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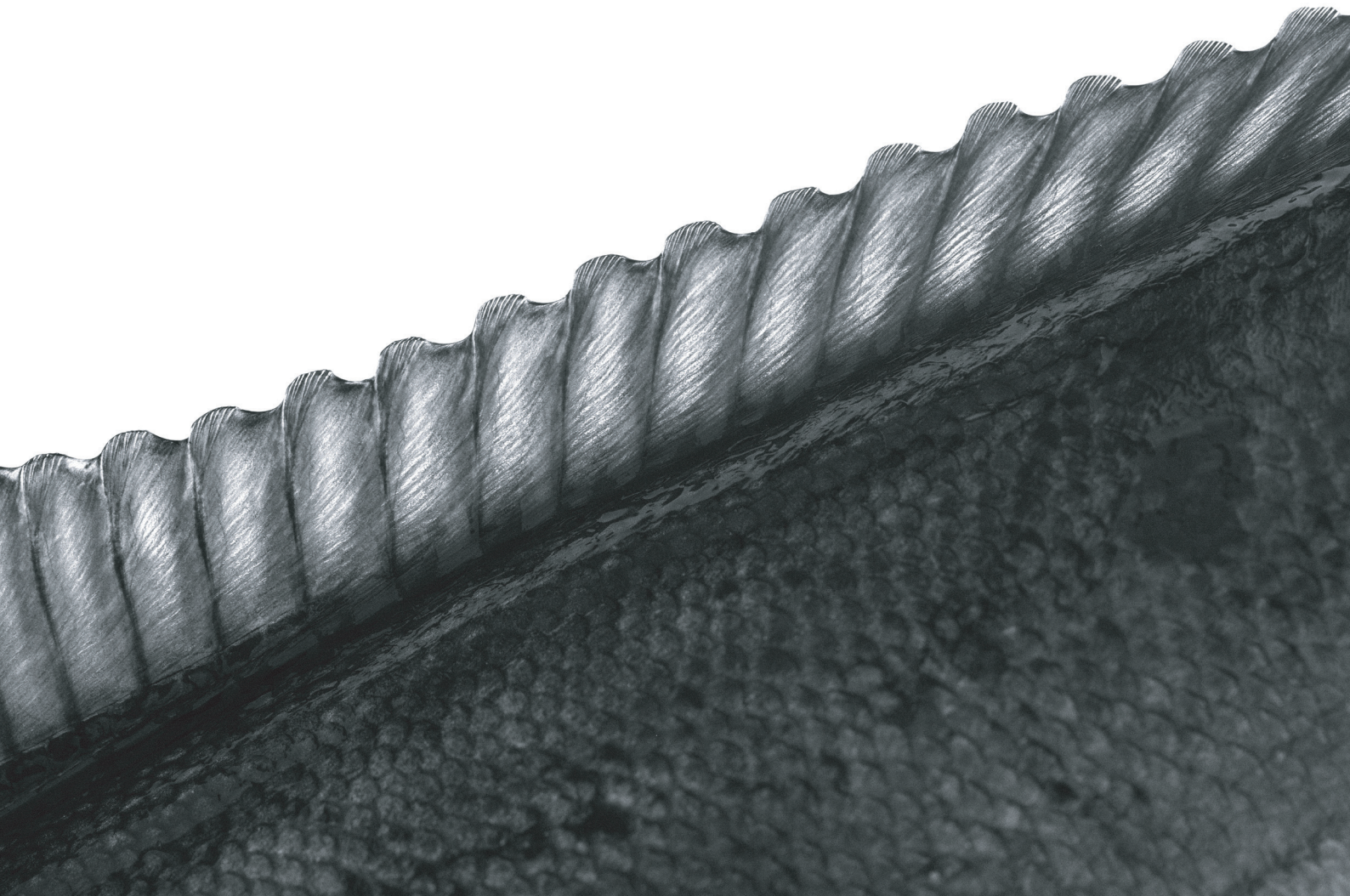

PhD Thesis

Skin associated effects of Neobenedenia girellae infection in greater amberjack and potencial use of functional diets for reducing parasite incidence

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A mi familia, los pilares mas sólidos sobre los que levantar mi ciencia

七転び八起き “Cae siete veces, levántate ocho” proverbio japonés

Abstract thesis

Nowadays, aquaculture is considered the fastest growing animal production sector. It follows consumer and market demands as well as adapts to climatic and geographic circumstances in harmony with nature and society. However, the European aquaculture needs a diversification on the number of species intensively produced, as there is a reduced number of marine species produced. Thus, it is necessary to find new candidate species to diversify the European aquaculture industry. This can be achieved by enhancing husbandry, welfare, technology and management knowledge of the candidate species.

Greater amberjack *(Seriola dumerili)* is considered one of the best candidate species for European aquaculture due to its fast-growing condition, flesh quality and acceptance in the market. Nevertheless, ectoparasite infections suppose an important bottleneck during greater amberjack on-growing period, being the monogenean ectoparasite *Neobenedenia girellae* infection considered the most frequent disease of this species. Moreover, *N. girellae* infection often is accompanied of bacterial secondary infections and, consequently, of high production losses due to high mortalities.

Thus, the objective of the present thesis was to investigate the effects of *N. girellae* infection and its relationship with the robustness of the greater amberjack juveniles, using functional diets as a tool for boosting fish health, stress resistance and reducing the parasite incidence. For that purpose, the following specific objectives were addressed: i) to evaluate greater amberjack stress response to face variations in culture conditions (Chapter 3), ii) to determine the effect of *N. girellae* infection in the morphology of greater amberjack skin (Chapter 4) and in the protein profile of its skin mucus (Chapter 5); iii) to evaluate the potential of functional additives: a) to boost the immune system and to increase the stress response through the validation of specific biomarkers and b) to improve the resistance of greater amberjack to *N. girellae* infection (Chapters 6, 7 and 8).

The lifecycle and the outbreaks of *N. girellae* n aquaculture are regulated by environmental and rearing conditions, like temperature, stocking density or the nutritional status of fish. To assess how greater amberjack's robustness is affected by variations in culture conditions, a first study was conducted using three different trials (Chapter 3). First trial consisted on a long-term rearing of 120 days at different temperatures (17°C, 22°C or 26°C) to assess if

long-term acclimation to rearing temperature is affecting the stress response of greater amberjack juveniles and to determine the basal level of circulating cortisol concentration and the expression of *muc-2* gene. The second trial was designed to study the stress response of greater amberjack after 90 days of rearing at different stocking densities (4 $kg \cdot m^{-3}$ or 8 $kg \cdot m^{-3}$) and were subjected to different protocols of handling (one group was sampled weekly and other group was sampled monthly), by inducing a shallow water stress test after the rearing period for evaluating the circulating cortisol response and the expression of *muc-2* gene in skin. The third study assessed the effect of a short-term (10 days) fasting challenge on greater amberjack stress response, by challenging fish against a shallow water stress test and evaluating the circulating cortisol response and the expression of *muc-2* gene in skin. A long-term acclimation period to temperature did not affect plasma or mucus cortisol levels and *muc-2* gene expression, basal levels of plasma cortisol being determined as 7.5 $ng \cdot ml^{-1}$ for juveniles of this species. A positive correlation between plasma and skin mucus cortisol was also found, denoting the potential of skin mucus cortisol concentration as indicator of stress in this species. Furthermore, final high stocking density (11.5 $kg \cdot m^{-3}$) induced an elevation ($p<0.05$) of plasma cortisol, a slower recovery after 24 hour after stress of basal cortisol levels and a reduction ($p<0.05$) of weight gain, although did not induce differences in the expression of *muc-2* gene in skin. However, a tendency to an up-regulation was observed in fish subjected to the high-density rearing treatment. Short-term fasting induced an increase ($p<0.05$) of plasma and skin mucus cortisol during stress acute phase together with a down-regulation and a lower responsiveness of *muc-2* gene in the skin.

In order to understand the effects of *N. girellae* infection in the skin of greater amberjack, a parasitization trial was conducted for studying the morphological alterations caused in the cranial and dorso-lateral regions pre and post parasite infection (Chapter 4). Morphological studies were conducted using optical, transmission and scanning electron microscopy. The studies carried out demonstrated clear differences between the structure of cranial and dorso-lateral region, being the cranial region the most susceptible region to be parasitized due to an absence of scales and a lower goblet cells density.

Furthermore, *N. girellae* fixation disrupted the structure of epidermal epithelial cells by overpressure. The epithelial cells from the surface of the *stratum superficiale* located near the parasite presented a clear cell degradation process, associated in some cases with epithelial cell detachment. Surrounding the fixation area, it could be observed a focal epidermal

hydropic degeneration and in the most severe cases punctual development of spongiosis. Moreover, focal mobilization of leucocytes to the fixation areas could be observed.

To determine the changes induced by *N. girellae* infection in greater amberjack skin mucus, a proteomic study was conducted by combining 2-DE-MS/MS and LC-MS/MS methodologies (Chapter 5). In addition, protease activity was determined, and proteases characterized. Skin mucus microbiome was obtained by specific blast of the LC-MS/MS data against bacterial database. The 2-DE-MS/MS identified 69 and 55 proteins in non-infected and infected fish respectively, showing an increase of structural proteins, mainly keratins, which were specifically degraded in parasitized fish. Additionally, and in agreement with the described selective degradation of structural proteins, skin mucus of infected fish showed a higher protease activity due to a higher serine and metalloprotease activities. Moreover, LC-MS/MS showed 959 and 357 proteins in the mucus of healthy and parasitized greater amberjack, respectively. The functional analysis of these proteins demonstrated an over-representation of proteins related to cytoskeleton in *N. girellae* infected fish. Other proteins related with apoptosis, certain metabolic pathways such as protein, carbohydrate or lipid metabolism, stress response and immunity were detected in parasitized and no parasitized greater amberjack skin. The bacterial distribution of the skin mucus microbiome was not affected at least at a taxonomic genus level, being *Pseudomonas* the most representative genus found.

Once the alterations induced by *N. girellae* to greater amberjack skin were described, three different studies were conducted with the main objective of boosting the immune system and reducing *N. girellae* infection incidence.

Firstly, after the cloning and characterization of greater amberjack piscidin as part of the group 3 piscidins (Chapter 6), baseline piscidin gene expression was analyzed in different tissues, being spleen and gut the tissues which presented a higher gene expression. A recombinant greater amberjack piscidin was produced and challenged against *Aeromonas salmonicida, Vibrio anguillarum, Lactococcus garvieae, Photobacterium damselae, Yersinia ruckeri* and *Escherichia coli,* obtaining a marked bacteriostatic effect (collaboration study with the University of Aberdeen within the context of the Diversify project). Moreover, an in vivo PAMPs stimulation was conducted using poly I:C, LPS and flagellin. Flagellin induced an increase of piscidin transcript in in the gills and the head kidney, whereas poly I:C induced an increase of piscidin transcript in the gut, the head kidney and the spleen. LPS only

induced an increase of piscidin transcript in the head kidney. On the other hand, *in vitro* head kidney PAMPs stimulation resulted in piscidin up-regulation after 24 hours especially with flagellin and LPS. In contrast, spleen cells up-regulated piscidin gene expression after 4 h of stimulation with poly I:C or flagellin, and remained up-regulated to 24 h after flagellin exposure, but returned to baseline levels by 12 h using poly I:C which highlights the different stimulation patterns of those tissues (Chapter 6). The validation of the expression of the piscidin gene as a biomarker was studied through the use of functional additives in the diet. For that purpose, the diets for greater amberjack juveniles were supplemented with dietary MOS (5g·kg^{-1}), cMOS (2g·kg^{-1}) or a combination of both additives (5g·kg^{-1} and 2g·kg^{-1} respectively) during 30 days. Dietary MOS increased the expression of piscidin gene in the spleen, whereas dietary cMOS up-regulated piscidin transcript levels in the gills and the head kidney, whilst the dietary supplementation of the blend of both the MOS and the cMOS up-regulated piscidin gene expression in the gut, when compared to fish fed the non-supplemented diet.

Taking into account the positive effect of dietary functional additives in the modulation of piscidin gene expression, and in order to boost systemic and mucosal immune system to reduce *N. girellae* infection, a study for 90 days of dietary supplementation with MOS (5g·kg^{-1}), cMOS (2g·kg^{-1}) or a blend of both additives was conducted (Chapter 7). Within this study, the expression of several immune-related genes was analyzed in skin, gills, posterior gut, spleen and head kidney together with an analysis of serum and skin mucus lysozyme and bactericidal activities. Furthermore, a 15 days parasitization challenge was conducted after the functional diets supplementation to evaluate the parasitization load and the possible effects on the parasite size. Although dietary additives did not induce effects in growth performance after 90 days of supplementation, cMOS significantly increased serum bactericidal activity, and specifically reduced greater amberjack skin parasite load, parasite total length and the number of parasites detected per unit of fish surface, whereas no effect of MOS was detected on those parameters. Regarding the 17 immune genes studied, dietary cMOS tendered to increase the response of mucosal tissues such as skin and gills, up-regulating the expression of hepcidin, defensin, Mx protein, interferon-γ (*ifn* γ), mucin-2 (*muc-2*), interleukin-1β (*il-1β*), ***il-10*** and immunoglobulin-T (*IgT*) genes. On the other hand, dietary MOS induced an up-regulation of the expression of spleen and head kidney immune-related genes, including piscidin, defensin, *iNOS, Mx protein*, interferons, *il-1β, il-10, il-17* and *il-22.* In posterior gut, both dietary MOS and cMOS both induced and up-regulation of *il-10, IgM* and *IgT* transcripts, MOS also

increasing piscidin, *muc-2,* and *il-1β,* whilst cMOS increased the expression of hepcidin, defensin and *IFNγ* genes. Fish fed the dietary mixture of both additives presented similar gene expression levels to those fish fed the non-supplemented diet in all tissues, probably in relation to an overstimulation effect.

For the last study, a different functional additive was assayed. Two trials were conducted to assess the effects of the dietary use of a phytogenic (HERB, 200ppm) or cMOS (2g·kg^{-1}) for 70 days on greater amberjack growth performance, stress response, parasitization and immune response (Chapter 8). In the first one a stress challenge test was performed after 70 days of dietary supplementation of the functional additive. For the second trial, after 70 days of dietary supplementation of functional additives, a parasitization challenge by cohabitation with experimentally infected fish was conducted for 15 days, removing the parasites of the experimental fish for obtaining the number of parasites per surface unit and parasite total size. Additionally, skin from parasitized greater amberjacks was sampled for gene expression analyses. The functional additives had no effect on greater amberjack growth performance. However, HERB dietary supplementation significantly reduced plasma cortisol levels after 3 hours of cage confinement compared with the other treatments, whereas dietary cMOS reduced the parasite load and total parasite size. Moreover, both additives increased the skin mucus protease activity before parasitization and the lysozyme activity after parasitization. Regarding the immune-related genes studied, HERB up-regulated the expression of *piscidin* previously to challenging the experimental fish with *N. girellae*. However, after parasitization, HERB induced an up-regulation of *muc-2* and *pis*, whereas the expressions of *tnf-α, il1- β, hep, c3, cd8* and *casp3* genes were up regulated by both functional additives. Moreover, the expressions of hep*, IgT, cath, c3, cd8* and *casp 3* genes were negatively correlated with the parasite load.

In conclusion and according with the data obtained on this thesis, the stocking density for greater amberjack juveniles must be lower than 11.5 kg·m^{-3} to keep the growth performance and to improve the recovery after stressful situations. After acclimation to culture conditions, 7.5 ng·ml^{-1} can be considered as the basal plasma cortisol concentration for greater amberjack juveniles, being circulating plasma cortisol correlated to mucus cortisol concentration under the different culture conditions assayed. A short-term starvation induced deleterious effects on greater amberjack skin mucin production and circulating cortisol after a stressful situation.

N. girellae fixation induced epidermal thickening, increased of goblet cells density, and alteration of the *stratum superficiale* structure, favoring the presence of hydropic degeneration and leucocyte-type cells foci in *stratum basale.* Moreover, due to its fixation, an increase of the number of cytoskeletal proteins in skin mucus was detected, whereas skin mucus microbiome profile was not altered at a taxonomic level of genus. In addition, *N. girellae* degraded greater amberjack skin mucus structural proteins, mainly keratins, and increases the activities of mucus metallo- and serine-proteases.

The use of dietary cMOS at 2 g·kg^{-1} for 90 days reduced the incidence of *N. girellae* in relation with an up-regulation of immune-related biomarkers in skin, including proinflammatory cytokines, *hep, muc-2* and *IgT* transcripts. In contrast, the use of dietary MOS (5 g·kg^{-1}) for 90 days up-regulated the expression of selected biomarkers in head kidney, spleen and posterior gut, including ***pis*** and proinflammatory cytokines genes, with no remarkable effects on other mucosal tissues such as skin or gill. However, the booster capacity of the immune system induced by the single dietary use of either cMOS or MOS is limited by the combination of these two functional additives in greater amberjack diets. In addition, dietary cMOS reduced *N. girellae* load, which is correlated with an up-regulation of *hep, IgT, cathelicidin, cd8* and *c3* transcripts in skin, validating them as the best biomarkers for greater amberjack against this parasite infection. Other additives as the phytogenic “HERB” at 200 ppm for 70 days ameliorated the acute stress response of greater amberjack after stress in terms of concentration of plasma cortisol. Furthermore, in response to *N. girellae* infection, HERB and cMOS boosted the SALT immune response of greater amberjack through the up-regulation of AMPs, *muc-2, IgT*, and pro-inflammatory cytokines transcripts.

List of abbreviations

GnRHa	Gonadotropin releasing hormone agonist
PAMP	Pathogen associated molecular pattern
DAMP	Damage associated molecular pattern
MALT	Mucosal associated lymphoid tissue
SALT	Skin associated lymphoid tissue
GALT	Gut associated lymphoid tissue
APP	Anti-parasitic peptide
ANOVA	Analysis of variance
PERMANOVA	Permutational analysis of variance
cDNA	Complementary DNA
CT	Cycle threshold
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization
FCR	Food conversion ratio
FI	Feed intake
LC-MS/MS	Liquid chromatography-mass spectrometer
2D-MS/MS	2 dimensional gel- mass spectrometry
H&E	Hematoxylin and eosin
HK	Housekeeping
PCR	Polymerase chain reaction
qPCR/RT-PCR	Quantitative real time polymerase chain reaction
SD	Standard deviation
SGR	Specific growth rate
Tm	Melting temperature
ULPGC	Universidad de Las Palmas de Gran Canaria
Ca2+	Calcium
Fe2+	Iron
c3a	Complement 3 a protein
HPI	Hypothalamus-pituitary-interrenal
PRR	Pattern recognition receptor
TLR	Toll like receptor

CLR	C-type lectin receptors
NF-κβ	Nuclear factor-κβ
MAPK	Mitogen-activated protein kinase
MOS	Mannan oligosaccharides
cMOS	Concentered mannan oligosaccharide
PCTM	Parque científico tecnológico marino
BOE	Boletín oficial del estado
RAS	Recirculation aquaculture system
GIA	Grupo de investigación en acuicultura
PAS	Periodic acid-Schiff stain
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
il1-β	Interleukin 1β
il-8	Interleukin 8
il-10	Interleukin 10
il-17af	Interleukin 17f
il-17d	Interleukin 17d
il-22	Interleukin 22
def	Defensin
hep	Hepcidin
pis	Piscidin
IgM	Immunoglobulin M
IgT	Immunoglobulin T
mx	Interferon-induced mx protein
iNOS	Inducible nitric oxide synthase
ifn-d	Interferon type I
ifn-γ	Interferon γ
tnfα	Tumor necrosis factor α
ef-1α	Elongation factor-1α
β-actin	β-actin
muc-2	Mucin-2
cath	cathelicidin
c3	Complement component 3

cd8	Cluster of differentiation 8
casp-3	Caspase 3
StAR	Stereidogenic acute regulatory protein
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
HCl	Chlorhydric acid
TRIS	trisaminomethane
CHAPS	3-[(3cholamidopropyl)dimethylammonio]-1-propanesulfonate
EDTA	Ethylenediaminetetraacetic acid
PMFS	Phenylmethylsulfonyl fluoride
BSA	Bovine serum albumin
VIE	Visible implant elastomer
PCA	Principal components analyses
PCO	Principal coordinates analyses
NMDS	Non-metric multidimensional scaling
AMPP	Antimicrobial polypeptide
LDLM	Low density low manipulation
LDHM	Low density high manipulation
HDHM	High density high manipulation
HDLM	High density low manipulation
A+	Apparent satiety feeding
A-	Starving fish
ATP	Adenosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
AA	Anterior attachment organ (adhesive glands)
PH	Pharynx
HAP	Haptor
AS	Posterior attachment structures
M	Marginal valve
H	Hooks (hamuli)
AN	Anchor (accessory sclerites)
PA	Parasite
EP	Epidermis
DER	Dermis

SC	Scales
SS	Stratum superficiale
SSP	Stratum spinosum
SB	Stratum basale
IS	Intercellular spaces
N	Nucleus
C	Cytoplasm
AJ	Adherens junction
TJ	Tight junctions
D	Desmosomes
MR	Micro-ridges
EL	Epidermal lacerations
GC	Goblet cell
MS	Mucosome
SM	Secreted mucus
CHR	Chromatophores
HD	Hydropic degeneration
IEL	Intraephitelial lymphocyte
NP	No-parasitized
P	Parasititized
PSM	Peptide spectral matches
FDR	False discovery rate
PMF	Peptide mass fingerprinting
GO	Gene ontology
MBS	Marine biosecurity station
MCMC	Markov chain Monte Carlo
PBS	Phosphate buffered saline
LPS	Lipopolisaccharide
p perm	Permutational p-value
TGFb	transforming growth factor b
MHCII	Major histocompatibility complex II
TCRb	T-cell receptor b
EO	Essential oil

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Stratum superficiale presented flat-shaped cells with irregular a centric/parallel to the basal membrane nucleus. Observe the higher density of goblet cells (*) on greater amberjack dorso-lateral region compared to cranial region (C vs D). Alcian blue-PAS-GIEMSA and toluidine blue, pH: 2.5. Scale bar= 50 µm. EP: epidermis; DER: dermis; SC: scales; SB: *stratum basale;* SSP: *stratum spinosum*; SS: *stratum superficiale.* ***Page 79.***

Figure 4.4. (A) Semithin section of *Neobenedenia girellae* haptor fixed to greater amberjack *(Seriola dumerili)* skin. Observe the induced cellular disruption and disorganization of epidermal *stratum superficiale* (®) in relation to a non-parasitized greater amberjack (B). Alcian blue-PAS-GIEMSA, pH=2.5. Scale bar= 25 µm. HAP: haptor; EP: epidermis; DER: dermis. ***Page 80.***

Figure 4.5. Detailed micrographs of greater amberjack (*Seriola dumerili)* parasitized and non-parasitized skin. (A) surface epithelial cells of a greater amberjack parasitized skin. Observe the partially digested surface epithelial cells (*) compared to non-parasitized fish skin (B). Alcian blue-PAS-GIEMSA, pH=2.5. Scale bars= 25 µm. (C) *Stratum superficiale* epithelial cells of non-parasitized skin areas, observe the maintenance of the cellular structure and micro-ridges. Scale bar= 2 µm. (D) Detail of the cell structure of non-parasitized areas with tight junctions, adherens junctions and desmosomes still maintaining their structure and cells cohesion. Scale bar= 500 nm. (E) TEM micrograph of surface parasitized greater amberjack (***Seriola dumerili)*** epithelial cells in contact with *Neobenedenia girellae* haptor, where focal acantholytic processes represented as intercellular spaces (Δ) could be observed with degraded cells (*). Scale bar= 10 µm. (F) Detailed TEM micrograph of parasitized greater amberjack degraded cells (*) due to the parasite attachment. Observe the necrophanerosis signs as digested apical membrane, pyknotic or karyorrhexic nucleus (®) and the absence of intercellular junctions. EP: epidermis; IS: intercellular spaces; N: nucleus; C: cytoplasm; AJ: adherens junctions; TJ: tight junctions; D: desmosomes; MR: micro-ridges. ***Page 81.***

