GONÇALO LOPES LEAL RIBEIRO

Potential immunological biomarkers for Gilthead seabream (*Sparus aurata*) welfare when exposed to an acute infection of *Amyloodinium ocellatum*

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Declaro ser o autor deste trabalho que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Declaration authorship

I declare to be the author of this work, which is original and unprecedented. Authors and works consulted are duly cited in the text and are included in the list of references.

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Resumo

O *Amyloodinium. ocellatum* é um ectoparasita obrigatório dinoflagelado que afeta muitas produções de aquacultura no sul da Europa. É uma preocupação central em aquacultura devido à sua capacidade para causar mortalidade súbita nos peixes, especialmente em produções semi-intensivas. A dourada (*Sparus aurata*) é uma das espécies em produção mais afetadas, e apesar de a sintomatologia e causas de mortalidade estarem bem descritas e documentadas, a reação fisiológica e imunológica do hospedeiro ao parasita ainda são pouco conhecidas. Além disso, os tratamentos existentes são pouco eficazes contra todos os estadios de vida do parasita, podem provocar stress no hospedeiro, e impossibilitar a venda e consumo destes animais. Neste estudo o objetivo foi clarificar os mecanismos moleculares da resposta fisiológica da dourada a infecções de *A. ocellatum*. De modo a identificar e selecionar candidatos a biomarcadores que possam ser usados para monitorizar o bem-estar e saúde dos peixes em aquacultura.

De modo a alcançar este objetivo, foi desenhado um ensaio de infeção. Três tanques controlo e três tanques de infeção foram montados em sistema fechado com apenas arejamento. Estes tanques foram colocados numa sala termicamente isolada a 23ºC durante todo o ensaio. Quarenta e duas douradas juvenis foram divididas pelos 6 tanques (7 em cada). Água com uma concentração de $78x10^3$/ml de *A. ocellatum* foi adicionada aos tanques de infeção. De modo a avaliar o progresso da infeção e o estado dos peixes, foram feitas 3 amostragens em três pontos temporais: 24h, 48h e 72h. Nestas, o primeiro e segundo arcos branquiais dos indivíduos foram removidos e observados ao microscópio numa amplitude de 40x. Três locais da branquia foram aleatoriamente escolhidos e o número de *A. ocellatum* foi contado. Em cada uma destas amostragens foi removido 1 peixe de cada tanque.. Na amostragem final todos os peixes *n*=30 foram sacrificados ebranquias, fígados, baços, e intestinos foram recolhidos e colocados a -80ºC, até serem processados. As branquias e baços de 6 indivíduos, 3 controlo e 3 infetados, foram sujeitas a uma análise via RT-qPCR. Seis genes candidatos a biomarcadores foram escolhidos (TNF- α, *IL-1β*, *HIF1-α*, *Cox-2*, *PPAR-α*, e *IgM*) e a sua expressão foi analisada.

Nas branquias apenas o *IgM* e o *HIF1-α* foramsobre expressos, sendo a diferença estatisticamente significativa apenas para o *IgM*. *IgM* é uma imunoglobulina que é expressa em diversos tecidos e é essencial para proteção contra diferentes agentes patogénicos. *HIF1-α* é um biomarcador habitualmente utilizado em peixes para avaliar hipoxia aguda e crónica e em ambas estas condições se têm demonstrado haver uma sobreexpressão. Algumas da possíveis razões para uma falta de significância estatística podem ser o facto de ter sido analisado uma quantidade de amostras insuficiente, ou uma análise feita numa janela temporal incorreta, já que outros estudos feitos com este gene admitem que uma exposição prolongada a condições de hipoxia causa a sobreexpressão deste marcador.

As duas citoquinas pro-inflamatórias estudadas estavam sub-expressas nos peixes infetados, apesar de não ter sido observada significância estatística. Este resultado não era esperado, mas a maior parte dos estudos com citoquinas parecem estar ligados a exposições crónicas e correlacionados com os danos observados nos tecidos. Assim, é possível que a exposição feita neste estudo não tenha sido suficientemente longa para provocar danos significativos e uma sobre expressão destes genes.

A expressão do *Cox-2* não foisignificativamente alterada, apesar de os peixes infetados parecerem ter níveis de expressão menores que peixes controlo. A expectativa inicial seria que houvesse um aumento dos níveis de mRNA de *Cox-2* em indivíduos infetados. No entanto, estudos feitos com este gene apontam para resultados

contraditórios que podem estar relacionados com uma aparente interligação entre os genes *Cox-2*, *IL-1β* e *TNF-α*.

Finalmente, o *PPAR-α* apresentou-se tal como *IL-1β*, *TNF-α* e *Cox-2*, ligeiramente sub-expresso, no entato sem significância estatística. Neste caso, os resultados não são surpreendentes visto que este gene é predominantemente expresso no fígado e não nas branquias. Aliás em dourada o PPAR-γ aparenta ser mais expresso do que o *PPAR-α*, sugerindo a utilização do primeiro ofereça em estudos futuros para avaliar o potencial deste gene como biomarcador para o catabolismo de lípidos em resposta a uma infecção.

A importância do baço durante infeções parasítárias tem sido descrita em muitos estudos como uma resposta sistémica a infeções secundarias e participação no processo de regeneração de feridas.

Neste estudo não foram detetadas nenhumas diferenças significativas em nenhum dos genes analisados no baço de peixes infetados quando comparado com o controlo. No entanto, todos os genes (excepto *Cox-2*) aparentam estar ligeiramente sobre-expressos. Apesar disto, devido a grande variabilidade nos valores de expressão dos indivíduos infetados, nenhum apresentou diferenças estatísticas.

No entanto, a aparente sobre-expressão dos genes analisados neste estudo estão de acordo com as nossas expectativas e com a literatura. Para o*HIF1-α*, por exemplo, já foi reportada uma ligeira sobre-expressã após 12 e 24h, e ainda uma sobre-expressão significativa aquando de uma exposição a longo prazo. Assim, tal como nas branquias, a janela temporal de exposição ao parasita parece ser um fator chave para detetar respostas imunitárias no hospedeiro. Isto pode explicar o porque de não ter disso detetada nenhuma resposta na expressão deste gene no baço.

Contrariamente ao observado nas braquias, a sobre-expressão de *IgM* não foi estatísticamente. Isto pode dever-se ao facto de ter sido detetada uma grande variabilidade nos níveis de expressão genética entre os indivíduos infetados. Todavia, esta pequena sobre-expressão está de acordo com as expectativas, já que alguns estudos feitos com circunstâncias similares apontam para uma resposta imunológica sistémica mais tardia. Assim é possível hipotetizar que em 72h *A. ocellatum* ativa uma resposta imunológica relativamente forte no local de infeção, mas não uma resposta sistémica completa.

Como dito anteriormente a expressão das citoquinas pro-inflamatórias no baço não foi estatisticamente significativa. Apesar disso notou-se uma ligeira sobre-expressão. A resposta destas citoquinas a infecções está bem descrita em peixes, e o tempo ao qual essa é activada pode variar de acordo com o estímulo e espécie estudada. Por isso a falta de significância revelada neste estudo pode indicar que se tivéssemos analisado as amostras noutra escala temporal poderíamos ter encontrado resultados similares as expectáveis.

Tal como nas branquias *Cox-2* não apresentou diferenças estatisticamente significativas entre infectados e controlo. Isto pode ser explicado pela mesma razão que nas branquias.

Por fim, a expressão de *PPAR-α* também se demonstrou estatisticamente insignificante, mesmo que tenha sido o gene com maior resposta. De qualquer forma, devido a alta variância nos níveis de expressão entre indivíduos do grupo de infectados estes resultados não podem ser conclusivos.

Em conclusão este estudo focou-se nas respostas iniciais (72h) das branquias e baços de dourada a uma exposição aguda de *A. ocellatum*. Este apenas demonstrou uma resposta forte de *IgM* no local de infecção (branquias), e nenhuma resposta significante foi detectada nos baços. No entanto, devido ao facto de só termos analisado um n=3 dos grupos infectados e de controlo, é possível que alguns dos genes estejam de facto a

responder ao parasita, mas a falta de amostras individuais não providencie poder estatístico suficiente para o detectar.

Para estudos futuros um aumento do tamanho da amostra, pontos de amostragem antes e depois das 72h, analise de mais tecidos tais como fígado, intestino e sopra-renal, alteração da carga de parasitas, desenhar a experiência para uma exposição crónica em vez de aguda, e estudar novos genes candidatos; podem ser formas de obter mais e melhores resultados.

Palavras chave: *Amyloodinium ocellatumi,* dourada, exposição aguda, marcadores, brânquias, baço, imunoglobulina M

Abstract

A. ocellatum is an obligate ectoparasitic dinoflagellate that affects many aquaculture productions in southern Europe. It is considered a central concern in fish farming due to its ability to cause sudden mortality to the fish, especially in semi-intensive productions. Gilthead seabream is one of the fish species productions affected, and although the symptomology and mortality causes are well described and documented, the host physiological and immunological responses to the parasite are poorly understood. In addition, the treatments that exist are ineffective against all stages of the parasite's life cycle and can induce stress on the host. In this study the objective was to clarify the molecular mechanisms underlying the seabream physiological response to *A. ocellatum* infestations hoping to pinpoint candidate biomarkers that may be used to monitor the fish welfare and health in production systems. In order to do so, seabream infected with an acute exposure to *A. ocellatum* were dissected 72h post initial infection and their gills and spleen analysed via RT-qPCR. Six candidate biomarkers were chosen: *TNF-α*, *IL-1β*, *HIF1-α*, *Cox-2*, *PPAR-α*, and *IgM*. In the gills *IgM* was the only to be up-regulated, which went in favour of our expectations. The rest did not present statistical significance. In the spleen no genes showed a statistically significant change in their expression. However, all, but *Cox-2* seem to have a tendency to be up-regulated in the infected groups.

