UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Mechanisms involved in the inflammatory process of gilthead seabream (*Sparus aurata*)

Mecanismos implicados en el proceso inflamatorio de la dorada (*Sparus aurata*)

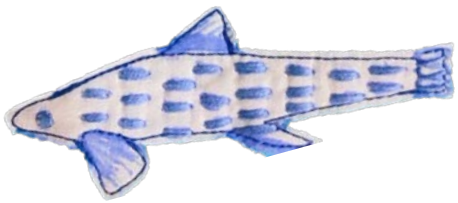
D. Jose Carlos Campos Sánchez

2022

“Hazlo o no lo hagas, pero no lo intentes”
Yoda, el imperio contraataca, 1980

まっすぐ自分(じぶん)の言葉(ことば)は曲(ま)げねぇ。それが俺(おれ)の忍道(しのぶみち)だから
(Avanzo sin arrepentirme nunca de mis palabras, porque ese es mi camino)
ナルト-, 2014

“La vida consiste en una serie de momentos en los que debes elegir el camino a seguir. El camino fácil no es el de vivir, así que coge lo necesario para pasar por el camino difícil”
JoseC, 2015



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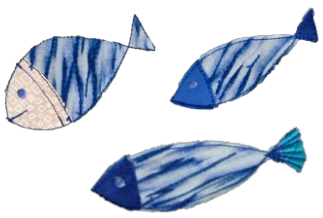

PROJECTS

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PUBLICATIONS

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Campos-Sánchez, J. C., Mayor-Lafuente, J., Guardiola, F.A., Esteban, M.A. (2021). *In silico* and gene expression analysis of the acute inflammatory response of gilthead seabream (*Sparus aurata*) after subcutaneous administration of carrageenin, *Fish Physiology and Biochemistry*. *47*, 1623–1643. https://doi.org/10.1007/s10695-021-00999-6.

Campos-Sánchez, J. C., Vitarelli, E., Guardiola, F. A., Ceballos-Francisco, D., García Beltrán, J. M., Ieni, A., Esteban, M. A. (2021). Implication of mucus-secreting cells, acidophilic granulocytes and monocytes/macrophages in the resolution of skin inflammation caused by subcutaneous injection of λ/κ-carrageenin to gilthead seabream (*Sparus aurata*) specimens, *Journal of Fish Diseases*. *45*, 19-33. https://doi.org/10.1111/jfd.13528.

Campos-Sánchez, J. C., Mayor-Lafuente, J., González-Silvera, D., Guardiola, F. A., Esteban, M. A. (2021). Acute inflammatory response in the skin of gilthead seabream (*Sparus aurata*) caused by carrageenin, *Fish & Shellfish Immunology*. *19*, 623-634. https://doi.org/10.1016/j.fsi.2021.10.009.

Campos-Sánchez, J. C., García-Carrillo, N., Guardiola, F. A., Ceballos-Francisco, D., Esteban, M. A. (2022). Ultrasonography and X-ray micro-computed tomography characterization of the effects caused by carrageenin in the muscle of gilthead seabream (*Sparus aurata*), *Fish & Shellfish Immunology*. *123*, 431-441. https://doi.org/10.1016/j.fsi.2022.03.013.

Publications related to the present Thesis (9):

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Campos-Sánchez, J. C., Guardiola, F. A., Esteban, M. A. (2022). *In vitro* effects of cantharidin on gilthead seabream (*Sparus aurata*) head-kidney leucocytes. *Fish & Shellfish Immunology*, *123*, 20-35. https://doi.org/10.1016/j.fsi.2022.02.045.

Campos-Sánchez, J. C., Guardiola, F. A., Esteban, M. A. (2022). *In vitro* effects of λ-carrageenin in the head-kidney leucocytes of gilthead seabream (*Sparus aurata*). Submitted to *Fish & Shellfish Immunology*: 3/12/2021.

Campos-Sánchez, J. C., Guardiola, F. A., Esteban, M. A. (2022). *In vitro* effects of *Chiliadenus glutinosus* (L.) Fourr. extracts in fish cells and pathogenic bacteria. Manuscript in preparation.

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Campos-Sánchez, J. C., Guardiola, F. A., Esteban, M. A. Are aqueous extract of *Chiliadenus glutinosus* (L.) Fourr. or cantharidin able to reduce the inflammation produced by λ-carrageenin in head-kidney leucocytes from gilthead seabream (*Sparus aurata*)? Manuscript in preparation.

Campos-Sánchez, J. C., González-Silvera, D., Gong, X., Broughton, R., Guardiola, F. A., Betancor, B. B., Esteban, M. A. Implication of adipocytes and fatty acids from subcutaneous tissue in gilthead seabream (*Sparus aurata*) skin inflammation caused by λ-carrageenin. Manuscript in preparation.

Campos-Sánchez, J. C., Serna-Duque, J. A., Guardiola, F. A., Esteban, M. A. Roles of hepcidins in carrageenin-induced gilthead seabream (*Sparus aurata*) inflammation. Manuscript in preparation.

Other publications/collaborations (1):

Otmani, I., Kahalerras, L., **Campos-Sánchez, J. C.,** Albaladejo-Riad, N., Abdennour, C., Esteban, M. A. Activities of bitter and sweet honey on sperm and testicular oxidative stress markers of Wistar rat exposed to inorganic mercury. Submitted to *Journal of Trace Elements and Minerals*: 25/3/2022.

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Campos-Sánchez, J. C., Albaladejo, N., Piñera, R., González-Silvera. D., Esteban, M. A. *"Efectos de la carragenina en la piel de la dorada (Sparus aurata L.)."* Oral communication in "V *Jornadas Doctorales de la Universidad de Murcia"*, 29-31 May, 2019. Murcia, Spain.

Campos-Sánchez, J. C., Mayor-Lafuente, J., Guardiola, F. A., Esteban, M. A. Acute inflammatory response 24 hours after subcutaneous administration of carrageenan in the skin of gilthead seabream (*Sparus aurata* L.). Oral communication in "VIII *Congreso Ibérico de Ictiología* (SIBIC)", 15-19 June, 2020. On line. Galicia, Spain.

Campos-Sánchez, J. C., Guardiola, F. A., Ceballos-Francisco, D., García Beltran, J. M., Esteban, M. A. Recruitment of acidophilic granulocytes after subcutaneous carrageenin injection to gilthead seabream specimens. Oral communication in Spanish Portuguese Advanced Optical Microscopy (SPAOM), 24-27 November, 2020. Valencia, Spain.

Campos-Sánchez, J. C., Guardiola, F. A., García Beltran, J. M., Ceballos-Francisco, D., Esteban, M. A. Effects of subcutaneous injection of carrageenin to gilthead seabream (*Sparus aurata*) specimens on skin mucus humoral immunity. Oral communication in Aquaculture Europe (AE), 12-15 April, 2021. Online.

Campos-Sánchez, J. C., Guardiola, F. A., Esteban, M. A. "*Efectos in vitro de λ-Carragenina en leucocitos de riñón cefálico de dorada* (*Sparus aurata*)". Oral communication in "VI *Jornadas Doctorales de la Universidad de Murcia"*, 21-24 June, 2021. Murcia, Spain.

Campos-Sánchez, J. C., Guardiola, F. A., Esteban, M. A. Pro- and anti- inflammatory *in vitro* effects of cantharidin in gilthead seabream (*Sparus aurata*) head-kidney leucocytes. Poster communication in Aquaculture Europe (AE), 4-7 October, 2021. Madeira, Portugal.

Campos-Sánchez, J. C., Guardiola, F. A., Esteban, M. A. *In vitro* hemolytic, hemagglutinating, cytotoxic and bactericidal effects of λ-carrageenin. Poster communication in Aquaculture Europe (AE), 4-7 October, 2021. Madeira, Portugal.

Campos-Sánchez, J. C., Guardiola, F. A., Esteban, M. A. I*n vitro* effects of C*hiliadenus glutinosus* (L.) fourr. aqueous extract in head-kidney leucocytes of gilthead seabream (*Sparus aurata* L.). Poster communication in Aquaculture Europe (AE), 4-7 October, 2021. Madeira, Portugal.

Campos-Sánchez, J. C., Guardiola, F. A., Esteban, M. A. Is cantharidin able to reduce the inflammation produced by λ-carrageenin in head-kidney leucocytes from gilthead seabream (*Sparus aurata*)? Oral communication in "IX *Congreso Ibérico de Ictiología* (*SIBIC*)", 20-23 June 2022. Porto, Portugal. Abstract accepted.

Serna-Duque, J. A., **Campos-Sánchez, J. C.,** Espinosa-Ruíz, C., Martínez-López, S., Esteban, M. A. Changes in morphology of hepatocytes and tissue-biodistribution in iron-overload gilthead seabream (*Sparus aurata*) Poster communication in "IX *Congreso Ibérico de Ictiología* (*SIBIC*)", 20-23 June 2022. Porto, Portugal. Abstract accepted.

García-Álvarez, M. Á., Cervera, L., González-Fernández, C., Arizcun, M., **Campos-Sánchez, J. C.,** Esteban, M. A., Chaves-Pozo, E., Cuesta, A. Potential impacts in the gilthead seabream larviculture by nodavirus. Poster communication in "IX *Congreso Ibérico de Ictiología* (*SIBIC*)", 20-23 June 2022. Porto, Portugal. Abstract accepted.

INDEX

List of abbreviations

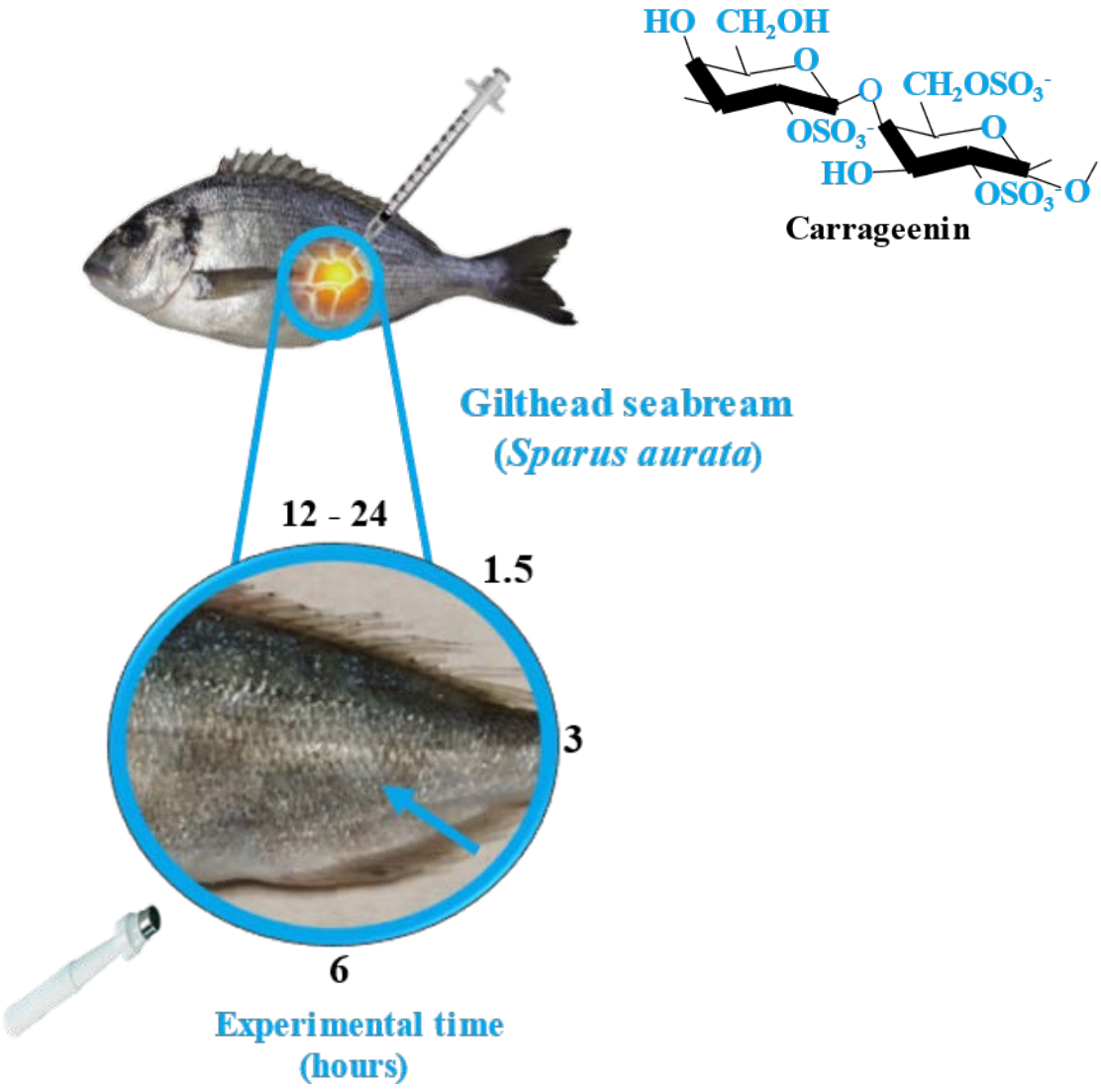
AB	Alcian blue
ache/ACHE	Acetylcholinesterase
actb	Actin beta
AGs	Acidophilic granulocytes
AID	Activation-induced cytidine deaminase
ANOVA	Analysis of variance
APCs	Antigen presenting cells
***bche*/BCHE**	Butyrylcholinesterase
B-mode	Brightness mode
cDNA	Complementary deoxyribonucleic acid
CE	*Conformite Europeenne*
CFA	Complete Freund's adjuvant
***chrna7*/CHRNA7**	Cholinergic receptor, nicotinic, alpha 7
***cox2*/COX2**	Cyclooxygenase 2
***c-rel*/c-REL**	v-rel avian reticuloendotheliosis viral oncogene homolog
***csf1r*/CSF1R**	Colony stimulating factor 1 receptor
CTAB	Cetyltrimethyl ammonium bromide
***ctsd*/CTSD**	Cathepsin D
***ctsl*/CTSL**	Cathepsin L
***ctss*/CTSS**	Cathepsin S
DC	Dendritic cell
ddc	DOPA decarboxylase
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
ef1a	Elongation factor-1 alfa
FAO	Food and agriculture organization
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FMM	Flexibacter maritimus medium
GALT	Gut-associated lymphoid tissue
GIALT	Gill-associated lymphoid tissue
grb	Granzyme B
HBSS	Hank's buffer salt solution
HE	Haematoxylin–eosin
hdc	Histidine decarboxylase
HEWL	Hen egg white lysozyme

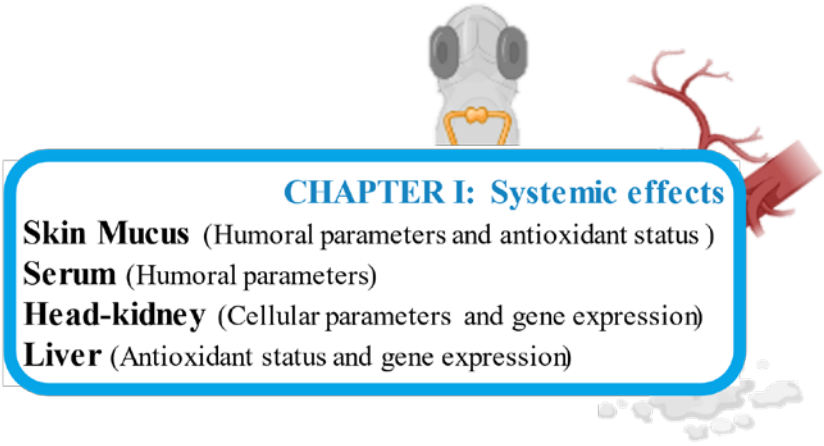
HK	Head-kidney
HKL	Head-kidney leucocytes
***ikbkg*/IKBKG**	Inhibitor of nuclear factor kappa B kinase regulatory subunit gamma
***il1b*/IL-1β**	Interleukin-1 beta
***il6*/IL-6**	Interleukin-6
***il-8*/IL-8**	Interleukin-8
***il-18*/IL-18**	Interleukin-18
igmh	Immunoglobulin M heavy chain
Igs	Immunoglobulins
ILT	Interbranchial lymphoid tissue
***irak1*/IRAK1**	Interleukin-1 receptor-associated kinase 1
kng1	Kininogen 1
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
lyz	Lysozyme
MALT	Mucosal associated-lymphoid tissue
Micro-CT	X-ray micro-computed tomography
MHC	Major histocompatibility complex
***mhciia*/MHCIIa**	Major histocompatibility complex class IIa
***mpo*/MPO**	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MTT	3-(4,5 dimethyl-2- yl)- 2,5- diphenyl tetrazolium bromide
MYA	Million years ago
***myd88*/MYD88**	Myeloid differentiation primary response 88
NALT	Nasopharynx-associated lymphoid tissue
NCC	Nonspecific cytotoxic cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
***nfkb1*/NF-κB1**	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
***nfkb2*/NF-κB2**	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2
NK	Natural killer
NLR	Nod-like receptor
***nlrc3*/NLRC3**	NLR family CARD domain containing 3
***nlrc5*/NLRC5**	NLR family CARD domain containing 5
***nlrx1*/NLRX1**	NLR family member X1
NOD	Nucleotide-binding oligomerization domain
oligo-dT	Oligo deoxy-thymine nucleotides
ORF	Open reading frames
PAMP	Pathogen associated molecular pattern
PAS	Periodic acid–Schiff
PBS	Phosphate buffer saline

PEG	Polyethylene glycol
PGDS1	Prostaglandin D synthase 1
***phox22*/PHOX22**	NADPH oxidase subunit Phox22
***phox40*/PHOX40**	NADPH oxidase subunit Phox40
PMA	Phorbol myristate acetate
Poly I:C	Polyinosinic:polycytidylic acid
RAG	Recombination activating gene
***rela*/RelA**	v-rel avian reticuloendotheliosis viral oncogene homolog A
***relb*/RelB**	v-rel avian reticuloendotheliosis viral oncogene homolog B
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
rps18	Ribosomal protein S18
RT-PCR	Real-time polymerase chain reaction
SALT	Skin-associated lymphoid tissue
SEM	Standard error of the mean
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Science
***stat3*/STAT3**	Signal transducer and activator of transcription 3
TCA	Trichloroacetic acid
TCR	T-cell receptor
TEM	Transmission electron microscopy
***tgf1b*/TGF1β**	Transforming growth factor 1 beta
Th2	T-helper 2
TLR	Toll like receptor
***tlr2*/TLR2**	Toll like receptor 2
***tlr5*/TLR5**	Toll like receptor 5
***tlr7*/TLR7**	Toll like receptor 7
***tlr8*/TLR8**	Toll like receptor 8
***tlr9*/TLR9**	Toll like receptor 9
***tlr13*/TLR13**	Toll like receptor 13
TMB	Tetramethyl benzidine hydrochloride
***tnfa*/TNF-α**	Tumour necrosis factor α
***tnfrsf1a*/TNFRSF1A**	Tumor necrosis factor receptor superfamily, member 1a
***tnfrs1b*/TNFRSF1B**	Tumor necrosis factor receptor superfamily, member 1b
***traf6*/TRAF6**	TNF receptor-associated factor 6
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VLRs	Variable lymphocyte receptors
WGD	Whole genome duplication

List of figures

Graphical abstract





CHAPTER I: Systemic effects

Skin Mucus (Humoral parameters and antioxidant status)
Serum (Humoral parameters)
Head-kidney (Cellular parameters and gene expression)
Liver (Antioxidant status and gene expression)

CHAPTER II: Local effects

Skin (Immunohistochemistryand gene expression)
Skin/Muscle (Ultrasound and micro-CT)

CHAPTER III: Genes involved

Skin (Gene expression)
***In silico* search of molecules**

SUMMARY

Inflammation is a non-specific response triggered by the innate immune system against a stimulus of any etiology (injuries, trauma, infections, irritating products, etc.) to defend the organism, in our work the fish, and restore it to the initial homeostasis. It presents a series of very characteristic symptoms called "*cardinal signs of inflammation*" by which it can be easily identifiable: swelling, redness, sensation of heat, pain, and functional alterations. Two types of inflammation can be differentiated: *i*) acute inflammation, or immediate response, which can be developed briefly upon detection of the initial stimulus, and last for days or weeks until its final resolution; and *ii*) chronic inflammation, produced by the inefficiency of the defensive mechanisms to resolve an acute inflammation and whose effects are highly related to the appearance of related chronic diseases. In this sense, inflammation is a very common event that occurs in the aquaculture industry due to the intense production conditions (overcrowding, water quality, deterioration of environmental conditions) to which fish are subjected to meet market demands. Under these situations, fish become more susceptible to wounds, trauma, and pathogens, that could be related to inflammation and the appearance of associated diseases. These negative situations are often translated into economic losses for the sector itself. Inflammation is an essential process to return fish to their initial state of health, since it participates directly in the repair of the damaged tissues. However, to date no model of fish inflammation has been developed that allows to delve into its intrinsic mechanisms in an adequate way.