Figure 4.6. (A, B) Transversal section of *Neobenedenia girellae* attached to greater amberjack (*Seriola dumerili*) skin. Observe how anchors almost reach the dermis, induce lacerations and pack epidermal cells while hooks are fixed more superficially. Alcian blue-PAS-GIEMSA, pH=2.5. Scale bars= 200 µm and 50 µm. (C) Detailed SEM micrograph of the laceration produced by *N. girellae* attachment. Scale bar= 100 µm. PA: parasite; EP: epidermis; H: hooks (hamuli); HAP: haptor; AN: anchor (accessory sclerites); DER: dermis; SC: scales; EL: epidermal lacerations. ***Page 82.***

Figure 4.7. Semithin sections micrographs detailing the differences between non-parasitized fish (A) and infected (B) greater amberjack (*Seriola dumerili)* epidermal goblet cells density (®). Alcian blue-PAS-GIEMSA, pH= 2.5. Scale bars= 50 µm. Observe the higher density of goblet cells in parasitized fish skin. (C) TEM micrograph of a greater amberjack skin goblet cell. Scale bar= 5 µm. EP: epidermis; DER: dermis; GC: goblet cell; CHR: chromatophores; MS: mucosomes; MR: micro-ridges; SM: secreted mucus: N: nucleus. ***Page 83.***

Figure 4.8. (A) Transversal section of parasitized cranial skin of greater amberjack, *Seriola dumerili.* Observe, the parasite-induced water imbalance on *stratum spinosum* and *basale* cells with hydropic degeneration (®). Intraepithelial lymphocytes could be also observed (*) (B). Alcian blue-PAS-GIEMSA, pH=2.5. Scale bars= 100 and 50 µm. (C) Longitudinal section of greater amberjack cranial skin. Observe the hydropic degeneration morphological pattern covering extensive areas of the tissue (spongiosis). Alcian blue-PAS-GIEMSA, pH=2.5. Scale bar= 100 µm in relation to a transversal section of no parasitized greater amberjack cranial skin (D) where this symptomatology is absent. Observe cellular cohesion and conserved structure of *stratum basale* (*) with oval nuclei disposed in the center of the cell. Alcian blue-PAS-GIEMSA, pH=2.5. Scale bar= 100 µm. PA: Parasite; HAP: haptor; EP: epidermis; HD: hydropic degeneration. ***Page 84.***

Figure 4.9. (A, B) Longitudinal section of *Neobenedenia girellae* haptor fixed to greater amberjack, ***Seriola dumerili*** skin. Observe the lymphocyte infiltrations (®) near the parasite anchors and hooks. Alcian-blue-PAS-GIEMSA, pH= 2.5., Scale bars= 100 and 50 µm. (C) TEM micrograph of intraepithelial lymphocyte (IEL) in the epidermis of a parasitized fish. Scale bar= 2 µm. (D) Detail of an epidermis area presenting IEL infiltrations (®) near the basal membrane associated to hydropic degeneration processes (*), near the parasite adhesion regions and mainly in *stratum basale and spinosum.* Alcian-blue-PAS-GIEMSA, pH= 2.5. Scale bar= 50 µm EP: epidermis; HAP: haptor; AN: anchors (accessory sclerites); H: hooks (hamuli); IEL: intraepithelial lymphocyte; N: nucleus; DER: dermis. ***Page 85.***

Figure 4.10. (A) Detail of greater amberjack *(seriola dumerili)* cranial region transversal section. Observe the focal dermic extravasation of intraepithelial lymphocytes (IELs) (®) associated a focal point near blood vessels. Alcian blue-PAS-GIEMSA, pH= 2.5. Scale bar= 100µm. (B) Detail of IELs focuses in the dermis *stratum spongiosum.* Hematoxilin-eosin,

as Group 1 - Green, Group 2 – Red and Group 3 - yellow. The accession numbers for the piscidin sequences incorporated into the tree are as follows : Amazon molly moronecidin (XP_014834599.1), Amazon molly moronecidin 1 (XP_016518673.1), Amazon molly moronecidin 2 (XP_014834599.1), Amazon molly piscidin (XP_007572108.1), American plaice pleurocidin 1 (AAP55793.1), American plaice pleurocidin 2 (AAP55794.1), American plaice pleurocidin 3 (AAP55795.1), Antarctic icefish piscidin (CBX55949.1), Barred knifejaw moronecidin (BAM99885.1), Barred knifejaw piscidin 1 (AMB38762.1), Barred knifejaw piscidin 4 (ATU75059.1), Barred knifejaw piscidin 5 (ATU75060.1), Brown marbled grouper piscidin (ADE06665.1), Clown anemonefish pleurocidin (XP_ 023120021.1), Copper rockfish piscidin (GE811382.1), Copper rockfish pleurocidin (GE814250.1), Duskytail grouper piscidin (ADY86110.1), European flounder pleurocidin (DV566089.1), European seabass dicentractin (P59906.1), Fairy ciclid mononecidin (XP_006805732.1), Fairy ciclid moronecidin (XP_006805731.1), Gilthead seabream piscidin 1 (FM145418.1), Gilthead seabream piscidin 2 (FM149199.1), Gilthead seabream piscidin 3 (FM154367.1), Gilthead seabream pleur- ocidin (AM973057.1), Gulf killifish piscidin (JW547320.1), Guppy pleurocidin (XP_017157770.1), Hongkong grouper piscidin (ACE78290.1), Japanese amberjack piscidin (DC609456.1), Large yellow croaker piscidin 1 (AIL82388.1), Large yellow croaker piscidin 2 (AIL82389.1), Large yellow croaker piscidin 3 (AQS27931.1), Large yellow croaker piscidin 4 (ACE78289.1), Large yellow croaker piscidin 5 (AGN52988.1), Malabar grouper piscidin 1 (AFS68802.1), Malabar grouper piscidin 2 (AFS68801.1), Mandarin fish moronecidin (AAV65044.1), Meagre piscidin (ASW20416.1), Medaka piscidin (DK151805.1), Medaka piscidin 2 (DK161574.1), Medaka piscidin 3 (DK192306.1), Mummichog dicentractin (JAR66906.1), Orange spotted grouper epinecidin (AAQ57624.1), Orange spotted grouper piscidin (AFM37317.1), Orange spotted grouper piscidin 2 (ADY86111.1), Orange spotted grouper piscidin 3 (AKA60776.1), Orange spotted grouper piscidin 4 (AKA60777. 2), Antarctic dragonfish moronecidin (AOW44479.1), Red drum piscidin (AFV40526.1), Red seabream piscidin (DC607430.1), Sabelfish dicentracin (ACQ58110.1), Sablefish moronecidin (ACQ57928.1), Sailfin molly moronecidin (XM_015038741.1), Sailfin molly piscidin (XP_014878007.1), Sheepshead minnow pleurocidin (GE334746.1), Shortfin molly moronecidin (XM_014977986), Shortfin molly piscidin (XP_014834599.1), Spiny chromis moronecidin (XP_022059843.1), Spotted seahorse piscidin (AAX58115.1), Stickleback piscidin (EG588124.1), Striped seabass moronecidin (Q8UUG0.1), Striped seabass piscidin (Q8UUG0.1), Striped seabass piscidin 3 (APQ32046.1), Striped seabass piscidin 4 (APQ32049.1), Striped seabass piscidin 6 (APQ32043.1), Striped seabass piscidin 7 (APQ32054.1), Tiger tail seahorse pleurocidin (XP_019738230.1), White bass moronecidin (Q8UUG2.1), White bass moronectin (AAL57318.1), White bass piscidin 3 (APQ32047.1), White bass piscidin 4 (APQ32050.1), White bass piscidin 5 (APQ32052.1), White bass piscidin 6 (APQ32044.1), Winter flounder pleurocidin (P81941.2), Winter flounder pleurocidin 2 (AAG10397.1), Winter flounder pleurocidin 3 (Q90VW7.1), Witch flounder pleurocidin (AAP55799.1), Witch flounder pleurocidin 2 (AAP55800.1), Yellowtail amberjack piscidin (ARK85994.1) and Zebra mbuna moronecidin (XP_004550684.1). ***Page 136.***

Figure 6.4. Transcript expression of greater amberjack piscidin. A) shows the relative constitutive expression of piscidin between gill, gut, head kidney (HK) and spleen (SP) tissues. B) shows the fold change in piscidin expression relative to values from PBS control fish for each tissue, for fish stimulated with poly I:C (IC), LPS or recombinant Yersinia ruckeri flagellin (FLA) for 24 h. C) shows the fold change in piscidin expression after 4, 12 and 24 h following in vitro stimulation of HK cells with poly I:C, LPS and flagellin, compared to PBS treated cells at each time point. D) shows the fold change in piscidin expression after 4, 12 and 24 h following in vitro stimulation of spleen cells with poly I:C, LPS and flagellin, compared to PBS treated cells. Piscidin transcripts were detected by qPCR and normalised to EF1α. Bars are either mean arbitrary units ± SEM, or mean fold-change ± SEM. N = 10. Letters denote significant differences ($P \leq 0.05$) between tissues, whilst asterisks denote significant differences relative to PBS controls. ***Page 139.***

Figure 6.5. Piscidin expression of greater amberjack on different diets. Greater amberjack were fed diets A) Skretting Seriola base diet (control), B) MOS enhanced diet, C) cMOS enhanced diet, and D) MOS and cMOS enhanced diet for 30 days. Piscidin expression was then quantified by qPCR and normalised to EF1α in the gills, gut, head kidney and spleen. Bars are mean arbitrary units ± SEM, N = 6. Letters denote significant differences ($p \leq 0.05$) in piscidin expression between diets, within an organ. ***Page 140.***

Figure 6.6. The effects of synthetic greater amberjack piscidin on bacterial growth. The growth of bacterial strains NCIMB 12260 *(Escherichia coli)*, MT423 (*Aeromonas salmonicida* - virulent), MT004 (*Aeromonas salmonicida* - avirulent), MT1741 (*Vibrio anguillarum* –serogroup 1), MT1742 (*Vibrio anguillarum* –serogroup 2), MT252 (*Yersinia ruckeri*), MT2055 *(Lactococcus garvieae* - noncapsulated), MT2291 *(Lactococcus garvieae* - capsulated) and MT1415 (*Pho-*

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CHAPTER 01

INTRODUCTION

1.1.
The greater amberjack (*Seriola dumerili*) as a promising candidate species for marine aquaculture.

The genus *Seriola* is composed by nine species of the family *Carangidae* commonly named as amberjacks. These fish species are pelagic and carnivorous and are highly appreciated by its commercial value and its popularity as game fish. All nine species cover most of the globe in terms of distribution, usually distributed in coastal waters, and can be found from the surface to 200 meters depth. The reproduction season varies depending on the species, being dioecious and pelagic spawners. Most *Seriola* species present a schooling behavior for protection, breeding and feeding of fish, squid and other invertebrates. As detailed in FAO statistics of 2019, miscellaneous pelagic fish category fisheries reached 7,931,000 t and only 347,000 t were produced by aquaculture, including all *Seriola* species.

Nowadays the need to diversify the aquaculture market and invest in fast growing species suppose a priority for the actual aquaculture sector strategy (Teletchea and Fontaine, 2014). In particular for the genus *Seriola*, there are 4 species *(S. dumerili, S. rivoliana, S. quinquerradiata and S. lalandi*) that stand out because of its high commercial value and interest in the worldwide market. These four species are actually being produced in aquaculture, being Japanese amberjack (*S. quinquerradiata)* and yellowtail kingfish (*S. lalandi*) the ones in which most of the studies have been focused, especially in Japan and Australia (Ikenoue and Kafuku, 1992; Nakada, 2002; Ottolengh *et al,* 2004). Nowadays, Japan, Korea, Taiwan and China are the main *Seriola* producers, notwithstanding *Seriola* rearing is also being produced in the US, Mexico, Chile, Ecuador, Spain, Portugal, Italy, Greece, Tunis, Malta, Turkey, Japan, Korea, Taiwan, China, Australia and New Zealand (Mazzola *et al*., 2000; Papandroulakis *et al*., 2005; Jerez*et al*., 2006; Haouas *et al*., 2010; Miegel *et al*., 2010; Öksüz, 2012; Fernández-Palacios *et al*., 2015, Mylonas, 2019). Traditionally, the production of *S. quinqueradiata, S. lalandi* and *S. dumerili* was based on the collection of wild-caught juveniles (Nakada 2000; Watanabe and Vassallo-Agius, 2003). However, the development of new broodstock management protocols, hormonal inductions and adaptation to captivity of these species lead to a successful spawn and larvae production, at least in *S. rivoliana, S. lalandi* and *S. dumerili* (Mylonas *et al*., 2004; Fernandez-Palacios *et al*., 2015; Mylonas, 2019; Sarih *et al*., 2019).

The aquaculture of the Japanese amberjack *(S. quinqueradiata*) (Temminck and Schlegel, 1845) started in the forties in offshore cages mainly in Japan, Korea and Taiwan, always related to wild-caught juveniles for rearing in culture conditions (FAO, 2009). *S. quinqueradiata* (Fig.1.1) represents the 80% of the total aquaculture production of the genus *Seriola*, and during the 80s and the 90s its annual production raised up to 140,000 t, a 70% of the total finfish production of Japan (Watanabe *et al*., 2001) while in 2017 its production reached 160,000 t (FAO, 2019).

Figure 1.1. *Japanese amberjack (Seriola quinqueradiata). Source: www.IGFA.com*

Yellowtail amberjack *(Seriola lalandi)* (Valenciennes, 1833) (Fig. 1.2) is considered the second biggest species in size and weight within the genus *Seriola*. During the 90s, from Australia and New Zealand made huge investments in research and technology with the objective of starting up the a commercial scale culture of these species in offshore cages from juveniles of captive-bred (Fielder, 2013). Nowadays, its production is focused in the US, Mexico, Australia, New Zealand, Chile, Taiwan, China, Japan and Korea. However, its production is lower than *S. quinqueradiata* production, almost reaching 10,000 t per year (FAO, 2019)

Figure 1.2. *Yellowtail amberjack (Seriola lalandi). Source: getfishing.com*

Almaco jack (*Seriola rivoliana*) (Valenciennes, 1833) (Fig. 1.3) is more related to tropical waters than the other *Seriola* species, being a good candidate for aquaculture in countries with a warm water temperature. At the end of the 90s almaco jack research started in Ecuador, Mexico and Chile for trying to produce this species in captivity. Since 2005, the US and several private companies have invested in the production of this species in Hawaii, reaching 750 t of production in 2008.

Figure 1.3. *Almaco jack (Seriola rivoliana). Source: www.fishingbbbooker.com*

Greater amberjack (*Seriola dumerili*) (Risso, 1810) (Fig. 1.4) is the biggest species from the genus ***Seriola*** in size and weight. This species shows a circumglobal distribution through warm waters and present a great acceptance in worldwide markets because of its flesh quality. In addition, greater amberjack has shown to perform 10 times better than, for example, the European seabass *(Dicentrarchus labrax)* (Muraccioli *et al.,* 2000), being proposed as a new emerging species for European aquaculture (Mylonas *et al.,*2019).

Greater amberjack culture in the Mediterranean region started in the 80s, and the first studies were mainly focused on determining the optimal culture conditions, on increasing the knowledge on the basic biology of this species and on dealing with nutritional and feeding practices, based mainly on fresh fish and moist feed, During the 90s, the first trials with dry feeds were conducted, and greater amberjack performed extremely good, gaining more than one kilogram during the first year of growing (González *et al.*, 1995; Jover *et al.*, 1999) and highlighting the good adaptation and acceptance of manufactured feeds (García-Gómez, 2000; De la Gándara, 2006). Nowadays, the knowledge on standard culture conditions (Fernandez-Montero *et al.*, 2018), nutrition (García-Gomez, 2000, Vidal *et al.*, 2008), larval rearing (Roo *et al.*, 2019) and reproduction (Sarih *et al.*, 2018, 2019) has significantly

increased, however the commercial production of greater amberjack in Europe is still very limited (Fig. 1.5). This is mainly associated to the appearance of several bottlenecks during its production that affect all the steps of the life cycle, including reproduction, larval performance and the on-growing period. Among the bottlenecks affecting juveniles, those related with health and welfare are of especial importance, as this species is highly susceptible to ectoparasites (Ogawa *et al.*, 1995; Sicuro and Luzzana, 2016).

Figure 1.4. *Greater amberjack (Seriola dumerili). Source: www.mrgoodfish.com*

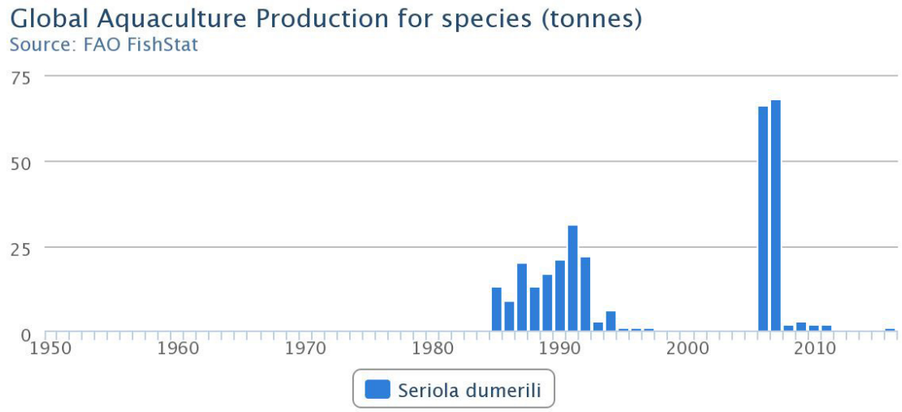


Figure 1.5. *Greater amberjack (Seriola dumerili) production (tonnes) from 1950 to 2016. Source:www.FAO.com*

1.2.
From a candidate to a real product for aquaculture: main bottlenecks associated to greater amberjack production

Despite of the potential of greater amberjack for aquaculture, this species has four main bottlenecks to be solved for a proper success during its production.

The first one is associated with reproduction, as occurs with most of the new cultured species under controlled conditions, which exhibit reproductive dysfunctions (Mylonas & Zohar, 2007). The use of wild caught fish, nutritional imbalances, and/or inappropriate management and culture conditions could lead to a mismatch in the environmental factors required to promote complete gonad development or spawning (Carrillo *et al.*, 1995; Sarih *et al.*, 2018; Sarih *et al.*, in press). Hormonal induction with exogenous gonadotrophin-releasing hormone synthetic analogue (GnRHa) has been shown to be effective in inducing maturation and multiple spawns (Fernández-Palacios *et al.*, 2015; Sarih *et al.*, 2018). Hormonal implants also have induced maturation and spawns of greater amberjack with minimal manipulation of fish (Mylonas *et al.*, 2004). However, spawning quality could be compromised by the hormonal induction methods and further research must be conducted to improve the spawning quality and the efficacy of the spawning induction protocols. In addition, spontaneous spawning has been reported rarely (Sarih *et al., 2018, 2019, in press*), obtaining a better spawning quality and larval survival than induced spawns. In addition, recent studies focused on enhancing the spawning quality of hormonally induced greater amberjacks by modifying broodstock nutrition, have obtained similar or even better results than those corresponding to natural spontaneous spawns (Sarih *et al.*, 2019; Sarih *et al.*, in press).

The second bottleneck associated to greater amberjack production is related to larval husbandry. Spawn quality is known to condition larvae surveillance during the first days after hatching (Fernandez-Palacios *et al.*, 1995; Sarih *et al.*, 2018). Furthermore, the limited knowledge on larval nutritional requirements of this species is related to a low larval survival rate and to a high incidence of skeletal anomalies (Roo *et al.*, 2019). Larvae culture conditions have also demonstrated to be decisive in this process, increasing surveillance and larvae quality by applying semi intensive rearing techniques (Papandroulakis *et al.*, 2005; Roo *et al.*, 2019).

Nutrition is also considered a bottleneck in all the life stages of cultured greater amberjack (Sicuro and Luzzana, 2016). As has been previously mentioned, a correct balanced nutrition for broodstock and larvae improves spawn quality and larvae surveillance. Additionally, greater amberjack juveniles have traditionally been fed in Europe with commercial diets for gilthead seabream *(Sparus aurata*) (40% protein and 22% lipids) and European seabass (42-48% protein and 20% lipids). Lately, turbot (*Scophthalmus maximus*) diets have been used because of the their higher protein and the lower lipids content (49% protein and 10% lipids). However, nowadays some of the main feed producers are recommending a higher content of protein and lipids (around 55% protein and 21% of lipids) in order to achieve a proper growth performance for this fish species. In addition, supplementation with several aminoacids, such as taurine or lysine, as for other carnivores, has been demonstrated to improve greater amberjack performance (Matsunari *et al.*, 2005, Papandroulakis, 2019).

The last bottleneck, and the most important one during the on growing period, involves the occurrence of pathological outbreaks. Those problems are mainly derived from inadequate culture conditions and management, a non-optimized nutrition and seasonality (Sicuro and Luzzana, 2016). The main associated disease to greater amberjack culture is related with capsalid monogenean ectoparasites (Ogawa *et al.*, 1995).

Capsalids are a family of monogeneans formed by more than 200 species, which cause important infections in fish (Ogawa and Yokoyama, 1998; Whittington, 2004). Their oral cavity is normally associated to suckers or to adhesive structures for fixing and helping in their feeding process. Adults present ovaries and testis, being hermaphrodites with internal fecundity. These parasites present attachment organs in the aboral pole, the haptor, which differ among the different taxonomic groups. The infections with monogenean ectoparasites are also a common pathology of all *Seriola* genus, nonetheless, differences in susceptibility among *Seriola* species could be observed, being greater amberjack especially sensitive to these infections (Nagakura *et al.*, 2006; Ohno *et al.*, 2008). Monogeneans show a simple lifecycle, with no intermediate host, monoxens and with only one larval stage. During adult stage, those parasites produce a high quantity of piriform eggs with fine filamentous structures to get entangled. After hatching, the single larval stage (oncomiracidia) starts to find its definitive host to complete their lifecycle (Ogawa *et al.*, 1995).