Keywords: *Amyloodinium ocellatum* Gilthead seabream acute exposure, biomarkers, gills, spleen, Immunoglobunin M.

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Abbreviation List

actβ: β-Actin

Cox 2: Cyclooxygenase 2

HEP1: Hepcidin 1

HIF1-α: Hypoxia inducible alpha subunit

IgM: Immunoglobulin M

IL-1β: Interleukin 1-beta

IL-8: Interleukin-8

L13: ribosomal protein *L13*

LDH: Lactate dehydrogenase

PPAR-α: Peroxisome proliferator activated receptor alfa

RPS18: Ribosomal protein S18

TGF-β: Transforming growth factor beta

TNF-α: Tumor necrosis factor alfa

1. Introduction

1.1 State of aquaculture

In a world where the population keeps growing and the demand for fish and its products is not slowing down, aquacultures are key contributors to the animal food production sector. According to the latest worldwide statistics compiled by FAO, world aquaculture production attained another record of 114.5 million tons in live weight in 2018 (**Fig. 1.1**) (FAO 2020). The farming of aquatic animals in 2018 was dominated by finfish (54.3 million tons), harvested from inland aquaculture (FAO 2020).

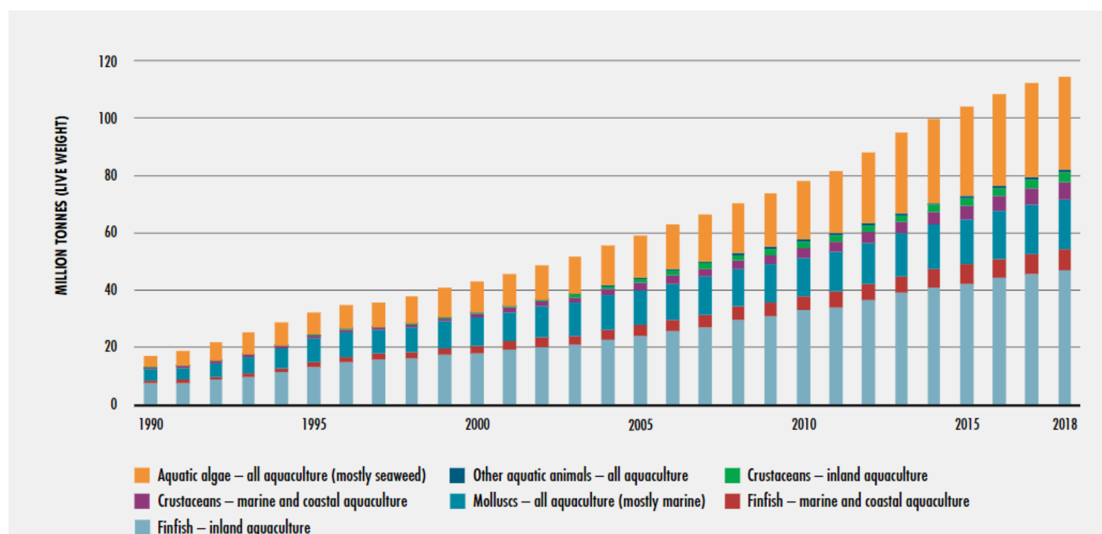

Figure 1.1 World aquaculture production of aquatic animals and algae, from 1990 to 2018 (FAO, 2020).

However, large scale production is facing problems regarding the quality of the fish. Fish infection with diverse pathogens and parasites are one of the main problems in this sector, representing severe annual costs to producers (Moreira *et al.*, 2017), especially in intensive fish farming where increased fish densities in the tanks increase their vulnerability to the spread of pathogens and parasites (Rosa et al., 2012). Parasitic diseases caused by obligate or opportunistic pathogens are a prominent issue with major impact on global finfish and shellfish production, constraints to the production, sustainability, and economic viability of in many regions (Moreira *et al.*, 2017). Mediterranean aquacultures are particularly at risk due to global warming. Indeed, in this region it is expected that pathogen range expansions will stimulate the growth, transmission, and survival of aquatic parasites, and risk overall production in this region

(Rosa et al., 2012). In Portugal, this is also a major risk as we face changing climates even though the majority of the fish production systems are centered in earthen ponds (semi-intensive production), not off-shore systems. Thus, the main external parasites of concern observed in fish farmed in earthen ponds are *Amyloodinium ocellatum* (Dinoflagellate), *Trichodina sp.* (Ciliata), Trematoda (Platyhelminthes), sea lice (Crustacea) and *Gnatia sp.* (Crustacea) (IPMA, 2016).

1.2 Species of interest: seabream (*Sparus aurata*)

One of the most important fish species cultivated in Mediterranean and Portuguese waters is the Gilthead seabream, *Sparus aurata* (Linnaeus, 1758), a saltwater teleost from the sparidae family (Worms, 2022). This species is common in the Mediterranean Sea, being distributed along the Eastern Atlantic coasts from Great Britain to Senegal, and in the Black Sea (FAO, 2009). It can be found in both marine and brackish water environments such as coastal lagoons and estuarine areas (De innocentiis *et al.,* 2005). Artificial breeding was successfully achieved in Italy in 1981-82 and large-scale production of seabream juveniles implemented in 1988-1989 in Spain, Italy, and Greece (FAO, 2009). This species demonstrated a quick adaptability to intensive rearing conditions, both in ponds and cages, and its annual production increases regularly (Oliva-Teles., 2000). The major disease infections reported in these rearing systems are pasteurellosis, vibriosis (*Vibrio sp*.) and lymphocystis (*Lymphocystivirus)*, parasitic enteritis caused by endoparasites (FAO, 2009) and amyloodiniosis caused by the dinoflagellate *Amyloodinium ocellatum* (Moreira *et al*., 2017).

In this present study, we will only focus on the *Amyloodinium ocellatum* (*A. ocellatum*), an obligate ectoparasitic dinoflagellate, that causes sudden mortality in marine fish and represents a central concern in marine fish farming (Nozzi et al., 2016), especially for semi-intensive aquaculture in southern Europe (Moreira *et al*., 2017).

1.3 The lifecycle of *Amyloodinium ocellatum*

A. ocellatum's lifecycle consists in three phases (Fig.1.2). Firstly, the parasites feed as stationary trophozoites (trophonts) on the epithelial surfaces of the skin and gills (Noga, 2012). Trophonts remain attached to the fish by root-like structures (rhizoids) that firmly anchor the parasite to the epithelium. After reaching maturity, the trophonts

detaches from the host, forming a reproductive 'cyst' or tomont. Lastly, this tomont divides, forming up to several dozen free-swimming individuals (dinospores) that can then infest a new host (Noga, 2012). At optimal conditions this process can take between 4 to 5 days (Paperna, 1984).

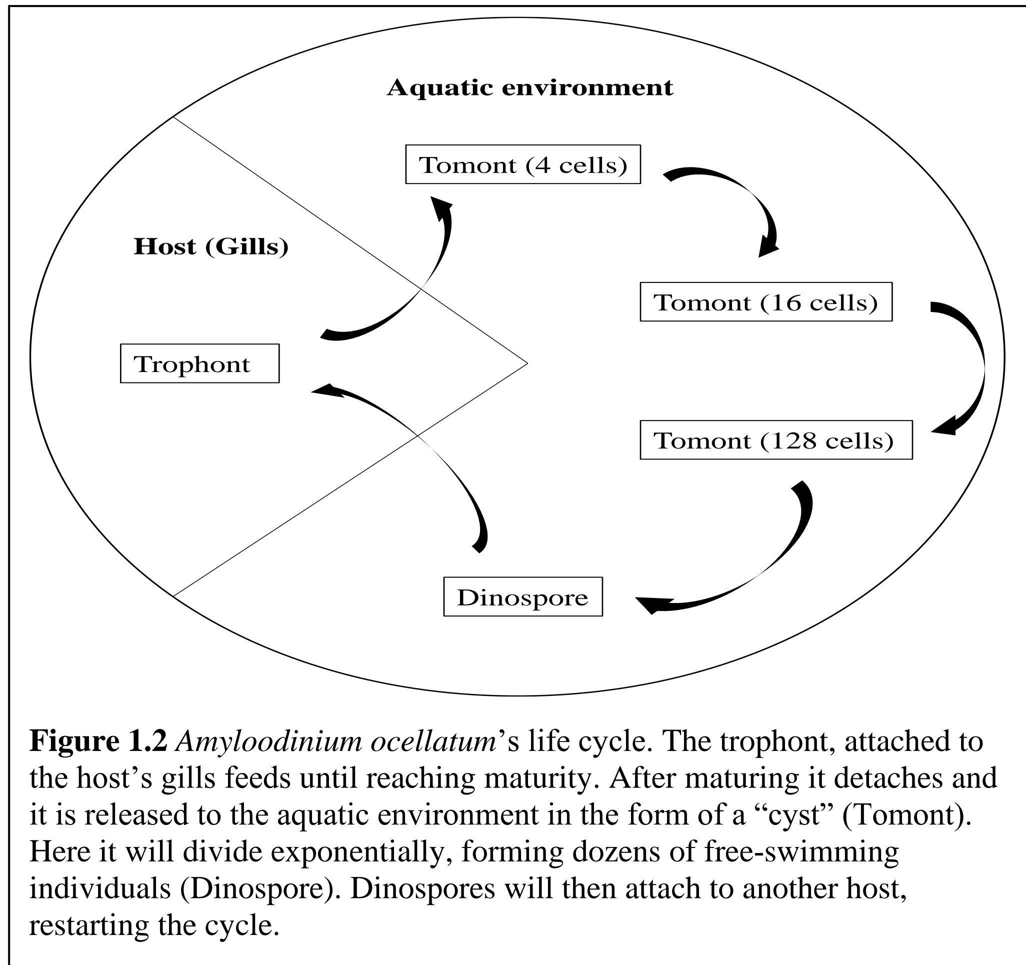

Figure 1.2 *Amyloodinium ocellatum*'s life cycle. The trophont, attached to the host's gills feeds until reaching maturity. After maturing it detaches and it is released to the aquatic environment in the form of a "cyst" (Tomont). Here it will divide exponentially, forming dozens of free-swimming individuals (Dinospore). Dinospores will then attach to another host, restarting the cycle.

