSLVLTSCNVVTIRGLIIRCKTKSGTSQSSKHWERLT	TETVIQLLGIMCVLLICW	280
Y.croaker	GLGIFSLVLTSCNVVTIRGLIIRCKTKSGTSQSSKHWERLT	TETVIQLLGIMCVLLICW	280
A.salmon	VLGIFSLVLTSCNVVTIRGLIIRCKTKSGTSQSSKHWERLT	TETVIQLLGIMCVLLICW	260
O.darter	VLGIFSLVLTSCNVVTIRGLIIRCKTKSGTSQSSKHWERLT	TETVIQLLGIMCVLLICW	281
Zebrafish	ALGIFSLVLTSCNVVTIRALVTRCKTKSSSTQSSKHWERLT	TETVIQLLGIMCVLLICW	282
Frog	CLGLLSFTTTFACNLSTI VALVSRCRRSSANASRQWERIT	LETLIQLLGIMCVLFCW	263
Chicken	CLGLLSFTTTFACNLSTI VALVSRCRRSSANASRQWERIT	LETLIQLLGIMCVLFCW	272
Mouse	CLGLLSFTTTFACNLSTI VALVSRCRRSSANASRQWERIT	LETLIQLLGIMCVLFCW	271
Human	FLGLLALTITVTFSCNLATIKALVSRCRAKATASQSSAQWGRIT	TETATQLLGIMCVLFCW	295
	EL3	TMD7	
Seabream	SPLLVLML-RMITKVSSSHCDNLGNP-IQTGTQEDHFSCNFFLT	ATRLASLNQILDWPV	338
Y.croaker	SPLLVLML-TMISTQVSSHCDNSTAVASNHTTGRDVLDCNFFLT	ATRLASLNQILDWPV	339
A.salmon	APLLVLML-RMISTQVSSHCHCKPAEV--SFTPSQDIQVDCNFFLT	ATRLASLNQILDWPV	317
O.darter	SPLLVLML-RMISNQVSSHCDNSTAVSSHTSTRDVLDCNFFLT	ATRLASLNQILDWPV	340
Zebrafish	SPLLVLML-KMITLHTSSHCHCYAGSL---SPQELQKDCNFFLT	ATRLASLNQILDWPV	317
Frog	SPLLVLML-KMNSNHTSVEHCKPKAS---EQNTELQKDCNFFLT	ATRLASLNQILDWPV	338
Chicken	SPLLVTML-KMIFNRTSFESCKGFS---ETQSSLEYKCNFFLT	ATRLASLNQILDWPV	327
Mouse	SPLLMNNLKWTFIAPVPSLGLRIS-----S-PREG-----		302
Human	SPLLIMMLK-MIFNQTSVEHCKQHT---EKQKECNFFLT	ATRLASLNQILDWPV	345
	YLLFREILLRKFCIVANAVSNCSIDDRKETQTALDALNKQNHDDNNIHKPQSS-----		391
Seabream	YLLFREILLRKFCIVANAVSNCSIDDHKENQTALDALNKQNHDDNNIHKPQSN-----		392
Y.croaker	YLLFREILLRKFCQVAVSNCSIDDKQDTQTALDALNKQPSDNTGL-----		364
A.salmon	YLLFREILLRKFCIMATVAVSNCIDEEQKTAALNKNQDGNNGNKLKQCS-----		393
O.darter	YLLLRHILLRKFCQVAVSNCSDVQKQTCQPLDVQNKAIQNDVHVKQSLNVENSILN		377
Zebrafish	YLLLRHILLRKFCQVAVSNCSDVQKQTCQPLDVQNKAIQNDVHVKQSLNVENSILN		381
Frog	YLLLRKILLQKFCQVAVSNCSDVQKQTCQPLDVQNKAIQNDVHVKQSLNVENSILN		370
Chicken	YLLLRKILLQKFCQVAVSNCSDVQKQTCQPLDVQNKAIQNDVHVKQSLNVENSILN		370
Mouse	YLLLRKILLQKFCQVAVSNCSDVQKQTCQPLDVQNKAIQNDVHVKQSLNVENSILN		302
Human	YLLLRKILLQKFCQVAVSNCSDVQKQTCQPLDVQNKAIQNDVHVKQSLNVENSILN		374
	-----	391	
Seabream			

7

Table 3

Amino acid sequence identity (%) between seabream Ptger1-4 and other vertebrates Ptger subtypes.

	Ptger1	Ptger2a	Ptger3	Ptger4
Human	42.8	46.7	61.3	56.8
Mouse	43.8	47.1	60.4	56.2
Chicken	–	44.3	63	58.6
Frog	46.1	45.5	65	64.9
Zebrafish	zEP1a: 67.8 zEP1b: 63.8 zEP1c: 47	zEP2a: 59.8 zEP2L: 58 zEP2b: 49.4	76.1	zEP4a: 66.6 zEP4b: 62 zEP4c: 49
Atlantic salmon	65.8	53.3	79.5	65.2
Yellow Croaker	87.1	74.4	86.2	62
Orange throat darter	88.1	71.3	80.4	84.7

triplicate samples.