Nowadays there are three monogenean species that suppose an important impact on greater amberjack culture (Fig. 1.6). On one side, *Zeuxapta seriolae* belongs to the polyopisthocotylea group, which has a characteristic complex haptor composed by several clamps. This ectoparasite normally shows an elongated morphology and is a blood feeder, being mainly found on greater amberjack gills. *Z. seriolae* is highly virulent, and it is considered the main bottleneck for greater amberjack production in the Mediterranean (Grau *et al*., 2003; Montero *et al*.,2004), causing also high mortalities in wild greater amberjack populations (Lia *et al*., 2007). Another monogenean directly related to *Seriola* and especially to greater amberjack is *Benedenia seriolae.* This monogenean is a monopisthocotylea, with a single and simple haptor that feeds from mucus and epithelial cells from the skin of its host. *B. seriolae* has been studied in depth due to its relation with *S. quinqueradiatta* culture in Japan (Ogawa *et al*., 1995, Whittington *et al*., 2001), and to the date no infections of this parasite have been reported in Mediterranean region (Whittington, 2011). In the case of greater amberjack, the most extended monogenean parasite and the main responsible of an important bottleneck for its production is *Neobenedenia girellae.*

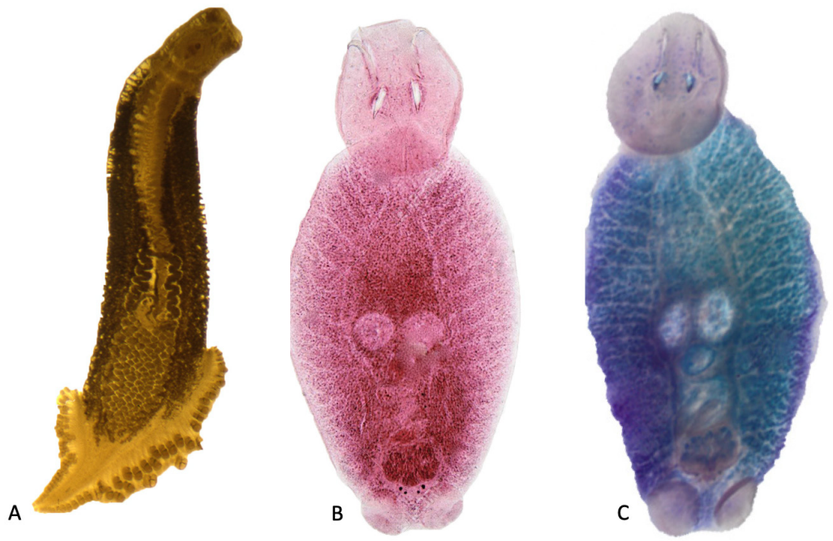


Figure 1.6. *Image of the three main ectoparasites potencially affecting greater amberjack (Seriola dumerili) culture; A: Zeuxapta seriolae (source:www.fishparasite.fs); B: Benedenia seriolae (source:www.alami.es); C: Neobenedenia girellae*

1.3.
The ectoparasite *Neobenedenia girellae*: an obstacle for the success of greater amberjack culture in sea cages. Lifecycle and infection mechanisms

Neobenedenia girellae is a marine monogenean of special importance for marine cultured species due to its low host specificity. It is due to its circumglobal distribution in warm waters, being considered the primary parasitic monogenean in global fisheries and aquaculture (Ogawa *et al*., 1995; Ogawa and Yokoyama, 1998; Brazenor *et al*., 2018). In addition, its distribution map is similar to greater amberjack, and supposes a big impact for this species when cultured (Ogawa *et al*., 1995; Hirayama *et al*., 2009; Whittington, 2011). *N. girellae* looks similar to *B. seriolae*, however, they differ in their anterior and posterior attachment organs (Kinami *et al*., 2005; Whittington, 2011). Indeed, *Neobenedenia* group has experimented several taxonomical modifications during last years. Firstly, *N. girellae* was considered a synonym with *N. melleni* and was re-described by Whittington and Horton in 1996. Moreover, due to the high morphological variations, *Neobenedenia* species were considered a complex, similarly to *B. seriolae* (Whittington *et al*., 2004; Sepúlveda and Gonzalez, 2014). Latest studies supported the theory about considering *N. melleni* synonym of *N. girellae* (Wang *et al*., 2004). In contrast, the last work conducted in this topic detaches *N. melleni* from *N.girellae,* proposing a more restricted distribution for *N. melleni* and considering *N. girellae* as the main monogenean problem involved in global fisheries and aquaculture (Brazenor *et al*., 2018).

N. girellae lifecycle is similar to other capsalids and it is strongly influenced by water temperature (Brazenor and Hutson, 2015) (Fig. 1.7.). Considering a medium water temperature of 22ºC, eggs would hatch after 7 days, releasing the oncomiracidia, which will start immediately to look for a definitive host. If oncomiracidia does not find a host 48 hours after hatching, the larvae would die. Once oncomiracidia fixes to its host, it takes about 9-10 days to become an adult and then, it will complete its whole lifecycle in a total of 16 days (Brazenor and Hutson, 2015).

Due to the temperature-dependence of *N. girellae* life cycle (direct-transmitted ectoparasites), any increase in temperature leads to an increase in their population densities (Chubb, 1977). Moreover, other environmental conditions as photoperiod are directly related with

N.girellae outbreaks. *N. girellae* eggs show a daily hatching pattern associated to the first light hours of the day, being the oncomiracidia photosensible, and thus, the opportunities for finding a host during the first hours after hatch increase (Shirakashi *et al.*, 2013; Hoai and Hutson, 2014 Hirano *et al.*, 2015).

In spite of its importance, little information is known about *N. girellae* fixation induced damage to greater amberjack skin. After hatching, oncomiracidia needs to find a suitable host in the next few hours for fixing and continuing its development to adult (Whittington, 2001; Brazenor and Hutson, 2015).

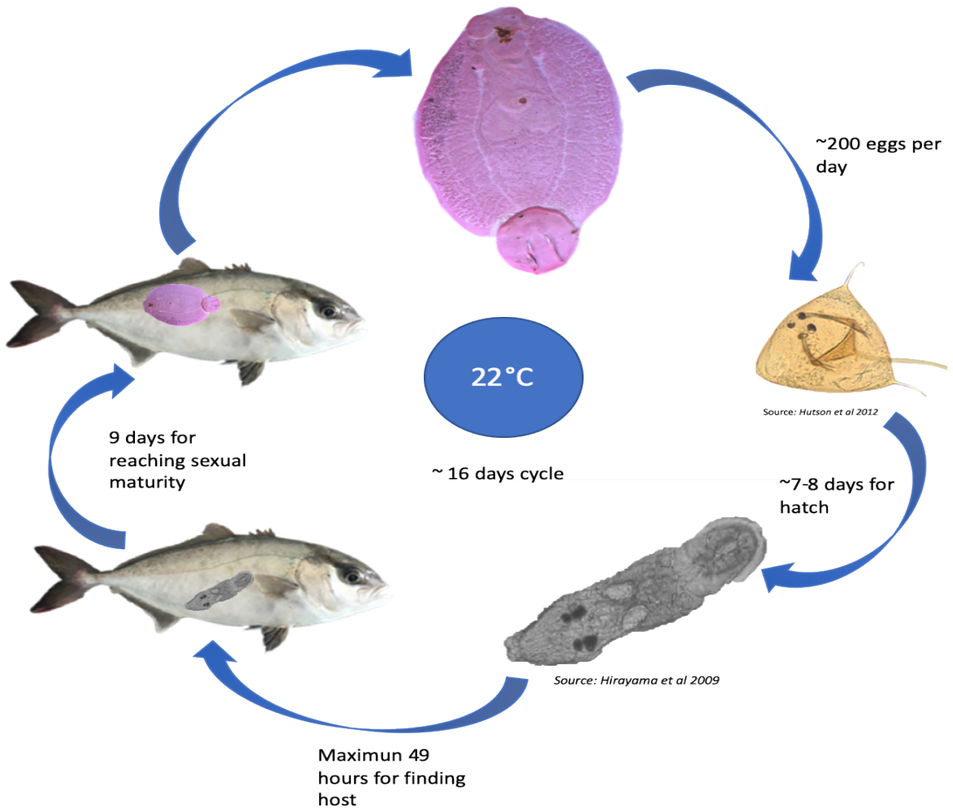


Figure 1.7. *Neobenedenia girellae lifecycle. Due to its strong relation with temperature, is represented for 22°C, the average temperature in canary islands.*

Skin cellular injury derived from pathogen attachment causes cell membrane damage, an imbalance of epithelial cells structure and limits mitochondrial ATP production (Esteban, 2012). Moreover, some pathogens as *Moritella viscosa* or some hematophagous parasites as sea lice (*Lepeophtheirus salmonis*) inhibit the epidermal regeneration capacity of keratocytes (Karlsen *et al.*, 2012) although this mechanism has not been described for *N. girellae* infection in greater amberjack. The effects of ectoparasites on fish skin are specific of each host and parasite. For example, the effects of sea lice infection on Atlantic salmon skin produces an ulcerative process caused by the second antennae, and an inflammatory process of the skin characterized by epidermal thickness variations, cell detachment, necrotic areas and leucocytes mobilizations (Jones *et al.*, 1990). Whereas *Gyrodactylus derjavini* infection in rainbow trout (*Oncorhynchus mykiss)* and Atlantic salmon is characterized by hydropic degenerated epidermal cells, cellular reorganization, changes in the density of goblet cells and variations of the epidermis thickness (Appleby *et al.*, 1997; Buchmann and Bresciani, 1997). Previous studies conducted in *N. girellae i*nfected greater amberjack reported alterations on greater amberjack skin epidermis thickness accompanied of an increased density of goblet cells (Hirayama *et al.,*2009; Hirazawa *et al.*, 2010; Hirazawa *et al.,* 2016), in relation to an unbalanced osmoregulatory and respiratory functioning of the skin (Hirayama *et al.*, 2009; Hirazawa *et al.*, 2016). In addition, the wound and ulcers produced by *N. girellae* attachment and scratching behavior of greater amberjack for trying to detach the parasites is a potential gateway for bacterial secondary infections (Fig. 1.8). The co-occurrence of secondary bacterial outbreaks together with ectoparasite infections at fish farms is very common in other species such as Atlantic salmon or rainbow trout (Buchmann and Bresciani, 1997). In addition, Bandilla *et al.*, (2006) found after challenging rainbow trout with *Argulus sp* and *Flavobacterium columnare* that ectoparasite infections can increase susceptibility of fish to a serious bacterial disease, increasing notably the associated mortality.

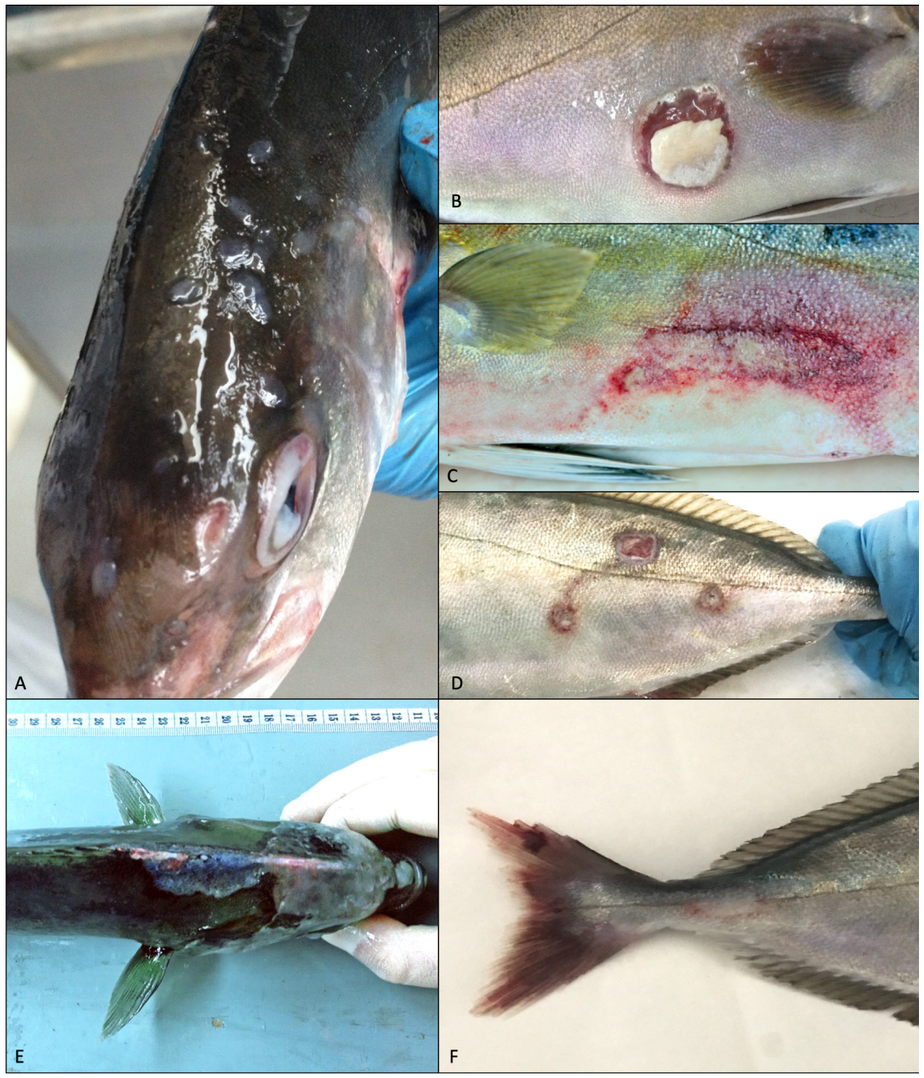


Figure 1.8. *Greater amberjack ulcerations. The nature and severity of these ulcerative processes could vary and affect different regions. Ulcerations caused by monogenean infection and scratching behavior could lead to produce the destruction of the eyeballs related with secondary bacterial infections (A). This ulcerative process could reach the dermis and muscle layer (B), cover wide areas with one single spot (C) or present several ulcers around its skin (D). In addition, cranial region (E) and fins (F) could also be affected by the ulcerative process related with Neobenedenia girellae infection.*

1.4.
Fighting against *Neobenedenia girellae:* improvement of the physical barrier, boosting the fish local and systemic immune potential and promoting fish welfare.

During the first contact between the oncomiracidia and greater amberjack, the skin and the mucus layer, play a key role to prevent the attachment of the parasite. The skin is a multifunctional organ and itself is also a physical barrier with roles of protection, excretion, locomotion, communication, respiration, sensory perception and thermal and ion regulation (Whitear, 1986). Furthermore, skin mucus is considered the first physical and immunological barrier in fish protecting from variety of chemical, physical and biological stressors (Ellis, 1999). Mucus is mainly composed of water and proteins, and it is produced by goblet cells located in the fish epidermis (Pittmann *et al.*, 2011). Within skin mucus proteins, mucins are the most abundant structural proteins, which play a key role in mucus viscosity, which could be modified with the types and quantity of mucin glycosylation (Koch *et al.*, 1991; Roberts and Powell, 2005). Moreover, the presence of other structural proteins such as keratins are also important determining fish mucus consistency (Alibardi, 2002). Skin mucus thickness varies within species and skin regions of fish, being also conditioned to its turnover rate for preventing the pathogen adherence, providing a medium in which antibacterial mechanisms may act (Esteban, 2012; Guardiola *et al.*, 2014). Mucus contains a series of humoral immune components such as immunoglobulins, defensins, lysozyme, lectin-like agglutinins, and a variety of antimicrobial peptides that provides a broad spectrum of protection against potential pathogens (Merrifield and Rodiles, 2015). Thus, mucus represents the first physical and immunological barrier that *N. girellae* oncomiracidia will have to fight against once finds the host. After that, oncomiracidia must fix to greater amberjack skin epidermis, and manage to resist to the immunological response of the whole skin associated lymphoid tissue (SALT). Thus, AMPs (antimicrobial peptides), APPs (antiparasitic peptides), complement proteins, proteases, immunoglobulins, lysozyme and lectins are released to skin mucus for arranging a defensive layer against pathogens (Shepard, 1994; Esteban, 2012). Some of those immune compounds have been described to have antiparasitic properties. APPs are the AMPs that show antiparasitic activity (Pretzel *et al.*, 2013) and the potential role of these APPs are pore-formation in parasite cells, disruption in cellular membranes, and alterations of Ca^{2+} or Fe^{2+} metabolism (Pretzel *et al.*, 2013). Hepcidin is one of the most important APPs because of its relationship with iron metabolism, important for parasites development (Valenzue-

la-Muñoz *et al*., 2017). Other molecules and proteins associated with antiparasitic properties are secreted to the skin mucus as the specialized immunoglobulins of mucosal tissues like IgT (Zhang *et al*., 2010). Moreover, complement c3a protein has been described to participate in pore-forming activity during monogenean infections (Buchmann, 1999).

Fish skin differs from other vertebrates because their non-keratinized outer layer of the epidermal *stratum superficiale*, being the active and viable epithelial cells in direct contact with the aquatic environment (Fig. 1.9). On the other hand, skin is covered with scales, what give to the fish an extra protection. The fish skin is divided in epidermis and dermis. The epidermis includes epithelial cells, goblet cells, and some specialized immune cells as IELs (intra epithelial lymphocytes) and rodlet cells. On the other hand, the dermis is composed by connective tissue, scales, fibroblasts, and chromatophores. As a physical barrier, ectoparasites, as *N. girellae* get fixed to epidermis, inducing a mechanical cellular damage, which triggers a cytokine production response by damaged epithelial cells and leucocytes. These cytokines act as messengers to trigger the immune response, as TNFα and IL-1β trigger an inflammatory response, leucocytes recruitment, AMPs production and stimulation of skin mucus production (Buchmann, 1999).

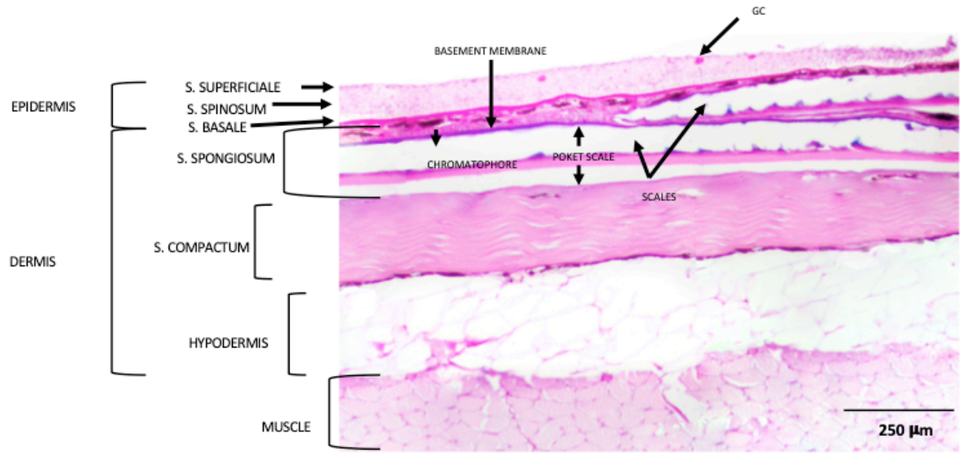


Figure 1.9. *Skin micrograph section showing some of the major morphological characteristics of the skin of greater amberjack (Seriola dumerili). Includes the epidermis, dermis, scales, hypodermis and muscle. Epidermis is composed by stratum superficiale, stratum spinosum and stratum basale, also including goblet cells (GC). Basement membrane separates epidermis from the dermis. Dermis is composed by the stratum spongiosum or loose connective tissue and the stratum compactum, Hypodermis separates dermis from muscle.*

Immune response is also mediated by the stress response, which triggers the innate immune response and leukocyte mobilizations during the adaptive phase, including an increased skin mucus production (Tort, 2001; Vatsos *et al*., 2010). However, the effect of a stressor depends of its intensity and duration, as well as to the physiological capacity of the fish to adapt to it, recovering allostatic equilibrium or developing to a non-adaptive phase, with deleterious effects. In particular, stress has been related with immune depletion under chronic situations (non-adaptive stress), where process that demands continuous energy from the fish like antibody production, immune proteins synthesis like complement, proteases, AMPs, or leukocyte differentiation would be depleted, dealing with a general immune depression (Sunyer *et al*., 1995; Ortuño *et al*., 2001; Tort *et al*., 2011). The main corticosteroid in fish is cortisol, that could be considered as a good indicator of stress in fish and is produced by the interrenal cells after the activation of the hypothalamus-Pituitary-Interrenal (HPI) axis as a consequence of a stressful event (Tort *et al*., 2011). Increases of circulating plasma cortisol have a direct impact on the immune system and systemic and SALT immune response could be conditioned by high levels of cortisol because of a chronic stressor (Tort, 2011). Besides, cortisol has also been detected in the skin mucus of different species including the gilthead seabream and has been proposed as a stress indicator (Fernandez-Alacid *et al*., 2018, 2019). Ectoparasite fixation has been described to increase plasma cortisol and to induce a long-term stress in rainbow trout *(Oncorhynchus mykiss)* parasitized with *Ichthyophthirius multifiliis* (Jorgensen and Buchmann, 2007) and in Atlantic salmon parasitized with sea lice (Bowers *et al*., 2000). The physiological condition of immune suppression caused by stress together with the immunological exhaustion caused by a parasite outbreak could be determinant for understanding the associated mortality of greater amberjack parasitized with *N. girellae,* although no studies have been conducted in that line for this *Seriola* species.

1.4.1. Reinforcement of skin physical barrier

Visual wounds in fish skin negatively affects the market price of the product with the subsequent important economic losses (Ravichandran *et al*., 2010). Because of that, some studies have focused on skin wounds and ulcers and its regenerative process, especially in Atlantic salmon (Roubal and Bullock, 1988; Bullock and Roberts, 1992; Jensen *et al*., 2015). However, the effect of skin wounds is not only related with the devaluation of fish market prize, as it has been previously mentioned, skin acts as a physical barrier which avoid the entrance

of pathogens. Moreover, a relationship between wounds and mortality of Atlantic salmon has been associated with *Vibrio anguillarum* and *Aeromona salmonicida* infections (Svendsen and Bøgwald, 1997). Sea lice induce wounds and ulcers on its host, and also act as a vector of other pathogens (Barker *et al.*, 2009; Jakob *et al.*, 2011). Nevertheless, the attachment structures of sea lice penetrate all the epidermis and reach the dermis, inducing bleeding and anemia on the host, which has not been associated to monogeneans due to the smaller size of its attachment organs (Jones *et al.*, 1990; Jonsdottir *et al.*, 1992; Whittington, 2011). In addition, a chronic stress produced by parasitosis induces increased circulating plasma cortisol levels, which could retard epidermal and specially dermal repair, particularly fibrosis and closure of the *stratum compactum,* indicating an impaired fibroblast activity (Roubal and Bullock, 1988). Nutritional supplementation with vitamin C, B, E, zinc, iron, copper or selenium has been demonstrated to reduce the timing of the wound healing process (Hardie *et al.*, 1991; Tenaud *et al.*, 2000). Moreover, some functional feeds have been used for reducing the timing of wound healing process in *Heterobranchus bidorsalis* (Akanmu *et al.*, 2016). However, little information is still available for greater amberjack wounds generated by *N. girellae* infection (Hirayama *et al.*, 2009).

1.4.2. Reinforcement of immune system

There are several studies addresing the effects of different ectoparasites in the immune system of the host. The immune response of Atlantic salmon against sea lice infection has been deeply studied, denoting an inflammatory process associated to AMPs production, increase in lysozyme and complement activity, iron metabolism regulation and immunoglobulin production (MacKinnon, 1993; Robledo *et al.*, 2018). Moreover, the infection of European sea bass infected with copepods (*Lernanthropus kroyeri*) is associated with an increase in lysozyme and alternative complement activity, followed by classical complement activation and respiratory burst (Henry *et al.*, 2009). However, little information is available about greater amberjack immune system (Milne, 2018), being nonexistent in the case of *N. girellae* infection. There is some information on the immune system of similar species, such as Japanese yellowtail and kingfish yellowtail (Darawiroj *et al.*, 2008; Choi *et al.,* 2016), but to the date no studies have been conducted addressing greater amberjack SALT response, being one of the objectives of this thesis. Although few information is available, it is known that the development stage of *N. melleni* conditions the immune status of the kingfish yellowtail

(Reyes-Becerril *et al.*, 2015, 2017), and some studies have demonstrated the role of complement activity against *B. seriolae* in the same species (Leef and Lee, 2009).