Temperature and salinity are the primary environmental modulators of *A. ocellatum*'s pathogenicity, as greater parasitic capacity is observed at higher temperatures (Noga, 2012) and due to this fact, it is more prevalent in the warmer months in regions with a more temperate climate (Levy, 2007). The optimal temperature for its development is between 23ºC and 28ºC, and reproduction stops at about 15º to 17ºC (Levy, 2007). Salinity tolerance ranges from 1 ppt to 70 ppt, but the optimal values for infection are between 10 to 60 ppt (Paperna, 1984).

1.4 *Amyloodinium ocellatum's* infection and treatments

The symptomatology of this disease is characterized by sudden changes in fish behavior, with jerky movements, swimming at the water surface, decreased appetite, increased respiratory rate, and gathering at the surface or in areas with higher dissolved oxygen concentrations (Moreira *et al*., 2017). Fish mortalities are normally attributed to anoxia (associated with serious gill hyperplasia, inflammation, hemorrhage, and necrosis) (Moreira *et al*., 2017), osmoregulatory impairment, and opportunistic bacterial infections associated with the lesions to the gills and skin caused by the parasite (Moreira *et al.,* 2022).

A. ocellatum's infestation can devastate aquaculture businesses (Floyd. R et al., 2011), and fish's morbidity and mortality from amyloodiniosis can be severe, sometimes with a rapid onset over a period of a few days (Floyd. R et al., 2011). Because of this, treatment strategies must be quick and effective. Nowadays the treatments available range from the use of certain drugs like chloroquine diphosphate (Noga, 2012) and treatments with freshwater (Nozzi et al., 2016), copper, and hydrogen peroxide (Floyd. R et al., 2011). These treatments are not effective on all stages of the parasite's life cycle, some cannot be used on fish meant for consumption, and overall have the drawback of inducing severe stress, which impacts the welfare and quality of the fish.

Despite the symptomology and mortality causes are well described and documented, the host physiological and immunological responses to *A. ocellatum*'s infestation are poorly understood (Moreira *et al*., 2017). Acquiring detailed knowledge on this parasite, mechanisms of infection and defense mechanisms of the fish immune system at the gene level will allow to identify biomarkers that may be used to monitor the health of the fish stock and detect in advance eventual outbreaks at fish facilities.

1.5. Candidate biomarkers for detecting A*myloodinium ocellatum* infection

As infection is associated to diverse cellular responses, genes associated to inflammatory responses (e.g. pro-inflammatory cytokines: *Tumoral Necrosis Factor Alpha* (*TNF-α*) and *Interleukin 1 Beta* (*IL-1β*) (Alvarez-Pellitero, 2008)),of hypoxia related-genes (e.g. *Hypoxia Inducible Factor* (HIF) (Piazzon et al., 2019)), oxidative enzymes (e.g. *Cyclooxygenase 2* (*Cox-2*) (Byadgi et al., 2019)), lipid and carbohydrate metabolism (e.g. *Peroxisome Proliferator Activated Receptor Alpha* (*PPAR-α*)) and

immune response genes (e.g. *Immunoglobulin M* (*IgM*) (Byadgi et al., 2019)), are strong candidates for biomarkers of *A. ocellatum* infection.

Pro-inflammatory cytokines are very useful when looking at the inflammatory response of fish following a pathogenic infection (Covello *et al.,* 2009). *TNF-α* is a known mediator of inflammatory and immune defense mechanisms that activates an array of cellular responses including phagocytosis, chemotaxis (Kadowaki *et al.,* 2009), apoptosis, and necrosis (Covello *et al.,* 2009) to eliminate pathogens. As such, it is considered a biomarker to assess the innate immunity of fish (Kadowaki *et al.,* 2009). *IL-1β* is normally produced and released at the early-stage response to infections, lesions or stress and acts as an initiator of pro-inflammatory response (Khansari *et al.,* 2017). It can also stimulate T cell proliferation (Feghali *et al.,* 1997), macrophage proliferation, and leucocyte migration (Covello *et al.,* 2009).

HIF1-α is the oxygen-sensitive and responsive component subunit of the *HIF's*, which are the main regulators of the response to oxygen changes (Zhang *et al.,* 2019). Its functions, in teleost fish, are mainly associated to the response to acute and chronic hypoxia situations (Zhang *et al.,* 2019), as seen in *A. ocellatum*'s infections.

Cox-2 is an inducible inflammation-related enzyme involved in the conversion of arachidonic acid (polysaturated fatty acid present in the phospholipids) into prostaglandins (small lipid molecules with important immuno-modulating properties (Lindenstrøm *et al.,* 2004)) and is involved in the innate immune response (Paredes *et al.,* 2014).

PPAR-α is a nuclear receptor that has an important role in the regulation of beta-oxidation of fatty acids and carbohydrate metabolism (Salmerón *et al.,* 2016). It is predominately expressed in the liver (Oku *et al.,* 2008) and plays a role in homeostatic functions during chronic inflammation responses (Nozzi *et al* 2016).

Finally, *IgM* is the most common immunoglobulin in teleost fish and is considered essential for immune protection against many different pathogens and their routes of infection (Salinas *et al.,* 2021) and it is expressed in all organs (Picard-Sánchez *et al.,* 2020).

1.6 Objectives of this study

The objective of this study is to clarify the molecular mechanisms underlying the seabream physiological response to *A. ocellatum* infestations hoping to pinpoint

candidate markers that may be used to monitor the fish welfare and health in production systems. Thus, this work focused on analyzing the gene expression of candidate genes in gilthead seabream subjected to *A. ocellatum* infection using real-time qPCR.

2. Methodology

2.1 Infection trial

This study was conducted at Estação Piloto de Piscicultura de Olhão (EPPO), a facility of the Portuguese Institute for the Ocean and Atmosphere (IPMA, I.P) (Olhão, Portugal). Forty-two gilthead seabreams, with approximately 140g were transferred to six 70L tanks (n=7 in each tank). Two conditions were studied in this trial, untreated fish (3 replicate tanks) versus *A. ocellatum* infected fish (3 replicate tanks). The tanks were set on a rack, three on the top shelf (control uninfected fish) and three on the bottom shelf (infected fish) (**Figure 2.1**).

Figure 2.1 Trial tanks set-up. Three control tanks on the top, and three infection tanks on the bottom. Trial worked on a closed system, with only aeration being provided. Water was kept at 23ºC, and half of the volume was renewed 24h post infection.

The tanks worked on a closed system with only one aerator in each, and the water was maintained at approximately 23ºC. They were exposed to natural light, which at the time of the trial was the winter photoperiod (10:14h of light/dark).

At 14:30h of 24 January, 16 ml of water containing approximately $78x10^3$/ml of *A. ocellatum* in the dinospore stage of life were added to each of the infection tanks. These

parasites were obtained via previous infections, fish were sacrificed, the first and second gill arches removed and submerged in fresh water. The parasites would then fall off and be collected. Twenty-four hours after infection half of the volume of each tank was subjected to a change of water to ensure its quality.

To assess the infection progression, three fish, one from each replicate tank (infected and control) were sampled at three time points: 24h (infected n=3, control n=3), 48h (infected n=3, control n=3), and 72h (infected n=3, control n=3). And further sacrificed with the objective to analyze the *A. ocellatum* infection on the first and second branchial arch under a light microscope (magnification 40x). The control animals were taken out to keep experimental consistency. From this moment on, the trial remained with 30 individuals (n=5 per tank).

At 72h after infection all fish were sampled for the qPCR analysis. Thus, all fish from each replicate were sacrificed with a precise cut to the head then weighted, measured and samples of liver, spleen, intestine, and gills were extracted. All samples collected were then stored at -80ºC for posterior use and analysis. In total 30 fish were sampled. Only three fish per group were used for the qPCR analysis (infected n=3, control n=3).

2.2 Analysis of *A. ocellatum* infection

The infection of *A. ocellatum* was calculated by counting the trophonts on the first and second branchial arches of the infected fish (n=3). The magnification was 40x and each gill was counted in triplicate with randomized locations on every replicate. In the end, the total number of trophonts counted in each replicate was averaged, and a final concentration of trophonts on the gills was calculated.

2.3 RNA isolation and quantification

Six samples, three from the control group and three from the infected were chosen at random and the following protocol was implemented.

All the extractions were processed using the NZY Total RNA Isolation kit (NZYtech, Lisboa, Portugal).

The samples were taken from the -80ºC freezer and placed in an ice box. Then, an approximate 30mg portion was cut from each organ (gill and spleen). Then weighted, and quickly placed on a sterilized mortar with 40ml of liquid nitrogen. The samples were then

macerated to a fine powder with a pestle, while carefully maintaining the mortar with sufficient liquid nitrogen to cover the sample. Briefly, the tissue powder was transferred to lysis buffer, β-mercaptoetanol. The lysate was applied into a kit provided Homogenization column and centrifuged. Ethanol 70% was then added and mixed with the lysate. The mixture was transferred to a binding column (kit provided), centrifuged, and the flow-through discarded. A DNase digestion mix was then applied to the mix and incubated at room temperature for 20 min. The mix was then washed with kit provided buffers. And the final product (50µl) placed in a microcentrifuge tube. 1µl of RNA from each sample was then quantified using the spectrophotometer series DS-11 (DeNovix, U.S.).