This Doctoral Thesis aimed to improve our knowledge of the inflammation process on gilthead seabream (*Sparus aurata*), one of the most important Mediterranean farmed fish species. To get our objective we divided this Doctoral Thesis in 3 chapters:

In **Chapter I**, the systemic effects caused by an injection of carrageenin were evaluated. Thereby, after 1.5, 3 and 6 h of intramuscular injection of phosphate buffered saline (PBS; control) or carrageenin (1%), samples of skin mucus, serum, head kidney (HK) and liver were obtained to determine the immunological and antioxidant status of this fish. Skin mucus of fish injected with carrageenin showed increased humoral activities (superoxide dismutase, peroxidase, lysozyme, bactericidal activity against *Vibrio anguillarum* and *Photobacterium damselae*, and total immunoglobulins) at 3 h post-injection compared with those specimens from the control group. The increases in these activities suggested the release of preformed molecules and hydrolytic enzymes

from the zone of inflammation to the skin mucus by the recruited cells of the immune system [most probably acidophilic granulocytes (AGs) due to the increase of peroxidase activity, which is a cell marker of these cells]. However, fish injected with carrageenin and sampled at 6 h post-injection showed a decrease of peroxidase activity in head-kidney leucocytes (HKLs) and protease activity in skin mucus, compared to control, which could first suggest the cessation of migration of AGs from the HK to the site of inflammation and, on the other hand, the beginning of the inactivation of such cells in the inflamed site itself. Furthermore, carrageenin injection had no effect on the systemic immune system, but hepatic catalase activity was reduced at 3 and 6 h in the carrageenin group, suggesting that it could be a side effect produced by the molecules released to the proximity of the inflammation site to redirect the hydrogen peroxide so that it can be used for microbiocidal effects of peroxidases. This fact was supported by the non-variation of the expression of any of the gene studied in the liver. Otherwise, the up-regulation of *interleukin-1 beta* (*il1b*) and *prostaglandin d synthase 1* (*pgds1*) expression levels in the HK at 1.5 and 3 h, respectively, in the carrageenin group compared to the values seen in the control group, supported the leucocyte activation and early migration of cells from the HK to the inflamed area. On the contrary, the down-regulation of the expression of *NADPH oxidase subunit Phox40* (*phox40*) gene in the carrageenin group at 6 h, compared to the control group, pointed to the beginning of the cell inactivation or to the decrease in said cell recruitment, as it has been previously commented.

Considering these results, **Chapter II** of this thesis consisted in the study of the local effects triggered by carrageenin at the injection place. In this sense, skin samples obtained at 1.5, 3, and 6 h post-PBS/carrageenin injection, were processed for histology and immunohistochemistry procedures, and for gene expression analysis. Microscopic results indicated an increase in the number of skin-mucus secreting cells and AGs in the skin of fish studied at 1.5 and 3 h after the carrageenin injection, respectively, with respect to the data obtained in control fish. These results offered information about the rapid differentiation and maturation of the skin-mucus secreting cells in response of an inflammatory stimulus such as carrageenin, evidencing their role in the holocrine mucus secretion, and possibly acting as a phagocytic cell in the first line of defence. The increase of skin-mucus secreting cells agreed with our previous study in which preformed molecules were released from the zone of inflammation to the skin mucus at 3 h post-carrageenin injection. In addition, information on cell recruitment to the inflamed area

was obtained at 3 h post-injection, which appeared to be headed by the AGs. In the case of gene expression, the nonspecific cytotoxic cells [NCC; described as a heterogeneous population composed of lymphocytes, monocytes/macrophages, and AGs] marker *granzyme b* (*grb*), as well as other AGs markers and related-molecules such as *il1b, phox40*, *NADPH oxidase subunit Phox22* (*phox22*), and *myeloperoxidase* (*mpo*) were up-regulated after carrageenin injection in comparison to control fish, clearly evidenced the recruitment and activation of the AGs in the inflamed area from 1.5 h post-injection.

To support these previous results, it was carried out an assay in which fish were also injected with PBS or carrageenin, and the injection zone was evaluated on the same fish by real-time ultrasound and micro-CT at 1.5, 3, 6, 12 and 24 h post-injection. Results showed that fish injected with carrageenin increased the skin thickness at 1.5, 3, and 6 h post-injection, and showed small hyperechoic foci in the muscle underlying the injection site surrounded by an anechoic area (seen in the images acquired by micro-CT as areas with positive values close to 0 Hounsfield Units) that were not observed in the control fish at the same experimental times. Analysis of micro-CT acquisitions also revealed a dark area at the carrageenin injection site at 1.5, 3 and 6 h. These areas had an average density of -850 to -115 Hounsfield Units, which did not correspond to any tissue density of the rest of the body, but rather to the density of air. Furthermore, no such dark areas were observed at the injection sites in the control fish. These results pointed to the effects produced by the molecules released in the injection zone, which consequently produced edema (anechoic area) and swelling. On the other hand, the probable carrageenin degradation was evidenced by the hyperechoic foci of ultrasound and the dark area obtained by micro-CT, which could be related to the release of gas consequently. This fact could make sense, since the skin thickness of the injected area decreased to normal values at 12 and 24 h after carrageenin injection and these dark areas were smaller in the fish analyzed at longer times (12 h) and almost disappeared in the fish at 24 h post-injection, denoting the decrease of the inflammation. Surprisingly, these signs indicated after carrageenin injection [subcutaneous emphysema (gas), edema and increased skin thickness] represent the typical symptomatology observed in human necrotizing fasciitis, although more studies would be necessary to deepen into this topic.

Finally, **Chapter III** consisted in the analysis of the genes involved in the inflammatory response caused by carrageenin. Then, fish specimens were injected with

PBS (as a control) or carrageenin (1%) intramuscularly, and after 1.5, 3 and 6 h post-injection skin samples were obtained from the injected area and processed to evaluate the expression of 40 inflammatory-related genes. Thus, inflammatory cell markers (*csfr1, mhcii* and *phox40*), several pro-inflammatory cytokines (*il1b, tnfa, il6, il8* and *il18*) and other related molecules (such as *myd88* and *c-rel*) up-regulated their expression at 1.5 and 3 h in fish injected with carrageenin compared to the control levels. In addition, gene expression of some anti-inflammatory molecules (*nlrx1, nlrc5 isoform 1, ctsd* and *ctss*) was down-regulated in fish injected with carrageenin at 3 h after injection, again compared to the gene expression in control fish. These results showed that carrageenin was able to stimulate the expression of pro-inflammatory gene and decrease the expression of anti-inflammatory gene in the skin of gilthead seabream at the experimental times tested.

In an independent assay, fish were injected likewise with PBS (control) or carrageenin (1%), and after 12 and 24 h skin samples were obtained from the injection site and processed to perform a gene expression assay with the same 40 genes previously studied. At these times, only a down-regulation in the gene expression of the macrophage marker (*csfr1*) was observed in the skin of fish injected with carrageenin at 12 h compared to the control, supporting our hypothesis of cell inactivation from 6 h from the onset of inflammation. In addition, an up-regulation in the *c-rel* gene (NF-κB subunit) was observed in fish injected with carrageenin and sampled at 24 h compared to fish injected with PBS, a fact that could indicate the activation of mechanisms necessary to finish the inflammation response to carrageenin and restore skin homeostasis. Taking into account this assay, an *in silico* test was carried out to study the phylogenetic conservation of these 40 inflammation related-molecules from protein sequences of gilthead seabream in comparison to other teleost protein sequences. This assay showed high percentages of identity of these sequences with the protein sequences of large yellow croaker (*Larimichthys crocea* L.), evidencing the highest conservation in molecules involved in the regulation of inflammation and cell markers.

Considering all our data from a joint point of view, AGs seem to be recruited very quickly to the site of inflammation caused by the carrageenin injection and after activation, they could initiate the release of pro-inflammatory mediators such as cytokines and ROS. In addition, AGs could be the main cells involved in the degradation of carrageenin by the release of preformed proteolytic enzymes, since not only myeloperoxidase or lysozyme but also other proteases are stored in their granules and are released after an inflammatory stimulus. Furthermore, AGs could also mediate the regulation of the inflammation due to their role as NCCs by preventing excessive inflammation. Therefore, the crucial role of these cells in gilthead seabream was demonstrated. The combination of all these methods and results indicates that carrageenin is a good inductor of inflammation in gilthead seabream, so it could be used as a model in subsequent studies.

1. INTRODUCTION

1.1. Fish immunity.

The immune system is defined as "a complex network of cells, tissues, organs, and the substances they make that helps the body fight infections and other diseases" according to the National Cancer Institute Dictionary (National Cancer Institute, n.d.). In addition, it is composed of two general subsystems that emerged during the phylogenetic evolution and are known as **innate, natural, or nonspecific immunity** and **adaptive, acquired, or specific immunity** (Flajnik & Du Pasquier, 2004). Thus, while the innate system is evolutionarily older (arose >600 million years ago) and may be found in all multicellular organisms, the adaptive system seems to have arisen approximately 450 million years ago in the first jawed vertebrates (Gnathostomata) (Brazeau & Friedman, 2015; Flajnik & Kasahara, 2010). In this sense, while jawless fish (Agnatha) have an adaptive immune system based on variable lymphocyte receptors (VLRs), B-like and T-like cells, Gnathostomes [subdivided into Chondrichthyes (cartilaginous fish) and Osteichthyes (bony fish)] which diverged from a jawless common ancestor and are the most distantly related group to mammals, have an adaptive immune system based on immunoglobulins (Igs), T-cell receptor (TCR), and Major Histocompatibility Complex (MHC) (Fig. 1) (Flajnik, 2018; Flajnik & Kasahara, 2010).

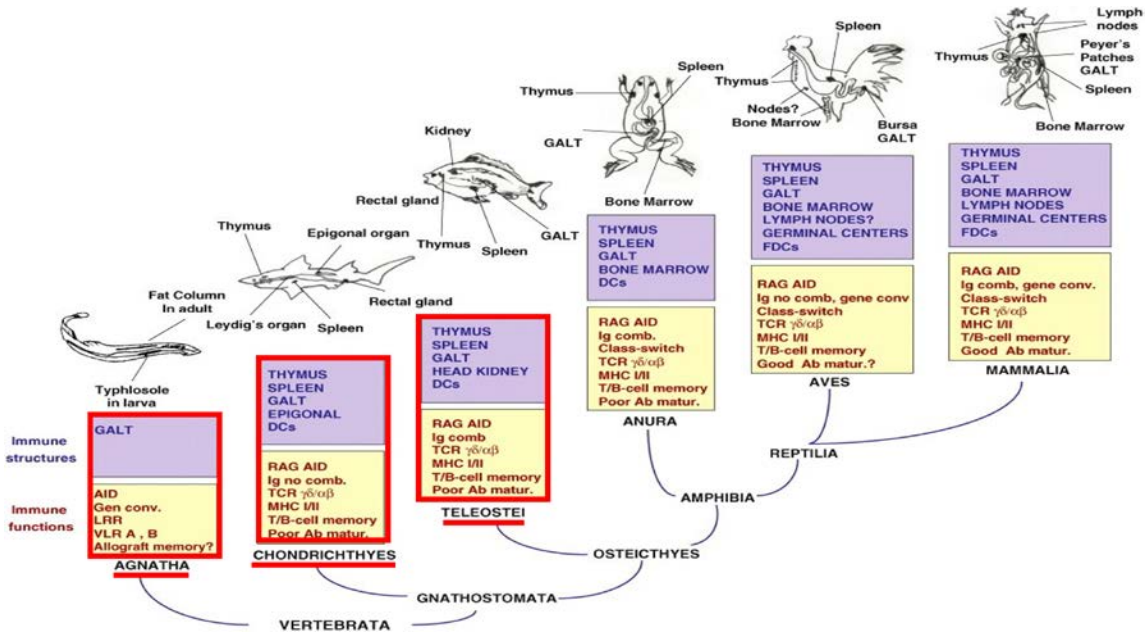


Figure 1. Lymphoid organs and immune responses across vertebrates [modified from (Hofmann, Greter, Du Pasquier, & Becher, 2010)]. Abbreviations: Ab matur, antibody affinity maturation; AID, Activation-induced cytidine deaminase; DC, dendritic cell; FDC, follicular dendritic cell; GALT, gut-associated lymphoid tissue; Gene conv, gene conversion; Ig comb, possibility of combinatorial association of V(D)J elements; LRR, Leucine-rich repeat; MHC, Major histocompatibility complex; RAG, recombination activating gene; TCR, T-cell receptor; VLR, Variable lymphocyte receptor.

One of the proposals for these evolutive differences is related to two episodes of whole genome duplication (WGD) that occurred early in vertebrate evolution, possibly before (1R) and after (2R) the jawless fish divergence, 500-430 million years ago (the 2R hypothesis) (Panopoulou & Poustka, 2005). In addition, evidence like the increase in the number of Hox gene clusters [discovered in assays developed with the fruit fly *Drosophila melanogaster* and responsible for the regulation of cell morphogenesis and differentiation during early embryonic development of most vertebrates (Hajirnis & Mishra, 2021)] point at additional WGD events, one in the teleost (the modern branch of bony fish) lineage divergence 320-350 million years ago (3R), another one in some fish species (4R), including salmonids and the common carp (*Cyprinus carpio*) (Fig. 2) (Glasauer & Neuhauss, 2014; Palti, 2011; Tsoi et al., 2006). These recent events could explain why teleost fish comprise the largest and more variable group of vertebrates with more than 20,000 species, in which essential components of immune system are conserved up to mammalians (Magor & Magor, 2001; Mulero et al., 2007; Swain & Nayak, 2009).

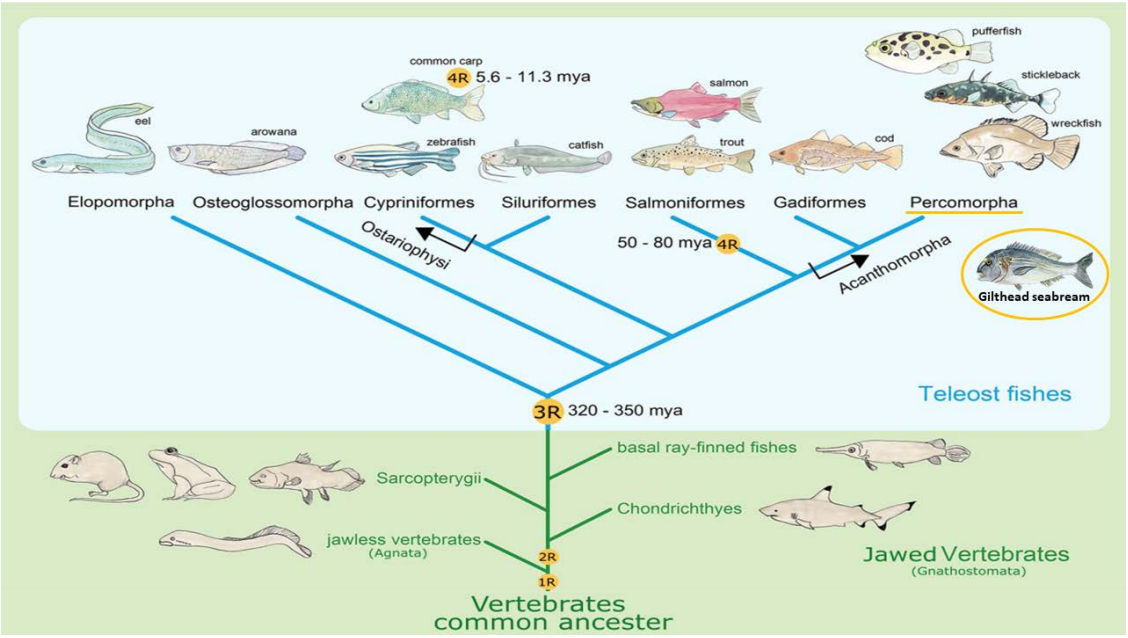


Figure 2. Simplified phylogeny of teleost fishes [modified from (Glasauer & Neuhauss, 2014)].

Unlike mammals which are homeotherms, fish are poikilotherms, and their internal temperature varies according to the temperature of the environment, so small changes in the environmental parameters can have a great influence on their welfare state (Delamare-Debouteville et al., 2006). This could be the reason why the fundamental defence mechanisms of these animals are mainly represented by the innate immune system with non-specific but rapid response, whereas the adaptative immunity (specific but low

responses capable of generating memory in successive encounters with the trigger) is more limited compared to those found in higher vertebrates. Furthermore, particular characteristics point to adaptations of fish to their environment to defend themselves against marine pathogens and foreign agents (Cuesta et al., 2011; Levraud & Boudinot, 2009; Muiswinkel & Wal, 2006). In this sense, as teleost fish lack bone marrow and lymph nodes, pronephros, also known as anterior or **head-kidney** (HK), is the main lympho-hematopoietic tissue where B cells develop and maturate, while the thymus is the primary site for the development and maturation of T cells (Manning, 1998). Furthermore, the spleen is the main secondary lymphoid tissue in fish, although dispersed leucocytes can be found in different anatomical locations forming mucosal associated-lymphoid tissue (**MALT**), such as in the skin (skin-associated lymphoid tissue; **SALT**), gills (gill-associated lymphoid tissue; **GIALT**, and interbranchial lymphoid tissue; **ILT**), nasopharynx (nasopharynx-associated lymphoid tissue; **NALT**) and gut (gut-associated lymphoid tissue; **GALT**) (Salinas et al., 2011; Bjørgen & Koppang, 2021). These mucosal surfaces are covered by a layer of mucus that acts as a barrier since it contains immune molecules (*e.g.*, lysozyme, peroxidases, proteases, Igs, etc.) involved in the protection and prevention against pathogens and their entry into the epithelium, as well as interact with the commensal microbial populations present on these surfaces (Griffin & Mitchell, 2007; Salinas et al., 2011).

At systemic level fish conserve the basic **hematological cellular pattern** of higher vertebrates constituted by erythrocytes, thrombocytes, and leucocytes, although the differences in this point are very remarkable: *i)* erythrocytes of fish are nucleated and participate in the immune response mainly mediated by hemoglobin since they possess the ability to detect pathogen associated molecular patterns (PAMPs), as well as transport oxygen and carbon dioxide and maintain the ion balance of the internal environment (like mammalian) (Pereiro et al., 2017; Yang & Su, 2021); *ii*) thrombocytes are nucleated leucocytes characterised by a canalicular system in their cytoplasm that play an important role in phagocytosis and are considered to be the functional counterparts of mammalian platelets (small cell fragments released from megakaryocytes which play an essential role in coagulation and hemostasis (Nagasawa et al., 2014); *iii)* leucocytes, although same cells than in mammals are found such as B and T lymphocytes, granulocytes (neutrophils, eosinophils and basophils) and monocyte-macrophages, there are some peculiarities among fish species (Esteban et al., 2013). Such is the case for fish granulocytes which

have a similar appearance to that of mammalian cells (neutrophils) or avian cells (heterophils), exhibiting a wide variation in morphology, distribution, functions, and types of cells among species and causing so much confusion due to their nomenclature (Smith et al., 2019). For instance, AGs from gilthead seabream (target fish species of the present Doctoral Thesis), are functionally equivalent to mammalian neutrophils (Meseguer et al.,1994; Morcillo et al., 2015; Sepulcre et al., 2002), while channel catfish (*Ictalurus punctatus*) neutrophils are reported as heterophils (Cannon et al., 1980). Cartilaginous fish granulocytes are majorly classified in three types based on size, shape, and staining properties as G1 (heterophils or fine eosinophilic granulocytes), G2 (neutrophils) and G3 (eosinophilic granulocytes) (Campbel T, 2015; Grant, 2015). Regarding fish monocyte-macrophages, although phagocytosis of these cells does not seem to be as effective as observed in mammals, surprisingly, the mechanism of macrophages to polarize to M1-M2 phenotype seems to be evolutionarily conserved in fish (Bird et al., 2007; Esteban et al., 2015; Secombes et al., 2011; Wang & Secombes, 2013). Likewise, the function of mammalian natural killer (NK) cells, which are involved in the lysis of virus-infected cells and tumor cells belonging to the innate immunity, is developed by a heterogeneous population of immune cells in teleost fish that collectively receive the name of NCC (Cuesta et al., 2005; Evans & Jaso-Friedmann, 1992). Gilthead seabream NCCs are composed of lymphocytes, monocyte/macrophages and AGs which express cytosolic FasL, being involved in cell-mediated cytotoxicity (Cuesta et al., 2005; Esteban, 1994; Evans & Jaso-Friedmann, 1992).