1.4.3. Cross talking among culture conditions, fish welfare and ectoparasites infection in *Seriola sp* culture

Culture conditions are directly related with fish stress response and subsequently, with fish welfare (Conte, 2004). However, few studies have been published on the relationship between variation of experimental conditions and ectoparasite incidences in fast growing species. As poikilotherm animals, fish are conditioned with the environmental temperature, and this must be inside its biological range of tolerance, as described for greater amberjack (Jobbling, 1981). Holding the fish out of the optimum range of rearing temperature could be a chronic stressor (Matthews and Berg, 1997).Temperature affects the development and prevalence in fish of monogenean parasites like *Gyrodactylus bullatarudis* on guppies *(Poecilia reticulata), G. salaris* on Atlantic salmon or *N. girellae* on greater amberjack (Scott and Nokes,1984;Jansen and Bakke, 1991; Hirazawa *et al.*, 2010). Furthermore, other environmental conditions, such as oxygen level are considered important stressors for fish (Matthews and Berg, 1997; Elliot, 2000) and environmental stress and diseases outbreaks are closely related (Snieszko, 1974; Walters and Plum, 1980). In relation with pathogen outbreaks, sea lice infection induces an increase of plasma cortisol in Atlantic salmon aside that high cortisol levels have not been related with a greater sea lice prevalence in a short-term induced infection (Fast *et al.*, 2006; Krasnov *et al.*, 2012). Photoperiod, as other culture conditions, is closely related with *N. girellae* outbreaks, since *N. girellae* eggs show a daily hatching pattern during the first light hours of the day (Shirakashi *et al.*, 2013; Hoai and Hutson, 2014 Hirano *et al.*, 2015). A high stocking density is also considered as one of the most important and common chronic stressors in aquaculture (Montero *et al.*, 1999) and it is also related with a higher ectoparasite incidence (Thoney and Hargis, 1991; Nowak, 2007). Besides, unbalances of other husbandry associated parameters, such as nutritional deficiencies or food deprivation, have been associated with changes in skin mucus composition and a depleted immune response in Atlantic salmon (Landeira-Dabarca *et al.*, 2013; Martin and Krol, 2017), in relation with a higher susceptibility of hybrid striped bass (*Morone saxatilis* x *M. chrysops*) to the ectoparasite *Ichthyophthirius multifiliis* (Corrales and Noga, 2011).

Little information is still available about the stress response of juvenile amberjacks, being some of the available studies focused on larvae (Sakakura *et al.*, 1998) and oxidative stress (Hossain *et al.*, 2017). However, Moran *et al.* (2008) observed a low stress response of kingfish yellowtail juveniles associated to handling and transport hypercapnia. Other studies with Japanese yellowtail highlighted the possibility of nutritionally modulate this species stress response, similarly as in other species, as for example by the inclusion of vitamin C in the diet (Ren *et al.*, 2008). However, no studies have been conducted on greater amberjack stress response or on its relation with *N. girellae* infection.

1.5. The use of functional diets as a tool for promoting fish welfare, enhancing immune system potential and reducing disease incidence

The development of fish feed formulation has change in order to meet not only fish nutritional requirements but also to add an extra health benefit or improved product quality. Furthermore, this change has been regarded as a promising approach to improve welfare and consequently aquaculture industry stability. These feeds are known as functional diets and are usually supplemented with specific ingredients known to directly or indirectly increase the efficiency of a given physiological mechanism. Moreover, these diets should be developed with ingredients economically attractive and environmentally friendly. Indeed, the development of functional feeds has been studied during the last 40 years and represents a great opportunity for the aquaculture industry (Gatlin, 2002; Olmos-Soto *et al.*, 2015).

Composition of functional additives is quite diverse, and their use in commercial diet formulations for aquatic species varies considerably (Encarnação, 2016). Functional diets have become an intrinsic part of the preventive health strategy in fish farms (Bowden 2008; Covello 2012). Thus nowadays, the use of functional feeds is a promising tool to reinforce fish immunity and to reduce disease incidence, and thus, allowing the reduction of the use of chemotherapeutics and antibiotics treatments, lowering the costs of disease treatment and management for aquaculture companies (Tacchi *et al.*, 2011). In the design of functional feeds, a wide range of feed additives can be used to improve growth and feed utilization, but also to support the health and stress resistance of the animals. Functional ingredients inclusion such as prebiotics, probiotics, phytogenics and herbal extracts, microbial derivatives, vitamins and nucleotides has been related with a better growth performance and feed conversion efficiency, as well as with positive effects on the intestinal health, stress response, immune system reinforcement and protection against bacterial infections (Tacchi *et al.*, 2011).

1.5.1. Dietary use of prebiotics: reinforcing mucosal tissues integrity, immune potential and fish welfare. Effects on ectoparasites resistance

A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the gut (Ringø *et al.*, 2010). They are composed by carbohydrates of variable size or degree of polymerization, and their chemical properties of the anomeric C atom (C1 or C2) of the monosaccharide units makes their glyosidic bonds non-digestible by the hydrolytic digestive enzymes of vertebrates (Roberfroid & Slavin 2000; Ringø *et al.*, 2010; Ringø *et al.*, 2014). The beneficial effects of prebiotics come along with the by-products originated by the fermentation of commensal bacteria, increasing the volatile short chain fatty acids, stimulating the growth of commensal beneficial bacteria as *Lactobacilus spp* and *Bifidobacterium spp* and inhibiting the colonization of pathogens by direct competition (Ringø *et al.*, 2014; Torrecillas *et al.*, 2014; Guerreiro *et al.*, 2018). Moreover, pathogens as bacteria and fungus present a membrane rich in peptidoglycans and other carbohydrates that are emulated by prebiotics. Furthermore, those carbohydrates are considered PAMPs, which are recognized by leucocytes and associated cells with PRRs (pattern recognition receptors) and thus, immediately generate an immune response against them (Carbone and Faggio, 2016). Differences in the structure of immunostimulants lead in different PRR activation of different PRR families, as for example, β-glucans activate signaling via toll like receptors (TLR) but there are also C-Type lectin receptors (CLRs) that recognize specifically lectins, converging all of them in a common group of inflammatory molecules including NF-κβ or MAPK (Bricknell and Dalmo, 2005; Hardy *et al.*, 2013; Hoseinifar *et al.*, 2015).

Mannan oligosaccharides (MOS) are carbohydrates obtained from yeast cell wall that have been studied as prebiotic functional ingredients in animal feed from the early 90s (Spring *et al.*, 2015). Dietary MOS induces modifications in microbiota communities and mediates bacterial colonization by simulating/mimicking lectins and specific carbohydrates of enterocytes cellular membrane, and thus getting attached to the dietary MOS instead (Spring et

al., 2015). Despite of the lack of studies conducted with these products in greater amberjack, MOS have demonstrated effects in other fish species boosting systemic immunity and GALT (gut associated lymphoid tissue) (for review see Torrecillas *et al.*, 2014). Moreover, MOS have been tested as a booster for SALT response and skin mucus production, in order to reduce sea lice infection in Atlantic salmon with successful results (Dimitroglou, 2010; Sweetman *et al.*, 2010; Refstie *et al.*, 2010; Jensen *et al.*, 2015).

1.5.2. Dietary use of Phytogenics: reinforcing mucosal tissues integrity, immune potential and fish welfare. Effects on ectoparasites resistance

Phytogenics are a large group of plant- derived compounds such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils that have been used as functional ingredients in aquaculture (Chakraborty and Hancz, 2011). Among their benefits as functional ingredients are to modulate the stress response, to act as antioxidants, as antimicrobials, as growth promoters and as immune modulators as well as to increase feed palatability in fish (Immanuel *et al.*, 2004; Citarasu, 2010; Reverter *et al.*, 2014). The use of herbal additives has traditionally been used in Asia (Ji *et al.*, 2009), and its use has extended to a large number of aquaculture species (Chakraborty and Hancz, 2011; Reverter *et al.*, 2014; Van Hai, 2015; Sutili *et al.*, 2018). Moreover, phytogenics exert a similar effect in gut than prebiotics, modulating the gut environment and bacterial composition and also inhibiting certain pathogenic bacterial groups (Laparra & Sanz 2010). Moreover, phytogenics (plant extracts, essential oils, or plant derived products) like *Eclipta alba* (Christybapita *et al.*, 2007), *Aloe vera* (Kim *et al.*, 1999), *Ocimum sanctum* (Logambal *et al.*, 2000), *Viscum album, Urtica dioica , Zingiber officinale* (Dügenci *et al.*, 2003)*, solanum trilobatum* (Divyagnaneswari *et al.*, 2007)*, Astragalus radix, Scutellaria radix* (Yin *et al.,* 2006), *Achyrantes aspera* (Rao and Chakrabarti, 2005)*, Allium sativum* (Shakya and Labh, 2014) and *Thymus vulgaris* (Jahanjoo *et al.*, 2018) have been associated with enhancement of nonspecific and specific defenses. However, in most of the cases the mechanism responsible for the enhancement of the immune system in fish is still unknown, but among them the potent antioxidant properties of phytogenics could be influencing notably fish immune response (Kim and Lee, 2008; Reverter *et al.*, 2014). The modulation of the immune system response in mucosal tissues as skin produces a hostile microenvironment for pathogens, similarly as prebiotics do. Furthermore, after oral administration of essential oils, their active compounds have been detected in skin mucus (Chakraborty and Hancz, 2011). In order with that, active compounds of essential oils have been observed to interact with biological membranes such as bacterial cell membrane changing its morphology and its lipid profile and increasing its permeability (Devi *et al.*, 2010; Lv *et al.*, 2011; Nazzaro *et al.*, 2013). Similarly, phytogenics have been studied for avoiding monogenean infection or disrupting its lifecycle. Thus, two seaweed extracts from *Asparagopsis taxiformis* and *Ulva sp.* reduced *Neoenedenia* infection in Barramundi *(Lates calcarifer)*, being *A. taxiformis* an inhibitor of embryonic development of the parasite (Hutson *et al.*, 2012) and dietary garlic produced a reduction of *Neobenedenia* parasitization in barramundi after 30 days of supplementation (Miltz *et al.*, 2013 a,b). Garlic has also been traditionally used by the Atlantic salmon industry for reducing sea lice infection in spite of not obtaining results as promising as in monogeneas (Costello, 1993). In addition, *in vitro* studies with *Neobenedenia* showed of ginger (*Zingiber officinale)* and basil (*Ocimum basilicum*) extracts (Trasviña-Moreno *et al.*, 2017) toxic properties. Particularly, in terms of reducing the incidence of parasitic pathologies, extracts of plant origin are less likely to produce drug resistance in parasites due to the high diversity of plant extract molecules (Olusola *et al.*, 2013; Reverter *et al.*, 2014).

Furthermore, phytogenics have been related with an improved stress response pattern by favoring the homeostatic recovery during the acute and the adaptive phase of cortisol response (Ji *et al.*, 2007, 2009; Souza *et al.*, 2019). The physiological mechanism for reducing stress by phytogenics is still unclear, however their sedative effects and interactions with the central nervous system and antioxidant effects have been proposed as potential routes of action (Bodur *et al.*, 2018; Souza *et al.*, 2019).

1.6. Objectives

The overall aim of this thesis was to investigate the effects of *Neobenedenia girellae* infection and its relationship with the robustness in greater amberjack, using functional diets as tool for boosting fish health, stress resistance and reducing the parasite effects. For that purpose, the following specific objectives were addressed:

1. To evaluate the stress response of greater amberjack to face variations in culture conditions.

2. To determine the effect of *Neobenedenia girellae* infection in the morphology of greater amberjack skin
3. To determine the effect of *Neobenedenia girellae* outbreak protein profile of greater amberjack skin mucus.
4. To evaluate the potential of functional additives to boost the immune system and to increase the stress response through the validation of specific biomarkers for greater amberjack.
5. To evaluate the potential of functional additives to improve the resistance of greater amberjack to *Neobenedenia girellae* infection.

CHAPTER 02

MATERIAL AND METHODS

2.1.
Experimental animals and experimental conditions

All the experiments conducted along this thesis have been performed at the Marine Scientific and Technologic Park (PCTM) of the University of Las Palmas de Gran Canaria (ULPGC) (Las Palmas, Canary Islands, Spain). The experiments described comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals. Most of the experiments conducted within the present thesis belonged to the DIVERSIFY project from the European Union Seventh Framework Program for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121): "Exploring the biological and socio-economic potential of new/emerging fish species for the expansion of the European aquaculture industry". This thesis has also been partially founded by the Spanish government, "Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente" and the "Junta Nacional Asesora de Cultivos Marinos, JACUMAR, Planes Nacionales de Acuicultura 2016 (BOE No145 del 16/06/2016), through the project "Diversificación de la acuicultura española mediante la optimización del cultivo de seriola (*Seriola dumerili)* - SERIOLA".

Within the present thesis, greater amberjack juveniles from different stocks have been used (Fig. 2.1). Those juveniles were obtained by mass spawning from the broodstock of greater amberjack held at PCTM. Briefly, greater amberjack broodstock was composed by wild-caught and captive breed fish ranging between 3.5-18 kg and maintained in groups of three female and three males in cylindroconical tanks of 40 m^3. Fish were fed with a commercial compound diet specific for broodstock maintenance (Vitalis Repro™ Skretting, Burgos, Spain) at 1% of their estimated total biomass, and once a week with Atlantic mackerel (*Scomber scombrus*) at 2%. During the spawning period (May-October in Canary Islands), the fish were injected intramuscularly with gonadotrophin-releasing hormone analogue (LHRHa, des-Gly10, [D-Ala6]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 µg/kg body weight, following the methodology described by Fernández-Palacios *et al.* (2015) and Sarih robustness . (2018). After that, tanks were prepared with 500µm pore diameter egg-collector nets, where the hormonal induced spawn uses to take place the next morning after the artificial induction. After 24-48 hours, greater amberjack eggs hatched in mesocosm tanks of 40 m^3, and larvae of greater amberjack were held at the hatchery from PCTM, under specific standardized protocols for this species developed by the Aquaculture Research group of ULPGC (Roo *et al.*, 2019).

Figure 2.1. *Greater amberjack juvenile reared in PCTM facilities.*

The experimental fish initial size is specified in each Chapter, ranging from 19.5g in Chapter 3 to 331 and 425g in Chapter 6 and 3 respectively.

The set of experiments were conducted in two different rearing conditions, depending mainly on the fish size used for each experiment. On the one hand, cylindroconical tanks in flow-through systems, either of 1000 l (Chapter 3 exp. 2 y 3, Chapter 6 and Chapter 7) or 500 l (Chapter 4, Chapter 5) of capacity were used depending on the fish size. Renovation rate was established at one full tank renovation per hour along the whole experiments of the present thesis , to maintain oxygen levels between 6 and 7.5 mg l^{-1}. On the other hand, tanks in Recirculation Aquaculture system (RAS) from the Marine Biosecurity Station (MBS) from PCTM (IUECOAQUA-ULPGC) were also used in Chapter 3, exp. 1 and Chapter 8. The RAS consists of independent RAS supplied with filtered water and with controlled temperature and pH. Each RAS consists of three 500 l tanks supplied with mechanical, biological and ultraviolet filtered water. Besides, in order to avoid loses of animals due to this species "jumping-out-the-tank" behavior, all the tanks were covered with 5-10 cm flesh nets.

All juveniles were fed on Europe 22 diet (Skretting, Burgos, Spain) composed by 52% of crude protein and 20% crude lipids when a dietary experiment with specific functional additives supplemented was not conducted (Chapter 3, 4 and 5). In those experiments in which a dietary experience was conducted, the details of each diet used are provided in its respective Chapter (Chapter 6, 7 and 8). All animals from the different experiments (except those fish from the group subjected to fasting in Chapter 3, experiment trial 3), were fed until apparent

Table 2.1. *Additives used for this thesis in the diet of greater amberjack juveniles and its concentration. All diets were produced by Skretting (Stavanger, Norway)*

Additive	Concentration	0 H
MOS (BIO-MOS®)	0.5%	6, 7
cMOS (ACTIGEN®)	0.2%	6, 7, 8
MOS + cMOS	0.5%+0.2%	6, 7
HERB	200 ppm	8

All diets were produced by Skretting (Stavanger, Norway). BIO-MOS® and ACTIGEN® were produced by Alltech (Kentucky, USA), and HERB was produced by DELACON (Steyregg, Austria)

satiation three times per day, 6 days per week. The different functional additives used are compiled in Table 2.1.

2.2. Anesthesia and other husbandry practices

For handling the fish, a standardized anesthesia protocol was used. Briefly, greater amberjack juveniles were placed in a tank with diluted clove oil as follow: 6 ml of clove oil (1:1 clove oil and ethanol 96%, 5ml/l; Guinama S.L; Spain, Ref. Mg83168) diluted in 100 l of water. After few minutes, depending on fish size, fish started to respond to the anesthesia and then were sampled (see details below). For recovery after anesthesia, fish were placed into a recovery tank with high aeration and water flow. For those animals that were subjected to euthanasia for the obtaining of samples, the euthanasia protocol consisted on an overdose of anesthetic (Clove oil and ethanol 96%, 5ml/l; Guinama S.L; Spain).

2.2.1. Anti-parasite preventive protocol

In order to avoid spontaneous parasitization after samplings with the subsequent associated fish mortalities, health and welfare problems, freshwater baths of 2-5 minutes (depending on greater amberjack size) were conducted when sampling the fish for routine measurement of

growth (usually monthly). The anti-parasite protocol used was a variation of the anesthetic protocol developed for this species (as mentioned in Fernandez-Montero *et al.*, 2018) and it consists on placing greater amberjack specimens in tanks containing freshwater with anesthetic (1-2 ml of clove oil in 100 l of freshwater). This protocol was established specifically to reduce the stress induced to fish due to the salinity change. Simultaneously, the experimental holding tanks and nets were thoroughly cleaned with bleach to avoid parasite *N. girellae* eggs fixation to the different structures.

2.3. General sampling protocols

2.3.1. Morphometric parameters

Individual whole body weight and total length were obtained for initial and final sampling points in all the experiments compiled in this thesis. Besides, depending on the experiment, a monthly sampling point to obtain data for analyzing fish growth performance was also done. Specific growth rate (SGR), weight gain (%) and feed conversion ratio (FCR), were calculated as follows:

SGR = (Ln (final weight) – Ln (initial weight)) *100/feeding time (days)
Weight gain= final weight – initial weight
FCR= (feed intake/weight gain)

2.3.2. Mucus extraction

Skin mucus was obtained in Chapter 3, 5, 6 and 8 following the methodology described by Guardiola *et al.* (2014) with some modifications. Skin mucus was collected by gently scrapping the surface of the fish skin with autoclaved glass slides (Fig. 2.2). This methodology was applied always on the left side of the fish and from the head to the caudal fin for avoiding epidermal damage. Thus, in case that histological or transcriptomic samples were taken, the right side was used. After that, skin mucus was diluted 1:1 with filtered and autoclaved saltwater and frozen at -80°C until analysis.

Figure 2.2. *Skin mucus extraction of greater amberjack left side using an autoclaved microscopy slide.*

2.3.3. Blood, serum and plasma extraction

Blood was obtained by caudal sinus puncture using 1 ml syringes. Blood samples were placed in 1.5 ml Eppendorf tubes. For plasma samples (Chapters 3 and 8), Eppendorf tubes and syringes were heparinized for avoiding blood coagulation, and blood was immediately centrifuged at 5000 g, during 5 min at 4°C. The plasma samples obtained were kept at -80°C until analysis. For serum samples (Chapters 6 and 8) no heparin was added and blood was left for clotting overnight at 4°C, after that, were centrifuged 5000 g for 5 minutes and the resultant supernatant stored at -80°C until analysis (common GIA standardized protocols).

2.3.4. Tissue collection

After euthanasia with an anesthesia overdose, fish were dissected to collect the different organs in the different experiments (Fig. 2.3). All the material used for sampling, as surgical material, was previously sterilized and between different samples, cleaned with propane AF, ethanol and diethylpyrocarbonate (DEPC) treated-water. Samples from head kidney and spleen (Chapters 6 and 7) were directly placed in RNA later (Sigma-Aldrich, San Luis, Missouri, US) in a ratio 1/5 (v/v). Skin samples (Chapters 3, 6 and 8) were obtained from the right side of the fish, from the same dorso-lateral region, and placed in 1/5 (v/v) proportion

in RNA later or prepared for morphological studies by fixing the target tissue in 4% buffered formaldehyde, ethanol 96% or glutaraldehyde (Chapter 4). Gills samples (Chapters 6 and 7) were obtained from the second gill arch of the right side and placed in RNA later in a ratio 1/5 (v/v). The whole intestine was dissected and a section of posterior gut was kept for transcriptomic (Chapters 6 and 7) analysis. Both sections were placed in RNA later at the same proportion as the other tissues mentioned above.

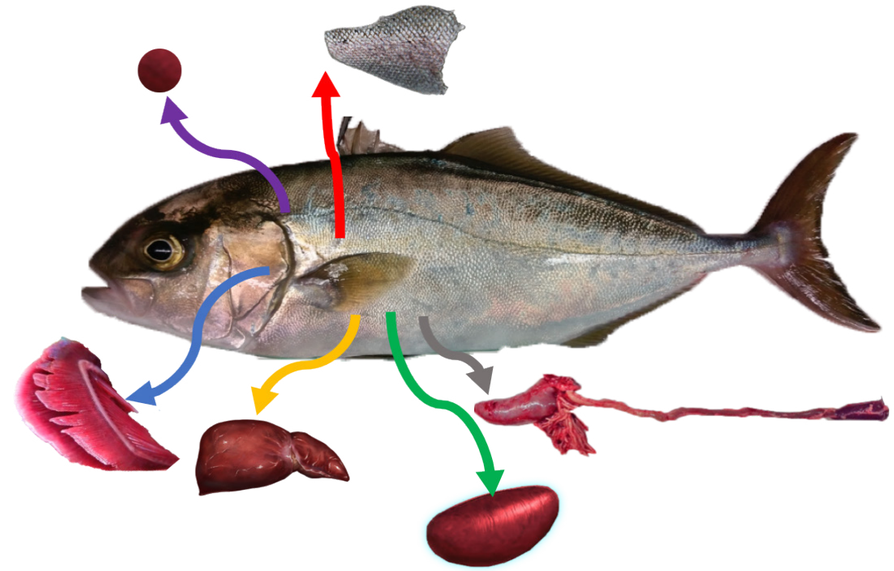


Figure 2.3. *Selected greater amberjack tissues used for this thesis during samplings: ⇒ skin (Chapters 3, 4, 6, 8); ⇒ head kidney (Chapters 6 and 7); gills ⇒ (Chapters 6 and 7); ⇒ liver; ⇒ spleen (Chapters 6 and 7); ⇒ gut (Chapters 6 and 7).*

2.4.
Morphological analyses. Optical and structural study

Samples for morphological studies (Chapter 4) were obtained by dissection and were fixed in buffered 4% formalin. After dehydration in a series of ethanol dilutions, samples for optical microscopy (Chapter 4), were embedded in paraffin blocks, processed and stained with haematoxylin-eosin (Martoja and Martoja-Pierson, 1970) or were embedded in Technovit

plastic (Electron Microscopy Sciences, PA, USA) following the method of Pittman *et al.*, (2013) and stained with Alcian Blue-PAS-GIEMSA (Martoja and Martoja-Pierson, 1970). In addition, micrographs were obtained with an adapted camera Olympus xc50 (Tokyo, Japan) for morphometric data collection using Image Pro Plus software (Media Cybernetics, Silver Spring, USA) (n=30 slides /region/treatment).