All samples were run on an agarose gel to check the integrity of the RNA samples. For this an 1.5% agarose gel was prepared using 110ml of TAE buffer, 1.65g of agarose, and 2.5µl of GreenSafe (nucleic acid stain, Nzytech) and 3µg of each RNA were run for 40 minutes at 100V. The integrity of the RNA samples was determined by visualization in a Molecular Imager® Gel Doc™ (Bio-Rad, U.S.).

2.4. cDNA synthesis

Each RNA, 7µl (1µg of RNA), was first treated with 1µl of DNase Buffer; 1µl DNase for 30 minutes at 37ºC in the thermocycler to eliminate any contamination of genomic DNA. The treated RNAs were denatured for 10 minutes at 65ºC. And cDNA was produced using 1µl Oligo DT, 1µl dNTP, 4µl FS 5X Buffer, 2µl DTT, 1µl RNase out, and 1µl MMLV-RT in a final volume of 20µl. The reactions were incubated for 50 minutes at 37ºC, following a 15-minute incubation at 70ºC to stop the reaction and inactivate the enzymes. The products were then stored in a -20ºC freezer.

2.5 Primer Design

The sequences of all genes were taken from the NCBI data base, with the exception of the Ribosomal protein S18 (*RPS18*), which was taken from the literature (R. Heredia *et al.*, 2018). Primer design was done using the NCBI BLAST (*BLAST: Basic Local Alignment Search Tool*, cited 2022 May 13) primer tool and with literature search. The primer product size was targeted between 70 to 200 bp and the melting temperature

between 60º and 63ºC. The maximum target amplicon size was kept at 200 bp (Table 2.1). Furthermore, the sequences were seabream specific.

Table 2.1 Gene name, symbol, forward and reverse primer sequences. Primers were either designed using GenBank or taken from literature.

Gene name	Symbol	Sequence (5'-3')	GenBank accession number
Interleukin-8	*IL-8*	(F) GAGCGAGAAGGCAGCTGAA (R) TGCTGCTCATCATTGTGCCT	JX976619
β-Actin	*actβ*	(F) CCCAAAGCCAACAGGGAGAA (R) GCCTGGATGGCAACGTACA	AY362763
Ribosomal protein S18	*RPS18*	(F) AGGGTGTTGGCAGACGTTAC (R) CTTCTGCCTGTTGAGGAACC	R. Heredia, et al, 2018
Cyclooxygenase 2	*Cox 2*	(F) ATCATCAACACTGCCTCCCTG (R) TAACGTCGGGCACGTGAAAG	AM296029
Tumour necrosis factor alpha	*TNF-α*	(F) CAGCGTACCTTGATTTGGTGC (R) ACACGGGTGGAAGAACGAGT	AJ413189.2
Immunoglobuli n M	*IgM*	(F) GCTATTGTCAGGTCCATCGGG (R) AGGTTGACCAGGTTGGTGTTTC	AM493677.1
Interleukin 1-beta	*IL-1β*	(F) ACAGGACAACTGCTGGGAAAA (R) GTGATGGGATGGTGGGCAAT	AJ277166.2
Hepcidin 1	*HEP1*	(F) GTGCTCACCTTTATTTGCCTTCAG (R) CCATACCCCATCTTCCATGACTCT	EF625901.1
Lactate dehydrogenase	*LDH*	(F) ATCCCCTTTGGCACTGGAAC (R) TTCAATGCACCGACTCCTCC	XM_030427018.1
Transforming growth factor beta	*TGF-β*	(F) AGAAATGCGAGCGGACTGAA (R) TCTGTCTCCGAAAGTGACGC	XM_030406468.1
Peroxisome proliferator activated receptor alpha	*PPAR-α*	(F) ACAGTGACCTGGCCCTTTTC (R) AAAAGGAAGGTGTCGTCGGG	AY590299.1
Transferrin	*Transferrin*	(F) ACAGTTGATGGGTGCCTGAAA (R) ACACTTTCCGGCTGTGTACG	GU219852.1
Ribosomal protein *L13*	*L13*	(F) TTCTACTTCCTTTTTCCTTTCGGC (R) CTTTCTGGCTGGCTGGTTG	XM_030415490
Hypoxia inducible alpha subunit	*HIF1-α*	(F) CCCCTCATCTTGCCACCATT (R) TCCCGAGCGACACTTCTTTG	XM_030443698.1

2.6 Real Time- Quantitative Polymerase Chain Reaction (RT-qPCR)

2.6.1 Primer Efficiency Test

The following primers were tested for its efficiency to amplify the target genes (≥90%): β-actin, *RPS18*; *IL-8*, *COX-2*, *TNF-α*, *IgM*, *IL-1β*, *HEP1*, *LDH*, *TGF-β*, *PPAR-α*, *Transferrin*, *L13*, *HIF1-α*. Of these, only the sets of primers for *PPAR-α*, *IL-1β*, *COX-2*, *IgM*, *TNF-α*, and *HIF1-α* were used to analyse gene expression. *RPS18* and *L13* were used as reference genes since their expression is prevalent in the cells consistently and it is not affected by the infection. The rest of the primer pairs did not pass the efficiency test and thus were discarded from this study.

To test the efficiency of the PCR primers and cDNA dilution to use in the RT-qPCR we started by preparing dilutions of the cDNAs (1:1, 1:5, 1:10: 1:25, 1:125 and 1:625). For each of the primer pair to test a PCR mix was prepared containing 11.2µl Primer Forward, 11.2µl Primer Reverse, 89.6µl water and 140µl Sensifast.

The PCR plates were setup as shown below (**Table 2.2**), each well contained 18µl of PCR mix and 2µl of sample (milliQ water in the case of the negative controls) for a total of 20 µl. The Plates were then placed in a thermocycler Biometra TOne series (Analytikjena, Germany) with the following protocol: 3 min at 95ºC, followed by 32

Table 2.2 PCR plate layout. Six primer pairs tested at a time. C (-) represents the negative controls.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1
B	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5
C	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10
D	1:25	1:25	1:25	1:25	1:25	1:25	1:25	1:25	1:25	1:25	1:25	1:25
E	1:125	1:125	1:125	1:125	1:125	1:125	1:125	1:125	1:125	1:125	1:125	1:125
F	1:625	1:625	1:625	1:625	1:625	1:625	1:625	1:625	1:625	1:625	1:625	1:625
G	C(-)	C(-)	C(-)	C(-)	C(-)	C(-)	C(-)	C(-)	C(-)	C(-)	C(-)	C(-)
H												
	Tested Primer 1		Tested Primer 2		Tested Primer 3		Tested Primer 4		Tested Primer 5		Tested Primer 6	

cycles of 10 seconds at 95ºC, 30 seconds at 63ºC, and 10 seconds at 72ºC with a final 5 minutes at 72ºC.

To calculate the primer efficiency, the replicate measurements for each primer, and each dilution, were averaged and plotted in a scatterplot against the dilutions scale in logarithmic format. From these graphs the slope was taken, and the efficiency calculated with the following formula: (10^(-1/slope)-1) x100. The percentage of efficiency were only considered valid when between 90% and 120% and any primer pair below or above that value were discarded.

2.6.2. Gene expression analysis of target genes

The samples utilized were control fish C1, C2, and C3; and infected fish I1, I2, and I3. And the genes tested were *L13*, *RSP18*, *PPARα*, IL-β, *COX-2*, *IgM*, *TNF-α*, and *HIF1-α*.

The cDNA samples were diluted 10x (1:10), to a final volume of 50µl. Next a master mix was prepared for every primer pair with the following protocol: 6 µl Primer Forward, 6 µl Primer Reverse, 96 µl water, and 150 µl Sensifast.

Each well contained 18µl of master mix and 2µl of sample (water, in the case of the negative control) for a total volume of 20µl. Since there was no room for all genes at once there was a need to load a second plate for every replicate, hence why rows C, D, E, and F have two possibilities. The plates were then inserted in a Real-Time PCR thermocycler (Bio-Rad, U.S.).

2.6.3. Data analysis

The data was extracted from the computer, exported, and analysed in excel with the following formula: $2^{-\Delta\Delta CT}$. And later with R. Statistical analysis of the gene expression was performed with a paired t-test for genes that showed a normal distribution, and a Wilcoxon non-parametric test for those that did not exhibit a normal distribution. Significance was set at $p < 0.05$. All statistical tests were performed with R commander.

3. Results

3.1 Analysis of *A. ocellatum* infection

The first and second branchial arches, that were taken from the sampled fish during the infection period, were observed and images acquired. (Figure 3.1) The trophonts were counted, and the total numbers averaged. It is important to note that only the gills of the infected fish were analysed. However, at the end of the trial, the control tank was also checked for potential *A. ocellatum*, contamination. With the averaged numbers the graph below was created (Figure 3.2). It illustrates the evolution and the growth of the infection. It starts at 24h with a concentration of 17±4 trophonts, at 48h there is a peak in the concentration of trophonts with 57±10, and finally at 72h a decline with 43±23 trophonts.