2.6. In silico analysis of seabream PGE₂ receptor sequences

Nucleotide sequences of seabream *ptger1*, *ptger2a*, *ptger2b*, *ptger3* and *ptger4* (Accession numbers in Table 2), were obtained from a non-public EST data base. The deduced amino acid sequences were obtained via EMBOSS: Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). Areas of seabream protein sequences similar to those of other vertebrate sequences obtained from the NCBI data base were compared after multiple amino acid sequence alignments within the website tool Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), provided by the European Bioinformatics Institute (EMBL-EBI), in our case: fish (large yellow croaker and zebrafish), reptiles (frog), bird (chicken), mammals (mouse and human). Phylogenetic reconstructions were performed with phylogeny software (<http://www.phylogeny.fr/>) (Dereeper et al., 2008). The molecular weight of each protein was determined using the Science Gateway tool (http://www.sciencegateway.org/tools/prot_einmw.htm). Finally the structure and domain organization of each receptor were determined using the psipred protein structure predictor, (<http://bioinf.cs.ucl.ac.uk/psipred/>), SMART program, and TMHMM Software (<http://www.cbs.dtu.dk/services/TMHMM/>) and with the HMMTOP server (<http://www.enzim.hu/hmmtop/>).

2.7. Cloning the full-length cDNA of seabream Ptger1-4

Sequences of seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4 were obtained by PCR amplification with Pfu DNA polymerase (Fermentas) using cDNA from seabream head kidney as template. The specific primers used for amplification (listed in Table 1) were designed by using the Primer Designing Tool of NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). PCRs were performed at low melting temperature: first 5 cycles at 58 °C followed by 30 cycles at 72 °C. The PCR amplified fragments were cloned between *Bam*HI and *Not*I restriction sites into the plasmid pcDNA6/V5-His C (Life Technologies) in frame with the sequence coding for the V5 epitope. All constructs were sequenced by using ABI PRISM 377 (Applied Biosystems).

2.8. Statistical analysis

Data were analyzed by ANOVA and Tukey's multiple range tests to determine differences between groups. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Identification and characterization of genes encoding Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4 in gilthead seabream

Searches within a non-publicly EST database allowed us to identify two genes encoding for gilthead seabream Ptger2 designated as Ptger2a

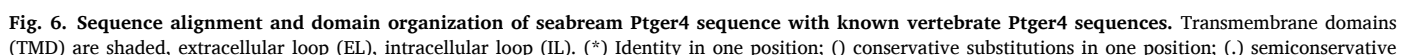
and Ptger2b (ENA accession numbers XM_030410287.1 and XM_030410288.1, respectively) and one for Ptger1, Ptger3 and Ptger4 (ENA accession numbers XM_030412901.1, XM_030434233.1 and XM_030436477.1, respectively). The full-length open reading frame (ORF) of *ptger1* (Fig. 1A), *ptger2a* (Fig. 1B), *ptger2b* (Fig. 1C), *ptger3* (Fig. 1D) and *ptger4* (Fig. 1E) are 1140, 1140, 885, 1203 and 1341 base pairs (bp), respectively, which translated into the putative 379, 379, 294, 391 and 446 amino acid polypeptides with estimated molecular weights of 41.34, 42.71, 33.46, 43.46 and 49.54 kDa, respectively. The coding sequences for Ptger2a and Ptger2b showed 73% nucleotide identity and differed in the last 312 nucleotides before the stop codon (Fig. 1B and C). The 5' and 3' UTR sequences of *ptger1* had 381 and 855 bp, respectively (Fig. 1A). However, the 5' and 3' UTR sequences of *ptger2a* are not available in public databases. The sequences of *ptger2b* 5' and 3'UTR had 90 and 330 bp (Fig. 1C), while *ptger3* had 78 and 1203, respectively (Fig. 1D). By contrast, *ptger4* receptor had the longest UTR sequences: 860 bp for 5'UTR and 1414bp for 3'UTR (Fig. 1E).