Samples for Scanning Electron Microscopy (SEM) analyses (Chapter 4) were dehydrated through a graded series of ethanol, followed by a critic point dried (Hitachi HCP-2, Chiyoda, Tokyo, Japan), mounted in aluminum stubs and metalized with argon-gold (Gibbons, 1986). After that, samples were examined and photographed with a JEOL JSM-6335F field emission scanning electron microscope (JEOL USA, Inc, USA).

For Transmission Electron Microscopy (TEM) samples were fixed in 2.5% glutaraldehyde, embedded in resin blocks and cut with an ultramicrotome (detailed in Chapter 4). Ultrathin (50 nm) sections were contrasted with lead citrate and examined with a JEOL JEM-1011 Transmission Electron Microscope (TEM; JEOL USA, Inc, USA) equipped with a digital camera MegaView III soft imaging system CCD Camera (EMSIS GmbH, Germany).

2.5. Molecular and proteomic analyses

2.5.1. Primers design

Primers used (Chapter 6 and 7) were obtained from Milne (2018). However, for studies detailed in Chapter 8, other specific primers were designed from target genes found in GenBank from species phylogenetically related with *S. dumerili,* following the methodology described in Milne (2018). Primers used in Chapter 8 were designed using the recently described genome of greater amberjack (Araki *et al.*, 2018). The Table 2.2 shows all the primers used within the present study.

2.5.2. Total RNA extraction and qPCR

Transcriptomics from Chapters 6 and 7 were conducted in the Scottish fish immunology research center of the University of Aberdeen, while transcriptomics from Chapters 3 and 8 were conducted in PCTM of ULPGC. Total RNA (Chapters 6 and 7) was extracted using Trizol reagent method (Invitrogen, Carlsbad, California, United States) according to the manufacturer's instructions. For removing contaminating genomic DNA, a DNase treatment was applied (AMPD1–1 KT, Sigma–Aldrich, Broendby, Denmark). For Chapters 3 and 8, total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions.

For both methodologies, RNA concentration and purity were checked by nanodrop (Thermo Fisher Scientific, Madrid, Spain) and by electrophoresis in agarose gels. Total RNA was reverse transcribed using a ThermoScript TM Reverse Transcriptase (Invitrogen) kit for obtaining complementary DNA (cDNA) and diluted 1:20 in miliQ water and stored at –20 °C. Moreover, qPCR was conducted with SYBRgreen and Truestar Taq following a program of: 1 cycle of 6 min denaturalization at 95 °C, 45 cycles of amplification (25 s at 95 °C, 30 s at the annealing temperature, 25 s at 70 °C for the extension, and 5s at 82°C), 1 cycle for the melting curve of 5s at 95°C and 1min at 75 °C, ending with 1 cycle of cooling for 1 min at 40 °C. For Chapters 6 and 7 cycle threshold (Ct) values were normalized in relation to the standard concentration value, and for Chapters 3 and 8 data is expressed as relative expression regarding to the control diet following the Livak methodology (Livak and Schmittgen, 2001).

2.5.3. Proteomic analyses

As detailed in Chapter 5 mucus samples were solubilized in urea buffer 8M, centrifuged and re-suspended in Tris-HCl 50mM buffer. In parallel, samples for 2-DE gel electrophoresis were prepared with a Clean-Up kit (GE Healthcare) and re-suspended in 6 M urea and 200 mM ammonium bicarbonate for gel-free LC-MS/MS.

For developing the 2-DE gels, proteins were separated by isoelectric point and SDS-PAGE. The most abundant spots were selected, digested and purified by C18 microcolumn (Zip-Tip, Millipore) and spotted onto a plate and analyzed using a 4800 Plus MALDI-TOF/TOF Analyzer (AB Sciex). The eight strongest precursors from the MS scan were isolated and fragmented by collision-induced dissociation system. Protein identification was performed by the combination of MS and MS/MS spectra against public NCBInr database (Chapter 5).

Alternatively, LC-MS/MS was conducted using a Dionex Ultimate 3000 nano UHPLC system (Thermo Fisher Scientific) connected to a mass spectrometer Orbitrap Fusion (Thermo Fisher

Table 2.2. *Primers designed and used along the present thesis (Chapters 3,6,7 and 8). Interleukins (IL), IL1-β, IL-8, IL-10, IL-17af, IL-17d, IL-22, defensin (def), hepcidin (hep), piscidin (pis), immunoglobulins (Ig), IgM, IgT, Mx protein (mx), inducible nitric oxide synthase (iNOS), Interferon type I (IFNd), Interferon γ (IFNγ), tumor necrosis factor α (tnfα), elongation factor-1α (ef-1α) and β-actin primers were developed by Milne, 2018. *annealing temperature*

Gene	Name	Ann. temp. (°C)*	Forward Sequence	Reverse Sequence
il1-β	Interleukin 1β	62	TGATGGAGAACATGGTGGAA	GTCGACATGGTCAGATGCAC
il-8	Interleukin 8	58	GAAGCCTGGGAGTAGAGCTG	GGGGTCTAGGCAGACCTCTT
il-10	Interleukin 10	58	CTCAAGAGTGATGTCACCAAATG-TAGAAACT	AGCAAATCC AGC TCG CCC ATT
il-17af	Interleukin 17f	62	GGTGGCCCCAGAGGATCTCC	GGAGGACCAAAACCTGGTAGTAGATGG
il-17d	Interleukin 17d	62	CGGTCTACGCTCCCTCCGTG	GCGGCACACAGGTGCATCCC
il-22	Interleukin 22	61	GCCAACATCCTCGACTTCTACCT-GAAC	TGGTCGTGGTAGTGAGTCACATTGC
def	Defensin	58	ATGAGGCTGCATCCTTTCCATG	AGAAAATGAGATACGCAACACAAGAAGCC
hep	Hepcidin	61	GATGATGCCGAATCCCGTCAGG	CAGAAACCGCAGCCCTTGTTGGC
pis	Piscidin	58	ATCGTCCTGTTTCTTGTGTTGTCAC	CGCTGTGGATCATTTTTCCAATGTGAAA
IgM	Immunoglobulin M	58	CTCTTTGATAGGAATACCGGAG-GAGAG	CAACTAGCCAAGACACGAAAACCC
IgT	Immunoglobulin T	59	TGGACCAGTCGCCATCTGAG	GGGAAACGGCTTTGAAAGGA
mx	Interferon-induced mx protein	61	GGCTACATGATTGTGAAGTGCA-GGG	CTTCCAGTCGAGGCAGAGATTTCTCA-ATGT
iNOS	Inducible nitric oxide synthase	60	TGTTTGGCCTTGGCTCCAGGG	GCCCAAGTTCTGAATGACTCCTCCTG
ifn-d	Interferon type I	59	GTCAGGGTGCAGCTGAGTTA	ACAGAAACGGCAGCTCAAAC
ifn-γ	Interferon γ	59	AACTTGGTTTCACGGTGCAG	TCACAACACCGAGAAAGTCCT
tnfα	Tumor necrosis factor α	62	GAAAACGCTTCATGCCTCTC	GTTGGTTTCCGTCCACAGTT
ef-1α	Elongation factor-1α	60	TGCCATACTGCTCACATCGCCTG	ATTACAGCGAAACGACCAAGAGGAG
β-actin	β-actin	61	TCTGGTGGGGCAATGATCTT-GATCTT	CCTTCCTTCCTCGGTATGGAGTCC
muc-2	Mucin-2	62	ATTGAGTTTGGCAACAAACA-GAAAGCCC	TACAGCACAGAACTGAGGTGTCCTC
cath	cathelicidin	58	TGAAGATGAAGGTCCAGGTGA	GAATTGGACGGAAAGCCTGC
c3	Complement component 3	59	ATAGGTGATGTGCGTGGCAA	AAGGGTTGATTAGGCTGGGC
cd8	Cluster of differentiation 8	59	AAAATGTCGCCACCACTTCG	GTCCTTCATGCTCCAACGGA
casp-3	Caspase 3	60	GCTCGGCCTCATTCATCTGT	AGAGTTTGGGCTTTCCCACC
StAR	Stereidogenic acute regulatory protein	59	GGAACATGGAGCAAATGGGC	ACAAAATCCCTCGGTCCCAC

Scientific) equipped with nanoelectrospray ionization interface. The peptide mix of skin mucus was concentrated and cleaned with a 300 µm x 5 mm Acclaim Pepmap precolumn (Thermo Scientific). The trapping column was switched on-line with the separation column and peptides were separated at 300 nL/min. The mass spectrometer was operated in the positive mode and for protein identification mass spectrometry raw data were processed using Proteome Discoverer v2.1.0.81 (Thermo Fisher Scientific). Moreover, MS2 spectra were searched with SEQUEST HT engine against UniprotKB database restricted to Actinopterigii class and bacteria for the microbiome analysis (Chapter 5). All the proteomic analyses were performed at the Proteomics Unit, SCAI (Central Facilities for Research Support), University of Córdoba, Spain.

2.6. Fish welfare and Immune parameters

2.6.1. Serum and skin mucus Bactericidal activity (Chapter 6)

Bactericidal activity was measured with a modification of the method described by Sunyer and Tort (1995) using *Photobacterium damselae* obtained from infected greater amberjack. This methodology evaluates the effect of the serum and skin mucus sample on the bacterial growth curve. The bactericidal activity was calculated as follows: % Bactericidal activity = (absorbance of sample with bacteria – absorbance of sample) *100/absorbance of bacteria solution

2.6.2. Serum and skin mucus lysozyme activity (Chapters 6 & 8)

Lysozyme activity was determined as described by Ellis (1990). Lysozyme activity was expressed in units ml^{-1}. One unit of lysozyme was considered as the quantity of enzyme needed for reducing absorbance by 0.001 per milliliter of serum and skin mucus per minute.

2.6.3. Protease activity (Chapters 5 & 8)

As detailed in Chapters 5 and 8, skin mucus was diluted 1:1 in a solubilization buffer (urea 8 M, CHAPS 2%, β-mercaptoethanol 10 mM) and centrifuged twice for removing mucins. After that, protease activity was quantified using the azocasein hydrolysis assay according to the

method of Ross *et al.* (2000). Protease characterization (Chapter 5) was conducted using the azocasein hydrolysis assay with specific inhibitors of proteases: 10 mM ethylenediaminetetraacetic acid (EDTA as inhibitor of metalloproteases) or 5mM phenylmethylsulfonyl fluoride (PMSF as inhibitor of serine-proteases).

2.6.4. Protein quantification

Protein quantification was conducted following the methodology described by Bradford (1976) using bovine serum albumin (BSA) as a standard. Data of protease activity was normalized by mg of protein.

2.6.5. Concentration of skin mucus and circulating plasma cortisol (Chapters 3 & 8)

Plasma and skin mucus cortisol from Chapter 3 were analyzed in Universidad Autónoma de Barcelona (UAB) using radioimmunoassay (RIA) based on the trypsin/antitrypsin method detailed by Rotllant *et al.* (2001). The antibody used for the assay was purchased from M.P. Biomedicals LLC (OH, USA) and used in a final dilution of 1:4500, where cross-reactivity with cortisol was 100%. The radioactivity was quantified using a liquid scintillation counter. On Chapter 8 cortisol determination was conducted by immunoassay (Access Immunoassays system, Cortisol ref: 33600, Beckman Coulter, Inc., USA).

2.7.
Standardized challenge test used

2.7.1. Standardized challenge test against Neobenedenia girellae (Chapters 4, 5, 6 & 8)

2.7.1.1. *Standardized Neobenedenia girellae culture*

N. girellae for experimental challenge tests were obtained from a greater amberjack parasitized tank. For maintaining the parasite source, three different 500 l tanks with greater amberjacks were used. Once one of the tanks was parasitized, eggs were collected with a 0.14 mm pore net (5 x 5 cm) that was fixed to the tank (Fig. 2.4), the net was checked and the number of eggs for cm^2 of net was obtained. If the number of eggs per cm^2 was over 10, the

Figure 2.4. *Own-designed tool for Neobenedenia girellae egg collection and evaluation. The mesh of the nets (A) was 5 or 0.14 mm depending on water conditions and was introduced in the tanks as figure 2.4 B shows.*

net was introduced inside another tank as source of parasites, and the previous parasitized tank was cleaned with bleach and the fish sampled and treated with a freshwater bath for 3 minutes, following this protocol the parasite source was maintained along the whole year.

2.7.1.2. *Parasitization challenge test*

For the parasitization challenge, a selected flow-through tank with greater amberjack juveniles was considered as the parasite source (provider), no anti-parasite prevention treatment protocol was applied (described in 2.2.1) and fish reached a high parasitization level by natural infection. Furthermore, three nets of 0.14 or 5 mm mesh (5 x 5 cm) were placed in the parasite source tank, and checked after 24 hours to evaluate that the number of eggs per cm^2 was at least, up to 40 for each net. Once the desired density of eggs in the nets was reached, the nets were placed inside a 1,000 l tank with selected non parasitized greater amberjack juveniles, considering them as parasite-carriers, over 50-100 g body weight, to obtain a homogeneous parasitization of all parasite-carrier fish. Then, after 10-15 days at 22-

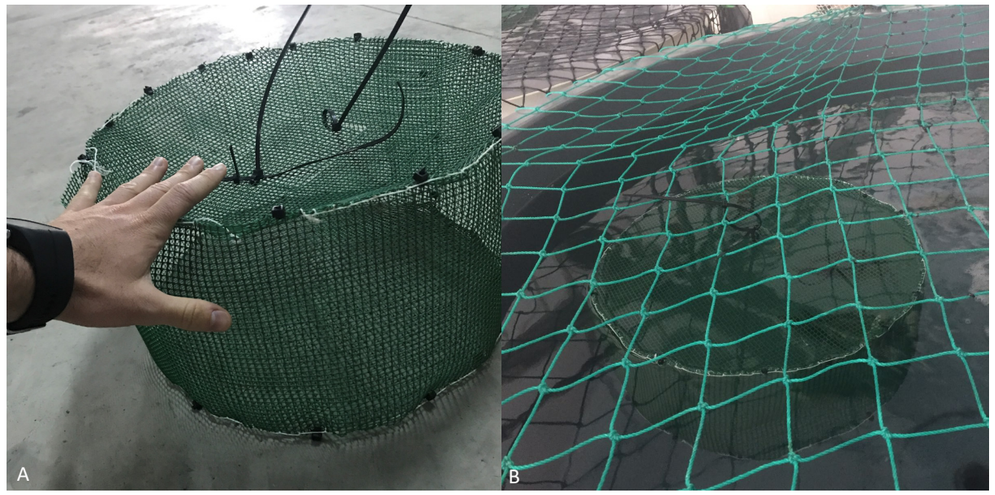


Figure 2.5. *(A) Cages of 0.03m³ used for the Neobenedenia girellae cohabitation challenges in Chapters 6 and 8. Notice that the cage is attached to the tank net for avoiding movements and reducing fish stress status (B).*

24°C, the parasite lifecycle was almost complete, and parasitized fish were placed into 0.03 m³ cages for 15 days, to enable a cohabitation challenge (Fig. 2.5).

As a variation, in Chapter 5 parasitized fish were not placed in cages and were released in the experimental tank with healthy fish in a direct cohabitation system. To properly identify them, cohabitant fish were marked with a Visible Implant Elastomer (VIE) (Astorga *et al.*, 2005) and the cohabitation process was maintained for 30 days in order to induce a long-term parasitization status. Moreover, in Chapter 4, parasitization was obtained by introducing nets with entangled eggs inside the experimental tanks due to the requirement of the highest parasitization level.

Fig. 2.6 shows the standardized protocol for challenging the experimental fish against *N. girellae* used along Chapters 4, 5, 6 and 8.

2.7.1.3. *Sampling collection of Neobenedenia girellae from infected fish after the challenge test.*

To collect the parasites after challenge test, infected fish were placed in 100 l plastic contai-

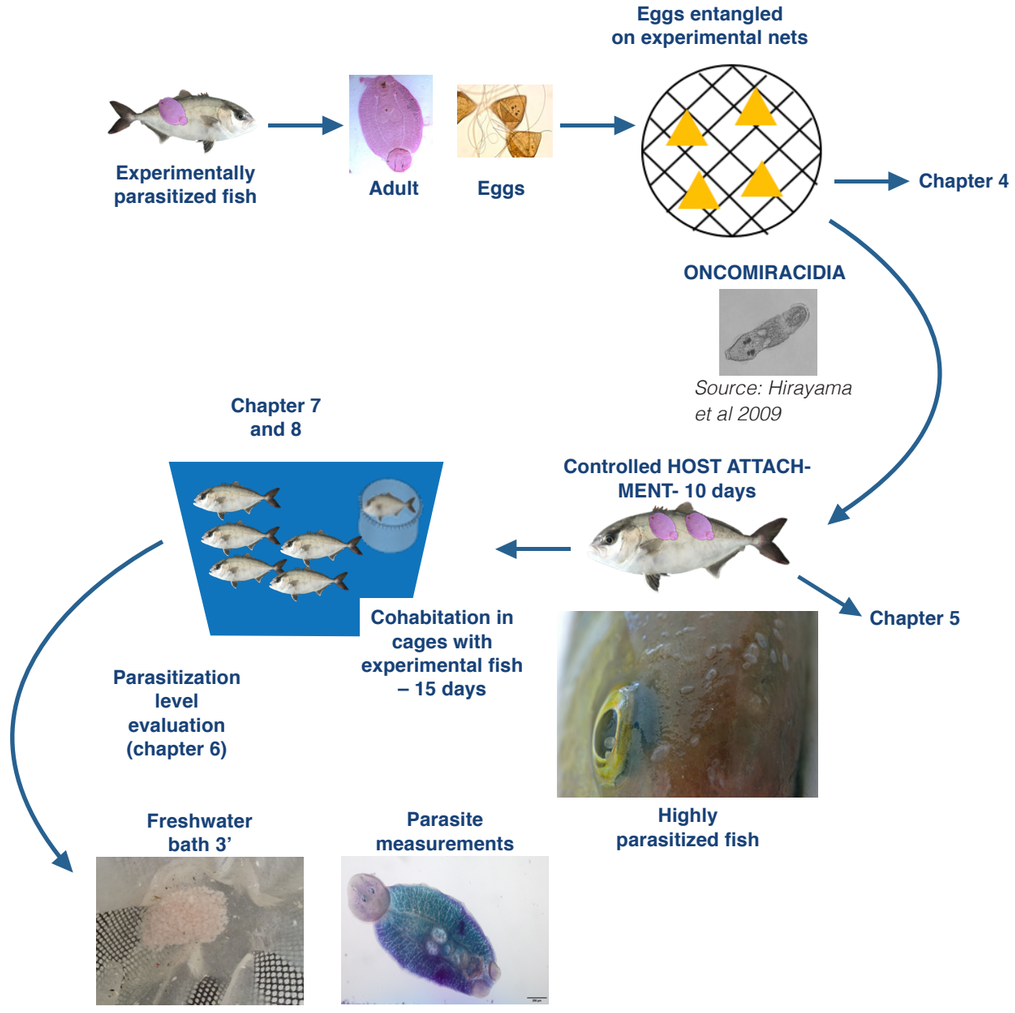


Figure 2.6. *Experimental protocol for Neobenedenia girellae controlled infections conducted in Chapters 4, 5, 6 and 8. For all the parasite challenge typologies used in the present thesis a controlled parasitized greater amberjack tank is the source of the parasites, from which N. girellae eggs are obtained after entangling to the egg collector net. Afterwards, nets are introduced for 10 days inside another greater amberjack tank (Chapter 4). The objective of this methodology is to infect all fish at the same time and with the same level of infestation (Chapter 5). After that, similarly parasitized greater amberjacks are used for the cohabitation model introducing the fish in cages inside the experimental tanks for 15 days (Chapter 6 & 8)*

ners with fresh water for 3 minutes. After the freshwater bath, *N. girellae* individuals change their color to white and unhook from greater amberjack skin, allowing their collection by filtering the water (0.14 mm mesh net). All the parasites were recovered and introduced in 96% ethanol to be counted and measured.

2.7.1.4. *Measurement and parasite load determination of Neobenedenia girellae (Chapters 6 & 8)*

Both, total greater amberjack surface area, and estimations on the number of parasites per cm^2 (Ohno *et al.*, 2008) were calculated as follows:

- Total greater amberjack surface area (cm^2) =0.158* (total length)$^{2.089}$ *2; (r=0.93)
- Number of parasites/cm^2 of fish surface area= number of parasites on fish/ fish surface area in cm^{2-}

A visual estimation of the parasitization level was conducted in Chapter 6 by three different researchers unaware of the experimental treatments, and following a previous established scale as follows: 0 (no parasites observed), 1 (1 to 5 parasites), 2 (6 to 15) and 3 (more than 15 parasites).

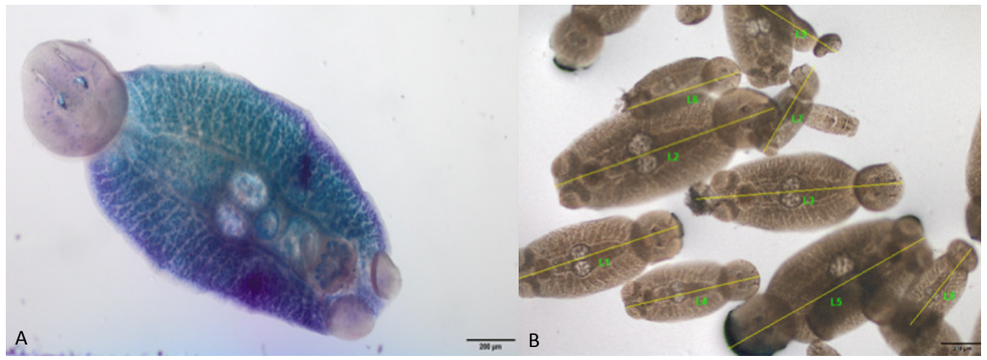


Figure 2.7. *(A) Neobenedenia girellae detail. (B) Detail of parasite total length measurements . Scale bars = 200 µm*

Parasites total length measurements were obtained using a profile projector (Mitutoyo, PJ-A3000, Kawasaki, Japan) by placing on microscopy slides and measuring the length from haptor to the oral suckers (Whittington, 2011) (Fig. 2.7).

2.7.2. Stress challenge tests (Chapters 3 & 8)

In order to use standardized stress protocols, a series of challenge tests were applied depending on the experiment objective, by adapting standardized protocols from PCTM routine experimentation (Rotllant *et al.*, 2003; Carvalho *et al.*, 2019; Torrecillas *et al.*, 2019).

2.7.2.1. *Shallow water stress challenge test (Chapter 3)*

Blood samples from three fish per tank were obtained before to the start of the challenge, which was considered as the initial sampling point (time 0). Total water volume was reduced from 1000 l to 200 l for 1 hour (Fig. 2.8.a). After this, and in order to obtain a time-course study of the plasma and skin mucus cortisol pattern, plasma and skin mucus were obtained from three fish per tank at 1, 3, 24 and 72 hours post stress

2.7.2.2. *Overcrowding stress challenge test (Chapter 8)*

Before the start of the challenge test, blood samples were obtained from three fish per tank, which was considered as initial sampling point (time 0). After this, three fish per tank were placed inside of 0.03m^3 cages, and two cages were placed in each challenged tank (Fig. 2.8.b)(for details see Chapter 8). Fish were sampled after 3- and 24-hours post cage-confinement and blood was obtained for plasma cortisol measurements.