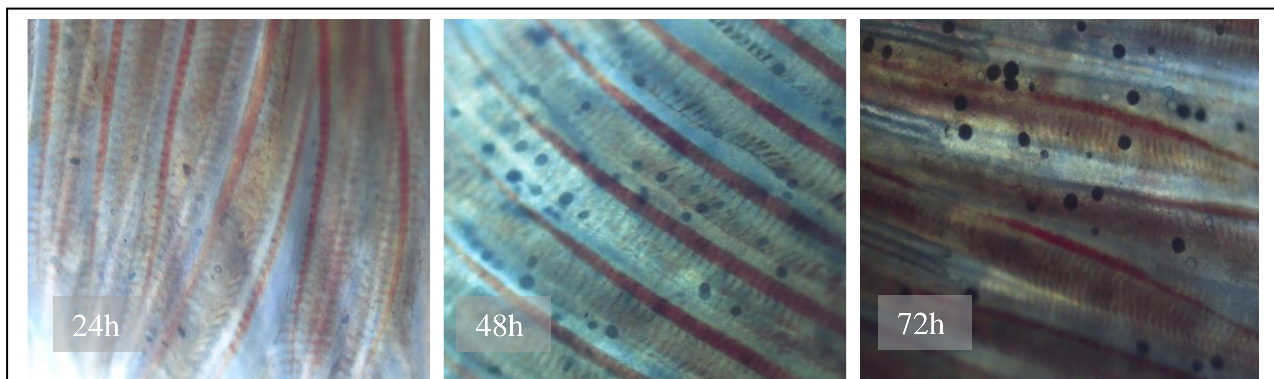

Figure 3.1 Microscope images of the infected fish gills (*Sparus aurata*) (40x). From the left to the right; 24h, 48h, and 72h. The black spots indicate individual trophonts.

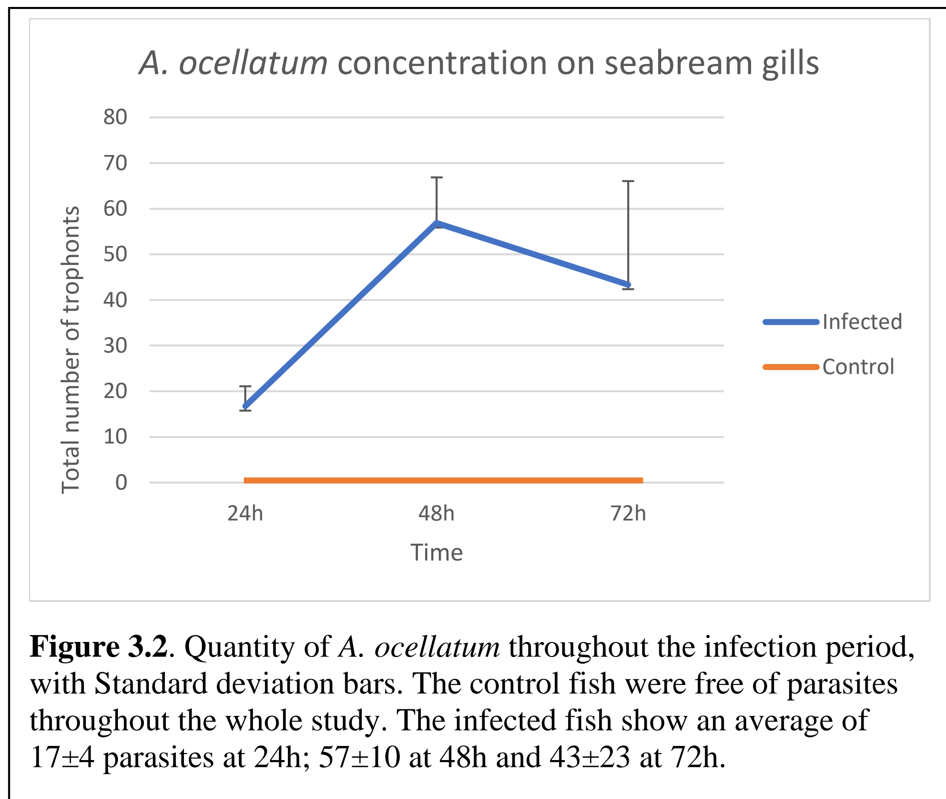

Figure 3.2. Quantity of *A. ocellatum* throughout the infection period, with Standard deviation bars. The control fish were free of parasites throughout the whole study. The infected fish show an average of 17±4 parasites at 24h; 57±10 at 48h and 43±23 at 72h.

3.2. Primer efficiency

The table below (**Table 3.1**) shows the results of the efficiency tests of the primers that were used in the study.

Table 3.1 Tested primers, with the calculated slope of the scatter plot linear regression, and the respective efficiency (%).

Primers	Slope	Efficiency (%)
RSP18	-3.47	93.86
Cox-2	-3.22	104.11
TNF-α	-2.48	152.31
IgM	-3.61	88.99
IL-1β	-2.64	138.95
PPAR-α	-2.97	116.93
HIF1-α	-3.44	95.19
L13	-3.27	102.07

All primers had an efficiency percentage of between 88.99% and 152.31%. And so, they all fell within the optimal value range, with the exception of *TNF-α* (152.31%) and *IgM* (88.99%). Nevertheless, they were used since it was decided that values were not too far from the optimal values.

3.3 Nanodrop and RNA integrity

The RNA concentration and purity of the samples isolated are displayed in (**Table 3.2**).

Table 3.2 RNA concentration (ng/µl) of gills and spleen tissues and ratios of 260/230 and 260/280.

Tissue	**Sample**	**Concentration (ng/µl)**	**[260/230]**	**[260/280]**
Gill	C1	154.504	2.087	2.060
	C2	186.259	2.182	2.074
	C3	72.041	2.184	1.953
	I1	279.063	2.271	2.038
	I2	273.869	2.173	2.062
	I3	247.453	2.085	2.053
Spleen	C1	732.069	2.266	2.202
	C2	360.593	2.216	2.140
	C3	352.481	2.203	2.134
	I1	377.026	2.251	2.129
	I2	228.504	2.074	2.132
	I3	368.490	2.238	2.136

The sample concentrations range from 72.041 to 732.069. The [260/280] ratio is used to access the purity of RNA, a ratio of ~2.0 is generally seen as pure. The RNA [260/230] ratio is a secondary measurement of nucleic acid purity, the expected values for samples to be considered pure is ~2.0-2.2. All samples are within the expected values for these ratios. The integrity was then checked with an electrophoresis gel. The gel images are shown below (**Figure 3.3**).

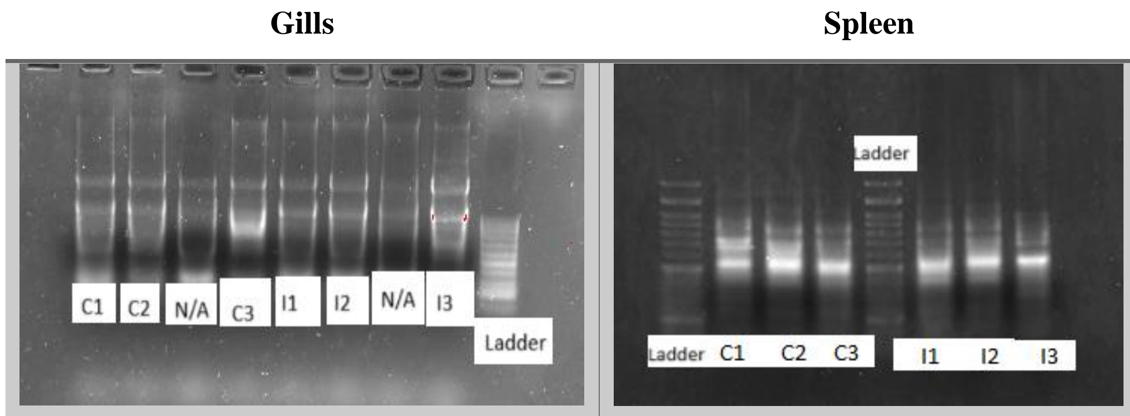

Figure 3.3 RNA integrity assessed by electrophoresis gel. The left graph displays the RNAs isolated from gills from control samples 1, 2, and 3; then infected samples 1, 2, and 3. The N/A represents pipetting errors. The last band represents a DNA ladder. The graph on the right displays the RNAs isolated from spleens. From left to right, the first lane contains a DNA ladder followed by control samples 1, 2, and 3; then infected samples 1, 2, and 3.