The pivotal regulatory elements found in each gene are shown in Fig. 1: i) two putative *ccat* boxes in *ptger2b* (one in the 5'UTR and one in 3'UTR) and three in *ptger4* (two in the 5'UTR and one in 3'UTR), ii) a polyadenylation signal, an important 3' end element involved in mRNA maturation, transport and translation in eukaryotes (Sachs, 1990), was found in *ptger1*, *ptger3* and *ptger4* 3'UTRs. iii) three motifs *attta*, associated with the instability of mammalian (Han et al., 1990) and fish (Roca et al., 2007) cytokine mRNAs, were found in *ptger1* 3'UTR, four in *ptger3* and six in *ptger4* (one located in the 5'UTR and five in 3'UTR). The presence of these motifs, especially the sequences with three or more ATTTA motifs, is concomitant with rapid post transcriptional modulation, splicing, mRNA export, degradation, translation activation or inhibition, and turnover (Akashi et al., 1994; Khabar, 2010; Moore et al., 2011). iv) a consensus sequence (ttatttat) of mammalian inflammatory mediator mRNAs was found in *ptger3* 3'UTR and v) a cytoplasmic polyadenylation element (tttttttttt) was identified in *ptger4* 3'UTR.

3.2. Polypeptide sequences and topology analysis of seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4

The in silico analysis of the deduced amino acid sequences of *ptger1*, *ptger2a*, *ptger2b*, *ptger3* and *ptger4* and of their domain organization revealed that they are G protein-coupled receptors, as has been documented in higher vertebrates (Narumiya et al., 1999). They consist of integral membrane proteins that possess seven transmembrane domains (TMD): the extracellular N-terminal, the intracellular C-terminal, three extracellular loops (EL) and three intracellular loops (IL) (Fig. 2A). However, Ptger2b possesses six instead of seven TMD, and hence two EL and a C-terminal on the extracellular side. (Fig. 2B).

In addition, multiple sequence alignments of Ptger1, Ptger2a, Ptger3 and Ptger4 with those of other vertebrates showed long areas of conserved amino acid residues in all the sequences analyzed. The most conserved regions were located within the seven putative transmembrane domains among the identified seabream Ptger1 (Fig. 3), Ptger2a (Fig. 4), Ptger3 (Fig. 5) and Ptger4 (Fig. 6) and their respective orthologs. The identities between whole-length seabream Ptger subtypes and other vertebrates Ptger ranged from 42.8% to 88.1% (Table 3). The motif TWCFI was identified in the second extracellular loop of seabream Ptger1 (Fig. 3), Ptger2a (Fig. 4), Ptger3 (Fig. 5) and Ptger4 (Fig. 6) and in those of the other sequences analyzed with the exception of zebrafish Ptger1c, frog Ptger1 and yellow croaker Ptger4, which contain leucine instead of isoleucine (Figs. 3–6). This motif constitutes part of the 12 highly conserved amino acid residues in Ptger2a, Ptger2b (YVQYCPGTWCFI) and in Ptger3 (QWPGTWCFISTG) of most of the vertebrates mentioned in this work, except chicken Ptger3, which lacks the G (Figs. 2C and 4). These invariant residues form part of the second extracellular loop in all the mentioned vertebrate sequences, and has been suggested to act not only as a link between the transmembrane domains, but also to have a conserved structural role (allosteric



substitutions in one position. The accession numbers for the sequences used are listed in Table 2. Conserved motifs are in bold and underlined. Signal peptides are in bold.