Concerning the **humoral components** of the fish immune response, although the innate molecules are quite similar to those found in higher vertebrates (*e.g.*, haemolytic complement, lysozyme, proteases and proteases inhibitors, and cytokines), the molecules from the adaptative system present clear differences in comparison. For instance, current studies have revealed the presence of different fish Igs isoforms to those found in mammals. So far, three Igs isotypes have been identified in the genome of different teleost fish species: IgM, IgD (orthologous to IgW in cartilaginous fish), and IgT/Z [specific of teleost and discovered in trout (*Oncorhynchus mykiss*), and zebrafish (*Danio rerio*), respectively], IgM being the predominant serum Ig type, IgD whose role remains largely unknown, and IgT/IgZ being involved in the mucosal immunity (analogous to mammalian IgA) (Hikima et al., 2011; Hsu & Criscitiello, 2006). Furthermore, structural differences have been found in teleost fish unlike mammals, IgD being either membrane-

bound or secreted in channel catfish (*I. punctatus*) and Japanese puffer (*Takifugu rubripes*) (although it is found most commonly in transmembrane form and co-expressed with IgM), and IgM lacking the J chain, so its monomers are associated by covalent (disulphide) bonds, grouping into tetramers instead of pentamers (Hikima et al., 2011; Hsu & Criscitiello, 2006; Smith et al., 2019).

1.2. Inflammatory process in fish.

Inflammation, from Latin "*inflammo*", which means "blaze or burn," is defined by the United States National Library of Medicine as "the body's immune system's response to stimulus" (Du et al., 2015). It is an unspecific intricated response of the innate immune system triggered to protect the organism against any chemical, physical or biological aggression from different aetiology (irritants, injuries, trauma, pathogen infections), and it is majorly characterized by a series of noticeable symptoms defined as "*The cardinal signs of inflammation*" (swelling, redness, heat sensation, pain and functional disorders or *function laesa*), which are associated with the increase of vascular permeability, blood flow and nerve fibre sensitisation (Fig. 3) (Bruce, 1913; Calixto et al., 2003; Chen & Nuñez, 2010; Hawiger & Zienkiewicz, 2019; Henson, 2005; Nathan, 2002).

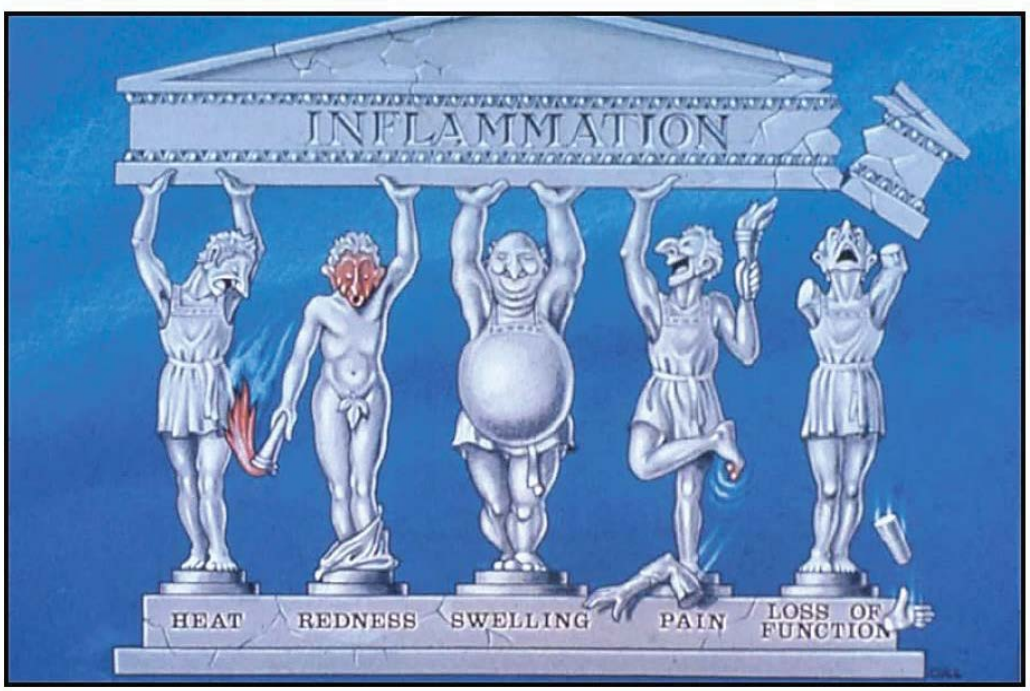


Figure 3. Illustration of five Greeks representing the cardinal signs of inflammation (Lawrence et al., 2002).

In fish, as in mammals, two types of inflammation have been described (Nathan & Ding, 2010): *i)* **Acute inflammation** is the initial immediate response that can be developed in minutes to hours and last for days or weeks; and *ii)* **Chronic inflammation** occurs when defensive mechanisms are insufficient to restore physiological homeostasis and acute inflammation cannot be resolved, being prolonged in time, and developing associated diseases (Fig. 4).

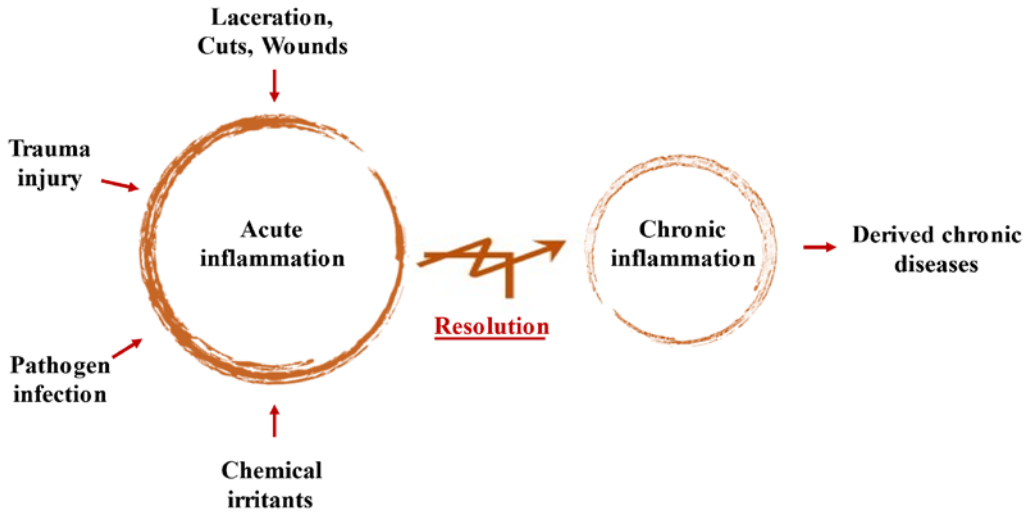


Figure 4. Schematic representation of the main contributors to fish acute inflammation-chronic inflammation and their main consequences [modified from (Esposito, 2019)].

Inflammation is a very common event that occurs in the aquaculture industry, one of the fastest-growing food-related sectors of the last decades according to the Food and Agriculture Organization (FAO) (FAO, 2020), after the intensive production conditions (overcrowding, water quality, deterioration of environmental conditions) to which fish are subjected to cover market demands. This scenario makes fish more susceptible to wounds, trauma, and pathogens, which could be related to the onset of inflammation and inflammation-associated diseases, that often result in economic losses for the sector itself (Balcázar et al., 2006; Esteban, 2012). Thus, as a response to any of these insults, resident non-immune cells, like epithelial and endothelial cells, alert immune cells to the presence of the exogenous occurrence to initiate acute inflammation, at the same time that sensory nerve fibres report the damage to the central nervous system (Fig. 5) (Al-Soudi, Kaaij, & Tas, 2017; Harris, 2014; Hehn, Baron, & Woolf, 2012).

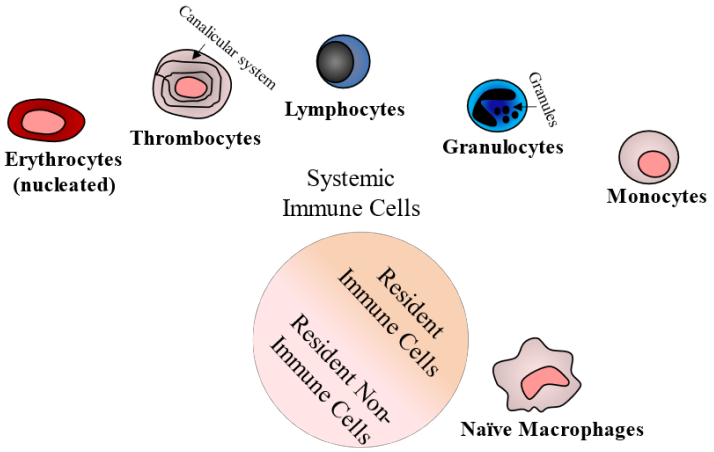


Figure 5. Schematic representation of the cell types involved in an inflammatory reaction in fish.

The acute inflammation in fish includes five successive stages: *i)* release of pro-inflammatory mediators; *ii)* effects of pro-inflammatory mediators, *iii)* cellular recruitment, *iv) r*egulation of inflammation; and *v)* reparation.

1. Release of pro-inflammatory mediators.

Tissue-resident immune and non-immune cells activate signaling cascades and transcription factors [such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)] that allow the release of inflammatory pre-formed mediators, such as vasoactive amines (mainly histamine and serotonin), proteolytic proteins, adhesion molecules, chemokines and prostaglandins. These molecules participate in the endothelial cell retraction and increase vasodilation and vascular permeability. Other molecules like pro-inflammatory cytokines [mainly interleukin-1β (IL-1β), tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6)], as well as neuropeptides and neurotransmitters will oversee developing the inflammatory response. These facts are majorly associated with the redness and swelling symptoms (Fig. 6) (Bordés-Gonzalez et al., 1994; Calder et al., 2013; Kolaczkowska & Kubes, 2013; Larsen & Henson, 1983; Medzhitov, 2008).

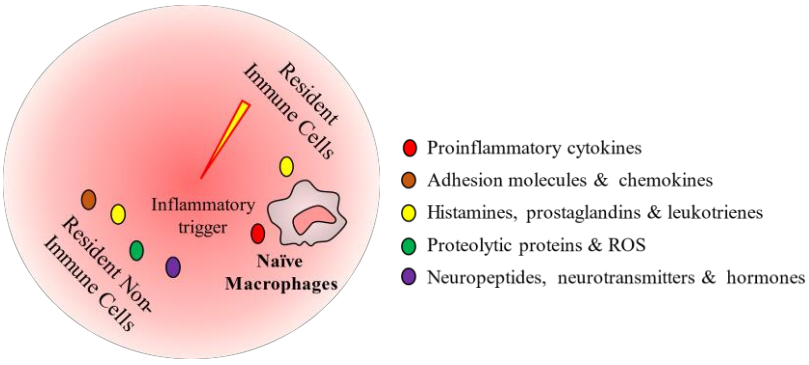


Figure 6. Schematic representation of some pro-inflammatory mediators released by resident cells after the trigger of an acute inflammation in fish. Key molecules involved are explained on the right box. Redness and swelling begin to be visible in the inflammation site.

2. Effects of pro-inflammatory mediators.

Mediators previously released bind to specific receptors and trigger the production of Igs, coagulation factors, activate the haemolytic complement and increase the blood flow towards the inflammation site. In addition, adhesion molecules and chemokines previously released induce the leucocyte activation, proliferation, and migration. The set of all these effects is represented as an inflammatory exudate that increase the redness and swelling symptoms and result in a local tumor in the affected area (Fig. 7) (Bordés-Gonzalez et al., 1994; Calder et al., 2013).

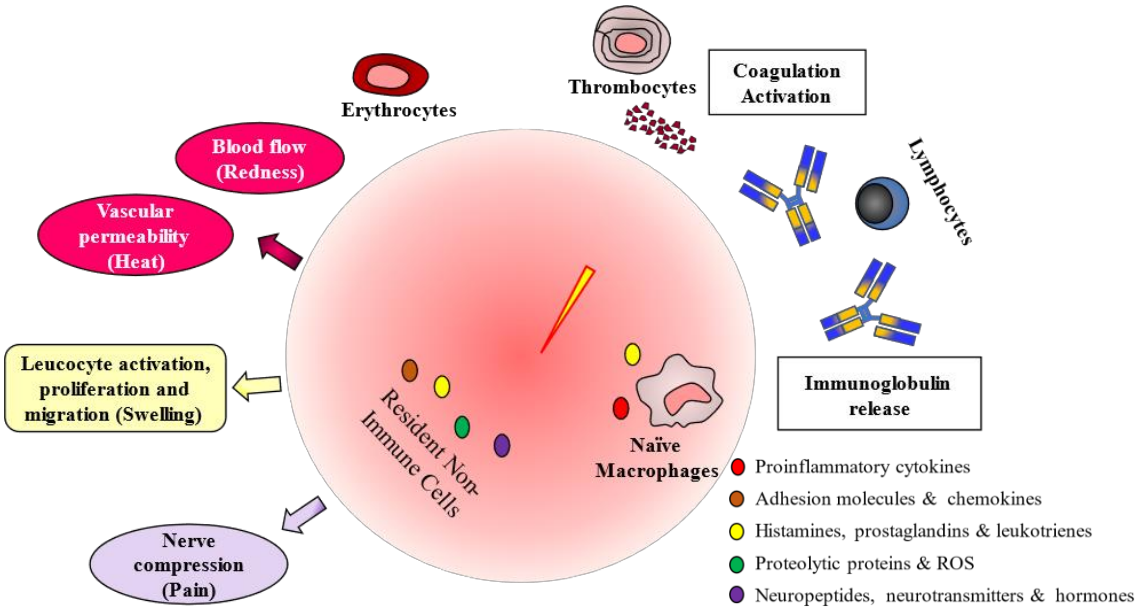


Figure 7. Schematic representation of the effects of pro-inflammatory mediators in an acute inflammation in fish. Key molecules involved are explained on the right box. Chemokines activate tissue-resident cells. Adhesion molecules released by the resident cells increase vascular permeability and produce local tumour in the affected area. Redness and swelling are visible in the site where the inflammation was initiated.

3. Cellular recruitment.

Leucocytes migrate from HK by rolling along the endothelium through the selectin-selectin ligand union, the subsequent adhesion to the endothelium and the final extravasation or diapedesis to the injured place (Bordés-Gonzalez et al., 1994; Calder et al., 2013; Ley et al., 2007; Meseguer et al., 1995). In sequential order, teleost granulocytes (mainly neutrophils or AGs, depending on the species of fish) are the first cells recruited at the inflamed site, followed by monocytes (which differentiate into macrophages and "activate classically" to pro-inflammatory M1-macrophages) and lymphocytes (Fig. 8) (Block et al., 2007; Nathan, 2006; Nguyen-Chi et al., 2015).

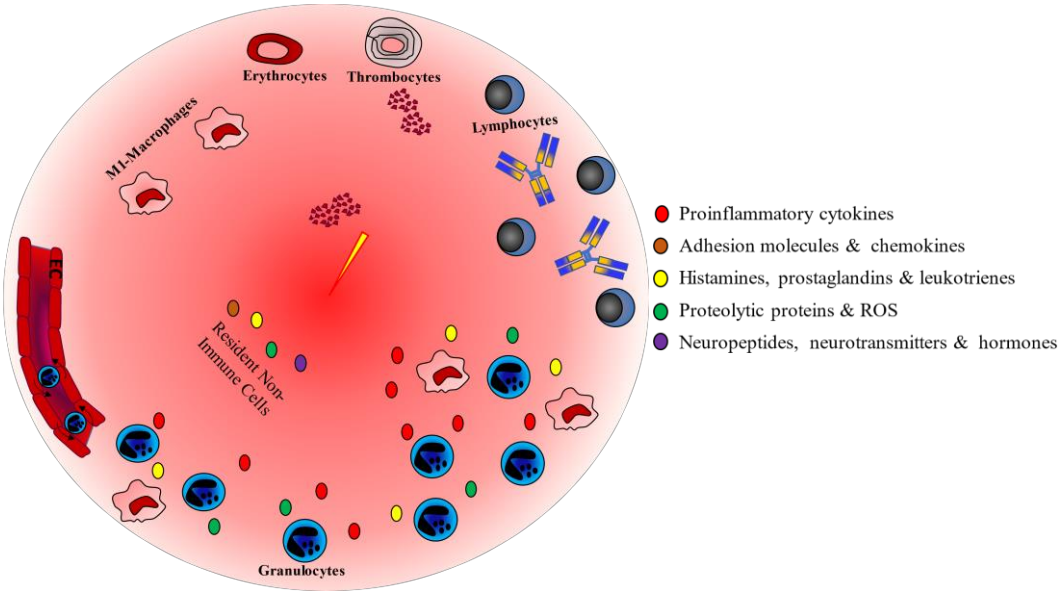


Figure 8. Schematic representation of the cellular recruitment in an acute inflammation in fish. Key molecules involved are explained on the right box. Blood leucocytes are recruited to the inflamed area.

4. Regulation of inflammation.

The resolution of inflammation starts with the apoptosis of leucocytes in the local affected area, and their following clearance. Simultaneously, inhibitory mechanisms, such as the progressive conversion of M1-macrophages to M2- like phenotype ('alternatively activated M2' macrophages), or the gene switching that leads to the release of anti-inflammatory molecules instead of pro-inflammatory ones, are activated in order to avoid the negative effects of excessive inflammation (Fig. 9) (Bordés-Gonzalez et al., 1994; Dalli & Serhan, 2017; Forlenza et al., 2011; Wiegertjes et al., 2016; Wynn et al., 2013).

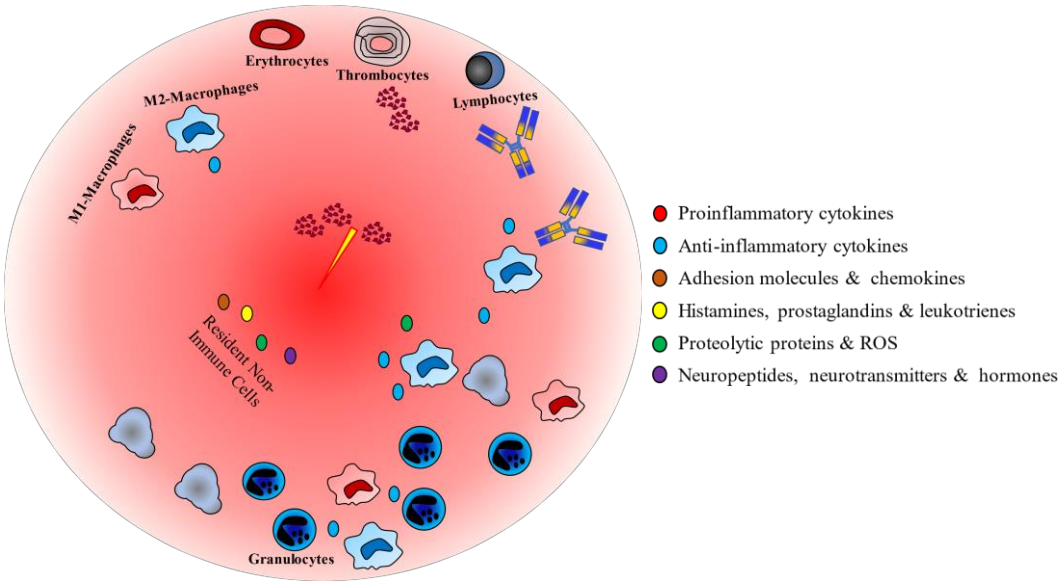


Figure 9. Schematic representation of the regulation of an acute inflammation in fish. Key molecules involved are explained on the right box. Inhibitory mechanisms are activated to compensate the effects of the pro-inflammatory molecules released previously. Redness and swelling begin to be less visible in the site where the inflammation was initiated.