2.7.2.3. *Stocking density plus handling (Chapter 3)*

These specific culture conditions were used in trial 2 of Chapter 3 for determining the stress response after a shallow water challenge test of fish reared under stressful culture conditions. In accordance to Mylonas (2019), the optimal culture density for greater amberjack was es-

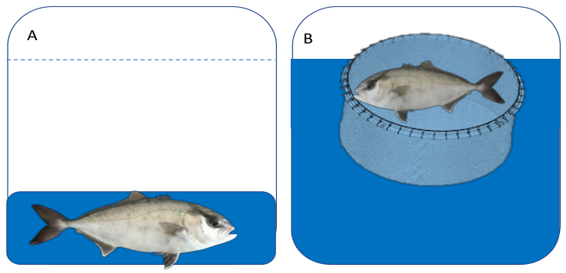


Figure 2.8. *Example of (A) shallow water stress model where a reduction from 1000 l to 200 l was used as stressor in Chapter 3, trials 2 and 3 and (B) overcrowding stress model in cages of 0.03m^3 used in Chapter 8.*

tablished at 4 kg m^3 as the low-density group and the high-density group was reared at 8 kg m^3. The handling stress protocol was established as a standard weight and length sampling, being the low handling level group only sampled every 30 days and the high handling group every 7 days. After that, a shallow water stress was applied (Chapter 3).

2.7.2.4. *Fasting challenge test (Chapter 3)*

Fasting was used as stressor in trial 3 of Chapter 3. Thus, control fish were fed to apparent satiety 3 times per day meanwhile fasting group remained starved for 10 days. After that, a shallow water stress challenge test was conducted (Chapter 3).

2.8.
Statistical analysis

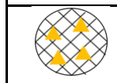
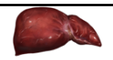
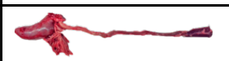
The statistical analyses followed the methods outlined by Sokal and Rolf (1995), with means and standard deviations (SD) calculated for each parameter measured. All data were tested for normality and homogeneity of variance. Data were subjected to one-way ANOVA if one factor was compared against more than 1 variable and differences were considered significant when $P < 0.05$. For determining significant differences between two samples, T-Student test was conducted considering significant when $P< 0.05$. Two-way ANOVA was conducted for evaluating the interaction among treatments if possible. If the variances were not normally distributed, data were transformed (log_{10}) and the Kruskall-Wallis non-parametric test applied. Kruskall- Wallis analysis was also used for range-comparison statistical analyses. A correlation matrix was produced considering significant differences when $p<0.05$ and consequently, a regression model was applied using Pearson correlation coefficient. All these analyses were performed using SPSS software (SPSS for windows 10).

Multivariant analyses and their plots were performed using PRIMER 7 and PERMANOVA. The number of permutations was established at 999. PERMANOVA analysis considered differences significant when the permutation p-value (p perm.) was below 0.05. Principal components analyses (PCA) were used when data did not show large differences among them due to the unconstrained nature of those analyses. Moreover, if data showed high variability, a resemblance matrix was produced and constrained analyses like principal coordinate analyses (PCO) or non-metric multidimensional scaling (NMDS) was developed.

2.9.
Schemes of the experimental designs used

Experimental protocols followed along this thesis are detailed in depth in each specific chapter. However, for a comprehensive lecture of the general material and methods, a collection of schemes has been included within this section. A legend of all the symbols used along the schemes are shown in Table 2.3

Table 2.3. *Summary of the symbols used whitin the schemes of the experimental*

	Greater amberjack		Stressor
	*Neobenedenia girellae*		Skin
	*Net-entangled N. girellae* eggs		Gills
	Parasitized fish		Liver
	Skin mucus		Head Kidney
	Blood		Intestine
	Plasma		Spleen
	Serum		Shallow water
	RNA later for transcriptomic		Cage confinement-overcrowding

2.9.1. Chapter 3. Stress response and skin mucus production of greater amberjack (Seriola dumerili) under different rearing conditions

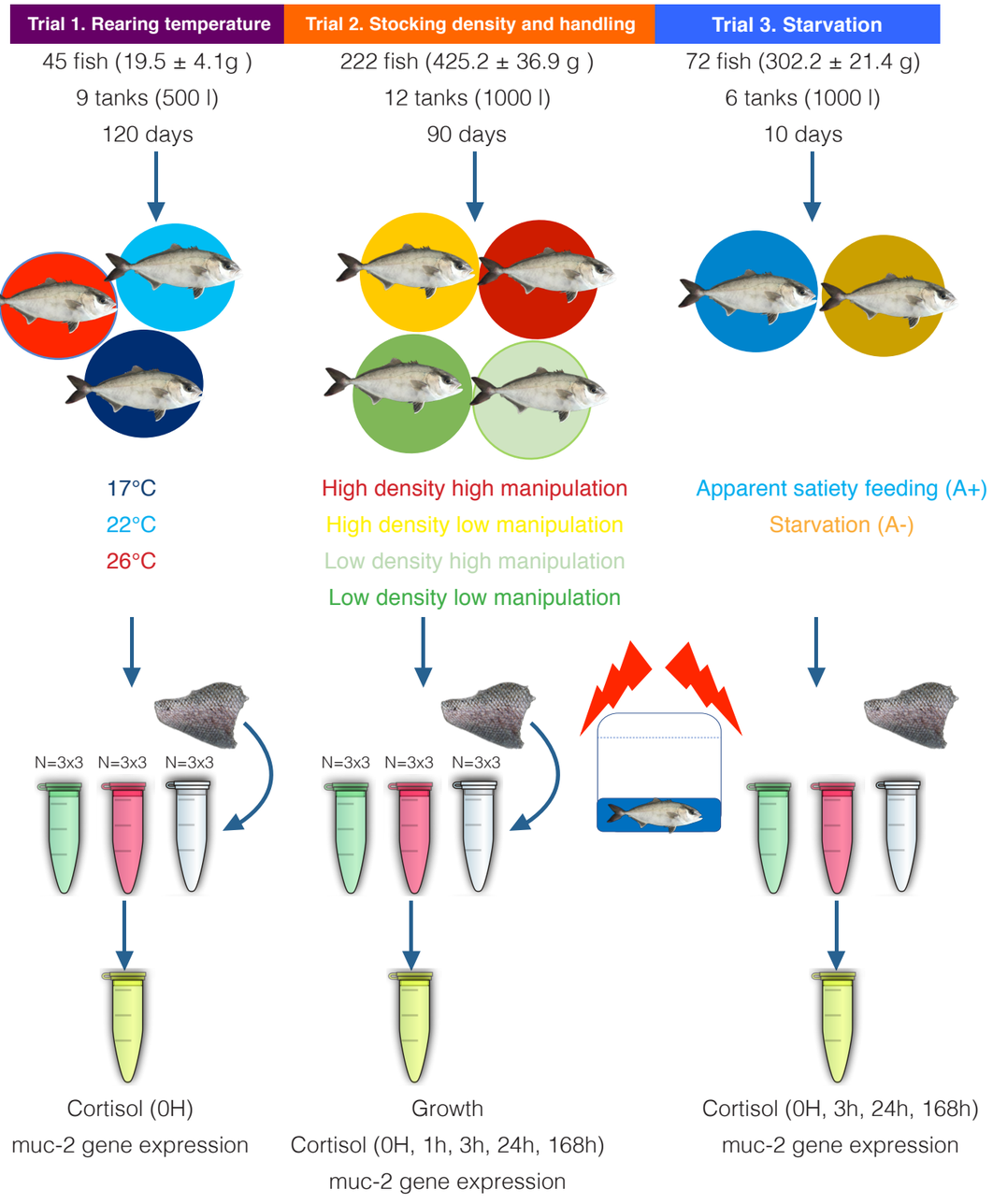


Figure 2.9. *Scheme of the experimental design from chapter 3*

2.9.2. Chapter 4. *Skin Infection of Greater Amberjack (Seriola dumerili) by monogenean ectoparasite Neobenedenia girellae: a morphological and descriptive study.*

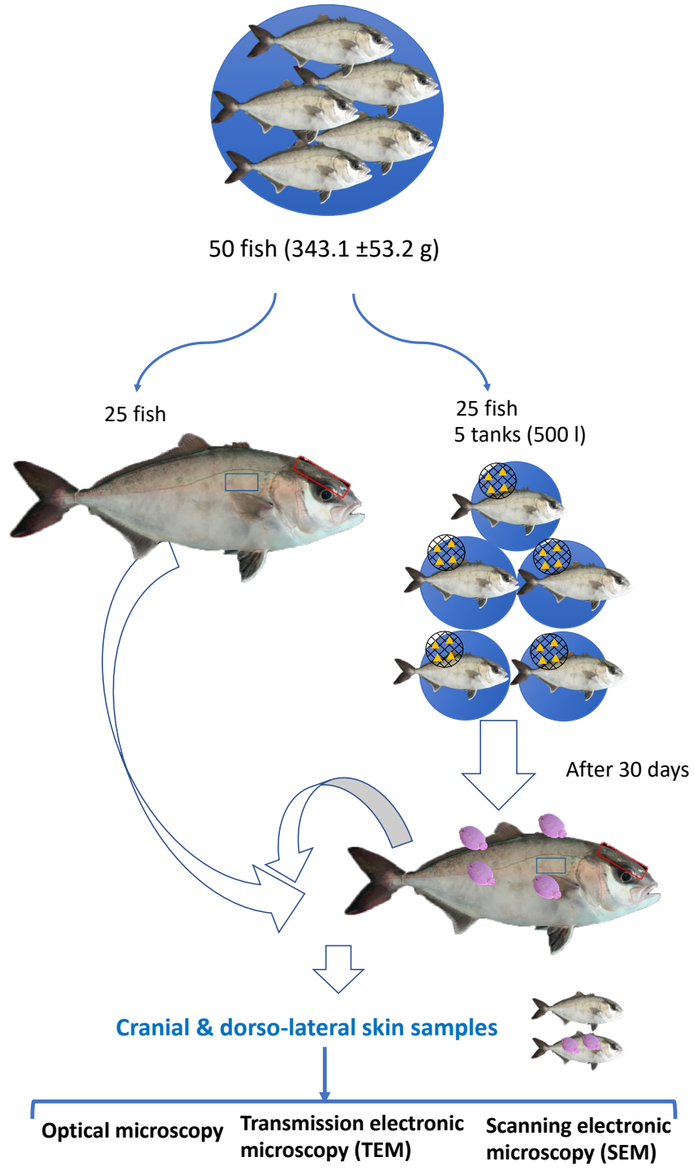


Figure 2.10. *Scheme of the experimental design from chapter 4*

2.9.3 Chapter 5. Proteomic profile, microbiota and protease activity of skin mucus in greater amberjack (Seriola dumerili) infected with the ectoparasite Neobenedenia girellae

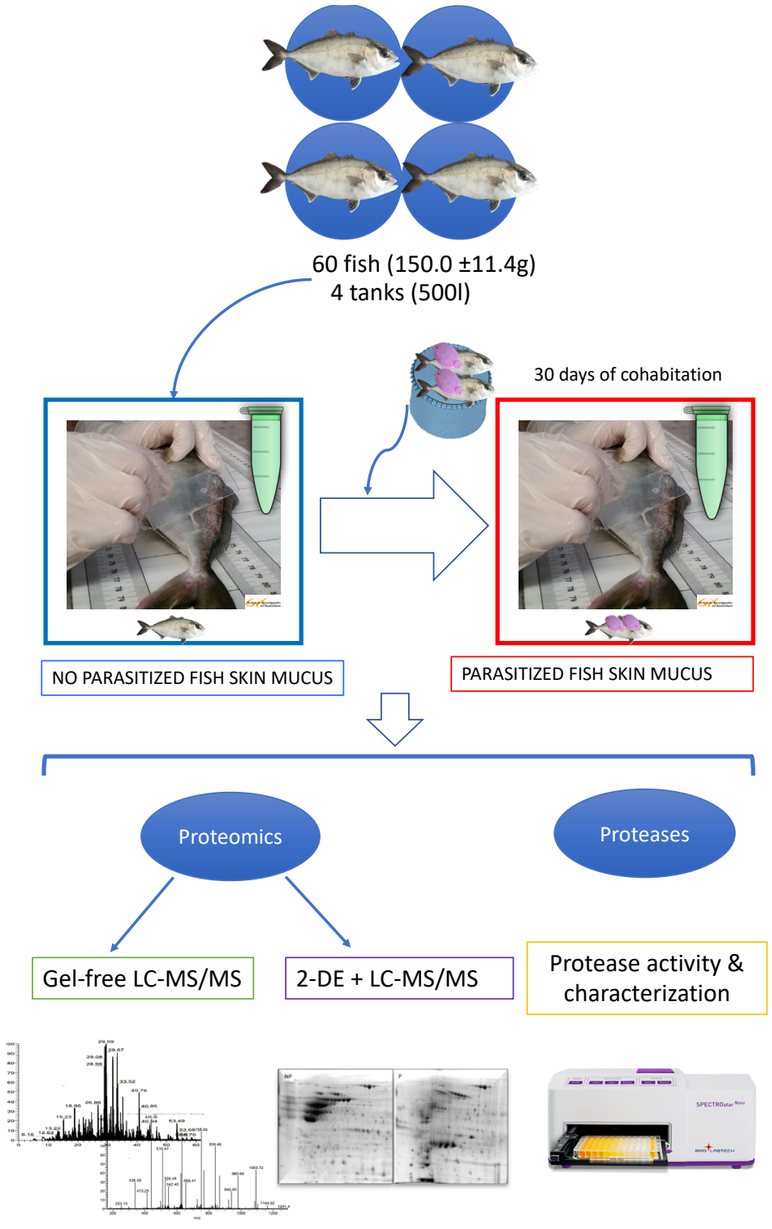


Figure 2.11. *Scheme of the experimental design from chapter 5*

2.9.4. chapter 6 An insight into piscidins: The discovery, modulation and bioactivity of greater amberjack, Seriola dumerili, piscidin

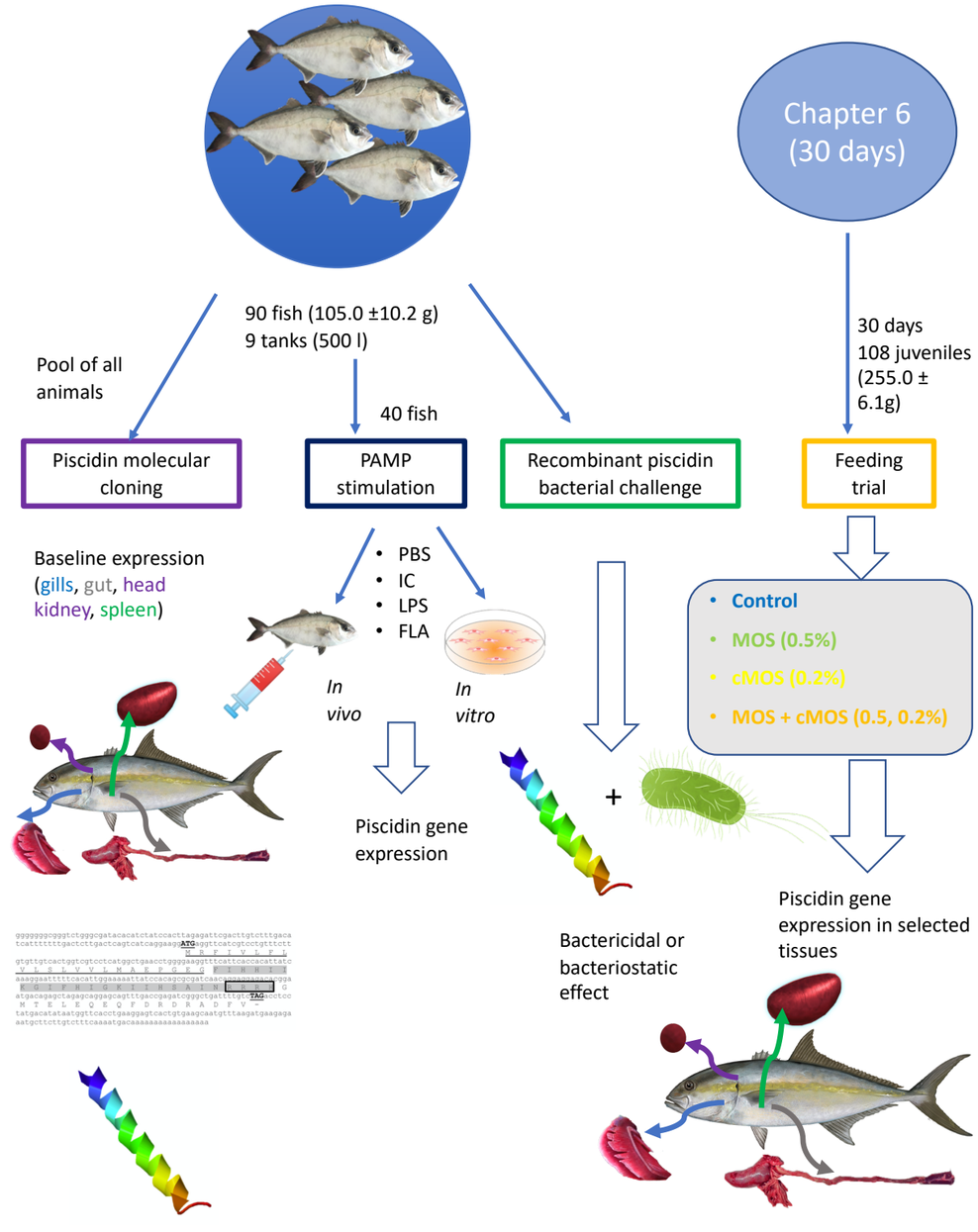


Figure 2.12. *Scheme of the experimental design from chapter 6*

2.9.5.Chapter 7. Increased parasite resistance of greater amberjack (Seriola dumerili Risso 1810) juveniles fed a cMOS supplemented diet is associated with upregulation of a discrete set of immune genes in mucosal tissues.

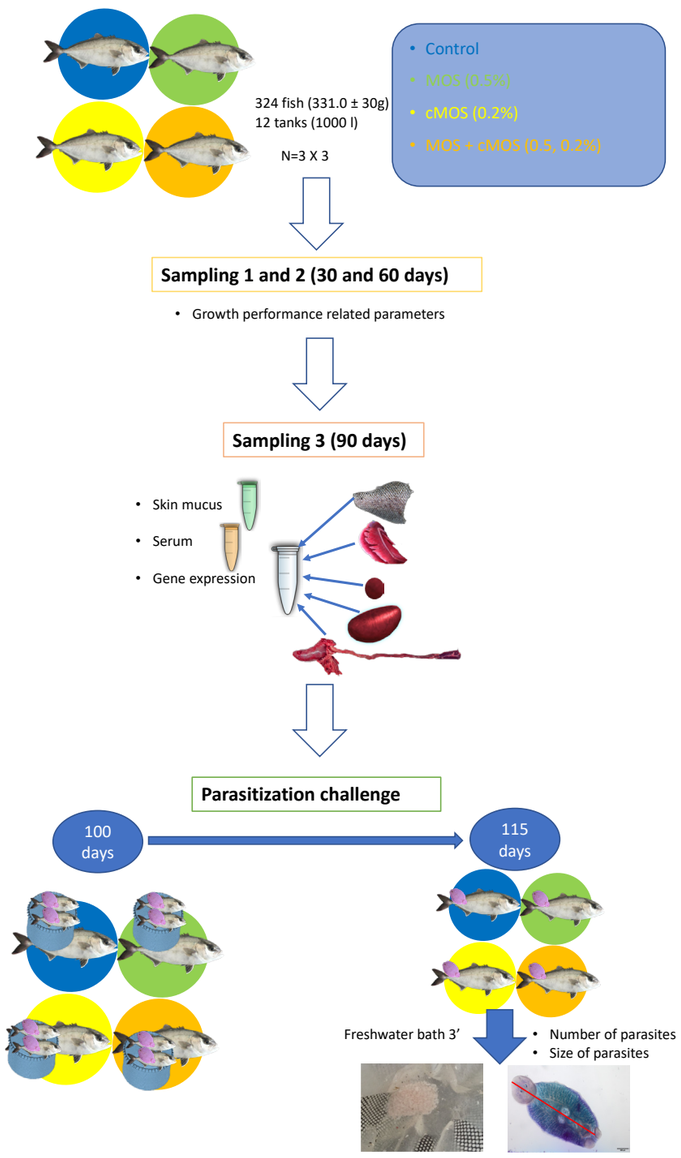


Figure 2.13 *Scheme of the experimental design from chapter 7*

2.9.6.Chapter 8. Improving Greater Amberjack (Seriola dumerili) defences against Neobenedenia girellae infection through functional dietary additives

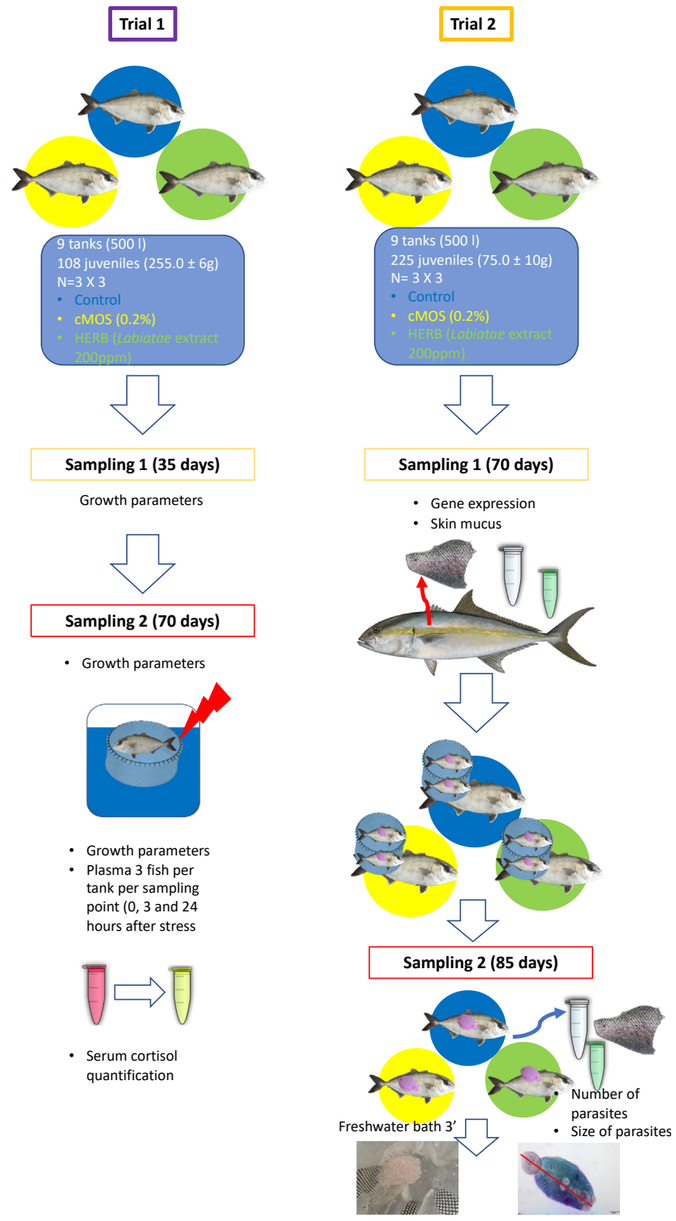


Figure 2.14 *Scheme of the experimental design from chapter 8*

CHAPTER 03

STRESS RESPONSE AND SKIN MUCUS PRODUCTION OF GREATER AMBERJACK *(SERIOLA DUMERILI)* UNDER DIFFERENT REARING CONDITIONS

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Abstract

The main objective of this study was to evaluate the stress response of the greater amberjack, *Seriola dumerili,* by determining plasma and skin mucus cortisol levels, as physiological indicator of fish stress, and skin mucin-2 gene expression as indicator of skin mucus production, under three different potential stressors related to aquaculture practices: temperature, handling and fasting. For trial 1, 45 greater amberjack juveniles (19.5 ± 4.1 g) were divided in three graded rearing temperatures (17, 22 and 26ºC) and maintained for 120 days, in order to define the basal cortisol level for this species after a long-term thermal acclimation within the range described for this species. Trial 2 was conducted under two different rearing densities and two different handling protocols with 222 fish (425.02 ± 36.9 g) divided in 4 treatments by triplicate for 90 days, in order to study the effect of routine handling protocols on the selected stress markers for this species. Trial 3 used 72 fish (302.27 ± 21.4 g) divided in two experimental groups, one fed to apparent satiety and another one subjected to fasting for 10 days (triplicates for each group). After trials II and III, a shallow water challenge was conducted to further study the adaptive response of greater amberjack juveniles to combined stress situations. Plasma and skin mucus were obtained for cortisol determination at 0, 1, 3, 24 and 72 hours post stress challenge. Additionally, skin was dissected out for determining *muc-2* gene expression. Long-term acclimation to temperature did not affect plasma or mucus cortisol levels and *muc-2* gene expression, being basal levels of circulating plasma cortisol around 7.5 ng/ml. A high stocking density (initial stocking density of 8 $kg \cdot m^{-3}$ and final 11.5 $kg \cdot m^{-3}$) induced an elevation ($p<0.05$) of plasma cortisol and a reduction ($P<0.05$) of weight gain. No differences were observed for the expression of *muc-2* gene, however, a tendency to an up-regulation was observed in fish subjected to the high-density rearing treatment. Short-term fasting induced an increase ($p<0.05$) of plasma and skin mucus cortisol together with a down-regulation of *muc-2* gene expression.