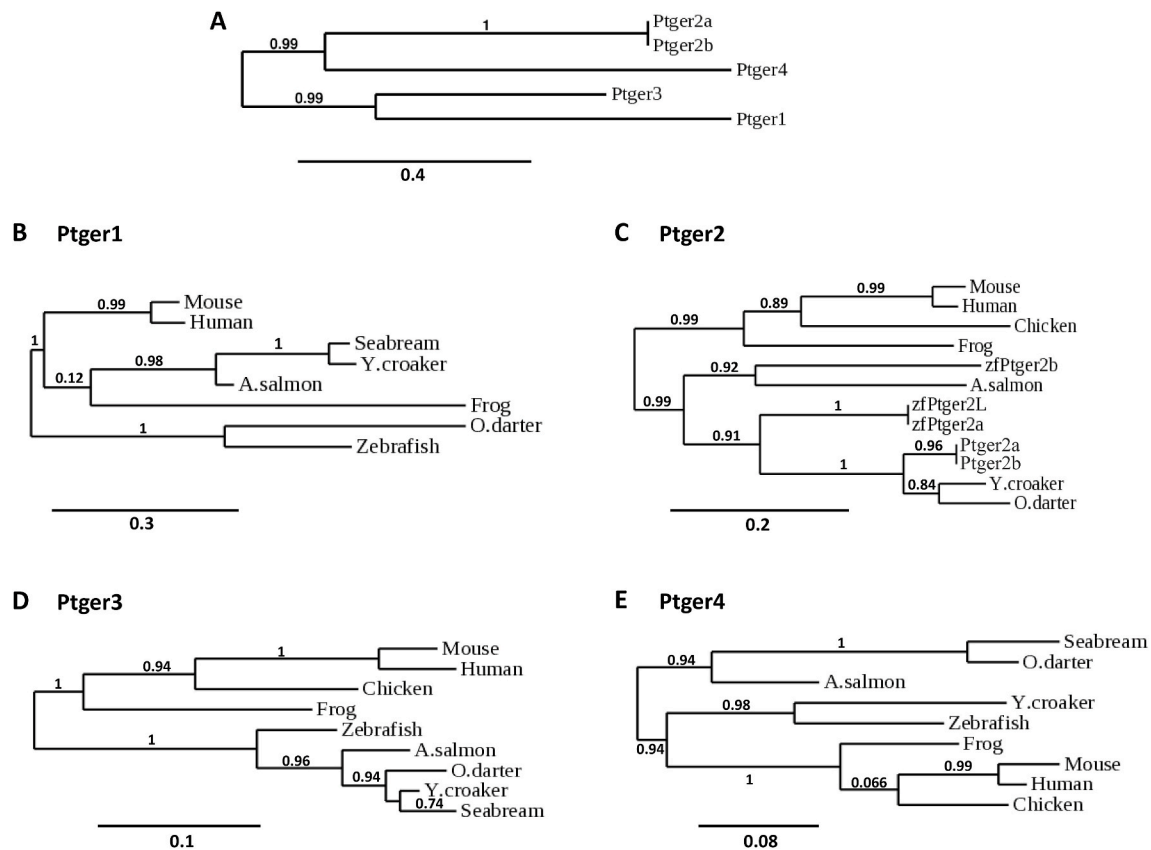


Fig. 7. Phylogenetic relationships seabream Ptger subtypes sequences. Phylogenetic tree of seabream Ptger1-4 (A) and vertebrate Ptger subtypes and seabream Ptger1 (B), Ptger2a and Ptger2b (C), Ptger3 (D) and Ptger4 (E) polypeptides. The tree was generated by phylogeny.fr software (Dereeper et al., 2008) using amino acid sequences. Numbers shown are percentages of 100 bootstrap replicates in which the same internal branch was observed. The horizontal lines are drawn proportional to the inferred phylogenetic distances, while the vertical lines have no significance. ENA accession numbers are listed in Table 2.

conformation) and functional actions related with GTP sensitivity as well as agonist binding interaction (Stillman et al., 1998). In this context, it is important to mention that the complex G protein/GTP plays an important role in the receptor's passage from inactive "R" to the active conformation "R*" in GPCR (De Lean et al., 1980; Nataraj et al., 2001; Samama et al., 1993; Stillman et al., 1999; Venkatakrishnan et al., 2013). Furthermore, seabream Ptger4 sequence has more highly conserved regions in their TMD, EL and IL than Ptger1, Ptger2a and Ptger3 (Fig. 6). Notably, key residues responsible for structural as well as functional aspects of mammalian Ptger, were also found in seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4. In particular, the conserved T (in EL1) (Stillman et al., 1999), D (TMD2), C (EL1 and 2) (Savarese and Fraser, 1992) and Y (TMD5) (Suganami et al., 2016), as well as the R (TMD7, a charged amino acid residue that is further conserved in the TMD7 of all PGE₂ receptors of the vertebrates mentioned in the present study except in zebrafish Ptger2b. In support of this finding, it was suggested previously that this arginine is one of the residues that may be involved in relevant functional aspects of these receptors, including conformation stability, ligand binding and signal transduction in zebrafish (Kwok et al., 2012) and in human (Audoly and Breyer, 1997; Neuschäfer-Rube et al., 2004; Wilson et al., 2001). The T and S residues of the C terminus of Ptger4, responsible for agonist-induced short-term desensitization (Bastepe and Ashby, 1999), were also found in seabream Ptger4 (Fig. 6).

3.3. Phylogenetic analysis of seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4

The phylogenetic tree constructed with the Neighbor-Joining method, based on full amino acid sequences of seabream Ptger1-4, revealed two branches. The first branch comprised Ptger2 and Ptger4 and the second branch comprised Ptger1 and Ptger3 (Fig. 7A). Furthermore, the phylogenetic tree constructed with mammalian and teleost Ptger sequences formed two clusters (Fig. 7B-E). Moreover, gilthead seabream Ptger1-4 have the closest phylogenetic relationship with their respective receptors of other fish species belonging to the Perciformes order, followed by Salmoniformes (Fig. 7B-E).