5. Reparation.

Hormones such as glucocorticoids secreted by the endocrine system together with growth factors and anti-inflammatory cytokines released by M2-macrophages, attract epithelial cells, fibroblasts, and blood vessels into the inflammation focus, as well as increase the phagocytic activity of apoptotic leucocytes and cellular debris to repair tissues damaged by the initial insult (already eliminated) or by the self-inflammatory response (Fig. 10). These events would culminate with the restoration of initial homeostasis (Bordés-Gonzalez et al., 1994; Shaw & Martin, 2009; Singer & Clark, 1999).

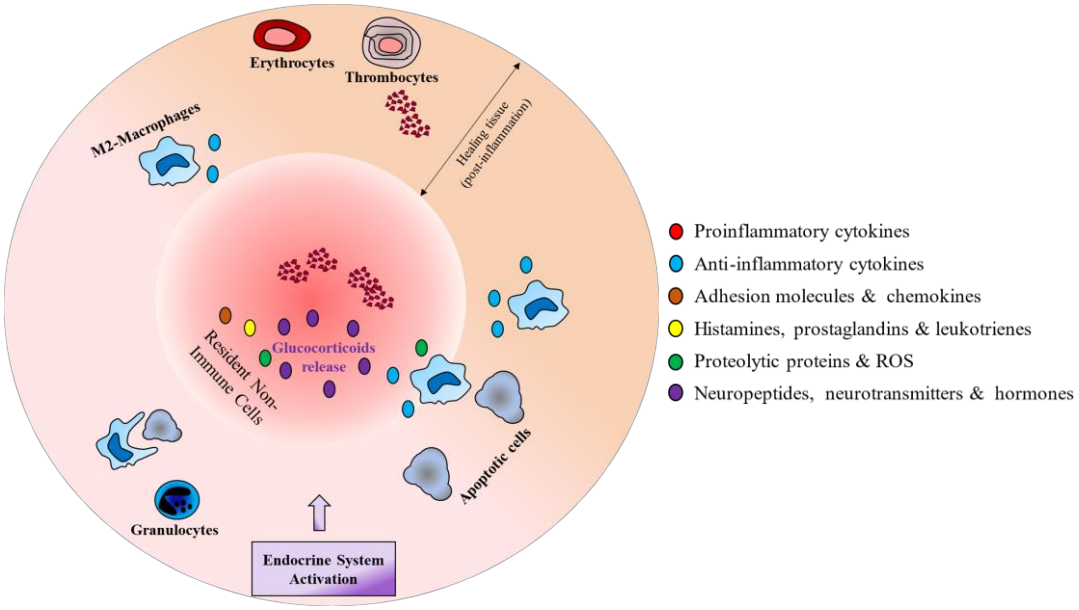


Figure 10. Schematic representation of the reparation phase of an acute inflammation in fish. Key molecules involved are explained on the right box. Mechanisms of damage repairment are activated in the affected tissue to recover physiological homeostasis. Redness and swelling begin to disappear in the site where the inflammation was initiated.

Here, it is important to highlight the necessary regulation of the mechanisms involved in the termination of inflammation to ensure effective immune protection, since any defect or failure in any of the stages previously explained would lead to the exacerbation of the inflammatory response, responsible in the long run for several pathologies. From this basis, in 2018, Dr. Charles Serhan introduced the concept of "*uncontrolled inflammation*" as the integral component of many diseases, and the differentiation between the anti-inflammatory and pro-resolution process as they can be confused (Serhan & Levy, 2018). For instance, some anti-inflammatory molecules, such as non-steroidal anti-inflammatory drugs and cyclooxygenase-2 (COX2) inhibitors are capable of reducing the amplitude of inflammation but prolonging the time needed for its resolution (Serhan & Levy, 2018).

Although acute inflammation was previously considered as a passive process, its natural resolution is currently associated with an active host response that coordinates multiple molecular mechanisms (Serhan, 2017). In this sense, NF-κB is the main molecule involved in the inflammatory axis in which multiple signalling pathways converge, so it is able to regulate tightly genes involved in immunity and the inflammatory cascade (Mulero et al., 2019; Napetschnig & Wu, 2013). The family of NF-κB molecules is constituted by five subunits RelA (p65), RelB, c-Rel, NF-κB1 (p50/ p105), and NF-κB2 (p52/p100), although the transcription factor is a cytosolic heterodimer comprising of two subunits, mainly RelA and NF-κB1, and an inhibitory subunit IκBα, whose association represent the canonical activation signalling pathway (Etemadi et al., 2015; Gugasyan et al., 2004; Mulero et al., 2019; Napetschnig & Wu, 2013). Upon activation by different inflammatory stimuli, the dimer is translocated to the nucleus where it binds to different promoter regions of pro-inflammatory genes (Kunnumakkara et al., 2020). Therefore, changes in its transcriptional activity are widely used as a biomarker of chronic inflammation diseases (Germolec et al., 2018).

Generally, most of the molecular mechanisms (via orthologs) and cells implicated in inflammation are conserved to a greater or lesser extent from fish to higher vertebrates (Zou & Secombes, 2016). Among the most important one, molecules from the NF-κB family transcription factor present a high conservation degree in the similarity and function throughout phylogenetic evolution (Etemadi et al., 2015; Gugasyan et al., 2004; Mulero et al., 2019; Napetschnig & Wu, 2013). However, the structure of other molecules originated later in evolution (such as cytokines) may be different from those of mammals, possibly as a consequence of WGD events previously explained, and therefore, their function might prevail or not prevail in most teleost fish as paralogue genes, which would explain the particular differences among species (Secombes et al., 2011; Wang & Secombes, 2013). Nonetheless, the minimal availability of fish antibodies implies an obstacle in the functional analysis of these molecules compared to those from mammals.

1.3. Importance of inflammation in health and disease of fish.

The inflammatory response plays a key role in the healing and homeostasis of fish, since in its last stages (regulation and reparation), it prepares the damaged area for the later phases of recovery, taking part directly in tissue repair processes (Elliott, 2000). Nonetheless, in the event that regulation of inflammation is not carried out properly, it can

become chronic, highlighting among the main reasons: *i)* the persistence of the causative agent or difficulty in removing it; *ii)* the prolonged exposure to toxins or secondary effect of another pathology; and *iii)* the excessive or misregulated immune response (Hawiger & Zienkiewicz, 2019). The development of inflammation will also be affected to a greater or lesser extent depending on external parameters which influence the basal conditions of the fish (Delamare-Deboutteville et al., 2006). Therefore, the pathogenesis of innumerable fish inflammatory diseases is closely related to the progression of inflammation, resolution being the critical step between health and disease (Fig. 11) (Feehan & Gilroy, 2019).

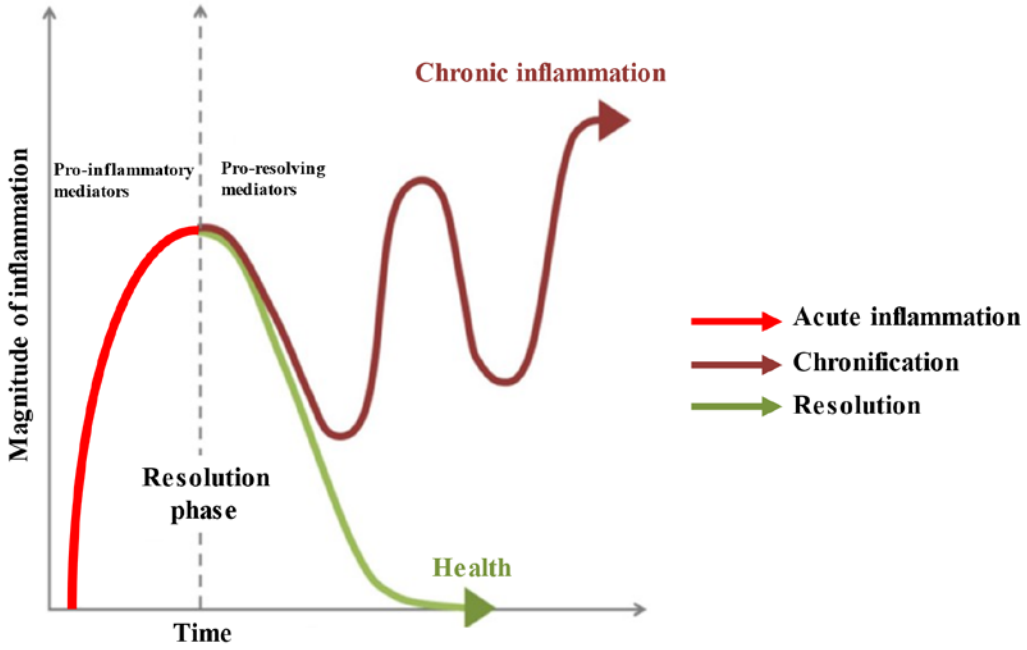


Figure 11. Dynamics of the inflammatory response in chronic inflammation [modified from (Barnig et al., 2019)].

Thus, the study of inflammation allows somehow to comprehend both the mechanisms of defense of fish against external damage and the initial steps of their recovery (Bruce, 1913; Calixto et al., 2003; Chen & Nuñez, 2010; Hawiger & Zienkiewicz, 2019; Henson, 2005; Nathan, 2002). However, no fish model of inflammation has been developed to date that allows one to deepen in the mechanism of fish inflammation and health properly. Although numerous drugs and products from diverse nature have been tested due to their possible anti-inflammatory properties (Ceballos-Francisco et al., 2020; García-Beltrán et al., 2017; Hall et al., 2014; Mansour et al., 2020), the lack of an inflammatory model make it impossible to understand their real mechanism of action, becoming them in unaccurate treatments. In addition, most of inflammatory related-diseases (mainly originting from pathogen infecctions) that affect

fish health have been faced until few years ago with the use of antibiotics and vaccines as main treatments and as measures of prevention, in spite of their negative effects (Cabello, 2006). In this sense, the harmful effects of antibiotics are mainly due to their non-biodegradability. Antibiotics are able to remain in low doses in the tissues of the fish and ending up in human consumption, as well as remain in the environment as a consequence of its excretion, generating in both cases the development of multi-resistant bacteria with the repercussions that this fact may entail (Cabello, 2006; Gullberg et al., 2011; Santos & Ramos, 2016; Smith et al., 1994). On the other hand, vaccination seems to be a method more effective than antibiotics against certain pathogens, since they are pathogen-specific, although this same characteristic greatly limits their action to other pathogens and makes their commercialization more difficult due to their high cost of production. Furthermore, its route of administration is stressful for fish (Gudding & Van Muiswinkel, 2013). Given the infeasibility of these treatments, their possible use is destined for disuse [although the regulation among countries varies throughout the world, in the European Union (EU) the use of antibiotics as additives for animal nutrition and for scientific purposes is banned from January 1 of 2006 by the "*Conformite Europeenne*" CE regulations (Official Regulatory Document No. 1831/2003 of the European Parliament and of the Council) and their use is only allowed in humans and veterinary medicine (except coccidiostats and histomonostats) just under prescription] (Cabello, 2006; Watts et al., 2017). Therefore, it would be necessary to look for alternative natural products and new treatments more directed at the inducing agent of the disease that were not as expensive as vaccines. Interestingly, since most pathogen infections are able to produce an inflammatory reaction (regardless of specific effects) easily identified by the cardinal signs of inflammation (Lawrence et al., 2002), the establishment of a general model of inflammation for fish could allow the development of more precise and targeted treatments. Then, the correct treatment of inflammation would avoid unnecessary losses for the aquaculture sector.

1.4. Methods or strategies used to study inflammation in mammals.

As it was commented, understanding inflammation is essential to develop effective interventions to arrest it and promote its resolution. With this purpose in mind, various techniques and methods have been used in mammals for years to detect and identify molecules and cells involved in this process (Patil et al., 2019).

Among them, *in vitro* assays represent important tools in pharmacological evaluations (Patil et al., 2019), from which cells of different origins (such as leucocytes) can be isolated, purified and cultivated to develop functional studies (Esteban et al., 1998; Salinas et al., 2007). In addition, stimulation of these cells with some pro-inflammatory agents (*i.e.*, endotoxin, as well-known as lipopolysaccharide, LPS), allows the detection of molecules and pro-inflammatory mediators in the culture supernatant by enzyme-linked immunosorbent assay (ELISA), by western blot assay, or by gene expression studies with real-time polymerase chain reaction (RT-PCR) (Eddouks et al., 2012; Liao, Guo, & Lin, 2011). In addition, recent advances in optical imaging like histo-cytometry or immunohistochemistry together with non-invasive techniques such photoacoustic imaging can be used to detect histological and metabolic perturbations in tissues, complete organs, or the whole organism (Gerner et al., 2012; Wang et al., 2003). Nevertheless, the amount of information that these techniques provide by themselves could be limited in the end, and other techniques would be necessary to verify the results obtained.

In contrast, traditional methods for studying acute and chronic inflammation have been developed using *in vivo* murine models such as the following ones:

- Formalin-induced paw edema (swelling caused by fluid accumulation) and complete Freund's adjuvant (CFA)-induced arthritis are well-characterized models of acute and chronic inflammation, respectively, that represent the clinical symptoms of human inflammation and arthritis and are primarily used to test drugs against arthritic conditions. However, some precautions must be taken, since formalin and CFA are severe irritating agents and their expose to animals produce them intense pain (Fig. 12) (Billiau & Matthys, 2001; Juma'a et al., 2009). In addition, these methods require sophisticated instruments like plethysmometer and Von-Frey apparatus necessaries to measure alterations in the paw volume and pain threshold, respectively (Mbiantcha et al., 2017).

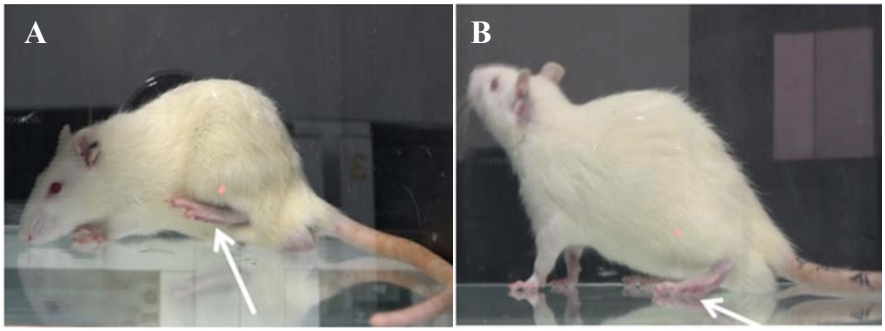


Figure 12. Representative photographs of inflammatory models in rats. Formalin test (A) and the complete Freund's adjuvant (CFA) (B) [modified from (Li et al., 2018)].

- LPS-induced inflammation method kwon as LPS-induced paw edema is a method that consists of subplantar injection of LPS into rodents' paw, which causes an acute localized inflammatory reaction, swelling of the injected paw, and hyperalgesia (an increased sensitivity to feeling pain) (Fig. 13) (Calil et al., 2014; Vajja et al., 2004). It is used to identify analgesic drugs and anti-inflammatory agents that act through cytokine modulation. However, the use of LPS is more focused on inflammatory stimulation in *in vitro* cultures (Patil et al., 2019).

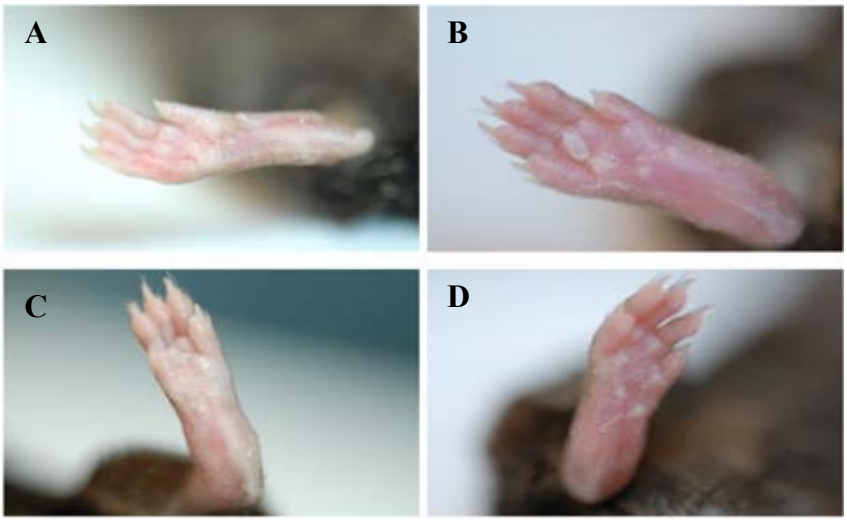


Figure 13. Photographs of the right rear paw of control mice (A, C) and LPS-induced inflammation mice (B, D) 8 days post-injection [modified from (Merrill et al., 2011)].

- In the last place, carrageenin-induced paw edema model is a distinctive model of acute inflammation with high reproducibility and widely used as preliminary test for the screening of anti-inflammatory drugs in preclinical evaluations (Winter et al., 1962). Carrageenin is a saccharide-nature phlogistic agent with sulphated groups and high molecular weight, responsible for producing an acute inflammatory response which is associated with hyperalgesia, edema (fluid accumulation), erythema (redness by vasodilation), and an exacerbated response to thermal and mechanical stimuli, but without visible systemic effects (it allows to use the non-injected paw as a control) (Fig. 14) (Posadas et al., 2004).

Figure 14. Representative photographs of rats' right hind paw before (Control; A), 5 (B) and 24 h (C) post-carrageenin injection (Hussein et al., 2012).

This characteristic inflammation is represented as a biphasic curve at molecular level: *i*) the first phase is partly assigned to injection trauma and the release of acute phase mediators (especially serotonin and histamine), which dilate postcapillary venules and result in exudation of inflammatory fluid and cells; and *ii*) the second phase occurs around 3 h after carrageenin injection mainly by the action of prostaglandins (Patil & Patil, 2017; Posadas et al., 2004). Chronification of this model causes granuloma formation (Chung et al., 2010; Patel et al., 2012). Otherwise, carrageenin can also be used as a model of pleurisy as well as other phlogistic agents like dextran or compound 48/80, and several phenomena like fluid extravasation, leucocyte migration and biochemical parameters in the exudate can be used to identify anti-inflammatory compounds (Patel et al., 2012; Rachmawati et al., 2016).

2. OBJECTIVES

The present Doctoral Thesis aims to improve our knowledge of the inflammation process on gilthead seabream (*S. aurata*).

The specific objectives are:

1. Evaluation of the systemic effects caused by an intramuscular carrageenin injection.
2. Characterization of the local effects caused by a carrageenin injection.
3. Study of the expression of genes involved in the inflammatory response caused by carrageenin.
4. *In silico* search of molecules conserved and involved in inflammation.
5. Establishment of an *in vivo* inflammatory model by using carrageenin in fish.

These objectives would allow us to know in depth the humoral and cellular mechanisms, as well as the modulation of the genetic expression that is carried out during the inflammatory process in gilthead seabream. This model could be used in the search of anti-inflammatory treatments in the aquaculture sector.

3. EXPERIMENTAL CHAPTERS

CHAPTER I. Systemic effects caused by carrageenin injection.

Campos-Sánchez, J. C., Guardiola, F. A., García Beltrán, J. M., Ceballos-Francisco, D., & Esteban, M. A. (2021). Effects of subcutaneous injection of λ/κ-carrageenin on the immune and liver antioxidant status of gilthead seabream (*Sparus aurata*), *Journal of Fish Diseases. 44*(9), 1449–1462. https://doi.org/10.1111/jfd.13452.

CHAPTER II. Evaluation of carrageenin effects at local level.

II. 1. **Campos-Sánchez, J. C.**, Vitarelli, E., Guardiola, F. A., Ceballos-Francisco, D., García Beltrán, J. M., Ieni, A., Esteban, M. A. (2021). Implication of mucus-secreting cells, acidophilic granulocytes and monocytes/macrophages in the resolution of skin inflammation caused by subcutaneous injection of λ/κ-carrageenin to gilthead seabream (*Sparus aurata*) specimens, *Journal of Fish Diseases. 45*, 19-33. https://doi.org/10.1111/jfd.13528.