3.1. Introduction

Fish skin mucus acts as a dynamic and semipermeable barrier, being the fish first external defense, protecting from environmental fluctuations and taking part of fish osmoregulation, respiration, nutrition and hydrodynamics (Shephard, 1994; Esteban, 2012). Mucus is secreted in skin by goblet cells in the epidermal layer, with an adequate composition to prevent stable colonization by potentially infectious microorganisms and infection by metazoan parasites (Nagashima *et al.*, 2003). Skin mucus is mainly composed by macromolecules like glycoproteins (mucins), which are the insoluble fraction and are responsible for rheological properties, giving a viscous consistency (Fletcher *et al.*, 1976). Besides, fish mucus contains components related with metabolism and immune defense, involving a series of antibacterial agents, such as lysozyme, immunoglobulin, complement or lectins, and antimicrobial polypeptides (AMPPs), which exert an inhibitory or a lytic activity against different pathogens (Parra *et al.*, 2015; Cordero *et al.,* 2015; Pérez-Sánchez *et al.*, 2017).

Additionally, other metabolites could be found in skin mucus such as free aminoacids that play a role in chemical communication for odorant-based schooling behavior (Ekman *et al.*, 2015), as well as those with implications in stress response, including glucose, lactate or cortisol (Fernández-Alacid *et al.*, 2018, 2019). Composition of fish mucus varies among fish species and with endogenous (sex and developmental stage) and exogenous factors (stress, water temperature, pH and infections) (Esteban, 2012). Among them, stress conditions such as temperature, handling, confinement or food deprivation among others can have a direct impact on fish mucus production and composition (Vatsos *et al.*, 2010), affecting directly the fish immune barrier (Terova *et al.*, 2011).

As poikilotherms, temperature is a crucial factor on fish physiology (Tort *et al.*, 2004) and a maladaptive response to changes of environmental temperature can induce a chronic stress in fish and harm fish skin and mucus immune potential (Aranchi*et al.*, 1999; Huang *et al.*, 2011, Sanahuja *et al.*, 2019). Both acute thermal stresses (either high or low temperature) have been shown to induce significant changes in skin mucus barrier, when a thermal shock occurs or acclimation fails to adapt to temperatures far away from the biological range for each species (Jensen *et al.*, 2015; Sanahuja *et al.,* 2019).

Other environmental parameters can act as stressors, inducing elevation of plasma cortisol, and influencing the functionality, structure or cellular composition of fish epidermis (Tacchi *et al*., 2015; Guardiola *et al.,* 2015). For salmonids, some evidences of correlations between stress or the subsequent plasma cortisol level and some mucus enzyme/protein profiles (Easy & Ross, 2010) and the discharge of mucus by the epidermal mucous cells both *in vivo* and *in vitro* (Van der Salm *et al.,* 2002) has been described. After four hours of confinement stress at a high biomass density, that induces an elevation of plasma cortisol (Vijayan *et al.,* 1997), antimicrobial polypeptides were altered in the skin of European sea bass (*Dicentrarchus labrax*) (Terova *et al*., 2011). Prolonged periods of high stocking density (120 days) inducing an elevation of plasma cortisol levels, have been shown to alter mucus composition in turbot (Jia *et al*., 2016). Other long-term stressors, such as repetitive handling stress, that also increases circulating cortisol in plasma, have been described to produce cell damage in the Atlantic salmon (*Salmo salar*) skin (Easy and Ross, 2010). One of the direct consequences of high stocking density is a poorer feed utilization or even a starvation status for variable periods of time, which cause a chronic stress situation (Santos *et al.* 2010), with a subsequent effect on fish skin structure, mucus production and functionality (Caruso *et al*., 2010).

The decreased skin mucus production and the immune alteration caused by stress is one of the main causes for the occurence of opportunistic or secondary pathologies (Wendelaar Bonga, 1997). Stress is also related with a depletion of mucosal immune response, increasing the occurrence of pathogenic outbreaks (Tort 2011; Niklasson *et al*., 2013). Skin becomes a target tissue to study when considering the main pathologies affecting species with high susceptibility to ectoparasite infections, such as the greater amberjack (Ogawa *et al*., 1995; 1998). This species is a good candidate for European aquaculture (Mylonas*et al*., 2019) but scarce information about greater amberjack juvenile management in culture conditions is yet available (Fernández-Montero *et al*., 2018).

Thus, the aim of this study was to evaluate the stress response in terms of plasma and skin cortisol, as well as the skin mucus production of greater amberjack (*Seriola dumerili)* under three different stressors related with standardized rearing conditions. In a first trial, greater amberjack juveniles were acclimated to three different and continous temperatures during 120 days to test if the rearing temperature is affecting the expression of *muc-2* gene or cortisol concentration in plasma or skin mucus. Once the temperature range was defined, a second trial was conducted, to test different aquaculture procedures, including both, high stocking density and routine handling procedures. In this second trial the stress response to standardized aquaculture-related protocols such as stocking density and handling, was measured not only in terms of plasma cortisol but also in terms of *muc-2* gene expression and skin mucus cortisol content. In a third trial, fasting was studied as a situation potentially derived from a high stocking density. Thus, an acclimation procces to a fasting period plus a shallow water stress test was assayed.

3.2. Material and Methods

The present study was conducted at the Scientific and Technologic Park of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain). The animal experiments described comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals. All fish used in this work have been raised in the facilities of Scientific and Technologic park of the University of Las Palmas de Gran Canaria.

3.2.1. Experimental fish, sampling and rearing conditions.

2.1.1. Trial 1. Rearing temperature trial

Forty-five greater amberjack juveniles (mean weight 19.5 ± 4.1 g) were distributed in 9 cylindroconical 500 L tanks (5 fish per tank). Afterwards, three temperature treatments (17, 22 and 26ºC (± 0.5)) were assayed in triplicate for 120 days; each three tanks for a given temperature were controlled by one recirculation system with oxygen values around 7.6 ± 0.9 ppm O_2. Fish were fed 3 times per day to apparent satiety. Growth performance, feed efficiency and rearing conditions have been previously published in Fernandez-Montero *et al.* (2018). After 120 days of rearing, plasma and skin mucus were obtained from the animals reared at each temperature without any stress stimulus.

3.2.1.2. Trial 2. Stocking density and handling trial

Two hundred and twenty-two fish (mean weight 425.02 ± 36.9 g) were distributed in twelve cylindroconical 1000 L tanks in an open water circulation. Water conditions were monitored daily, maintaining salinity at 37 mg L^{-1}, oxygen values at 7.2 ± 1 ppm O_2 and temperature at 22°C ± 0.9. Fish were fed by hand 3 times per day to apparent satiety.

Two different densities (4.2 kg·m^{-3} and 8.8 kg·m^{-3}) and two fish handling protocols were assayed, a high manipulation group (HM) and a low manipulation (LM) group, simulating standardized protocols to obtain fish weight and length every 7 days for HM group and every 30 days for LM group. For that, fish were anaesthetized with clove oil (1:1 clove oil and ethanol 96%, 5mL·L^{-1}; Guinama S.L; Spain, Ref. Mg83168). Growth parameters calculated were: Weight gain (g), specific growth rate (SGR) and feed conversion ratio (FCR). Experimental groups were defined as low density/low manipulation (LDLM), low density/high manipulation (LDHM), high density/low manipulation (HDLM) and high density/high manipulation (HDHM). After 90 days of rearing, a shallow water stress challenge test was conducted by reducing water volume down to 200 L for 1 hour, keeping flow-through to maintain water quality and dissolved oxygen for all the experimental groups assayed. Blood and skin mucus samples were obtained at time 0 and after 1, 3, 24 and 168 hours post shallow water stress.

3.2.1.3. Trial 3. Starvation trial

Seventy- two fish (mean weight 302.27 ± 21.4 g) were randomly distributed in six cylindroconical 1000 L tanks in a flow-through system. Temperature and oxygen were kept at 22.1°C ± 0.8 and 6 ± 1 ppm O_2 respectively. Experimental groups were designed as control group (A^+) feeding to apparent satiety 3 times per day, and a fasting group (A^-), submitted to starvation for 10 days. After this period, a shallow water challenge test was conducted following the conditions previously described for trial 2. Blood and skin mucus samples were obtained at time 0 and after 1, 3, 24 and 168 hours post shallow water stress.

3.2.2. Sampling protocols

Blood samples were extracted with a heparinized syringe for plasma extraction, where at least 1ml of blood was obtained from each fish. Skin mucus samples were obtained following the methodology described in Fernández-Montero *et al.* (2019) and immediately frozen at -80°C. Skin samples for gene expression analyses were obtained from the same dorso-lateral left side of all fishes. All samples were obtained at time 0 and 1 hour after stress.

3.2.3. Cortisol determination

Plasma was extracted from blood after centrifuging 5 minutes at 3000g at 4°C. Skin mucus was diluted 1:1 with autoclaved and filtered sea water and then centrifuged 2 times at 30000g at 4°C for 15 minutes. Cortisol determination was conducted using a RIA based on the trypsin/antitrypsin method detailed in Rotllant *et al.* (2001). The antibody used for the assay was purchased from M.P. Biomedicals LLC (OH, USA) and used in a final dilution of 1:4500, where cross-reactivity with cortisol was 100%. The radioactivity was quantified using a liquid scintillation counter.

3.2.4. Gene expression

After sampling, the tissue conserved in RNAlater was stored at 6ºC for 48 hours. After that, RNA was extracted using Trizol method (Invitrogen, Thermo Fisher Scientific, Madrid, Spain) according to the manufacturers instructions. RNA concentration and quality were checked by spectrophotometry at 260 and 280nm (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain) and by electrophoresis in agarose gels. For avoiding genomic DNA contamination, a DNase treatment was applied to the extracted RNA according to the manufacturer's specifications (AMPD1–1KT, Sigma–Aldrich, Broendby, Denmark). Thus, ThermoScript™ Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Madrid, Spain) kit was used for reverse transcription of total RNA, and cDNA was obtained in a thermocycler (Mastercycle ® nexus GSX1, Eppendorf AG, Hamburg, Germany) according to the manufacturer's instructions.

Specific primers for *muc-2* and *ef1* as housekeeping were described previously (Milne, 2018). qPCR was conducted with SYBRgreen and truestar taq under the following conditions: 1 cycle of 6 min denaturalization at 95°C, 45 cycles of amplification (25 s at 95°C, 30 s at the annealing temperature, 25 s at 70°C for the extension, and 5 s at 82°C), 1 cycle for the melting curve of 5 s at 95°C and 1 min at 75°C, ending with 1 cycle of cooling for 1 min at 40°C. Observing temperature was change to 84°C in *muc-2*, annealing temperature was 60°C for *ef1*α and 62°C for *muc-2*.

3.3. Results

3.3.1. Trial 1. Rearing temperature trial

Rearing temperature affects greater amberjack performance in terms of growth and feed

utilization. Partial data, without including cortisol and *muc-2* expression has previously been published in Fernández-Montero *et al.* (2018). Briefly, fish held at the lowest temperature (17°C) grew worst than the other groups held at 22°C or 26°C. Although there was no significant different feeding intake per 100g of fish, those fish held at 17°C utilized worst the feed, and being 22°C the medium temperature of Mediterranean and Macaronesia-Atlantic regions, this temperature was used for the next trials. Long-term acclimation at the different temperatures assayed, did not alter plasma cortisol of greater amberjack juveniles after 120 days, being plasma cortisol basal levels determined around 7.5 ng·ml^{-1} plasma (Table 3.1). Values obtained after 120 days of rearing at different temperatures, with no other stressful condition altered, could be considered as basal levels for this species. The acclimation to rearing temperature did neither alter the concentration of cortisol in mucus, being levels around 0.75 ng·ml^{-1} (Table 3.1). A significant ($p<0.05$; $r=0.22$) correlation was observed between plasma and skin mucus cortisol in spite of the low Pearson correlation index obtained. The acclimation process to different temperatures during 120 days did not induce changes in the skin mucin production, as no differences were observed in *muc-2* transcripts among the different temperatures challenged (Table 3.5).

3.3.2. Trial 2. Stocking density and handling procedures

At the end of the experimental period, high density group reached 11.5 kg·m^3 and low density 5.9 kg·m3. High stocking density induced a significant ($p<0.05$) reduction of growth performance in greater amberjack juveniles. Those fish held at high stocking density, either

Table 3.1. *Plasma and mucus cortisol (ng·ml^{-1}) after long-term acclimation to different temperatures. Data represent basal cortisol without an external stress stimuli (0H)*

	Treatment	0 H	
Plasma	17°C	5.45±2.55	
	22°C	7.56±3	
	26°C	8.56±3.61	
Mucus	17°C	0.48±0.11	
	22°C	0.79±0.11	
	26°C	0.75±0.14	
Correlation	R=0.22	P=0.048	Y=-0.021X+0.777

with low or high manipulation, showed the worst ($p<0.05$) growth performance or feed utilization in terms of weight gain, SGR or FCR, when compared to low density/low manipulation (LDLM) group. Two-way ANOVA analyses showed significant differences for the factor density ($p<0.05$), no differences for manipulation ($p>0.05$) and no interaction was detected between bboth factors ($p>0.05$) (Table 3.2)

Table 3.2. *Greater amberjack performance in trial 2, stocking density and handling procedures. LDLM: low density low manipulation; HDLM: High density low manipulation; LDHM: low density high manipulation; HDHM: High density high manipulation. Different letters denote significant differences in one-way ANOVA comparison ($p<0.05$). * denotes significant differences in two-way ANOVA ($p<0.05$)*

Treatment				
	LDLM	HDLM	LDHM	HDHM
Weight gain	135.56 ±5.71^{a}	102.76 ±10.09^{bc}	120.26 ±5.88^{ab}	91.01 ±7.84^{c}
SGR	0.99 ±0.11^{a}	0.75 ±0.07^{bc}	0.90 ±0.08^{ab}	0.71 ±0.08^{c}
FCR	1.24 ±0.14^{a}	1.57 ±0.17^{bc}	1.32 ±0.21^{ab}	1.76 ±0.11^{c}
Two-way ANOVA				
	Density	Manipulation	D X M	
Weight gain	P=0.01*	P=0.286	P=0.08	
SGR	P=0.02*	P=0.367	P=0.09	
FCR	P=0.01	P=0.472	P=0.11	

At the end of the experimental period, fish held at a high stocking density showed a higher ($p<0.05$) concentration of circulating plasma cortisol (23 ng·ml^{-1}), irrespective to the handling protocol assayed, compared to fish stocked at low density (9 ng·ml^{-1}). After one hour of the stress stimuli, fish held at high density, irrespectively of the handling protocol applied, showed higher cortisol level ($p<0.05$) compared with the low density treatments (Table 3.3), being values almost twice the values presented by juveniles acclimated to low stocking density and short-period routine practices (high handling). At 24hours post stress, HDHM group showed higher ($p<0.05$) cortisol values when compared to the rest of the experimental groups. After 72 hours of stress stimuli, all the experimental groups presented cortisol values around 20 ng·ml^{-1}, and no differences were observed among experimental groups. Two-way ANOVA test showed that at 1 h post stress, the individual factor density generated differences in cortisol levels regardless the manipulation grade applied and no interaction

between both factors combined (density x manipulation) was detected. At 24 hours post stress, no differences were obtained for density and handling, nevertheless, significant differences were observed in factors combined (density x manipulation). At 72 hours, no differences were detected for any factor.

Greater amberjack growth performance and feed utilization parameters in trial 2, stocking density and handling procedures. LDLM: low density low manipulation; HDLM: High density low manipulation; LDHM: low density high manipulation; HDHM: High density high manipulation. Different letters denote significant differences by one-way ANOVA comparison ($p<0.05$). * denotes significant differences after two-way ANOVA analyses ($p<0.05$).

3.3.2.1.Mucus cortisol after stress

No differences were recorded in mucus cortisol at time 0. However, after 1h of stress, LDHM group showed a lower ($p<0.05$) skin mucus cortisol concentration when compared to the other experimental groups (Table 3.3). No differences were recorded for 24 and 72 hours post stress. Besides that, significant differences ($p<0.05$) were observed among the different sampling points for all the groups with the exception of LDHM, that presented similar mucus cortisol concentration during all the time-course of stress. Two-way ANOVA showed no differences among factors at 1, 24 or 72 hours post stress. A significant correlation ($p<0.05$; $r=0.73$) was observed between plasma and mucus cortisol concentration parameters.

3.3.2.2.Skin mucin production

The different combinations of rearing protocols (high/low density, high /low handling) did not affect ($p>0.05$) the expression of *muc-2* gene at 1h post stress, values of fish held at high stocking density showed a tendency to higher (almost two fold higher) than those held at low stocking density (Table 3.5).

3.3.3.Trial 3. Short-term starvation trial

3.3.3.1.Plasma cortisol

No differences were obtained between fasted and unfasted fish at 0 and 1 h post stress (Table 3.4). The time-course of plasma cortisol was similar between both treatments, with a significant ($p<0.05$) elevation of plasma cortisol 1h after shallow water stress. However, fasted fish showed significantly ($p<0.05$) higher circulating plasma cortisol at 3h and 24h after shallow water stress.

Table 3.3. *Plasma and mucus cortisol (ng·ml⁻¹). Capital letter denotes differences ($p<0.05$) among time 1, 24 and 72 hours post stress. Lowercase different letters denotes significant differences among treatments at the same time point ($p<0.05$). * denotes significant differences ($p<0.05$) in the two-way ANOVA analyses. 1h: 1 hour post stress; 24h: 24 hours post stress; 72h: 72 hours post stress; LDLM: low density low manipulation; LDHM: low density high manipulation; HDLM: high density low manipulation; HDHM: high density high manipulation*

Sampling point (hours post stress)					
	Treatment	**0h**	**1h**	**24h**	**72h**
Plasma	LDLM	8.2 ± 3.2^{Aa}	88.8 ± 6.58^{bB}	28.0 ± 6.40^{bA}	25.5 ± 9.20^{A}
	LDHM	9.8 ± 2.3^{Aa}	76.1 ± 6.73^{bB}	17.7 ± 5.71^{bA}	23.8 ± 11.62^{A}
	HDLM	22.5 ± 7.7^{Ab}	122.5 ± 11.68^{aB}	18.2 ± 8.30^{bA}	33.0 ± 20.35^{A}
	HDHM	23.9 ± 7.2^{Ab}	116.3 ± 11.44^{aB}	51.4 ± 10.28^{aC}	20.4 ± 5.89^{A}
Mucus	LDLM	0.2 ± 0.12^{A}	1.5 ± 0.16^{aB}	1.2 ± 0.09^{B}	0.8 ± 0.28^{A}
	LDHM	0.3 ± 0.2	0.5 ± 0.09^{b}	0.8 ± 0.05	0.8 ± 0.23
	HDLM	0.8 ± 0.19^{A}	1.7 ± 0.39^{aB}	0.7 ± 0.05^{A}	0.7 ± 0.24^{A}
	HDHM	0.9 ± 0.18^{A}	2.2 ± 0.23^{aB}	0.7 ± 0.10^{A}	0.8 ± 0.09^{A}
Correlation	R=0.73		P=0.001		Y=73.161X-20.616
Two-way ANOVA					
	Treatment	**0h**	**1h**	**24h**	**72h**
Plasma	Density	P=0.021*	P=0.030*	P=0.086	P=0.951
	Manipulation	P=0.452	P=0.585	P=0.099	P=0.264
	D X M	P=0.084	P=0.850	P=0.010*	P=0.362
Mucus	Density	P=0.093	P=0.436	P=0.766	P=254
	Manipulation	P=0.352	P=0.258	P=0.486	P=594
	D X M	P=0.256	P=0.512	P=0.652	P=486

3.3.3.2.Mucus cortisol

Time course of skin mucus cortisol after stress showed similar trends than plasma cortisol, with a significant increase ($p<0.05$) for both experimental groups. However, after 1h of stress, fasted fish showed a significant ($p<0.05$) increase of mucus cortisol when compared with fed fish (Table 3.4). No differences ($p>0.05$) were found for 3 and 24 hours between

experimental groups (Table 3.4). A significant (p=0.002; r=0.38) correlation was observed between plasma and skin mucus cortisol levels (Table 3.4).

Table 3.4. *Plasma and mucus cortisol (ng/ml) recorded in trial 3. Capital letter denotes differences (p<0.05) among time 0 and 1, 3 and 24 hours post stress. Lowercase different letters denotes significant differences among treatments at the same time point (p<0.05). 0H: time 0, no stress; 1H: 1 hour post stress; 3H: 3 hours post stress; 24 H: 24 hours post stress; A+: apparent satiety feeding group; A-: fasting group*

Sampling point (hours post stress)					
	Treatment	**0h**	**1h**	**3h**	**24h**
Plasma	A+	8.84 ± 2.27^{A}	94.68 ± 12.23^{B}	21.94 ± 2.92^{bC}	19.37 ± 6.08^{bC}
	A-	8.44 ± 2.54^{A}	89.53 ± 16.06^{B}	39.6 ± 8.89^{aC}	32.22 ± 12.41^{aC}
Mucus	A+	0.2 ± 0.03^{bA}	1.54 ± 0.25^{bB}	1.11 ± 0.19^{B}	0.4 ± 0.08^{A}
	A-	0.43 ± 0.04^{aA}	3.11 ± 0.53^{aB}	1.02 ± 0.25^{B}	0.5 ± 0.1^{A}
Correlation	R=0.38	P=0.002	Y=0.008X+0.689		

3.3.3.3.Gene expression of muc-2 in skin

Fasting did not induced significant (p>0.05) changes in the relative expression of muc-2 gene (Table 3.5). However, after 1 h of appliying a shallow water stress, fasted fish showed an up regulation (p<0.05) of *muc-2* gene expression.