3.4. Gilthead seabream Ptger1-4 are differentially distributed and modulated upon bacterial infection

To gain further insight into the role of the Ptger1-4 immune response in gilthead seabream, RT-qPCR was used to analyze the expression pattern of their corresponding genes as well as their modulation upon bacterial challenge. Seabream *ptger1*, *ptger2a*, *ptger3* and *ptger4* were expressed in all the tissues examined, including such key immune organs as head kidney (HK), spleen, thymus and gills (Fig. 8). In particular, the highest mRNA levels of *ptger1* were found in spleen, followed by HK, thymus, gill and peritoneal exudate (PE, the site of bacterial injection) (Fig. 8B). In a similar way, the most abundant transcript levels of *ptger3* were also found in spleen and gill, followed by thymus, HK, and liver

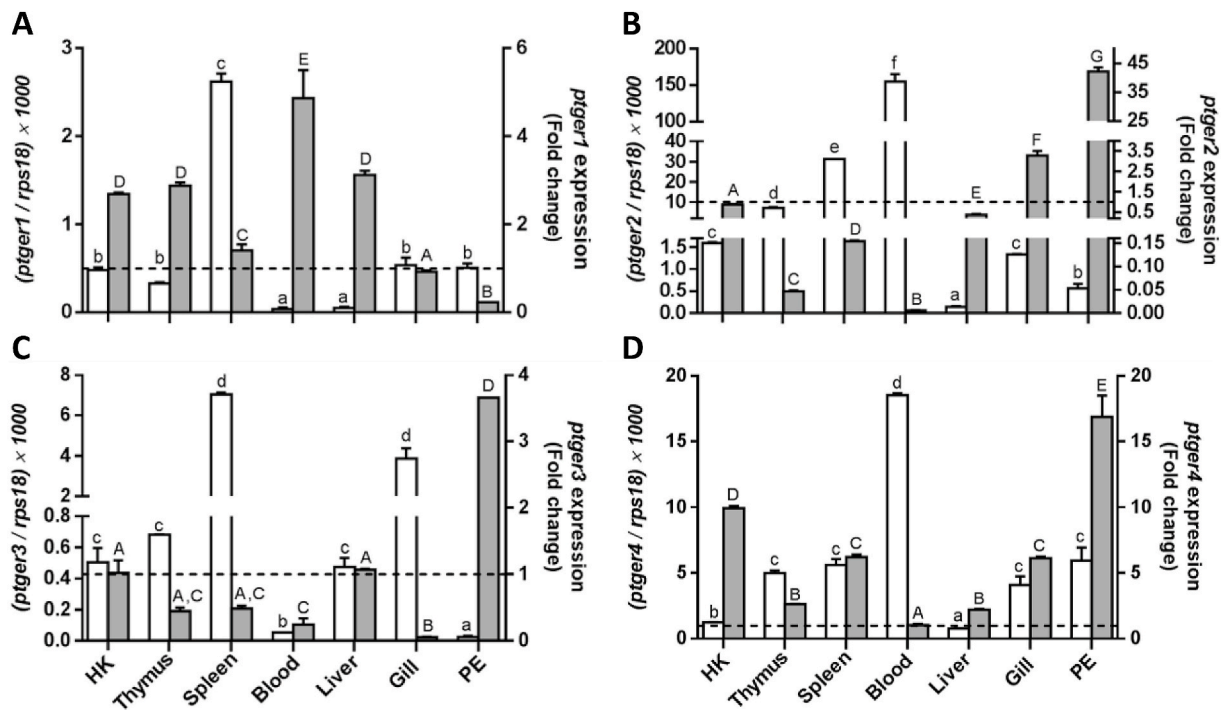


Fig. 8. Tissue expression pattern of seabream Ptger subtypes. Basal mRNA levels (left y axis, white bars) and those following bacterial challenge (right y axis, gray bars) of *ptger1* (A), *ptger2a* (B), *ptger3* (C) and *ptger4* (D) were determined by real-time RT-PCR in the indicated tissues of control adult specimens or following a 4 h challenge with 10^8 cells of *Vibrio anguillarum*. The results are representative of five fishes. The constitutive expression is shown relative to *rps18* and the regulation of the expression following infection is shown as the mean $\pm$ S.E. of the mRNA fold change in infected fish relative to control fish (indicated with horizontal dashed lines). Different letters denote statistically significant differences among the groups according to a Tukey test ($p < 0.05$). The groups marked "A" did not show statistically significant differences from non-infected fish.

(Fig. 8C). Interestingly, the weakest mRNA levels of *ptger1* and *ptger3* were found in blood (Fig. 8C). By contrast, the highest mRNA levels of *ptger2a* and *ptger4* were found in blood (Fig. 8B–D), followed by spleen and thymus for *ptger2a* (Fig. 8B) and by spleen, thymus, PE and gill for *ptger4* (Fig. 8D). However, it is important to note that the magnitude order of the mRNA levels of *ptger2a* is higher compared to those of *ptger4* and much higher compared to *ptger1* and *ptger3* ones (Fig. 8A–D).