II. 2. **Campos-Sánchez, J. C.**, García-Carrillo, N., Guardiola, F. A., Ceballos-Francisco, D., Esteban, M. A. (2022). Ultrasonography and X-ray micro-computed tomography characterization of the effects caused by carrageenin in the muscle of gilthead seabream (*Sparus aurata*), *Fish & Shellfish Immunology. 123*, 431-441. https://doi.org/10.1016/j.fsi.2022.03.013.

CHAPTER III. Genes involved in the inflammatory response caused by carrageenin.

III. 1 **Campos-Sánchez, J. C.,** Mayor-Lafuente, J., González-Silvera, D., Guardiola, F. A., Esteban, M. A. (2021). Acute inflammatory response in the skin of gilthead seabream (*Sparus aurata*) caused by carrageenin, *Fish & Shellfish Immunology. 19*, 623-634. https://doi.org/10.1016/j.fsi.2021.10.009.

III. 2 **Campos-Sánchez, J. C.,** Mayor-Lafuente, J., Guardiola, F. A., Esteban, M. A. (2021). *In silico* and gene expression analysis of the acute inflammatory response of gilthead seabream (*Sparus aurata*) after subcutaneous administration of carrageenin, *Fish Physiology and Biochemistry. 47*, 1623–1643. https://doi.org/10.1007/s10695-021-00999-6.

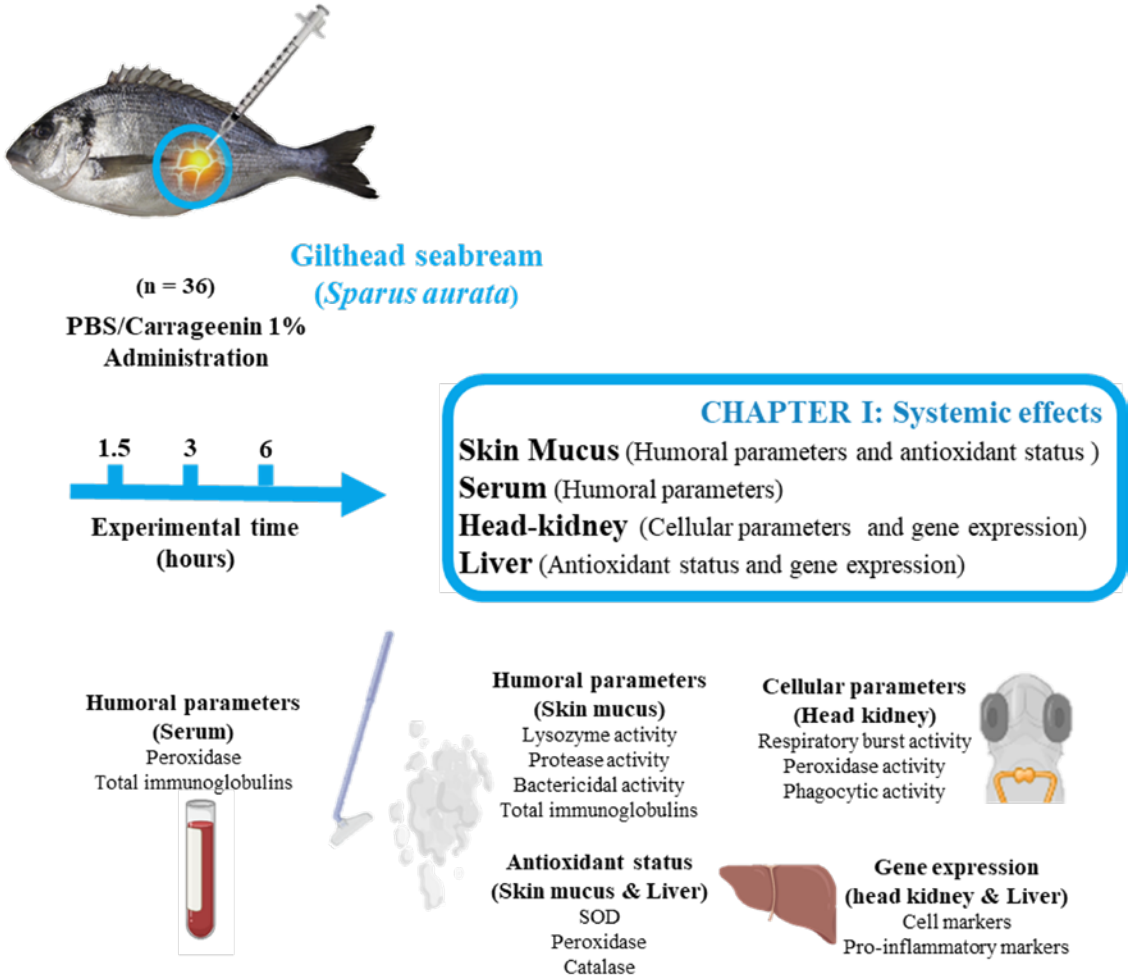
PhD contributions: for each publication, the PhD student conducted the experimentation and sampling, processes the samples, analysed the data, and wrote the manuscript.

CHAPTER I.

Systemic effects caused by carrageenin injection.

Graphical abstract

Effects of subcutaneous injection of λ/κ-carrageenin on the immune and liver antioxidant status of gilthead seabream (*Sparus aurata*).



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RESEARCH ARTICLE

Journal of Fish Diseases WILEY

Effects of subcutaneous injection of λ/κ-carrageenin on the immune and liver antioxidant status of gilthead seabream (*Sparus aurata*)

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Abstract

This study investigated the acute inflammatory response induced by subcutaneous injection of carrageenin (1%) or phosphate-buffered saline (control) in gilthead seabream (*Sparus aurata*). Skin mucus, serum, head kidney (HK) and liver were sampled at 1.5, 3 and 6 hr post-injection (p.i.) to determine the immune and antioxidant status of this fish species. The skin mucus of the carrageenin group showed increased superoxide dismutase and peroxidase activities, lysozyme abundance, bactericidal activity against *Vibrio anguillarum* and *Photobacterium damselae*, and total immunoglobulins compared with those of the control group. However, the carrageenin-injected fish sampled at 6 hr p.i. showed decreased protease activity in the skin mucus and peroxidase activity in the HK leucocytes compared with the control. Moreover, the carrageenin injection had no effects on the systemic immune system, but it reduced the liver catalase activities at both 3 and 6 hr in the carrageenin group relative to those in the control group. The expression levels of several proinflammatory and cell marker genes in the HK and liver were also determined. In the HK, the expression levels of *interleukin-1β* and *prostaglandin D synthase 1* were upregulated at 1.5 and 3 hr, respectively, in the carrageenin group compared with those in the control group. Contrarily, the expression of the NADPH oxidase subunit *phox40* (an acidophilic granulocyte marker) in the carrageenin group at 6 hr was downregulated compared with that in the control group. These results suggested that subcutaneous injection of κ/λ-carrageenin in gilthead seabream triggered an acute skin inflammation characterized by the rapid recruitment of acidophilic granulocytes and the release of humoral mediators into the skin mucus.

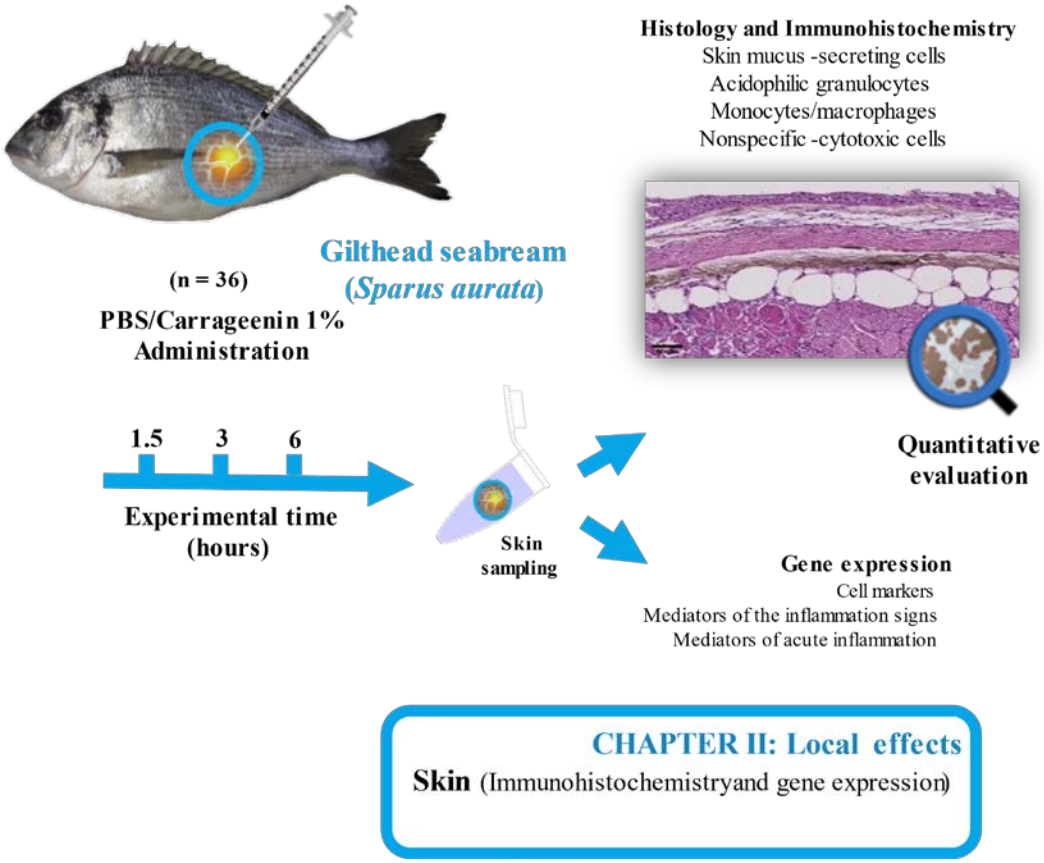
KEYWORDS
carrageenin, granulocyte recruitment, inflammation, innate immunity, marine aquaculture, teleosts

CHAPTER II.

Evaluation of carrageenin effects at local level.

Graphical abstract

II. 1. Implication of mucus-secreting cells, acidophilic granulocytes and monocytes/macrophages in the resolution of skin inflammation caused by subcutaneous injection of λ/κ-carrageenin to gilthead seabream (*Sparus aurata*) specimens.



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RESEARCH ARTICLE

Implication of mucus-secreting cells, acidophilic granulocytes and monocytes/macrophages in the resolution of skin inflammation caused by subcutaneous injection of λ/κ-carrageenin to gilthead seabream (*Sparus aurata*) specimens

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Funding information
Fundación Séneca; Ministerio de Economía y Competitividad con Fondos Feder

Abstract

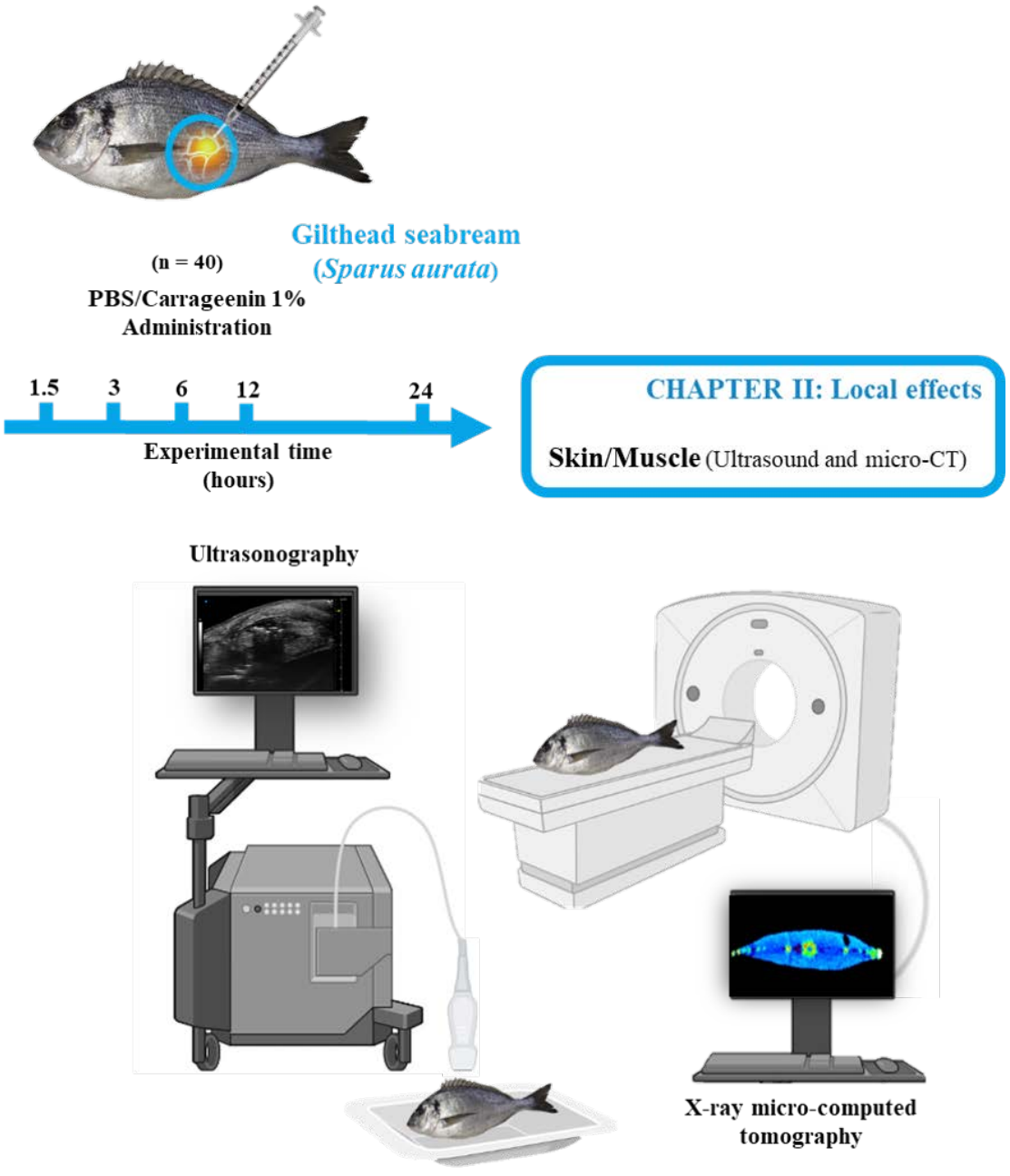
To date, the mechanisms of inflammation have been poorly studied in fish of commercial interest, due to the lack of development of appropriate experimental models. The current study evaluated a local inflammation triggered by a polymeric carrageenin mixture (a mucopolysaccharide derived from the red seaweed *Chondrus crispus*) in the skin of gilthead seabream (*Sparus aurata*). Fish were injected subcutaneously with phosphate-buffered saline (as control) or λ/κ-carrageenin (1%), and skin samples from the injection sites were collected 1.5, 3 and 6 hr post-injection, processed for inclusion in paraplast and stained with haematoxylin–eosin, Alcian blue or periodic acid–Schiff. Furthermore, immunohistochemistry and expression analyses of several cells' markers and proinflammatory genes were also analysed in samples of the injected sites. Microscopic results indicated an increased number of skin mucus-secreting cells and acidophilic granulocytes in the skin of fish studied at 1.5 hr and 3 hr post-injection with carrageenin, respectively, with respect to the data obtained in control fish. Otherwise, both the gene expression of the non-specific cytotoxic cell marker (granzyme B, *grb)* and the proinflammatory cytokine (interleukin-1β, *il-1β)* were up-regulated at 1.5 hr in the skin of fish injected with carrageenin compared with the control fish, whilst the gene expression of acidophilic granulocyte markers (NADPH oxidase subunit Phox22 and Phox40, *phox22* and *phox40*) was up-regulated at 3 and 6 hr in the carrageenin group, compared with the control group. In addition, the gene expression of myeloperoxidase (*mpo)* was also up-regulated at 6 hr in the skin of fish injected with carrageenin in comparison with control samples. The present results indicate the chronological participation of two important immune cells involved in the resolution of the inflammation in the skin of gilthead seabream.

KEYWORDS

aquaculture, carrageenan, gene expression, gilthead seabream (*Sparus aurata*), immunohistochemistry

Graphical abstract

II. 2. Ultrasonography and X-ray micro-computed tomography characterization of the effects caused by carrageenin in the muscle of gilthead seabream (*Sparus aurata*).



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Ultrasonography and X-ray micro-computed tomography characterization of the effects caused by carrageenin in the muscle of gilthead seabream (*Sparus aurata*)

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Ultrasound
X-ray micro-computed tomography (micro-CT)
Gilthead seabream (*Sparus aurata*)
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ABSTRACT

The current work aimed to carry out an *in vivo* study of the λ-carrageenin-induced inflammation in the skin of gilthead seabream (*Sparus aurata*). The fish were injected intramuscularly with phosphate-buffered saline (PBS, as control) or λ-carrageenin (1% in PBS), and the injection zone was evaluated by real-time ultrasonography (Vevo Lab, VisualSonics) at 1.5, 3, 6, 12, and 24 h post-injection (p.i.). Results demonstrated that the skin thickness was increased in fish injected with λ-carrageenin and sampled at 1.5, 3, and 6 h p.i. However, the skin thickness of the injected area decreased to the normal values in those fish sampled at 12 and 24 h p.i. In addition, fish injected with λ-carrageenin and analysed at 1.5, 3, and 6 h p.i. showed, in the underlying muscle at the injection place, several hyperechoic small foci surrounded by an anechoic area which were not observed in control fish. Furthermore, the fish were analysed by X-ray micro-computed tomography (micro-CT). The analysis of the micro-CT acquisitions revealed also a dark area in the place of the injection with λ-carrageenin at 1.5, 3, and 6 h. These areas were smaller in fish analysed at longer times (12 h p.i.) and were almost disappeared in fish sampled at 24 h p.i. These areas had an average density of −850 to −115 HU, which did not correspond with any tissue density of the rest of the body. Furthermore, similar dark areas at the injection zones were never observed in control fish. Present results support the use of both non-invasive techniques to study the inflammatory process in fish of commercial interest such as gilthead seabream.

1. Introduction

Inflammation is a nonspecific process triggered mainly by the innate immune system in response to various stimuli or insults and whose purpose is to restore initial homeostasis. This complex reaction usually occurs locally and temporarily [1,2] and it is resolved within minutes or hours (acute inflammation) after terminating the initial damage [3]. Nevertheless, if the event that causes this damage is not eliminated, inflammation can persist for weeks to months or years (chronic inflammation). In mammals, this intricated response has been studied for years, and a great variety of techniques to study it has been developed. Among them, carrageenin-induced paw edema is a classic model used to reproduce experimental inflammation in the paw of rodents (such as rats, mice, or guinea pig) [4–7]. Carrageenin is an unusual sulphated mucopolysaccharide of high molecular-weight, whose structure is composed of alternate subunits of D-galactose and 3,6-anhydrogalactose linked by α-(1, 3) and β-(1, 4) glycosidic bonds, with one (κ-), two (ι-) or three (λ-) sulphates per disaccharide unit [4]. The degree of sulphation, as well as the dosage and the administration way, play a really important role in the triggering of the local acute inflammatory response induced in rodents, being the λ-carrageenin the main and most used one for inducing acute inflammation [4–10]. Carrageenin is a model well characterized by the formation of hyperalgesia, erythema, and edema, and stands out for its high reproducibility, which allows it to be used for screening anti-inflammatory drugs in preclinical evaluations [5]. Furthermore, the local acute inflammatory response produced by

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CHAPTER III.

Genes involved in the inflammatory response caused by carrageenin.