3.4. Gene expression (qPCR analysis)

3.4.1. Gills

The expression of the selected housekeeping genes (*L13* and RSP18) in the gills did not vary amongst samples of infected and uninfected fish, and both follow the same

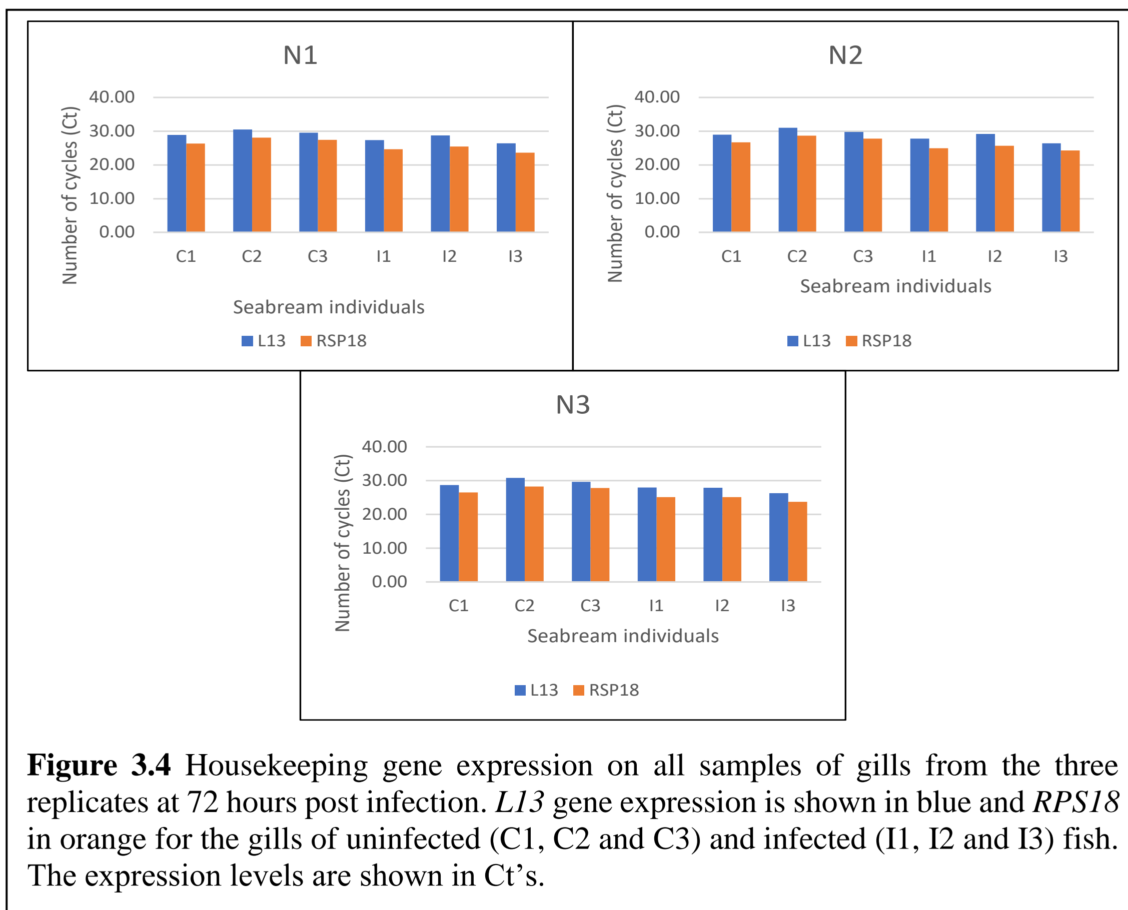

Figure 3.4 Housekeeping gene expression on all samples of gills from the three replicates at 72 hours post infection. *L13* gene expression is shown in blue and *RPS18* in orange for the gills of uninfected (C1, C2 and C3) and infected (I1, I2 and I3) fish. The expression levels are shown in Ct's.

trend (**Figure 3.4**). Because of this, we used the average of the housekeeping gene expression to linearize the gene specific expression of this trial. The figure below (**Figure 3.5**) shows these results.

Overall, we have only detected significant differences in expression between control and infected gills for *IgM* (*p*=0.03351), although in general all genes appeared less expressed in infected fish (except for *HIF1-α*). The *p*Values and fold change of this analysis is shown in **Table 3.3**.

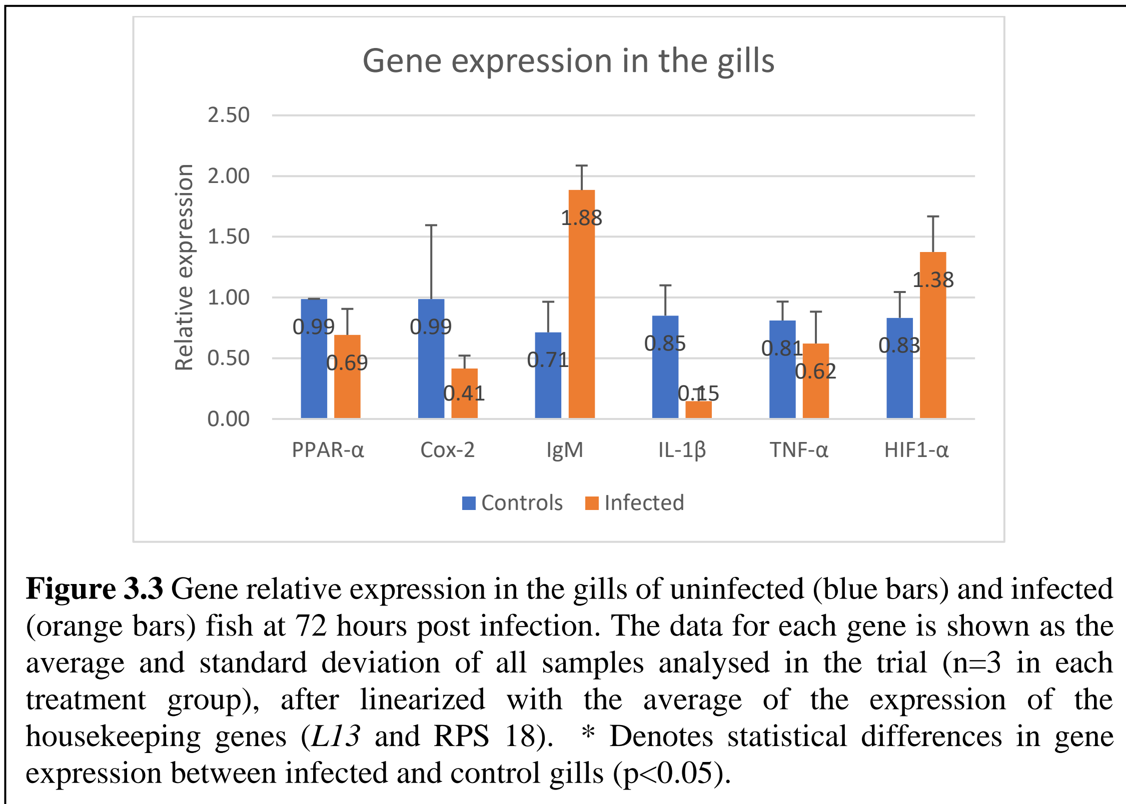

Figure 3.3 Gene relative expression in the gills of uninfected (blue bars) and infected (orange bars) fish at 72 hours post infection. The data for each gene is shown as the average and standard deviation of all samples analysed in the trial (n=3 in each treatment group), after linearized with the average of the expression of the housekeeping genes (*L13* and RPS 18). * Denotes statistical differences in gene expression between infected and control gills (p<0.05).

Table 3.3 Statistical differences in gene expression between control and infected gills. *p* < 0.05 and fold change.

Genes Control *vs.* Infected	**pValue**	**Significant differences**	**Fold change**
PPAR-α	0.25	No	0.70
Cox-2	0.1888	No	0.42
IgM	0.03351	Yes	2.64
IL-1β	0.25	No	0.17
TNF-α	0.3861	No	0.77
HIF1-α	0.1945	No	1.65

~3.4.2. Spleen

As seen in gill samples, both *L13* and *RPS18* were also stable genes to use as housekeeping genes, showing the same pattern and absence of difference between samples of all treatments as shown in **Figure 3.6**.

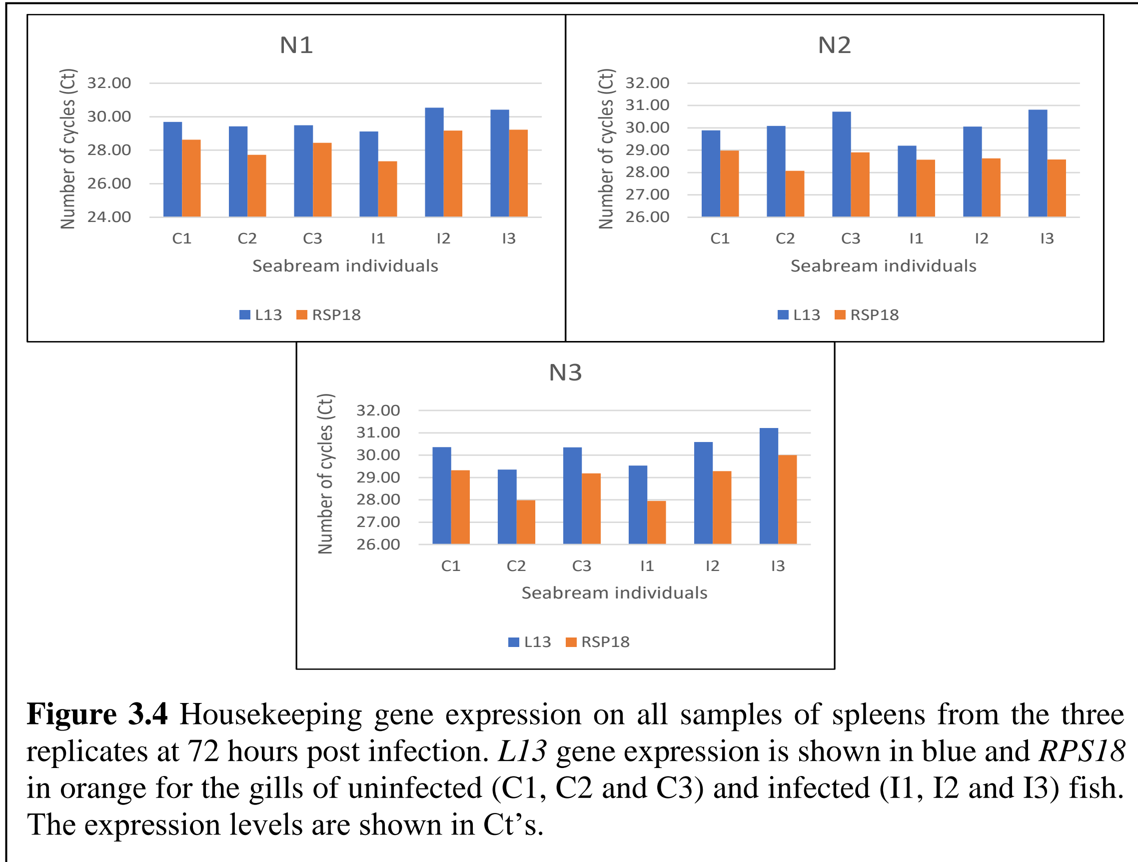

Figure 3.4 Housekeeping gene expression on all samples of spleens from the three replicates at 72 hours post infection. *L13* gene expression is shown in blue and *RPS18* in orange for the gills of uninfected (C1, C2 and C3) and infected (I1, I2 and I3) fish. The expression levels are shown in Ct's.