Furthermore, infection of fish with the pathogenic bacterium *V. anguillarum* resulted in increased mRNA levels of *ptger1*, mainly in blood followed by HK, thymus and liver (Fig. 8A). However, bacterial challenge led to decreased mRNA levels of *ptger1* in PE (Fig. 8A). By contrast, the mRNA levels of *ptger3* did not significantly change, or slightly decreased, in all the tissues examined, with the exception of PE, in which increased four-fold. Similarly, bacterial challenge resulted in increased mRNA levels of *ptger2a* and *ptger4*, mainly in PE (Figs. 8B–4D) followed by gill in the case of *ptger2a* (Fig. 8B), and HK, spleen and gill in the case of *ptger4* (Fig. 8D). However, the mRNA levels of *ptger2a* did not significantly change in HK, and even decreased dramatically in blood (the highest basal expression site), spleen and thymus after bacterial challenge (Fig. 8B). However, the mRNA levels of *ptger4* increased in all organ tissues analyzed, mainly in PE, but not in blood, where they did not change (Fig. 8D). Taken together, these results showed a different distribution and modulation pattern of the four PGE₂ receptors in the main immune organs of gilthead seabream.

3.5. Expression of gilthead seabream Ptger1–4 in professional phagocytes

We next analyzed the constitutive expression of *ptger1*, *ptger2a*, *ptger3* and *ptger4* in seabream professional phagocytes, macrophages and AGs, as well as the effect of their activation with VaDNA or/and PGE₂ (the receptor ligand) in the mRNA levels of those receptors. It is important to note that although the methods used for phagocyte isolation could induce some level of activation, previous studies showed that

this induction was minimal compared to those of VaDNA for other activation markers, like *il1b* and *tlr5* (Sepulcre et al., 2007) or *pge* (Montero et al., 2016). The results showed that the four receptors were constitutively expressed in both cell types, more strongly in macrophages than in AGs, with the exception of *ptger1* (Fig. 9A). Interestingly, while AGs showed similar mRNA levels of the four receptors, the transcription levels of the genes encoding for Ptger2a and Ptger3 in macrophages were much higher than those of Ptger1 or Ptger4 (Fig. 9A). Furthermore, the results revealed that, in macrophages, the mRNA levels of *ptger1*, *ptger2a*, *ptger3* and *ptger4* decreased upon stimulation with VaDNA (Fig. 9B–E). However, treatment of macrophages with PGE₂, resulted in a similar expression pattern of *ptger1* and *ptger3*, whose mRNA level decreased (Fig. 9B and D), and of *ptger2a* and *ptger4*, whose mRNA levels did not significantly change (Fig. 9C and E). In addition, the joint treatment of macrophages with PGE₂ and VaDNA had a synergistic effect in the reduction of the mRNA levels of *ptger1*, but led to a less pronounced decrease in the transcription levels of *ptger3*. Furthermore, the joint treatment of macrophages with PGE₂ and VaDNA abolished the effect that VaDNA alone had on the reduction of *ptger2a* and *ptger4* transcript levels.

In the case of AGs, stimulation by VaDNA or PGE₂ resulted in increased mRNA levels of *ptger2a* and *ptger4*, especially in the case of PGE₂ (Fig. 9C and E). In addition, treatment of AGs with PGE₂ and VaDNA together abolished the effect of PGE₂ in the induction of *ptger2a* and *ptger4* transcript levels (Fig. 9C and E). In the case of *ptger1* mRNA levels, the individual treatment of AGs with VaDNA or PGE₂ had a much weaker effect, and their combined use resulted in a dramatic decrease (Fig. 9B). However, the results differed in the case of *ptger3*, when VaDNA increased *ptger3* mRNA levels and PGE₂ had no effect on its own. However, the combination of both stimuli resulted in the lower induction of *ptger3* transcript levels compared with VaDNA alone (Fig. 9D). All these results further demonstrate that seabream phagocytic cells are armed with Ptger1, Ptger2a, Ptger3 and Ptger4, which allows them to