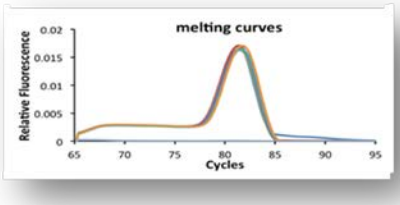
Graphical abstract

III. 1. Acute inflammatory response in the skin of gilthead seabream (*Sparus aurata*) caused by carrageenin.

Gilthead seabream
(*Sparus aurata*)

(n = 24)

PBS/Carrageenin 1%
Administration



1.5 3 6

Experimental time
(hours)

Skin
sampling

Gene expression
Cell markers
Pro-inflammatory markers
Anti-inflammarory markers

CHAPTER III: Genes involved
Skin (Gene expression)
***In silico* search of molecules**

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Acute inflammatory response in the skin of gilthead seabream (Sparus aurata) caused by carrageenin

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ABSTRACT

Although inflammation is a well-characterized process in mammals, few studies have dealt with the mechanisms involved in this process in fish. The present study evaluated the expression of inflammation-related genes in the skin of fish injected with carrageenin, which has previously been used in inflammatory models in mammals. In our case, fish were injected subcutaneously with PBS (as control) or carrageenin (1%), and skin samples from the injection site were collected 1.5, 3 and 6 h post-injection. The gene expression of inflammatory markers (*csfr1*, *mhc-ii* and *phox40*), several pro-inflammatory cytokines (*il1b, tnfa, il6, il8* and *il18*) and other molecules related (such as *myd88* and *c-rel*) were up-regulated at 1.5 and 3 h in fish injected with carrageenin compared with control levels. By contrast, the gene expression of anti-inflammatory molecules (*nlrx1, nlrc5* isoform 1, *ctsd* and *ctss*) was down-regulated in fish injected with carrageenin and sampled 3 h post injection, again compared to the gene expression in control fish. According to our results, carrageenin can be considered not only a good stimulator to study skin inflammation in gilthead seabream but also this method might be use to study the modulation of fish inflammatory process caused by internal or external factors.

1. Introduction

Inflammation is a complex reaction of the innate immune system that is frequently involved in different diseases and which affects all tissues [1]. It also plays a critical role in alerting cells to prepare effective immune responses, and initiate wound repair and healing processes to help recover physiological homeostasis [1]. Generally, this reaction is initiated as a response to a stimulus, such as the presence of pathogens or irritants, injury or trauma. Such stimuli trigger a series of cascading reactions in order to resolve the situation [2]. Among the most characteristic symptoms of inflammation are heat, redness, swelling, pain and loss of function [3]. Although these acute symptoms are usually temporary and local, in some circumstances, they may persist to become a chronic response [4]. In humans, chronic inflammation is related to numerous diseases, [5–7]. At molecular level, NF-κB, a pivotal transcription factor consisting of five subunits (p65/RELA, RELB, c-REL, p50/NF-κB1 and p52/NF-κB2), controls the gene expression of numerous inflammation-associated molecules, including proinflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, as well as genes involved in ROS production, playing a key role in the modulation of the inflammatory response [8]. Inflammation needs to be studied in greater depth in fish, particularly in those species that are of economic interest in aquaculture, one of the fastest-growing food-related sectors in the world in the last decades [9]. It is well known that intensive fish production conditions, whereby high numbers of fish are confined in a small volume of water, increase the occurrence of injuries and diseases in farmed animals. These, in turn, are very frequently associated with the appearance of wounds or ulcers in the fish skin, triggering an inflammatory response and causing serious economic losses in the aquaculture industry [10,11].

In order to improve our knowledge of the inflammatory process, carrageenin was used, as a possible inflammation trigger in fish. Carrageenin is a high-molecular-weight sulphated mucopolysaccharide obtained from the cell walls of a red seaweed(*Chondrus crispus*) [12]. In ionic solutions, κ- and ι-carrageenins self-associate into helical structures that form rigid or flexible gels, respectively, and these gels seem to be related to the immunostimulant properties in teleost fish against bacterial infections [13–15]. In contrast, λ-carrageenin does not form

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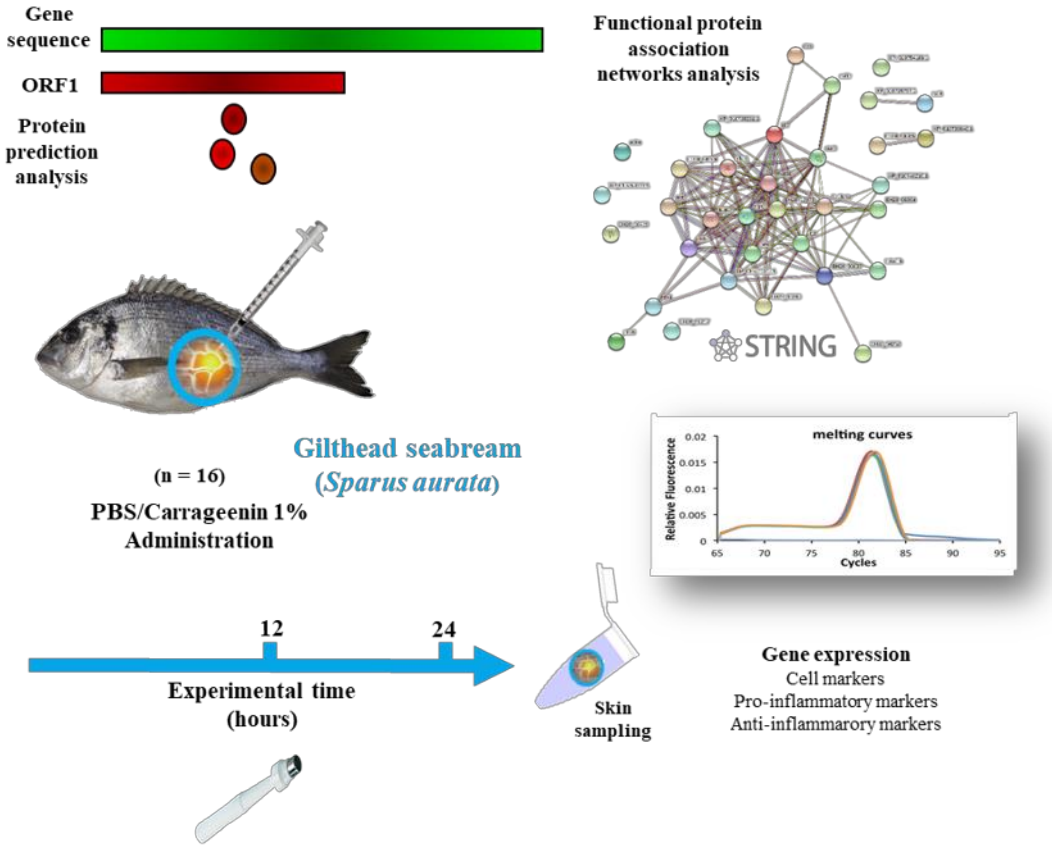
Graphical abstract

III. 2. *In silico* and gene expression analysis of the acute inflammatory response of gilthead seabream (*Sparus aurata*) after subcutaneous administration of carrageenin.



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In silico and gene expression analysis of the acute inflammatory response of gilthead seabream (*Sparus aurata*) after subcutaneous administration of carrageenin

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Abstract Inflammation is one of the main causes of loss of homeostasis at both the systemic and molecular levels. The aim of this study was to investigate in silico the conservation of inflammation-related proteins in the gilthead seabream (*Sparus aurata L.*). Open reading frames of the selected genes were used as input in the STRING database for protein–protein interaction network analysis, comparing them with other teleost protein sequences. Proteins of the large yellow croaker (*Larimichthys crocea L.*) presented the highest percentages of identity with the gilthead seabream protein sequence. The gene expression profile of these proteins was then studied in gilthead seabream specimens subcutaneously injected with carrageenin (1%) or phosphate-buffered saline (control) by analyzing skin samples from the injected zone 12 and 24 h after injection. Gene expression analysis indicated that the mechanisms necessary to terminate the inflammatory response to carrageenin and recover skin homeostasis were activated between 12 and 24 h after injection (at the tested dose). The gene analysis performed in this study could contribute to the identification of the main mechanisms of acute inflammatory response and validate the use of carrageenin as an inflammation model to elucidate these mechanisms in fish.

Keywords Carrageenin · Skin inflammation · In silico analysis · Gene expression · Teleosts · Aquaculture

Introduction

Inflammation is a temporary local response characterized by heat, redness, swelling, pain, and functional disorders (Nathan 2002). It is triggered by the innate immune system to protect the host against tissue damage, and its aim is to restore physiological functions when homeostatic mechanisms are insufficient (Medzhitov 2008). Inflammation consists of a sequence of events. Depending on diverse factors (such as the causal agent of the inflammation or the anatomical site where it is triggered), inflammatory mediators (including prostaglandins, histamines, cytokines, reactive oxygen species, and enzymes) are released by tissue-resident immune and non-immune cells to increase the blood flow and cause vasodilation (Calder et al. 2013). Capillary permeability is subsequently increased due to the retraction of endothelial cells, allowing access of many large and soluble molecules to the inflammation site (Calder et al. 2013). The third main event is the recruitment of leucocytes (granulocytes, neutrophils, eosinophils

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4. GENERAL DISCUSSION

4.1. Discussion.

Inflammation is a process of highly complicated mechanisms triggered by a damaging agent, whose cascade of events is summarized in the Figure 15. The importance of these mechanisms remains highly conserved throughout phylogenetic evolution, although their research has focused mainly on mammals. In comparison, the information available about the inflammation on teleost is quite limited. Therefore, the present Doctoral Thesis has focused on the characterization of the inflammatory process in a fish species of very high interest in marine aquaculture, the gilthead seabream. In addition, due to the increase in alternative *in vivo* methods, the general methodology followed in our work has been mostly implemented to comply with the 3R strategy (reduction, refinement, and replacement) (Doke & Dhawale, 2015).

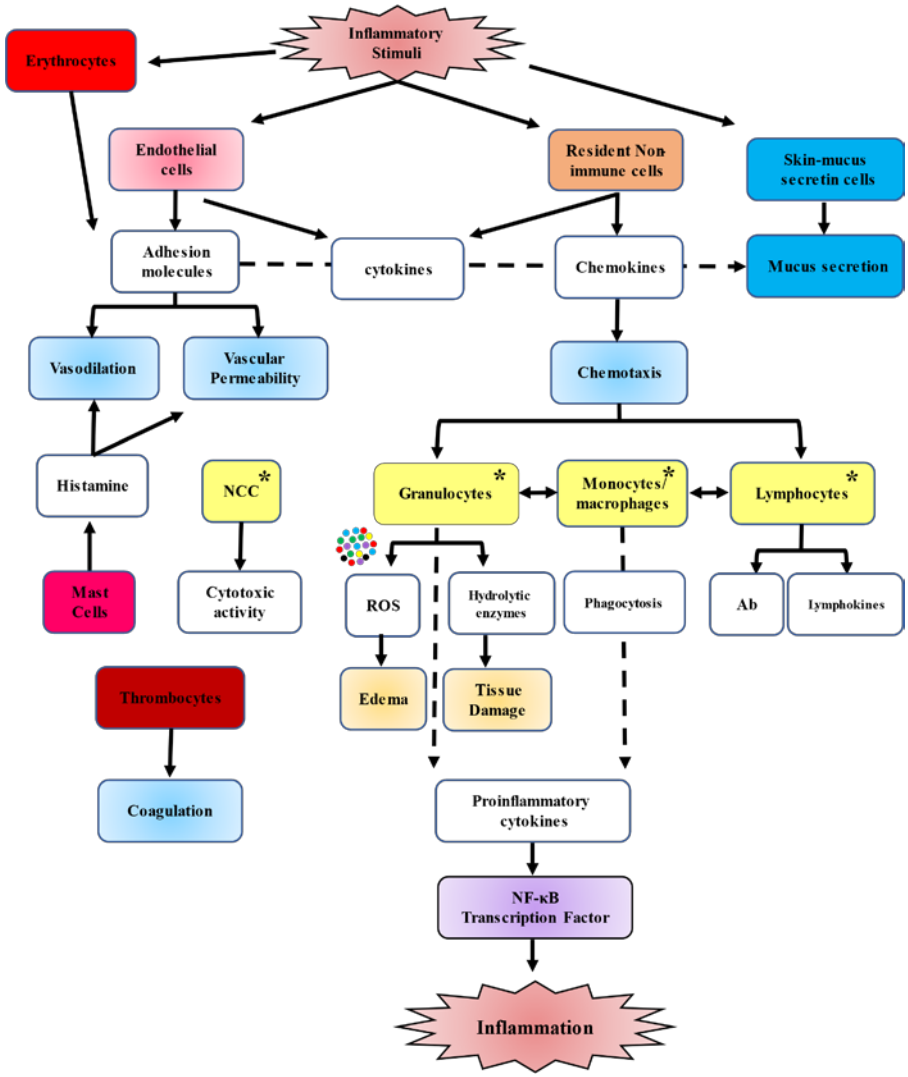

Figure 15. Potential inflammatory cascade in fish [modified from (Patil et al., 2019)]. *Nonspecific cytotoxic cells in gilthead seabream.

To study the inflammatory process, a specific methodology was required to design, being the first step the election of an inductor of inflammation. In this sense, the LPS-induced inflammation method is one of the most used to investigate inflammation in higher vertebrates, and its action in the NF-κB pathway cascade is mediated by the receptor TLR4. Nonetheless, the presence of TLR4 orthologs is curiously unknown in gilthead seabream (Sepulcre et al., 2009). This fact could explain the resistance of this fish species to the toxic effects of bacterial LPS, being these effects not as considerable as the ones seen in mammals (Antonopoulou et al., 2017). Then, among other different possible inductors of the inflammatory reaction from different origins it could be found: organic compounds (*e.g.,* formalin), PAMPs and compounds containing PAMPs (*e.g.*, peptidoglycan, flagellin, CFA), virus (*e.g.*, CpG motifs, double-stranded RNA, Polyinosinic:polycytidylic acid [poly I:C]), fungi (*e.g.*, β-glucan, zymosan), plants (*e.g.,* phytohemagglutinin), insects (*e.g.*, cantharidin) or algae (*e.g.*, carrageenin). In the present Doctoral Thesis, carrageenin was selected as a model of inflammation due to its noticeable and well-characterised effects and its high reproducibility in rodents (Attaya et al., 2018; Gábor, 2003; Hong et al., 2013; Winter et al., 1962). The concentration of carrageenin was selected by considering the results obtained from a previous *in vitro* assay carry out by our research group (unpublished data). This study evidenced that gilthead seabream head-kidney leucocytes (HKLs) exposed to increasing doses of carrageenin (0, 0.1, 1, 10, 100 and 1,000 μg mL^{-1}) at several experimental times (3, 6, 12 and 24 h) showed by transmission electron microscopy (TEM) changes in their morphology and increased the respiratory burst and phagocytic activities, as well as up-regulated the gene expression of pro-inflammatory cytokines (*il1b*, *tnfa*, *il6*). Therefore, these results suggested that carrageenin was effectively able to activate HKLs by acting like antigen due to its atypical structure with high molecular-weight, sulphated moieties and galactoses binds with an unusual α-1,3-galactosidic linkages (Galili et al., 1985; Klein et al., 1998; Thibaudeau et al., 1996; Younes et al., 2018). So, it would be remarkable that among the different doses tested, the dose of 1,000 μg mL^{-1} (1%) of carrageenin, which is the one normally used *in vitro* (Ai et al., 2018) and *in vivo* murine assays (Levy, 1969; Morris, 2003; Winter et al., 1962) to study inflammation, was the dose that produced a higher increase in the activities studied. Consequently, the dose of 1,000 μg mL^{-1} of carrageenin was selected to develop the *in vivo* studies, from which a characterization of the inflammatory process was obtained in the skin-muscle of gilthead seabream (carrageenin-induced skin inflammation model).

Thus, **Chapter I** of the present Doctoral Thesis consisted in clarifying whether carrageenin was able to produce systemic effects in gilthead seabream. However, no relevant systemic repercussions were found after carrageenin injection into the skin of the fish specimens. Furthermore, any minor effect (*e.g.*, those observed in the liver) was considered as a side effect of the soluble factors released in this area due to its proximity to the injected site, according to the findings obtained in mammals by Fowler & Thomson (1978). Indeed, the decrease observed in the catalase activity in the liver of fish injected at 3 and 6 h post-carrageenin injection could have as a function to limit its own reaction in the liver, facilitating the use of the hydrogen peroxide by peroxidases to produce hypohalous acid in the inflamed area with microbiocidal effects (Klebanoff, 2005).

Regarding these interesting data, the evaluation of the local effects of carrageenin was carried out in **Chapter II,** following the general stages of fish inflammation: With these criteria in mind, the inflammatory process could be initiated as it happens in mammals in response to the damage produced by the injection or by the recognition of carrageenin by tissue-resident non-immune and immune cells [*e.g.*, macrophages, NCC, antigen presenting cells (APCs), etc.]. Considering the detection of antigens, it would be important to mention that gilthead seabream cells seem to present a higher recognition potential than mammals, since B and T lymphocytes, monocyte/macrophages, melanomacrophages, epithelial cells, dendritic-like cells and even AGs possess MHC-II being therefore, all of them considered as APC (unlike mammals whose APCs are basically dendritic cells, macrophages and B lymphocytes) (Cuesta et al., 2006; Glimcher & Kara, 1992). This fact could make it questionable whether the expression of non-classical MHC could facilitate the encounter of these cells with carrageenin which quickly activate both innate and adaptative systems in this species, although more studies would be required to delve into the recognition mechanisms of carrageenin. Regardless of this mechanism, once activated, these local cells could start to release preformed inflammatory mediators such as ROS [mainly superoxide dismutase (SOD)], hydrolytic enzymes like granzyme B, and pro-inflammatory cytokines such as IL-1β to promote the inflammatory response (Larsen & Henson, 1983). These molecules were detected in the skin mucus (**Chapter I**) and in the skin at genetic level (**Chapter II. 1**) at 1.5 h after injection of carrageenin. At the same experimental time, skin-mucus secreting cells seemed to differentiate into mature cells or mucosomes capable to secrete mucus in a holocrine way to the skin surface containing all the pro-inflammatory molecules

previously released into the skin. Maybe, these cells could also develop phagocytotic activities together with phagocytes and participate in the inflammatory response in a proactive way (Iger & Abraham, 1990). The set of all these events mentioned above and related to the effect of carrageenin could be translated in a located way into the increase of the skin thickness and the appearance of intramuscular edema at 1.5, 3 and 6 h, observed by real time ultrasound as areas with low acoustic impedance (anechoic), and by micro-CT as areas with positive values (~ 0 Hounsfield Unit) (Payne, 1978; Ríos, 2010), in the skin of fish injected with carrageenin (see **Chapter II. 2**). Interestingly, in these experimental times (from 1.5 to 6 h), carrageenin is able to induce in mammals the release of histamine, serotonin, and bradykinin, which are the responsible for producing the first visible effects related to inflammation (Posadas et al., 2004; Zadeh-Ardabili & Rad, 2019). Therefore, our findings would evidence the first difference in comparison with mammals, since only with the data obtained in our analysis of genetic expression and considering the possible molecular changes at protein level, it could not prove nor deny the participation of these molecules (histamine, serotonin, and bradykinin) in this process. Furthermore, the most distinguishable difference with mammals was the presence of emphysema (gas) in the underlying muscle from fish injected with carrageenin from 1.5 to 6 h post-injected [seen as hyperechoic foci by ultrasound and presenting density values close to -850 HU in the micro-CT, which are associated with air-related structures, according to the results obtained by Ceballos-Francisco et al. (2021) and Nelson (2004)]. The presence of this emphysema could be explained by the action of the previously released hydrolytic enzymes which could begin to degrade carrageenin. Surprisingly, the signs evidenced after carrageenin injection (subcutaneous emphysema, edema, and increased skin thickness) represent the typical symptoms observed in human necrotizing fasciitis (Buttar et al., 2017), although more studies would be necessary to deepen into this finding. On the other hand, the pro-inflammatory molecules previously commented (mainly IL-1β) would facilitate the recruitment of leucocytes from the HK to the inflamed area. According to genetic and immunohistochemistry results obtained in the present Doctoral Thesis, AGs, macrophages, and the rest of leucocytes seemed to be recruited to the inflamed area in fish injected with carrageenin from 1.5 to 6 h post-injection. This recruitment would correspond to the peak of inflammation, coinciding with the major increase of skin thickness and edema. These cells would be able to trigger the release of pro-inflammatory cytokines and enzymes such as SOD, peroxidase, lysozyme, as well as Igs into the skin mucus (see **Chapter I**). In this sense, carrageenin has been seen to show mitogenic

capacity in B lymphocytes in mouse (Fowler & Thomson, 1978), probably due to the fact previously commented of which the α-1,3-galactosidic linkage of its structure can be recognised as an epitope and then, induce the synthesis of Igs (Galili et al., 1985; Klein et al., 1998; Thibaudeau et al., 1996; Younes et al., 2018). At this point, it would be interesting to mention the role of the lysozyme enzyme, which could be pre-synthetized in the granules of AGs, explaining why we did not find significant differences either in the gene expression analysis nor in the immunohistochemistry assay. Then, it could be released after induction by carrageenin, what would explain its increment in the skin mucus at 3 h post-injection, enhancing the bactericidal activity in the skin mucus against *V. anguillarum* and *P. damselae* at the same time. In fact, lysozyme could not be limited only to its proteolytic function, but also it could act like an opsonin facilitating the cellular recruitment to the inflamed site, as well as participate in tissue repair and healing after terminating inflammation (Bulfon et al., 2015; McCarty & Percival, 2013; Saurabh & Sahoo, 2008).