We did not detect any significant differences in the genetic expression between the spleens of infected and uninfected gene, although in general all genes (except for *Cox-2*) appear to be slightly up-regulated in the infected group as shown in the **Figure 3.7**. The statistical analysis is shown in **Table 3.4**

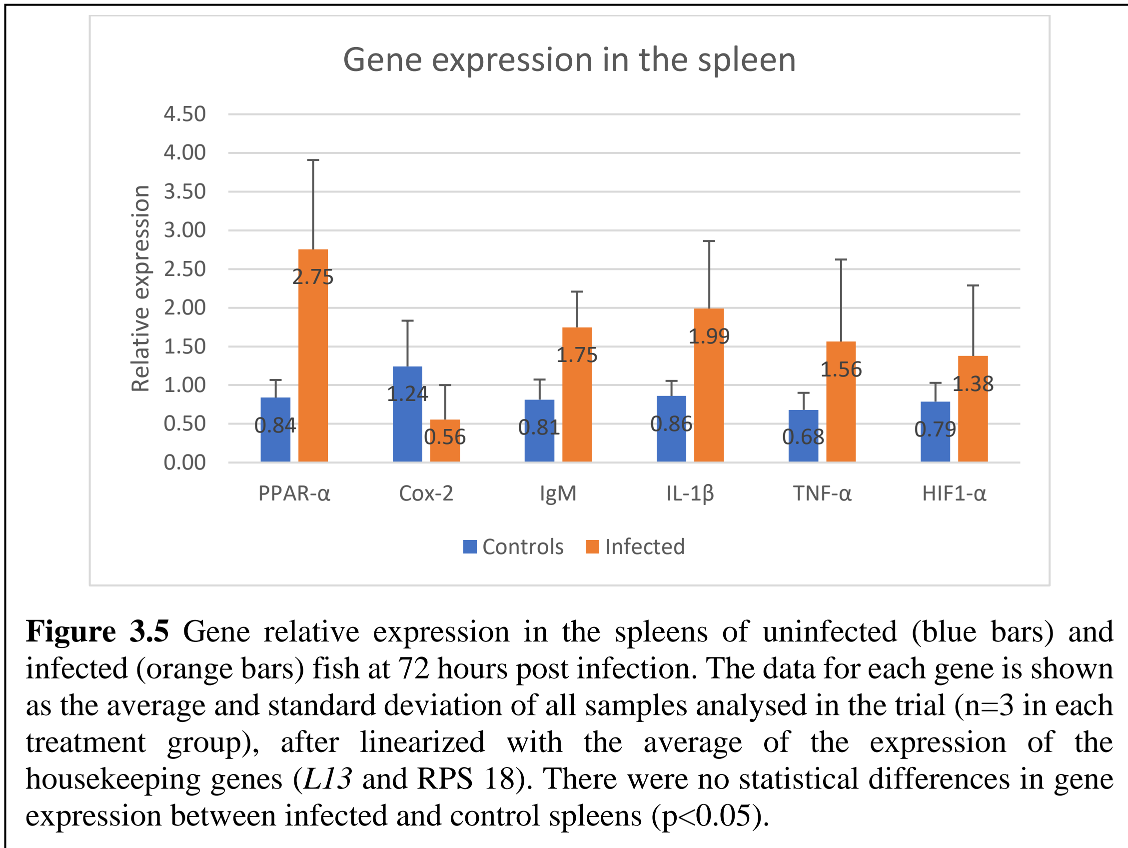

Figure 3.5 Gene relative expression in the spleens of uninfected (blue bars) and infected (orange bars) fish at 72 hours post infection. The data for each gene is shown as the average and standard deviation of all samples analysed in the trial (n=3 in each treatment group), after linearized with the average of the expression of the housekeeping genes (*L13* and RPS 18). There were no statistical differences in gene expression between infected and control spleens (p<0.05).

Table 3.4 Statistical differences in gene expression between control and infected spleens. $p < 0.05$ and fold change

Genes Control vs. Infected	pValue	Significant differences	Fold change
PPAR-α	0.25	No	3.28
Cox-2	0.3573	No	0.45
IgM	0.25	No	2.15
IL-1β	0.1091	No	2.32
TNF-α	0.2593	No	2.30
HIF1-α	0.2165	No	1.75

4. Discussion

4.1. Analysis of *A. ocellatum* infection

In this work we were able to successfully proceed with the infection of gilthead seabream with *A. ocellatum* and test its impact on the gene expression of different immune response related genes. Throughout the 72h, we were able to observe an increase in

numbers and size (not measured) of the trophonts as described in literature (Moreira *et al.,* 2022).

We observed an increase in the average number of trophonts in the gills between 24h and 48h (from 17 to 57, respectively), compatible with the progression of the infection. At 72h though, there was a smaller reduction in the number of parasites found in the gills, which might be due to experimental errors, since we also have high variability in the counts, as demonstrated by the high standard deviations. Nonetheless, we cannot discard the possibility that as *A. ocellatum* reaches the trophont stage they might detach from the gills, between day two and six after infection (Byadgi *et al.,* 2020), thus explaining the reduction in counts observed in this study at 72h. In fact, this detachment from the host's body is not voluntary once they reach maturation. Instead, detachment is triggered by the increasing incapability of the parasite to maintain its attachment on the host tissue substrate (Paperna, 1984), due to friction between the parasite and the water column.

4.2. Genetic analysis

4.2.1 Gills

As gills are the primary substrate for the attachment and maturation of *A. ocellatum*, we expected that this tissue would trigger its defences by increasing the expression of different immune related genes within the first 72h. Interestingly, from all the genes that we have tested, we only detected a significant up-regulation on the gene expression of *IgM* and the same response on the *HIF1-α*, although it did not reach statistical significance when compared to uninfected fish.

IgM is an immunoglobulin that is expressed in diverse tissues and is essential for immune protection against different pathogens and their routes of infection (Piazzon *et al.,* 2016). In the present study *IgM* expression responded to the parasite infection at 72h as initially hypothesized. However, these results are in contradiction with those found with the European sea bass infected with *A. ocellatum*, as only low or non-existent level of *IgM* expression were detected in the gills of infected fish (Byadgi *et al.,* 2019). In this latter study, the authors attributed the results to either a hypothesized negatively influence of the parasite toxins on the systemic specific immune response or to the fact that the disease might have reached its onset too rapidly and the fish did not have enough time to

activate a specific immunological response. Interestingly, in a recent study with gilthead seabream infected with *Sparicotyle chrysophrii*, (an ectoparasite that also attaches itself to the gills) the authors have also failed to link the *IgM* expression levels of infected fish with the infection of this parasite due to the amount of damage induced in the gills (Mladineo *et al.,* 2023). Nevertheless, although the literature studies contrast the results obtained in this study, we cannot discard the possibility that these differences may be related to either differences between fish species immune function, differences in time windows or differences in parasite load.

HIF1-α is commonly used as a fish biomarker for both acute and chronic hypoxia exposure (Hoem *et al.,* 2023), as under these conditions, fish have shown to respond with an up-regulation of this gene (Piazzon *et al.,* 2019). In the present study we have also detected an upregulation of *HIF1-α*, but it did not reach statistical significance. However, as we only analysed three individuals in control and infected fish, it is very likely that the expression levels did not reach statistical significance due to low number of samples analysed or to higher variation in this gene amongst individual fish. Nonetheless, we cannot discard the possibility that as we only measured the gene expression at 72h, when parasite counts started dropping, the expression of this gene might also be lowering and that if we had analysed the tissues at 48h we could have found significant levels of this gene in the gills, in concert with the higher number of trophonts attached to the gills. In fact, this hypothesis aligns with the fact that the time window that we use to link gene expression to any stress factor is determinant. For example, a study on the bottom dwelling carp (*Cirrhinus mrigala*) concluded that a 72h exposure to hypoxia was enough to raise *HIF1-α* expression only moderately, however continuous exposure, up to 168h raised the upregulation of this gene significantly (Varghese *et al.,* 2018). Thus, this study further corroborates that the lack of statistical significance of *HIF1-α* upregulation in the present study may be linked to the fact that we analysed this gene in the wrong time window.

Nevertheless, it is also possible that we focused on the HIF1 subunit, or this gene may be more relevant for the defence response in other tissues but not in the gill. In fact, it has been noted that, depending on the exposure duration to hypoxia, some HIF subunits can be significantly downregulated in some tissues such as the head kidney (Piazzon *et al.,* 2019). In the Nile tilapia (*Oreochromis niloticus*) while *HIF1-α* had higher expression levels in the brain and the heart, but not on the gills (Li *et al.,* 2017).