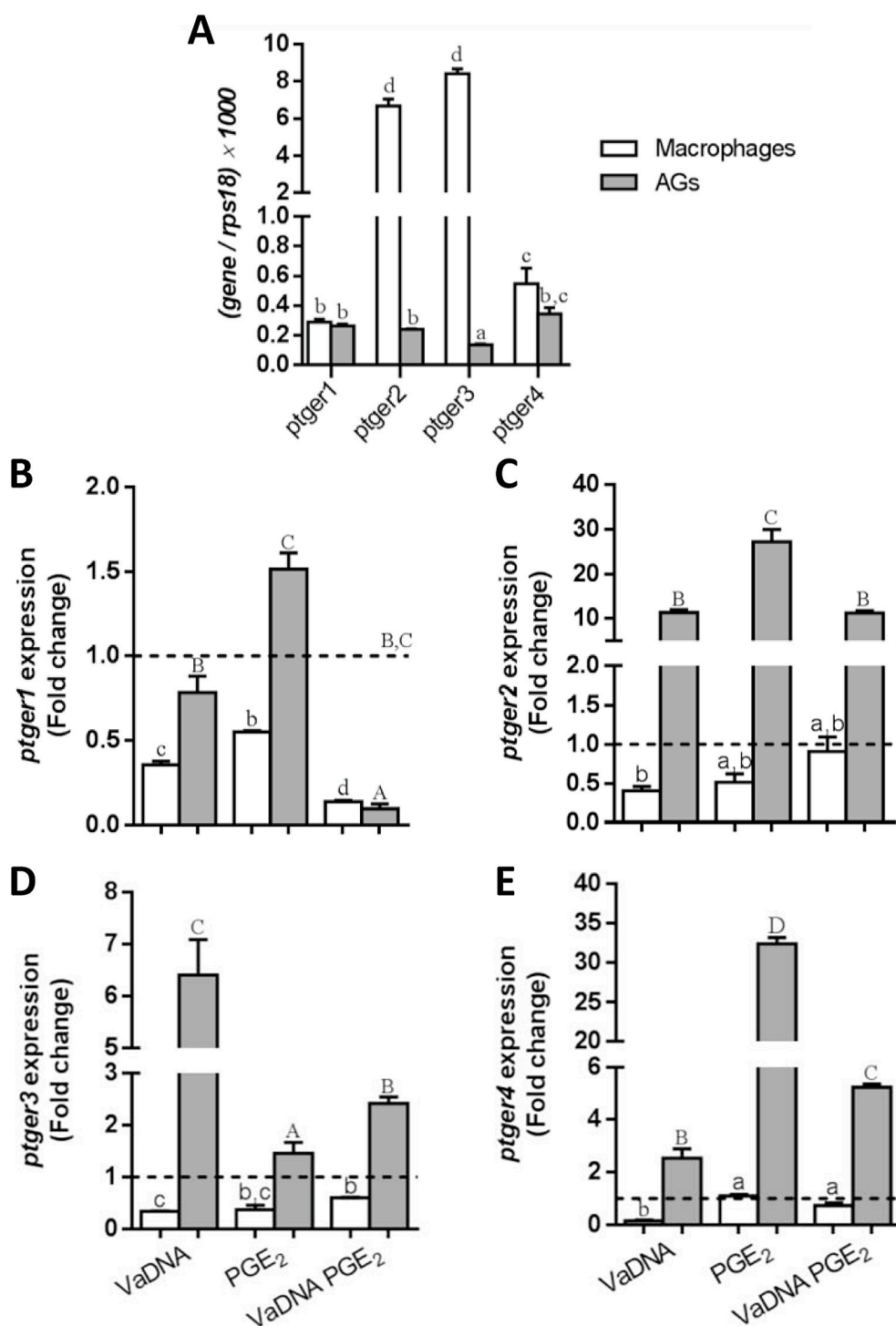


Fig. 9. Expression pattern of seabream Ptger subtypes in professional phagocytes. Basal mRNA levels of seabream Ptger subtypes in macrophages (white bars) and AGs (gray bars) (A) and following stimulation with VaDNA and/or PGE₂. Levels of *ptger1* (B), *ptger2a* (C), *ptger3* (D) and *ptger4* (E) were determined by real-time RT-PCR. The results are representative of six independent experiments. The constitutive expression is shown as the mean ± S.E. of the mRNA fold change in non-stimulated cells. Different letters denote statistically significant differences among the groups. The groups marked “a” for macrophages or “A” for AGs did not show statistically significant differences from non-stimulated cells with the exception of Ptger1 where this group is marked “B,C”.

respond to their ligand, PGE₂, which interfere in the regulation of the mRNA levels of each receptor by PAMPs. Furthermore, in the light of our data, it is tempting to speculate that each phagocyte population would play different roles in the innate immune responses through these receptors, however further functional studies are required.

4. Discussion

Recent studies from our group have shown that PGs, including PGE₂, play a key role in the inflammatory process in the most divergent group of vertebrates, teleost fish (Gómez-Abellán et al., 2015; Gómez-Abellán

and Sepulcre, 2016; Montero et al., 2016; Tyrkalska et al., 2016). However, studies concerning the PGE₂ signaling pathways are scarce. In the present study, seabream PGE₂ receptors, Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4, were cloned and characterized. Multiple alignment, structure domain and phylogenetic tree analysis revealed that the characterized receptors are the bona fide orthologs of mammalian PTGERs, with the typical domain organization of G protein-coupled receptors. Furthermore, a variant of seabream Ptger2a, called Ptger2ab, was also identified and found to be structurally composed of six TMD rather than the seven present in all PGE₂ receptor subtypes of the vertebrates mentioned in this study, similar to zebrafish Ptger2a and