To comprehend the genetic mechanisms that trigger this entire cascade of processes, **Chapter III** of this Doctoral Thesis had the purpose of studying the expression of genes involved in the inflammatory response caused by carrageenin. Thus, carrageenin would be able to activate the NF-κB pathway cascade at molecular level (probably expressed as the union of homodimers or heterodimers of c-REL) and trigger the release of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, IL-8 and IL-18 into the skin (see **Chapter III. 1**), producing the effects previously observed by ultrasound and micro-CT. In fact, the highest expression of these molecules was evidenced at 3 h post-carrageenin injection compared to control fish, coinciding with the peak of inflammation above-mentioned. At this experimental time (3 h), regulatory molecules such as cathepsins, nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) and molecules of the cholinergic system were down-regulated in fish injected with carrageenin, allowing the course of inflammation. However, the end of the inflammatory process could be evidenced among 12-24 h after of the fish carrageenin injection (see **Chapter III. 2**). This fact could be explained based on the existence of regulatory mechanisms that control inflammation precisely. Concretely, the up-regulation of the *c-rel* gene expression observed in the skin of fish injected with carrageenin at 24 h could pointed at the presence of these important mechanisms that in our study seemed to modulate the expression of pro-inflammatory and anti-inflammatory inducible molecules, which were unaltered at 12

and 24 h post-carrageenin injection, in order to promote the restoration of the physiological homeostasis. These data could be supported by the results obtained from ultrasound and micro-CT after 12 and 24 h of the carrageenin injection, which showed a decrease in the skin thickness, as well as the edema and gas produced in the underlying muscle. Therefore, both data clearly suggested the resolution of inflammation, although it would be necessary to study the mechanisms involved in the healing stage in more detail. To confirm the function of the studied molecules, an *in silico* assay (see **Chapter III. 2**) of the gilthead seabream protein sequences was carry out, evaluating the degree of conservation from mammals to teleost fish. Thus, the molecules most conserved were those involved in the regulation of inflammation, such as NF-κB subunits (NF-κB2, RelA, NF-κB1, C-Rel, RelB), adaptor proteins of the same pathway (STAT3, TRAF6, IκBKG, MYD88), AChE, cathepsins and NOD-like receptors, as well as the cell markers for macrophages (CSF1R) and AGs (PHOX40 and PHOX22). Therefore, these molecules could be considered orthologs (Konaté et al., 2017; Postlethwait et al., 2000) associated with inflammation and its regulation. Nonetheless, molecules which appeared later in evolution like cytokines or Toll-like receptors (TLRs), could have been subjected to WGD events as commented above, affecting possibly to both their structure and function among species or preserving them as paralogues (Smith et al., 2019). Thus, considering the conservation and homology of the molecules involved in the regulation of the inflammatory process along phylogeny, it could be postulated that the peak of inflammation occurs approximately at the same time in mammals and gilthead seabream. However, this species of fish seems to resolve the inflammatory process in a more effective way than mammals. This fact contrasts sharply with the model of inflammation produced by carrageenin in mammals (Levy, 1969; Morris, 2003; Winter et al., 1962). In this sense, it is well-known that in mammals, macrophages try to retire carrageenin from the extracellular medium little by little by phagocytosis. Other times, carrageenin tend to form macroaggregates of even higher molecular-weight and macrophages are unable to phagocyte them (McKim, 2014; Pawelec & Brons, 1978; Silva et al., 2010). In these cases, macrophages coalesce to form granulomas as a last attempt to degrade it, but generating a major inflammatory reaction in consequence (Fowler et al., 1980; Pawelec & Brons, 1978). Moreover, the high-molecular weight of carrageenin provokes osmotic swelling, rupture of lysosomal and release of hydrolytic enzymes into the cytosol of macrophages that manage to phagocytize it, which leads to their lysis (Medzhitov, 2008; Pawelec & Brons, 1978; Thomson & Fowler, 1981). In the end, all these events are

related to the development of chronic inflammation (Medzhitov, 2008; Pawelec & Brons, 1978; Thomson & Fowler, 1981). According to these facts, which were not reflected in our studies, we hypothesised that unlike mammals, carrageenin should be degraded anyway in the muscle of gilthead seabream, avoiding its harmful effects and the subsequent chronification of the inflammation, allowing the activation of regulatory mechanisms and ending the process.

Considering all our data from a joint point of view, it could be highlighted the role of AGs in the skin/muscle inflammation of gilthead seabream, which could participate in the inducement of the said process itself due to its rapid recruitment and the release pro-inflammatory mediators such as cytokines and ROS (Sies, 2017). Furthermore, AGs could be the main cells involved in the degradation of carrageenin by the release of preformed proteolytic enzymes, since not only myeloperoxidase or lysozyme but also other proteases are stored in their granules and are released after an inflammatory stimulus (Dovi et al., 2004; Rodríguez et al., 2003). In addition, AGs could also mediate in the regulation of inflammation due to its role as NCCs avoiding the excessive inflammation (Cuesta et al., 2005; Esteban, 1994; Evans & Jaso-Friedmann, 1992). Therefore, the role of these cells might be the main difference among gilthead seabream and mammals, which could give sense to all data from our studies.

4. 2. Contribution of present Doctoral Thesis to the state of the art.

The results obtained in the present Doctoral Thesis illustrate the importance and the complexity of inflammation in fish which to date were unknown. Therefore, the present results could have an impact on the aquaculture sector, since to date there was no established an inflammation model in fish which allowed to correctly understand the action mechanism of its trigger, and therefore, that effective anti-inflammatory treatments could be developed to combat it. In this sense, to the best of our knowledge, there are only some studies related to the inflammatory process in several fish species such as plaice (*Pleuronectes platessa*) (Timur et al., 1977), pacu (*Piaractus mesopotamicus*) (Martins et al., 2006), trahira (*Hoplias malabaricus*) (Ribas et al., 2016), Miiuy croaker (*Miichthys miiuy*) (Chu et al., 2019), Nile tilapia (*O. niloticus*) (Matushima & Mariano, 1996) and zebrafish (*D. rerio*) (Belo et al., 2021; Huang et al., 2014; Prata et al., 2020). However, there was no relation that explained the mechanisms that carry it out from its onset to its

resolution in any marine species, but rather loose data. In addition, only some of those studies used carrageenin as trigger to study inflammation (Belo et al., 2021; Huang et al., 2014; Martins et al., 2006; Matushima & Mariano, 1996; Prata et al., 2020; Ribas et al., 2016; Timur et al., 1977). In addition, there are few data on the inflammatory molecules involved in fish (Hong et al., 2013; Morimoto et al., 2021; O'Connor et al., 2014; Wiegertjes et al., 2016). Regarding the techniques used to study inflammation, histological sections comprise the main method to consider despite the fact that is an invasive technique which require the animal dissection or a biopsy of the tissue to be studied (Babaei et al., 2016; Romvári et al., 2002). On the other hand, although several studies have been developed to tests anti-inflammatory drugs or natural products (Ceballos-Francisco et al., 2020; García Beltrán et al., 2017; Hall et al., 2014; Mansour et al., 2020), they have only offered information about few anti-inflammatory parameters without explaining properly the action mechanism of the tested substances.

Therefore, the findings of the current Doctoral Thesis contribute to the state of the art providing new methodologies that have supposed the establishment of the first inflammatory model in a fish species of commercial interest, gilthead seabream (Fig. 16).

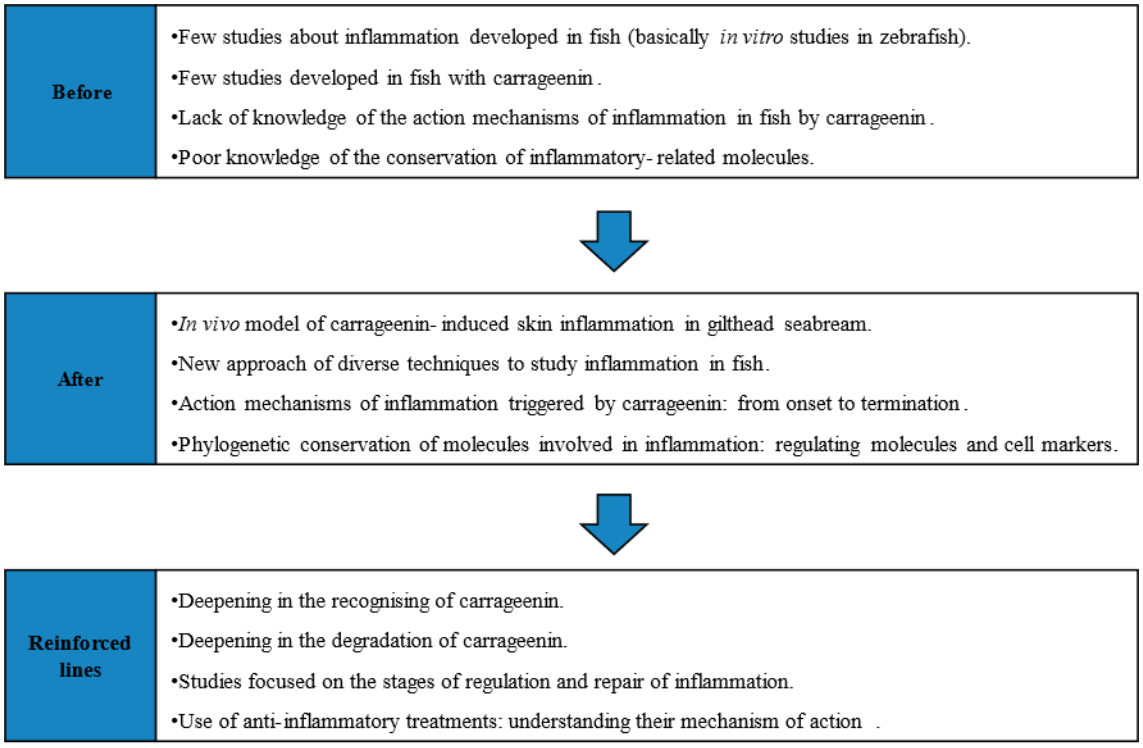

Figure 16. Schematic representation of the contribution of the current Doctoral Thesis to the state of the art.

In this sense, the implement of image advanced techniques such as real-time ultrasound and micro-CT to study the inflammatory reaction, make it possible to examine the entire body of fish in a low-invasive way, generating high-quality images of the affected zone/s, and allowing to quantify areas and/or volumes *in situ* to evaluate the grade or magnitude of inflammation. In addition, those techniques allowed us to evidence the sign of necrotizing fasciitis in those fish injected with carrageenin. Our studies tried to clear both the mechanisms and the effects of carrageenin, not only in the onset, but also in the development and the termination of inflammation. Moreover, our findings provide also more knowledge in the conservation of the inflammatory molecules along phylogeny.

The model of carrageenin-induced skin inflammation in gilthead seabream still requires more studies that help to elucidate how carrageenin is recognized in the tissue and how it is finally degraded. In addition, similar experiments could be developed in other fish species to compare and evaluate the variations among them. Nonetheless, all the results obtained, and the methodologies applied in the current Doctoral Thesis could be used as basis for deepening in the near future in the resolution of inflammation and the study of anti-inflammatory natural or alternative drugs to treat fish inflammatory related diseases properly, in which we still have limited knowledge.

5. CONCLUSIONS

Considering the results obtained in this Doctoral Thesis and studying the available literature on the present topic we were able to reach the following conclusions:

1. One intramuscular carrageenin injection of 1% in phosphate buffer saline is enough to provoke inflammation in gilthead seabream specimens.
2. The carrageenin injection has no impact on the gilthead seabream systemic immunity.
3. Although a decrease in the hepatic catalase activity was observed at 3 and 6 h post-injection, no significant variations were detected in the gene expression of none of the genes studied in the liver, evidencing that this organ was not affected by carrageenin.
4. The carrageenin injection caused an increase in the number of skin mucus-secreting cells and acidophilic granulocytes in the skin near the injection place at 1.5 and 3 h post-injection, respectively.
5. The humoral immune activities (superoxide dismutase, peroxidase, lysozyme, bactericidal activity against *V. anguillarum* and *P. damselae*, and total immunoglobulins) in the skin mucus increased at 3 h of the carrageenin injection. These increments could be due to the release of preformed molecules by the recruited immune cells to the inflamed area.
6. The up-regulation in the gene expression of several cell markers, as well as the microscopic evidences obtained in samples of skin studied at 3 h post-carrageenin injection, clearly demonstrated the recruitment of the acidophilic granulocytes to the inflamed area.
7. The carrageenin injection caused in the underlying muscle the typical symptoms observed in human necrotizing fasciitis (*e.g.*, increased skin thickness, edema, and emphysema) at 1.5, 3, and 6 h post-injection. This symptomatology seems to be due not only to the molecules released in the inflammation site but also to the possible degradation of the carrageenan at this place.
8. The up-regulation in the expression of markers cells and pro-inflammatory genes, as well as the down-regulation of anti-inflammatory-related genes studied in the skin of gilthead seabream in the interval of 1.5-6 h post-injection, indicate the duration of the induction of the inflammatory response caused by carrageenin.

9. All the changes already enumerated after the carrageenin injection in the gilthead seabream specimens decreased in fish sampled at 12 h post-injection and almost disappeared in fish analyzed at 24 h post-injection, pointing to the end of the triggered inflammatory response.

10. The proteins involved in inflammation of large yellow croaker (*Larimichthys crocea* L.) presented the highest percentages of identity with the protein sequences of gilthead seabream, according to the *in silico* study. The most conserved proteins of inflammation are those involved in its regulation. This fact also supports the hypothesis that regulatory gene mechanisms could be activated 24 h after carrageenin injection in order to control the inflammatory response.

11. Finally, all the findings of this Doctoral Thesis evidenced that one single injection of 1% carrageenin can be used to trigger a local inflammation in gilthead seabream, which constitute a model to continue studying this crucial process, both in health and disease, in this important fish species.

6. RESUMEN EN CASTELLANO

6.1. Introducción.

La inflamación es una respuesta inespecífica desencadenada por el sistema inmunitario innato frente a cualquier estímulo de diversa etiología (lesiones, traumatismos, infecciones por patógenos, productos irritantes, etc.) para defender el organismo, en este caso de un pez, y devolverlo a la homeostasis inicial (Du et al., 2015). Se trata de un proceso altamente conservado a lo largo de la escala filogenética. La inflamación presenta una serie de síntomas muy característicos por los que puede ser fácilmente identificable, como son la hinchazón, el enrojecimiento, la sensación de calor, el dolor y las alteraciones funcionales, cuyo conjunto recibe el nombre de "*Signos cardinales de la inflamación*" (Bruce, 1913; Calixto et al., 2003; Chen & Nuñez, 2010; Hawiger & Zienkiewicz, 2019; Henson, 2005; Nathan, 2002). Se pueden diferenciar dos tipos de inflamación (Nathan & Ding, 2010): *i*) inflamación aguda, o respuesta inmediata, la cual puede desarrollarse de forma breve ante la detección del estímulo inicial, y durar días o semanas hasta su resolución final; e *ii*) inflamación crónica, producida ante la ineficacia de los mecanismos defensivos para resolver la inflamación aguda y cuyos efectos están altamente relacionados con la aparición de enfermedades crónicas asociadas. La inflamación aguda de los peces, al igual que la de mamíferos, incluye cinco etapas sucesivas: *i*) la liberación de mediadores pro-inflamatorios; *ii*) el efecto de dichos mediadores; *iii*) el reclutamiento celular; *iv*) la regulación de la inflamación; y, por último, *v*) la reparación tisular. Además, se requiere de una gran regulación de dichas etapas y de los mecanismos implicados en su finalización con el fin de evitar patologías derivadas. En este sentido merece la pena resaltar a nivel molecular la acción del factor de transcripción NF-κB, que es el eje angular de la inflamación, ya que se encarga de activar y reprimir cascadas completas de genes pro- y anti-inflamatorios, así como de otros genes involucrados en su regulación (Mulero et al., 2019; Napetschnig & Wu, 2013).

La inflamación es un evento muy común que ocurre en peces de acuicultura debido a las intensas condiciones de producción (hacinamiento, calidad del agua, deterioro de las condiciones ambientales) a los que son sometidos los peces para cubrir las demandas del mercado. Este escenario hace a los animales más susceptibles a heridas, traumatismos e infecciones por patógenos, lo que podría estar relacionado con la inflamación y la aparición de enfermedades asociadas, que muchas veces se traducen en pérdidas económicas para el sector (Balcázar et al., 2006; Esteban, 2012). La inflamación supone

un mecanismo esencial para devolver a los peces a su estado inicial de salud, ya que participa de forma activa y directa en la reparación del tejido dañado (Elliott, 2000). En concreto, la regulación de esta respuesta inflamatoria es una etapa clave que, en la mayoría de los casos, supone la diferencia entre la salud y la enfermedad de dichos animales (Feehan & Gilroy, 2019). El estudio de la inflamación permitiría la compresión de los mecanismos de defensa ejercidos contra agentes externos y los estadios iniciales de su recuperación (Bruce, 1913; Calixto et al., 2003; Chen & Nuñez, 2010; Hawiger & Zienkiewicz, 2019; Henson, 2005; Nathan, 2002). No obstante, hasta la fecha no se ha desarrollado ningún modelo de inflamación en peces que permita profundizar en tales mecanismos. Así, la mayoría de las enfermedades de carácter inflamatorio o asociado (mayormente infecciones por patógenos) que afectan a peces se ha tratado hasta la fecha de forma inefectiva mediante el uso de antibióticos, los cuales además pueden tener efectos perjudiciales tanto para los peces, como para los humanos y el medio ambiente (Cabello, 2006). Las vacunas son un método más eficaz que los antibióticos ya que son patógeno-específicas, aunque su elevado coste de producción y su acción limitada contra otros patógenos para las que no son especificas complica su comercialización (Gudding & Van Muiswinkel, 2013). Además, aunque numerosos productos de diversa naturaleza han sido testados debido a sus posibles propiedades anti-inflamatorias (Ceballos-Francisco et al., 2020; García-Beltrán et al., 2017; Hall et al., 2014; Mansour et al., 2020), la falta de un modelo inflamatorio hace imposible entender correctamente los mecanismos de acción de dichos productos. En este sentido, dado que la mayoría de las infecciones por patógenos pueden producir una reacción inflamatoria (independientemente de sus efectos específicos) fácilmente identificable por los signos cardinales de la inflamación (Lawrence et al., 2002), el establecimiento de un modelo general de inflamación en peces podría permitir el desarrollo de tratamientos más precisos y específicos. Consecuentemente, el tratamiento correcto y efectivo de la inflamación evitaría pérdidas innecesarias para el sector acuícola (Bruce, 1913; Calixto et al., 2003; Chen & Nuñez, 2010; Hawiger & Zienkiewicz, 2019; Henson, 2005; Nathan, 2002).

Por otro lado, en mamiferos se han desarrollado multitud de técnicas y metodologias *in vitro* para detectar moleculas pro-inflamatorias, como ensayos inmunoabsorbentes ligados a enzimas (ELISA), ensayos de expresion génica mediante reacción en cadena de la polimerasa (PCR), histologia e inmunohistoquimica y otras técnicas de imagen no invasivas (Eddouks et al., 2012; Gerner et al., 2012; Liao et al.,

2011; X. Wang et al., 2003). A nivel *in vivo* se pueden destacar varios modelos como el "edema de pata" inducido por formalina y la artritis inducida por CFA, aunque ambos modelos están mas enfocados al estudio del dolor (Billiau & Matthys, 2001; Juma'a et al., 2009), el método de induccion de inflamación mediante LPS (cuyo uso está mas enfocado a la estimulación de cultivos *in vitro*) (Calil et al., 2014; Vajja et al., 2004) y el modelo de "edema de pata" inducido por carragenina (Winter et al., 1962). Resaltar la importancia de este ultimo modelo debido a su facil reproductividad y caracterización mediante la visualizacion de unos efectos macroscopicos muy específicos como son la aparicion de edema, hiperalgesia, eritema y una respuesta exacerbada a estímulos térmicos y mecánicos que alcanzan su grado máximo a las 3 h de la inyección, y dos fases bien definidas a nivel molecular por la liberación de mediadores de fase aguda y de prostaglandinas en segundo lugar (Patil & Patil, 2017; Posadas et al., 2004).