The two pro-inflammatory cytokines studied, *TNF-α* and *IL-1β*, were down regulated in the infected groups, although they did not reach statistical significance, with emphasis to *IL-1β* which showed the highest reduction in expression compared to the control. The downregulation of these cytokines was not expected but are in accordance with a similar study done with sea bass infected with *A. ocellatum* (Byadgi *et al.,* 2019). In contrast, a study done with striped trumpeter (*Latris lineata*) demonstrated that after an infection with *Chondracanthus goldsmidi*, an ectoparasite, led to the opposite effect as a significant upregulation of both *TNF-α* and *IL-1β* in the gills (Covello *et al.,* 2009) was detected. These results are more in line with the hypothesis set in this study since it is consistent with previous studies that involve the host response to ectoparasites. However, most studies where these cytokines are upregulated in response with parasite infections appear to be tightly linked with long term chronic exposure to infection and to the amount of damage that is done to the host's tissues. For example, in carp (*Cyprinus carpio L.*), it was proposed that the amount of damage done to the host tissues is the most relevant factor to trigger the stimulation of proinflammatory cytokines expression (Gonzales *et al.,* 2007) and that a certain level of mechanical damage must be done in order to trigger a pro-inflammatory response. This was further demonstrated by the fact that, when the authors mimicked mechanical injuries that would be caused by an ectoparasite infection, they found that it was enough to trigger a pro-inflammatory response, even in the parasite's absence. Thus, it is possible that the exposure to the parasite in present study was not long enough to cause the necessary damage to the gill tissue to induce the upregulation of pro-inflammatory cytokines.

In the present study we did not detect significant differences in *Cox-2* expression, although infected fish appeared to have lower levels than uninfected fish. Our initial hypothesis was that *Cox-2* would be stimulated in response to infection as described in previous studies (Byadgi *et al.,* 2019). Indeed, in sea bass infected with *A. ocellatum*, *Cox-2* was significantly up-regulated in the gills, which was suggested to be positively correlated with resistance of the host to the parasite. However, a subsequent study also made with the same species noted that *Cox-2* was unaffected during the infection (Byadgi *et al.,* 2021) as seen in the present study with gilthead seabream. These opposing observations may be related to the fact that *Cox-2* expression appears to be dependent on the expression of *IL-1β* and *TNF-α*, as suggested in rainbow trout infected with *Gyrodactylus derjavini* (Lindenstrøm *et al.,* 2004) and in the European sea bass (Byadgi *et al.,* 2019 and Byadgi *et al.,* 2021). Thus, our results are in line with this hypothesis as

IL-1β, *TNF-α* and *Cox-2* were all decreased in infected gills, although none passed statistical significance.

Lastly, *PPAR-α* as described for *IL-1β*, *TNF-α* and *Cox-2* was also slightly down-regulated in the gills, but again did not pass statistical significance. However, in this case, the results are not surprising as this gene is predominantly expressed in the liver (Oku *et al.,* 2008), not in the gills. In fact, *PPAR-α* has been shown to be less expressed in gilthead sea bream than its counterpart PPAR-γ (Oku *et al.,* 2008), suggesting that future analysis of the latter gene may provide a completely different picture and possibly underline a candidate role for lipid catabolism in this tissue in response to infection.

4.2.2. Spleen

The importance of the spleen during parasite infections has been shown in many studies as a systemic response to fight secondary infections and participate in the wound healing process (Fernández-Montero *et al.,* 2019).

In the present study we did not detect any significant differences in any of the genes analysed in the spleen of infected compared to uninfected fish. Overall, all genes (except *Cox-2*) appear to be slightly up-regulated, however due to the high variability in the expression values of individuals within the infected group, none passed statistical significance. As this was not detected in control fish, we suggest that either there was an experimental factor explaining the variability of this group or that the individuals may have differences in immune response capacity explaining the higher variability in gene expression within this group. This latter possibility would mean that these results should probably be analysed looking at the individual samples and not grouped altogether.

Nevertheless, the apparent up-regulation of the genes analysed in this study are in line with our initial hypothesis and according to the literature. For example, Li *et al.,* (2017) found that *HIF1-α* expression was slightly increased in fish exposed to hypoxia for 12h and 24h of exposure. In Indian catfish (*Clarias batrachus*), a significant increase in *HIF1-α* expression in the spleen was observed but only after long-term exposure to hypoxia (Eissa *et al.,* 2014). Thus, most studies pinpoint that, as referred for the gill's expression, the time window of exposure to the parasites or stress factor is likely key to detect the immune responses of the host, which may explain why we did not detect such prominent responses in the expression of gilthead seabream spleens.

Contrary to the *IgM* expression in the gills, we did not detect significant up-regulation in the spleen of infected gilthead seabream as seen in the gills, although it was slightly up-regulated. We also saw a big variability in expression levels among the infected individuals, which may suggest that the lack of statistical significance may be related to differences in response mechanisms or to low number of individuals analysed. Nonetheless, this slight up-regulation is in line with the results obtained in Atlantic salmon infected with salmon louse (*Lepeophtheirus salmonis*) where the *IgM* transcripts were up-regulated in the spleen at later stages of the infection (up to 15 days) (Tadiso *et al.,* 2011). Thus, as the authors suggest, the adaptive immune response may be triggered at later stages of infection, which could also explain the lack of significant stimulation of *IgM* in the present study after 72h. Nevertheless, the significant response of *IgM* in the gills at 72h, the site of infection, and the slight up-regulation in the spleen do suggest that if we had analysed the spleens of these animals a few days after we would have probably detected a significant response in the spleens of infected fish. This appears to be the case of European sea bass and *Ceratothoa oestroides* (a parasite found in the tong) (Piazzon *et al.,* 2021), where *IgM* levels were significantly higher in the tong (the site of infection) compared to the spleens. It seems then, for present study, that *A. ocellatum* triggers a relatively strong immune reaction at the site of infection but have yet not triggered its full systemic response by 72h.

The expression of both pro-inflammatory cytokines (*TNF-α* and *IL-1β*) was not significantly different between infected and uninfected fish as seen in the gills, despite slight up-regulation 72h after infection. The response of these cytokines to infection is well described in fish. For example, in rainbow trout (*Oncorhynchus mykiss*), infected with *Ichthyophthirius multifiliis*, a significant stimulation was seen in *IL-1β* and *TNF-α* expression in the spleen after 24h and 12h, respectively (Sigh *et al.,* 2004). In rainbow trout infected with amoebic gill disease, similar results were also found, although *IL-1β* stimulation was only detected at later infection stages (Bridle *et al.,* 2006). Thus, it appears that the expression of these cytokines responds strongly to infectious processes, although the timing at which the response is triggered may vary with the stimulus and possibly with the fish species studied. Thus, the lack of significant response in the present study may indicate that if we had analysed a different time scale, we would have possibly found results similar to those described in the literature.

As seen in the gills, *Cox-2* levels are not significant different between infected and uninfected fish. However, as referred previously, if in gilthead sea bream *Cox-2*

expression is linked to that of *IL-1β* and *TNF-α* as suggested by Lindenstrøm *et al.* (2004) and Byadgi *et al.* (2019, 2021) then, it is not surprising as none of these genes were significantly modified. However, we cannot discard the possibility that *Cox-2* may respond to the parasite within the first hours of infection as seen in rainbow trout infected with *Myxobolus cerebralis* (Severin *et al.,* 2007). Indeed, the authors found that there was an up-regulation of *Cox-2* until 2h of infection, after which it returned to control levels.

Finally, *PPAR-α* was also not significantly modified by parasite infection in the spleen, although this gene was the one that appeared to be up-regulated. However, due to the high variation in levels among individuals of the infected group the group average did not reach statistical significance. Nonetheless, these results suggest that *PPAR-α* may play a role in infection, possibly by modelling the energy levels of the spleens of gilthead sea bream through lipid metabolism as detected earlier in the livers of sea bass infected with *A. ocellatum* (Nozzi *et al.,* 2016). Nonetheless more samples and different time points should be analysed to support this hypothesis.

Altogether, this study focused on the early responses (72h) of the gills and spleens of gilthead sea bream to an *A. ocellatum* infection. The study of the expression of different immune-related factors in these tissues only showed a strong response of *IgM* in the site of parasite infection (gills), and no significant response was detected in the spleens. Nonetheless, given the fact that we could only analyse an *n*=3 in infected and uninfected groups it is possible that some of these genes may be responding to the parasite infection but, the lack of individual samples per group did not provide for statistical power to detect it. In addition, as we only focused one time point (72h post infection), it is possible that these genes may be responding significantly sooner (e.g., 6h, 12h and 18h) or later (e.g., 5 days, 7 days, and 15 days) as seen in the literature. For this reason, we suggest increasing the number of fish in the trial to be able to extend the sampling and increase the group *n* at each time point.

5. Conclusions

In this study it was aimed to search potential candidate for biomarkers that can be used to monitor fish health and welfare when exposed to *A. Ocellatum*. The results of the 72h parasitic exposure on the gill tissues showed an up-regulation in *IgM* expression indicating that a short but acute exposure to the parasite was enough to activate an immune reaction. *HIF1-α* appears to have a tendency to be up-regulated but had no statistical significance. *IL-1β*, *TNF-α* appear to have a tendency to be down-regulated, however they were also not statistically significant. In the case of *PPAR-α*, the fact that this gene is mainly expressed in the liver, and not the gills, can explain a statistically insignificant down-regulation. Finally, *Cox-2* had no significant differences in expression.

Regarding the spleen tissue no genes were found to have a statistically significant variations in expression. However, all genes have a tendency to be up-regulated in the infected fish, except *Cox-2* that appears to be down-regulated.

In this study it was not possible to clarify with certainty if any of these candidate genes are good biomarkers. However, *IgM* and *HIF1-α* seem to be the ones with most potential.

This work opened the door to interesting future study possibilities. Namely increasing the sample size; adding more sampling points before and after 72h; analysing more tissues, such as liver, head-kidney, and intestine; altering the parasite load or designing a chronic exposure experiment; and finally studying new genes, such as the ones excluded from this work or different subunits of studied genes.

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