mouse Ptger3, which have six TMD. It is possible that these two isoforms exhibit different degrees of sensitivity with regard to PGE₂ binding, resulting in different levels of action. Two isoforms, Ptger2a and Ptger2b, have been identified in zebrafish, in which both enhance cAMP synthesis (Tsuge et al., 2013). Interestingly, putative ccaat boxes and polyadenylation signals were found in the UTRs of ptger2a and ptger4, as they have been in higher vertebrates (Arakawa et al., 1996; Foord et al., 1996; Locker and Buzard, 1990; Smock et al., 1999). Furthermore, motifs associated with mRNA instability were identified in ptger1, ptger3 and ptger4; however, cytoplasmic polyadenylation elements were identified only in ptger4 UTRs. In addition, seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4 share with their vertebrate orthologs the key residues responsible for structural as well as functional aspects, namely the conserved threonine (Stillman et al., 1999), aspartic acid, cysteine (Savarese and Fraser, 1992) and tyrosine (Suganami et al., 2016) residues. Conserved arginine of the seventh TMD (Narumiya et al., 1999), which is preserved among vertebrates (Yokoyama et al., 2013) and targeted by N/O glycosylation, as well as the threonine and the serine residues of the C terminus responsible for agonist-induced short-term desensitization (Bastepe and Ashby, 1999) were only found in Ptger4. The motifs of the extracellular and intracellular loops involved in G protein coupling and/or ligand binding are conserved among fish and their orthologs (Kwok et al., 2012), which was further confirmed by the phylogenetic analysis which showed that seabream PGE₂ receptors clustered into two groups: one involving higher vertebrates and the other encompassing all fish species. These new findings, together with previous results (Margan et al., 2012; Stillman et al., 1999; Hagiwara et al., 2014; Guo et al., 2015), support the idea that PGE₂ signaling through Ptger1-4 subtypes predated the split of fish and tetrapods more than 450 million years ago.

Several reports exist about the effect of PGs on immune-related activities in fish, most of them focused on the biological activity of PGE₂ (Gómez-Abellán and Sepulcre, 2016). The variety of effects that PGE₂ can elicit, may reflect the presence of specific Ptger subtypes in different tissues and cell types. We have shown herein that gilthead seabream Ptger subtypes were expressed in all the immune tissues analyzed, including HK, spleen, blood, gill, thymus and PE, but with different distributions. In this respect, the results pointed to two distinct expression patterns: one for ptger1 and ptger3 and another for ptger2 and ptger4. The highest mRNA levels of the genes coding for Ptger1 and Ptger3 were found in spleen, while those coding for Ptger2a and Ptger4 were also observed in blood. However, ptger2a and ptger4 were the most abundant subtypes in most of the tissues analyzed, unlike in mouse, where Ptger3 and Ptger4 are the most robustly distributed. It is important to note that the basal expression profile of the four receptors differs in the main phagocytic cell types of seabream, being ptger2a and ptger3 more strongly expressed in macrophages than in AGs and showing AGs similar basal mRNA levels of ptger1-4. Hence, the basal expression profile of ptger1-4 in the different organs reflects the contribution of the breadth of cell types present in the specific organ, not only of macrophages and AGs.

Furthermore, seabream challenged with *V. anguillarum* modulated the expression of genes encoding the different Ptger subtypes, ptger2a and ptger4, showed similar expression profiles, PE (the site of bacterial injection) being the tissue with the highest induction rate of the mRNA levels of both receptors, and blood with lowest rate. In contrast, ptger1 showed a modulation profile that was the opposite of that shown by the rest of the ptger subtypes. Taken together, these results suggest not only that mRNA levels in the specific tissues are modulated in response to bacterial challenge but also could reflect the mobilization of different immune cells from one organ to another (Chaves-Pozo et al., 2005). To get further into this point, we also analyzed the constitutive expression of the genes encoding Ptger1-4 and their modulation by PAMP and ligand stimulation in the main innate immune cells of seabream. The results showed that stimulation of phagocytic cells with VaDNA and PGE₂, alone or together, up-regulated the mRNA levels of ptger2a, ptger3

and ptger4 in AGs, but not in macrophages, where the levels decreased very slightly or did not change. This data, together with those previously reported showing that seabream infection with *V. anguillarum* results in AGs recruitment and activation at the injection site at the time tested herein (Chaves-Pozo et al., 2005), support the hypothesis that mobilization of AGs from different organs contribute to the mRNA levels of ptger1-4 in the specific tissues after bacterial challenge. Furthermore, the results confirmed the *in vivo* results by demonstrating the similar regulation of ptger2a and ptger4 expression by VaDNA or/and PGE₂ in both AGs and macrophages. Nevertheless, ptger1 and ptger3 showed a similar degree of modulation in macrophages, but behaved in a totally different way in AGs. This differential regulation of ptger1-4 in macrophages and AGs is in agreement with the fact that PGE₂ promotes M2 polarization of macrophages but deactivates AGs.

In conclusion, the above results, taken together, suggest a role for G protein-coupled PGE₂ receptors, that is conserved from fish to mammals, thus adding for our understanding of their roles in the modulation of the teleost immune response.

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