6.2. Objetivos.

La presente Tesis Doctoral pretende mejorar nuestro conocimiento sobre el proceso de inflamación de la dorada (*S. aurata*).

Los objetivos específicos son:

1. Evaluación de los efectos sistémicos provocados por la inyección intramuscular de carragenina.
2. Caracterización de la respuesta local provocada por la inyección la carragenina.
3. Estudio de la expresión de genes implicados en la respuesta inflamatoria provocada por la carragenina.
4. Búsqueda *in silico* de moléculas conservadas e implicadas en la inflamación.
5. Establecimiento de un modelo inflamatorio en peces *in vivo* mediante el uso de carragenina como inductor de la inflamación.

Estos objetivos nos permitirían conocer en profundidad los mecanismos humorales y celulares, así como la modulación de la expresión genética que se lleva a cabo durante el desarrollo del proceso inflamatorio en la dorada, lo que podría tener repercusiones relevantes en la búsqueda de tratamientos anti-inflamatorios en el sector de la acuicultura.

6.3. Principales resultados y discusión.

La inflamación es un proceso muy complicado, cuyos mecanismos permanecen muy conservados a lo largo de la evolución filogenética, aunque su investigación se ha centrado principalmente en mamíferos. En comparación, la información disponible acerca de la inflamación de teleósteos es bastante limitada. Por ello, la presente Tesis Doctoral se ha centrado en la caracterización de la inflamación en la dorada, una de las especies de interés en acuicultura más importantes del Mar Mediterráneo. Para ello, y debido al aumento de métodos alternativos en los últimos años, la metodología implementada fue pensada para cumplir con la estrategia de las 3Rs (reducción, refinamiento y reemplazo). Para conseguir nuestro objetivo dividimos esta Tesis Doctoral en 3 capítulos.

En el **Capítulo I** se evaluaron los efectos sistémicos provocados por una inyección de carragenina. Los peces fueron inyectados intramuscularmente con PBS (control) o carragenina (1%) y tras 1,5, 3 y 6 h se tomaron muestras de moco de la piel, suero, riñón cefálico e hígado para determinar el estado inmunológico y antioxidante en la dorada. El moco de la piel de los ejemplares pertenecientes al grupo inyectado con carragenina mostró un aumento de las actividades de superóxido dismutasa y peroxidasa, lisozima, actividad bactericida contra *V. anguillarum* y *P. damselae* e inmunoglobulinas totales a las 3 h de la inyección en comparación con los ejemplares pertenecientes al grupo control. El aumento de estas actividades se relacionó con la liberación de moléculas preformadas y enzimas hidrolíticas desde la zona de inflamación al moco de la piel por parte de las células reclutadas del sistema inmunitario (muy probablemente AGs, debido al aumento de la actividad peroxidasa, que es un marcador de este tipo de células) (Meseguer et al., 1995). Sin embargo, los peces inyectados con carragenina y muestreados a las 6 h después de la inyección mostraron una disminución de la actividad de peroxidasa en los leucocitos de riñón cefálico y la actividad proteasa en el moco de la piel en comparación con los peces del grupo control, lo que podría sugerir en primer lugar el cese de migración de AGs desde el riñón cefálico al sitio de inflamación y por otro lado, el inicio de la inactivación de dichas células en el propio lugar inflamado, por el cese inflamatorio. Además, aunque la inyección de carragenina no tuvo efectos sobre el sistema inmunitario sistémico, se redujo la actividad catalasa hepática a las 3 y 6 h en el grupo de carragenina en relación con la del grupo control, sugiriendo que el efecto de la carragenina es solamente local, y que estos efectos podrían ser efectos secundarios solamente debidos a

su proximidad al sitio de inflamación y producidos por las moléculas liberadas para redirigir el peróxido de hidrógeno hacia la zona inflamada. De esta forma, el peróxido de hidrógeno podria continuar siendo utilizado como sustrato de enzimas peroxidasas para desarrollar efectos microbicidas (Klebanoff, 2005). También se determinaron los niveles de expresión de varios marcadores celulares y genes pro-inflamatorios en el riñón cefálico y el hígado. En el primero, los niveles de expresión de *il1b* y *pgds1* aumentaron a las 1,5 y 3 horas, respectivamente, en el grupo de carragenina en comparación con los del grupo control, apoyando la activación celular y la migración temprana de células desde el riñón cefálico hasta el lugar de inflamación (Meseguer et al., 1995). Por el contrario, la expresión de *phox40* en el grupo de carragenina a las 6 h disminuyó en comparación con la del grupo control, apuntando al inicio de la inactivación celular o a la disminución de dicho reclutamiento celular, como ha sido anteriormente comentado. No se observó ninguna variación en la expresión de los genes estudiados en el hígado, evidenciando de otra manera que este órgano no fue directamente afectado por la carragenina.

Teniendo en cuenta estos resultados, en el **Capítulo II** de esta tesis se estudiaron los efectos locales desencadenados por la carragenina en el lugar de la inyección. En este sentido, los animales fueron inyectados intramuscularmente con PBS (control) o carragenina, y después de 1,5, 3 y 6 h de la inyección, se obtuvieron muestras de piel que fueron procesadas por un lado para su inclusión en parafina y posterior tinción con hematoxilina-eosina, azul Alcián, ácido periódico de Schiff, o varios marcadores celulares (D2 para AGs, lisozima para AGs y monocitos/macrófagos, granzima B para células citotóxicas no específicas, inmunoglobulina M para linfocitos B) mediante inmunohistoquímica, y por otro lado se observó la expresión génica de los mismos marcadores celulares. Los resultados microscópicos indicaron un aumento en el número de células mucosecretoras de la piel y AGs en la piel de los peces estudiados a las 1,5 y 3 h después de la inyección con carragenina, respectivamente, con respecto a los datos obtenidos en ejemplares pertenecientes al grupo control. Estos resultados ofrecieron información acerca de la rápida diferenciación y maduración de las células mucosecretoras de la piel ante un estímulo inflamatorio como la carragenina, evidenciando su papel en la secrecion holocrina de moco, y posiblemente su actividad como células fagocíticas en la primera línea de defensa (Iger & Abraham, 1990). El aumento de las células mucosecretoras de la piel se pudo relacionar de manera muy coherente con nuestro estudio anterior en el que se liberaron moléculas preformadas desde

la zona de inflamación hacia el moco de la piel a las 3 h después de la inyección de carragenina. Además, se obtuvo información sobre el reclutamiento celular hacia la zona inflamada a partir de las 1,5 h de la inyección, el cual pareció estar encabezado por AGs (Meseguer et al., 1995). En el caso de la expresión génica, el marcador de células citotóxicas no especificas *grb* y la citoquina pro-inflamatoria *il1b* incrementaron su expresión a las 1,5 h en la piel de peces inyectados con carragenina en comparación con el grupo de peces control, mientras que la expresión génica de marcadores de AGs (*phox22* y *phox40*) se incrementó a las 3 y 6 h en los ejemplares del grupo tratado con carragenina, en comparación con los ejemplares del grupo control. Además, el gen *mpo* (otro marcador de AGs) también incrementó su expresión a las 6 h en la piel de peces inyectados con carragenina en comparación con las muestras control. El aumento en la expresión de estos genes evidenció claramente el reclutamiento de los AGs a la zona inflamada. Interesantemente, las células citotóxicas no especificas en la dorada se describieron como una población heterogénea compuesta por linfocitos, monocitos/macrófagos y AGs (Cuesta et al., 2005; Esteban, 1994; Evans & Jaso-Friedmann, 1992), por lo que el aumento de la expresión génica de *grb* podría indicar de la misma manera al reclutamiento y activación de estos granulocitos a este tiempo experimental (1,5 h).

Para respaldar los resultados anteriores se llevó a cabo un ensayo en el que se inyectó intramuscularmente a los peces con PBS (control) o con carragenina (1%), y la zona de inyección se evaluó mediante ecografía en tiempo real y micro-CT a 1,5, 3, 6, 12 y 24 h después de la inyección. Los resultados demostraron que en los peces inyectados con carragenina se produjo un aumento en el grosor de la piel a las 1,5, 3 y 6 h después de la inyección, y mostraron en el músculo subyacente al lugar de la inyección, unos pequeños focos hiperecoicos rodeados por un área anecoica (vista en las imágenes adquiridas por micro-CT como áreas con valores positivos cercanos a 0 Unidades Hounsfield) que no se observaron en los peces del grupo control a los mismos tiempos de estudio. El análisis de las adquisiciones de micro-CT reveló también un área oscura en el lugar de la inyección con carragenina a las 1,5, 3 y 6 h. Estas áreas tenían una densidad promedio de -850 a -115 Unidades Hounsfield, que no se correspondía con ninguna densidad de tejido del resto del cuerpo, sino con la densidad del aire, de acuerdo a los trabajos realizados por Ceballos-Francisco et al. (2021) y Nelson (2004). Además, no se observaron tales áreas oscuras en las zonas de inyección en los peces del grupo control. Estos resultados apuntaron por un lado a los efectos producidos por las moléculas

liberadas en la zona de inyección, produciendo consecuentemente edema (área anecoica) e hinchazón (Payne, 1978; Ríos, 1010), y por otro lado, a la posible degradación de la carragenina en el punto de inflamación, evidenciada por los focos hiperecoicos y el área oscura obtenida por micro-CT que podrían estar relacionado con la liberación de gas como una consecuencia de dicho proceso (Ceballos-Francisco et al., 2021; Nelson, 2004). Este hecho, podría tener sentido, ya que el grosor de la piel del área inyectada volvió a tener valores cercanos a los normales a las 12 y 24 h después de la inyección de carragenina y estas áreas oscuras fueron más pequeñas en los peces analizados a tiempos más largos (12 h) y casi desaparecieron en los peces muestreados a las 24 h después de la inyección, evidenciando de alguna manera el descenso de la inflamación. Sorprendentemente, estos signos hallados despues de la inyeccion de carragenina [enfisema subcutáneo (gas), edema y aumento del grosor de la piel] representan la sintomatologia tipica observada en la fascitis necrosante humana (Buttar et al., 2017), aunque serían necesarios más estudios para profundizar en este tema.

Finalmente, en el **Capítulo III** de la presente Tesis Doctoral se incluyeron los resultados obtenidos del análisis génico de la respuesta inflamatoria provocada por la carragenina en la dorada. Para ello, la expresión de 40 genes relacionados con la inflamación se evaluó *in vivo* en dos ensayos que se llevaron a cabo por separado. En el primer experimento, los especímenes de dorada fueron inyectados con PBS (como control) o con carragenina (1%) por vía intramuscular, y después de 1,5, 3 y 6 h se recolectaron las muestras de piel del área inyectada y se procesaron para evaluar la expresión génica. Con este ensayo se observó que marcadores celulares inflamatorios (*csfr1, mhcii* y *phox40*), varias citoquinas pro-inflamatorias (*il1b, tnfa, il6, il8* e *il18*) y otras moléculas relacionadas (como *myd88* y *c-rel*) incrementaron su expresión a las 1,5 y 3 h en peces inyectados con carragenina en comparación con los niveles observados en los peces del grupo control. Además, la expresión génica de moléculas anti-inflamatorias (*nlrx1, nlrc5 isoforma 1, ctsd* y *ctss*) estudiada en peces inyectados con carragenina y muestreados 3 h después de la inyección, disminuyó su expresión en comparación con la expresión génica observada en peces del grupo control. Estos resultados demostraron que la carragenina fue capaz de estimular la expresion de genes pro-inflamatorios y disminuir la expression de genes anti-inflamatorios en la piel de la dorada en los tiempos experimentales ensayados.

En el segundo ensayo se replicó el mismo diseño experimental, pero aumentando el tiempo de muestreo a 12 y 24 horas, y se analizó la expresión de los mismos 40 genes estudiados previamente. De entre todos los genes estudiados, solo se observó una disminución en la expresión génica del marcador de macrófagos (*csfr1*) en la piel de peces inyectados con carragenina a las 12 h en comparación con la piel de los peces del grupo control, lo cual apoya nuestra hipótesis de inactivación celular a partir de las 6 h del inicio de la inflamación. Además, se observó un incremento del gen *c-rel* (subunidad de NF-κB) en peces inyectados con carragenina y muestreados a las 24h en comparación con los peces inyectados con PBS, hecho que podría indicar la activación de mecanismos necesarios para terminar la respuesta inflamatoria a la carragenina y recuperar la homeostasis de la piel (Gugasyan et al., 2004). Teniendo en cuenta estos resultados, se realizó un ensayo *in silico* para estudiar la conservación filogenética de estas 40 moléculas inflamatorias a partir de secuencias proteicas de la dorada. Los marcos de lectura abiertos de los 40 genes se utilizaron como entrada en la base de datos STRING para el análisis de la red de interacción proteína-proteína, comparándolos con otras secuencias de proteínas de teleósteos. Nuestros resultados confirmaron que dichas moléculas están conservadas en mayor o menor medida desde mamíferos hasta teleósteos, siendo las proteínas de la corvina amarilla (*Larimichthys crocea* L.) las que presentaron los mayores porcentajes de identidad con la secuencia protéica de la dorada, y siendo además las más conservadas aquellas implicadas en la regulación de este proceso. Entre ellas cabe destacar las subunidades de NF-κB (NF-κB2, RelA, NF-κB1, C-Rel, RelB), proteínas adaptadoras de la misma vía (STAT3, TRAF6, IκBKG, MYD88), AChE, catepsinas y proteínas NOD, así como marcadores celulares para macrófagos (CSF1R) y AGs (PHOX40 y PHOX22). Por lo tanto, estas moléculas podrían considerarse ortólogos asociados con la inflamación (Konaté et al., 2017; Postlethwait et al., 2000). Sin embargo, moléculas que aparecieron más tarde en la evolución, como las citoquinas o los receptores de tipo Toll (TLR), podrían haber estado sujetos a eventos de duplicación del genoma (WGD) afectando tanto a su estructura como su función entre especies, o preservándolas como parálogos (Smith et al., 2019).

Teniendo en cuenta la conservacion de moleculas como las subunidades de NF-κB, implicadas en la regulación de la inflamacion desde peces a mamiferos, y en base a los ensayos *in vivo* en los que se llevó a cabo el análisis de los genes involucrados en la respuesta inflamatoria, se llegó a la conclusión de que el pico de inflamación ocurre

aproximadamente al mismo tiempo en la dorada y en mamiferos (Levy, 1969; Morris, 2003; Winter et al., 1962). Sin embargo, la dorada parece resolver el proceso de inflamación de una manera más efectiva, suceso que contrasta fuertemente con el modelo de inflamación producido por carragenina en mamíferos (Levy, 1969; Morris, 2003; Winter et al., 1962). Quizás, la diferencia pueda estar en las células implicadas en el proceso, ya que, en mamíferos, los macrófagos intentan fagocitar la carragenina, la cual tiende a formar macroagregados de mayor peso molecular, y debido a su ineficacia para fagocitarla adecuadamente, estos se reúnen formando granulomas, generando como consecuencia una importante reacción inflamatoria que en la mayoría de los casos acaba con su cronificación (Fowler et al., 1980; Pawelec & Brons, 1978). Teniendo en cuenta estos hechos, los cuales no se reflejan en los resultados obtenidos en nuestros estudios, llegamos al siguiente razonamiento o hipótesis, y es que, la carragenina es degradada de alguna manera en la zona donde fue inyectada evitando como consecuencia sus efectos nocivos y la consiguiente cronificación de la inflamación.

Considerando todos nuestros resultados, se podría destacar el papel de los AGs, los cuales parecen reclutarse muy rápidamente al lugar de la inflamación y tras su activación, podrían iniciar la liberación de mediadores pro-inflamatorios como citoquinas y ROS. Además, las mismas células podrían ser las principales implicadas en la degradación de la carragenina por la liberación de enzimas proteolíticas preformadas, ya que no solo la mieloperoxidasa o la lisozima, sino también otras proteasas se almacenan en sus gránulos y son liberadas tras un estímulo inflamatorio. Además, los AGs también podrían mediar en la regulación de la inflamación debido a su papel como células citotóxicas no específicas evitando la inflamación excesiva. Por tanto, el papel de estas células podría ser la principal diferencia entre la dorada y los mamíferos. La combinación de todos estos resultados indica que la inyeccion intramuscular de carragenina al 1% en la dorada puede ser usada para inducir una respuesta inflamatoria aguda que es capaz de resolverse en menos de 24 h, lo que podría utilizarse como modelo inflamatorio de peces en estudios posteriores.

6.4. Conclusiones.

Considerando los resultados obtenidos en la presente Tesis Doctoral y estudiando la literatura disponible sobre inflamación pudimos llegar a las siguientes conclusiones:

1. Una inyección intramuscular de carragenina al 1% en tampon fosfato salino es suficiente para provocar inflamación en ejemplares de dorada.
2. Dicha inyección de carragenina no tuvo repercusiones en la inmunidad sistémica de la dorada.
3. Aunque se observó una disminución de la actividad catalasa hepática a las 3 y 6 h despues de la inyección, no se detectaron variaciones significativas en la expresión génica de ninguno de los genes estudiados en el hígado, evidenciando que este órgano no se vio afectado por la carragenina.
4. La inyección de carragenina provocó un aumento en el número de células mucosecretoras de la piel y de granulocitos acidófilos en la piel cercana al lugar de la inyección a las 1,5 y 3 h después de la inyección, respectivamente.
5. Las actividades inmunitarias humorales (superóxido dismutasa, peroxidasa, lisozima, actividad bactericida frente a *V. anguillarum* y *P. damselae* e inmunoglobulinas totales) aumentaron en el moco de la piel a las 3 h de la inyección de carragenina. Estos incrementos podrían deberse a la liberación de moléculas preformadas por las células inmunitarias reclutadas en el área inflamada.
6. El aumento en la expresión génica de varios marcadores celulares, así como la evidencia microscópica obtenida en muestras de piel estudiadas a las 3 h después de la inyección de carragenina, demostraron claramente el reclutamiento de granulocitos acidófilos en el área inflamada.
7. La inyección de carragenina provocó en el músculo subyacente los síntomas típicos de la fascitis necrosante humana (p. ej., aumento del grosor de la piel, edema y enfisema) a las 1,5, 3 y 6 horas después de la inyección. Esta sintomatología parece deberse no sólo a las moléculas liberadas en el sitio de la inflamación sino también a la posible degradación de la carragenina en dicho lugar.

8. El aumento en la expresión de marcadores celulares y genes proinflamatorios, así como la disminucion en la expresión de genes anti-inflamatorios, estudiados en la piel de dorada en el intervalo entre 1,5 y 6 h tras la inyección, indican la duración de la inducción de la respuesta inflamatoria provocada por la carragenina.

9. Todos los cambios enumerados después de la inyección de carragenina en los ejemplares de dorada disminuyeron en los peces muestreados tras 12 h de la inyección y casi desaparecieron en los peces analizados tras 24 h, lo que demuestra el final de la respuesta inflamatoria desencadenada.

10. Las proteínas involucradas en la inflamación de la corvina amarilla (*Larimichthys crocea* L.) presentaron los mayores porcentajes de identidad con las secuencias proteicas de la dorada, según el estudio *in silico*. Las proteínas de la inflamación más conservadas son las implicadas en su regulación. Este hecho también apoya la hipótesis de que los mecanismos génicos reguladores podrían activarse 24 h después de la inyección de carragenina para controlar la respuesta inflamatoria.

11. Finalmente, todos los hallazgos de esta Tesis Doctoral evidencian que una sola inyección de carragenina al 1% puede ser utilizada para desencadenar una inflamación local en doradas, lo que constituye un modelo para continuar estudiando este importante proceso, tanto en la salud como en la enfermedad, en esta especie.

7. REFERENCES

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