Relative expression of *muc-2* gene (2^ ΔΔ ct) in skin for trials 1, 2 and 3. Lowercase different letters denote significant differences among treatments at the same time point (p<0.05). Capital letter denotes differences (p<0.05) between time 0 and 1 h post stress in trial 2. LDLM: low density low manipulation; LDHM: low density high manipulation; HDLM: high density low manipulation; HDHM: high density high manipulation; A+: apparent satiety feeding group; A-: fasting group.

3.4. Discussion

Rearing zootechnology for *S. dumerili* has been developed at experimental level by studying reproduction, ontogeny and nutritional aspects mostly at larval rearing level (Roo *et al.*,

Table 3.5. *Relative expression of muc-2 gene (2^ΔΔ ct) in skin for trials 1, 2 and 3. Capital letter denotes differences (p<0.05) between time 0 and 1 h post stress in trial 2. Lowercase different letters denotes significant differences among treatments at the same time point (p<0.05). LDLM: low density low manipulation; LDHM: low density high manipulation; HDLM: high density low manipulation; HDHM: high density high manipulation; A+: apparent satiety feeding group; A-: fasting group.*

	Treatment	**0 H**
Trial 1	17°C 0	5.32±0.86
	22°C 0	6.64±1.59
	26°C 0	5.21±1.78
Trial 2	LDLM 1h	6.26±2.03
	LDHM 1h	5.28±3.76
	HDLM 1h	7.35±1.50
	HDHM 1h	9.55±3.10
Trial 3	A+0	5.55 ± 0.78^{A}
	A-0	3.67±2.50
	A+1h	9.49 ± 3.21^{aB}
	A-1h	2.86 ± 0.87^{b}

2019) or broodstock management (Sarih *et al.,* 2019; Navarro- Guillén *et al.,* 2019). Studies focused in juveniles are scarce and in some cases those are based on captured wild juveniles (Jover *et al.,*1999; Mazzola *et al.,* 2000) and are mainly focused on protein/energy ratios and some nutritional aspects (Takakuwa *et al.,* 2006; Monge-Ortiz *et al.,* 2018). Not so much information is available about the effects of different rearing conditions for this species, being rearing temperature the most studied parameter. Nevertheless, most of the information related to rearing temperature has been reported for *S. lalandi* (Miegel *et al.,* 2010; Abbink *et al.,* 2012; Bowyer *et al.,* 2014) and only recently has been studied in *S. dumerili* (Fernández-Montero *et al.,* 2018). The optimal temperature range for those species has been determined to be 26ºC, but with a valid range down to 22ºC, which is the medium temperature of Mediterranean and Macaronesia-Atlantic regions, with no significant effects on growth or feed utilization (Fernández-Montero *et al.,* 2018). The minimum temperature for this species was determined to be around 17ºC, due to the deleterious effects on fish growth and shape observed (Fernández-Montero *et al.*, 2018).

Rearing temperature is considered an important stressor when thermal shocks occur or when long-term variations occur far away from the optimum temperature range for each species (Pérez-Casanova *et al.*, 2008). In the present study, no differences were observed after long-term acclimation to different temperatures among the fish groups at the selected rearing temperatures, which concur with Mediterranean and Atlantic culture temperature for this species, being this temperatures inside biological temperature range for greater amberjack (Nakada, 2000).

Extreme temperature conditions have a direct effect on the production of fish skin mucus, since extreme (both high and low) temperature are acting as a stressor for fish. The skin of Atlantic salmon (*Salmo salar)* adapted to high (16ºC) temperature showed variations in transcriptome, mucus composition and the coverage of skin mucous cells, when compared to 4ºC acclimated fish, which suggest a stress response in the skin at high temperature (16ºC) for this species (Jensen *et al.*, 2015). A chronic cold stress (14ºC) induced a partial loss of mucus functionality in the gilthead sea bream, with a described an up-regulation of the so-called "response to stress" proteins, such as heat shock proteins (HSPs) and transferrin, and a down-regulation of proteins with metabolic activity, evidencing depressed skin metabolism (Sanahuja *et al.*, 2019). Within the present study, no differences were found on the parameters studied after a long-time acclimation period at different temperatures, in a RAS with the rest of the parameters maintained constant during 120 days, being levels of cortisol around 7.5 $ng \cdot ml^{-1}$ for all the experimental groups, which suggest that the animals were well acclimated to the rearing temperatures and that it did not supposed a thermal shock for this species. Taking into consideration the low variation of plasma cortisol, a parameter that usually has a wide inter-individual variation, in fish acclimated at any of the rearing temperature assayed, the value of 7.5 $ng \cdot ml^{-1}$ of circulating cortisol could be consider as a basal level for this species after acclimation to rearing conditions.

Recently, the standard husbandry practices have been pre-determined for greater amberjack, with a value of aproximately 5 $kg \cdot m^{-3}$ considered acceptable for a pelagic fish since higher densities (7 $kg \cdot m^{-3}$) caused a reduction on fish specific growth rate (Mylonas *et al.*, 2019). Depending on the rearing temperature, the used stocking density for this and other *Seriola* species has been low in most of the studies, either with early juveniles in RAS (Kohbara *et al.*, 2003; Miegel et al., 2010; Hirazawa *et al.*, 2010; Abbik *et al.*, 2012; Takakuwa *et al.*, 2006) or juveniles in experimental sea cages (Yilmaz *et al.*, 2011; Takagi *et al.*, 1996). Most of those studies were focused in other parameters than stocking density and only few studies have been focused on the effect of stocking density on the welfare status of greater amberjack, pointing out the high susceptibility of this species to stocking density (Miki *et al.*, 2011). In the present study, and taking into account the results obtained on the trial 2, stocking density is a crucial factor for this species, decreasing greater amberjack growth and rising its plasma cortisol levels. Indeed, a high stocking density has been described to be an important factor rising the cortisol levels in an acute and, especially, in a chronic stress response in case of fish maladaptation occurs (Tort *et al.*, 2011), affecting negatively health, growth and feed utilization. Overcrowding associated to stocking density is a stressor, which affects different metabolic enzymes related to lipid, carbohydrate and protein metabolism, with a direct impact on fish growth performance (Costas *et al.*, 2008; Laiz-Carrión *et al.*, 2012; Montero *et al.*, 1999; Sangiao-Alvarellos *et al.*, 2005a).

In the present study, rearing this fish species at a high density has influenced the recovery after an acute response, with animals held at a higher stocking density showing higher values of plasma cortisol after shallow water stress and a slower recovery than fish reared at a lower stocking density, denoting the low availability of greater amberjack juveniles to recover from a stressful situation (shallow water in this case) when held at a high density. A synergic interaction of different stressors has been described for different species of fish and the associated deleterious effects have been pointed out to be related with a fish non-adaptation to the sum of stressors, as has been observed for changes in temperature and oxygen in other species (McBryan *et al.*, 2013).

Despite of assuming that the well known acute stress response to handling is to increase plasma cortisol in several species (Barton *et al.*, 2000; Davis *et al.*, 2002; Ramsay *et al.*, 2009), acclimation to long-term exposure to standardized handling protocols had no effect on cumulative plasma cortisol levels in greater amberjack juveniles, in agreement with results found for other species such as Atlantic salmon (Easy & Ross, 2010). Those authors described no effect on cumulative plasma cortisol after a long-term handling stress consisting on a daily handling of 15 seconds out of the water for 3 weeks, indicating that fish are able to acclimate to standardized protocols. Madaro *et al.* (2016) showed that Atlantic

salmon parr and post-smolts presented a lower cortisol concentration after chasing stress if being exposed to the same stressor for some days. Similarly, in Eurasian perch (*Perca fluviatilis)*, the stress response strongly indicated a habituation to the standardized handling stressor twice a week for an 8-week period (Jentoft *et al.*, 2005). Yang *et al.,* (2015) showed that repeated handling had no effect on basal cortisol overwintering gibel carp (*Carassius auratus gibelio*), but values of plasma cortisol post bacterial challenge were found to be lower in those animals habituated to handling stress. However, other authors such as Conde-Sieira *et al.* (2018) demonstrated that short-term (3 days of handling) trained Senegalese sole (*Solea senegalensis*) had an increased plasma cortisol level after a period of repeated handling protocols, but showed a post-stress evident inhibition of the dopaminergic activity and behavioral response to the stressor, indicating some habituation to the repeated stressor. The lack of an increase in cortisol levels for the long-term handling standardized protocol, suggested that greater amberjack may have the capability to habituate to such weekly stressor. Although the cortisol response always depends on the intensity of the stressor and the duration of the habituation period (Schreck, 2010), the stress response also depends on the predictability and controllability of the stressor (Schreck and Tort, 2016). Thus, if the fish are able to cope with these two factors, as it seems the case for the greater amberjack under the handling protocol, levels of cortisol would show a lower increase and a faster recovery.

Fasting is a direct consequence of non-adaptation to chronic stress situation, as crowding stress induces a reduction of fish intake (Santos *et al.*, 2010). Short term fasting could be derived from a maladaptation to crowding conditions, were individual fish can receive less feed than needed due to competence. Food deprivation induces energy-releasing catabolic processes that compensate for a reduced energy intake (Wunderink *et al.*, 2012), which vary depending on different factors such as age, size, and species metabolism adaptations or modulations in order to maintain homeostasis (Navarro and Gutiérrez, 1995; Montero *et al.* 1999), with subsequent effects on daily changes of several plasma metabolites and hormones including cortisol (Laiz-Carrión *et al.*, 2012; Menezes *et al.,* 2015). Fasting is a factor affecting stress response in terms of circulating cortisol (Davis and Gaylord, 2011). In the present study, starvation did not caused an increase in basal plasma cortisol after 10 days of food deprivation, in agreement with previous studies for other fish species (Leatherland and Farbridge, 1992; Pottinger *et al.*, 2003; Weber and Bosworth, 2005). However, the reported data on the effects of fasting on cortisol levels are very diverse, including decreased cortisol levels in fasted fish (Small, 2005) or increased cortisol levels (Barcellos *et al.,* 2010). Those

differences could be due to the species studied, the duration of the starving period or the metabolic status of the individual to cope with the metabolic challenge imposed by the restriction of food. However, other modulators such as synergic effects of different environmental factors cannot be rejected. In this way, fasting is affecting cortisol response after a shallow-water stress in the greater amberjack, starved fish showing increased plasma cortisol after 3 h of shallow-water stress. An accumulative effect of two different stressors has been described for gilthead sea bream subjected to both crowding and fasting stress (Laiz-Carrión *et al.*, 2009), although when the catabolic effect of the increased levels of corticosteroids after fasting is combined with another stressor, it would facilitate the mobilization of energy from body reserves in this new energy-demand situation (Navarro & Gutierrez, 1995).

In the present experiment, a positive correlation has been found between plasma cortisol and mucus cortisol concentrations, in agreement with previous studies done in marine fish (Carbajal *et al.,* 2019). The interest to measure cortisol in fish skin mucus has recently increased, especially because the sampling method is much less invasive compared to the blood extraction techniques (De Mercado *et al.*, 2018; Guardiola *et al.,* 2016). Information about cortisol transport to mucosal surfaces is scarce, but skin mucus cortisol levels have shown to reflect circulating concentrations in different species of aquacultured fish (Bertotto *et al.,* 2010; Carbajal *et al.*, 2019; Fernández-Alacid *et al.*, 2019; Simontacchi *et al.*, 2008). To the knowledge of the authors, this study is the first one obtaining data for skin mucus concentration of greater amberjack. Rearing temperature in the present study did not change basal concentration of skin mucus that was around 0.5 $ng \cdot ml^{-1}$. Acute changes in water temperature reduced the number of goblet cells and mucus production in channel catfish epidermis, but after acclimation to the new temperature within its biological rearing temperature range, mucus production returns to its normal basal values (Quiniou *et al.*, 1998). Sanahuja *et al.* (2019) found a significant effect of chronic low temperature in gilthead sea bream mucus interactome, with increased levels of so called “stress proteins”. In the present study, the acclimation to the lower temperature is not acting as a chronic stressor, although it is affecting fish growth performance (Fernández-Montero *et al.,* 2018). However, the concentration of mucus cortisol of greater amberjack changed depending on the different stressful situations, increasing significantly after stress in both time-course stress trials (2 and 3), with the highest values obtained 1 h after stress in both trials, which were fivefold higher than basal cortisol measured in the other trials. Fernández-Alacid *et al.*, (2019), found that skin mucus cortisol exudation is stressor dependent in meagre *(Argyrosomus regius*), being the response to hypoxia three-

fold higher than to netting stress for that species. The mucus cortisol concentration returned to initial values relatively faster after stress in the present study in agreement with Carbajal *et al.* (2019) who found that differences in mucus cortisol concentration between control and stressed rainbow trout were much more evident in the early phase of the stress response.

Stressful conditions are known to modify fish skin functionality (Sanahuja *et al.*, 2019) and structure (Tacchi *et al.*, 2015; Vatsos *et al.,* 2010). Stressful situations have a direct effect on mucus production with an increment of amorphous goblet cells at the epidermal surface after stress, that are exocytosing their content (Iger *et al.*, 1994). Tacchi *et al.* (2015) showed an overexpression of *muc-2* in the skin of rainbow trout after stress transportation, denoting the increase of mucus production. Although in the present experiment no significant differences were found in skin *muc-2* gene expression after appliying high density or repeated handling protocols, the higher value obtained for *muc-2* gene expression after a combination of high density-high manipulation group, could be related with the increase of mucus production. Such dynamics shows a trend in accordance with cortisol data, in agreement with Van der Salm *et al.* (2002) who showed that cortisol treatments had no effect on the number of epidermal mucous cells of rainbow trout, although the discharge of mucus increased.

The expression of *muc-2* gene increased in greater amberjack subjected to shallow water stress (trial 3), however, fasted fish fails to increase mucus production. Starvation induced a reduction in the average thickness of the epidermis and a reduction on the abundance of epidermal goblet cells on European eel (*Anguilla anguilla*) (Caruso *et al.*, 2010). Food deprivation has been described also to cause a rapid decrease in the density of epidermal mucous cells in Atlantic salmon, mainly in the flank of the fish (Landeira-Dabarca *et al.*, 2014), this region requiring a higher production of mucus to attenuate the frictional force during swimming (Somejo *et al.* 2004). Besides, starvation has been also described to induce important changes in the composition of mucus, increasing ketones content (Heming and Paleczny, 1987), changing glucidic composition (Landeira-Dabarca *et al.*, 2014), affecting the diversity of the associated microbiota (Landeira-Dabarca *et al.*, 2013) and increasing pathogen adhesion (Yan *et al.*, 2007). The changes in the mucus properties caused by fasting and the failure of greater amberjack to respond properly to shallow water stress, could affect its disease resistance, given that the primary reason for mucus secretion in fish is to protect themselves against infections.

In conclusion, a long-term acclimation (120 days) of greater amberjack juveniles to different temperatures within their biological range did not affect plasma cortisol levels, being basal levels around 7.5 $ng \cdot ml^{-1}$ when additional stressful situations are not present. Moreover, rearing density showed to be an important parameter for achieving an optimal growth and welfare conditions of greater amberjack juveniles. Results show that stocking density must be lower than 8 $Kg \cdot m^{-3}$ to avoid deleterious effects on growth performance and plasma cortisol concentration rise. This species also showed a good adaptation to standardized handling protocols, without affecting fish growth or stress parameters. A short-term fasting induced a slower recovery of basal cortisol levels after shallow-water stress compared to standard feeding, and induced a reduced responsiveness in terms of *muc-2* gene expression.

CHAPTER 04

SKIN INFECTION OF GREATER AMBERJACK *(SERIOLA DUMERILI)* BY MONOGENEAN ECTOPARASITE *NEOBENEDENIA GIRELLAE*: A MORPHOLOGICAL AND DESCRIPTIVE STUDY.

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Abstract

Neobenedenia girellae is considered an epizootic infection for intensively cultured fish species. Particularly for greater amberjack *(Seriola dumerili), N. girellae* causes high mortality rates and supposes a bottleneck during its on-growing period. Thus, the objective of this work was to describe the skin morphological alterations caused by a *N. girellae* infection on greater amberjack. Greater amberjack juveniles were sampled pre and post being exposed to an experimental infection with *N. girellae.* Cranial and dorsal region skin samples were processed for morphological and structural studies. Both regions clearly differed structurally, proposing the cranial region as the most susceptible region to be parasitized due to an absence of scales and the lower goblet cells density. *N. girellae* adhesion disrupted the structure of epidermal epithelial cells by causing an overpressure effect. *Stratum spongiosum* surface-epithelial cells located near the parasite presented a clear cell degradation process, associated in some cases with a cellular detachment process. *N. girellae* infection induced, in some cases, epidermal hydropic degeneration and focal spongiosis. Tissue ulcerative lesions caused by the parasite's attachment structures were characterized by a specific mobilization of leucocytes to the fixation areas.

4.1. Introduction

Fish skin is continuously exposed to external pathogens and stressors. Skin is well-organized in a pre-epithelial barrier mainly composed by skin mucus, which constitutes the first physical barrier against external pathogens (Whitear, 1986), an epithelial barrier which differs in structure depending on the fish region studied, and a scattered skin associated lymphoid tissue (SALT) (Salinas *et al*., 2011). An altered biochemical and immunological composition of the fish skin

mucus may result in an increased susceptibility to pathogen infections or to winter syndrome (Fast *et al*. 2002a; Contesi *et al*., 2006), as described in a wide range of fish species, such as Coho salmon *(Oncorhynchus kisutch)*, Atlantic salmon *(Salmo salar),* rainbow trout *(Oncorhynchus mykiss)* or gilthead seabream *(Sparus aurata).* Besides, the physical status of fish skin epithelial layer and the capacity of response of its SALT contribute to modulate the susceptibility to pathogen infections in relation to prevent pathogen physical attachment (Ourth and Chung, 2004; Griffin and Mitchell, 2007). However, fish skin presents a limited morphological response to injury (Esteban, 2012). Skin cellular injury derived from pathogen attachment causes cell membrane damage, an imbalance of epithelial cells and limits mitochondrial ATP production (Esteban, 2012). Moreover, some pathogens as *Moritella viscosa* or some hematophagous parasites as sea lice *(Lepeophtheirus salmonis)* inhibit the epidermal regeneration capacity of keratocytes (Karlsen *et al*., 2012). Therefore, skin integrity and composition are key features to maintain fish health (Caruso *et al.* 2010; Esteban, 2012; Fernández-Montero *et al*., 2019), especially in species with high susceptibility to ectoparasites.

Ectoparasites interaction with fish skin supposes an arm race of coevolution (Buchmann, 1999). Ectoparasites sensory structures are able to detect chemical and mechanical stimuli on the host surface, facilitating its recognition and the fixation process (Jones *et al*., 1990; Buchmann and Lindenstrøm, 2002).

The effects of ectoparasites on fish skin are specific of each host and parasite. For example, a sea lice infection on Atlantic salmon skin produces an ulcerative process caused by the second antennae and an inflammatory status of the dermis characterized by epidermal thickness variations, cell detachment, necrotic areas and leucocytes mobilizations (Jones *et al*., 1990). Whereas *Gyrodactylus derjavini* infection is characterized by hydropic degenerated epidermal cells, cellular reorganization, changes in the density of goblet cells and variations on the epidermis thickness (Appleby *et al*., 1997; Buchmann and Bresciani, 1997).

N. girellae, is a marine warm water monogenean ectoparasite of special importance for marine culture species, which significantly affects to cultured greater amberjack *(Seriola dumerili),* representing the main bottleneck for its production (Shirakashi *et al*., 2013). *N. girellae* infection induces variations on greater amberjack skin epidermis thickness accompanied of an increased density of goblet cells (Hirayama *et al*., 2009; Hirazawa *et al*., 2010; Hirazawa *et al.,* 2016), in relation to an unbalanced osmoregulatory and respiratory functioning of the

skin (Hirayama *et al.*, 2009; Hirazawa *et al.*, 2016). Moreover, *N. girellae* infection in this fish species usually is associated with skin secondary bacterial infections, derived from an altered fish behaviour characterized by scratching with the tanks and nets and thus, causing important skin wounds.

Thus, the objective of this study was to describe the associated morphological skin alterations on greater amberjack parasitized experimentally with *N. girellae* as a potential tool to predict the possible strategies to enhance the skin defences of this specie.

4.2. Material and methods

The present study was conducted at the Scientific and Technologic Park of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain). The animal experiments described comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals.

4.2.1 Experimental fish and experimental conditions

The initial weight of experimental greater amberjack juveniles was 343.0 ± 53.0 g. Fish were divided randomly in two experimental groups of twenty-five individuals. First group was euthanized with an anaesthetic overdose (clove oil, 5mL/L; Guinama S.L; Spain, Ref. Mg83168) and sampled as control non parasitized fish. The other twenty-five fish were distributed in 5 cylindroconical 500 L tank (5 fish/tank), feeding to apparent satiety during 30 days with a commercial diet (Europe 22, Skretting). Temperature (22ºC ± 0.5) and dissolved oxygen (7.4 ± 0.9 mg L^{-1}) were monitored daily.

4.2.2 Neobenedenia girellae experimental infection

Neobenedenia girellae infection was performed with some modifications of the methodology previously described in Fernández-Montero *et al.* (2019). Briefly, eggs were collected from a tank with parasitized greater amberjack individuals (5 mm pore net), leaving the net for 24 hours for egg entanglement. Nets with the eggs were disposed in the experimental tanks for 30 days to let them hatch and the oncomiracidia fix to the fish.

4.2.3 Sampling procedures

Initial sampling was conducted pre-parasitation and final sampling was conducted after 30 days, when all the animals showed visual signs of parasitation. Skin samples were obtained from two different regions, cranial and dorso-lateral (Fig. 4.1), according to the differences observed in parasite fixation and prevalence by Hirayama *et al.* (2009) for this fish species. Sampling was conducted in the same way for the non-parasitized fish group.

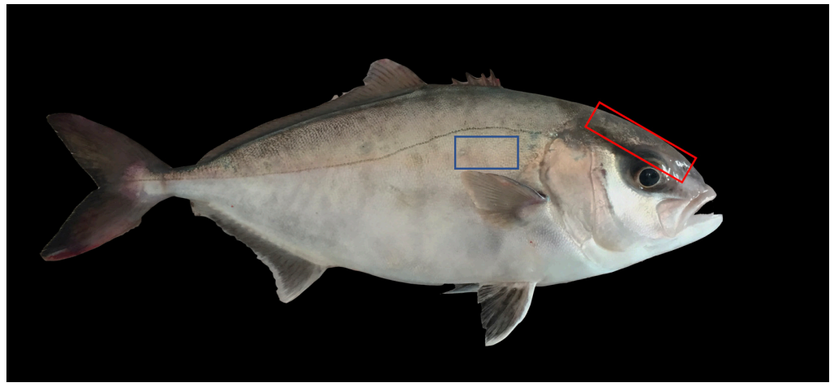

Figure 4.1. *Greater amberjack selected skin sections for histological analyses. Red colored corresponds with cranial region; blue-colored corresponds with dorso-lateral region*

4.2.4 Morphological studies

Sections of skin (Fig. 1) from 25 fish per group were removed and fixed in 4% buffered formalin. Samples were dehydrated in a series of ethanol dilutions and embedded progressively in Technovit (Electron Microscopy Sciences, PA, USA) following the method of Pittman *et al.*, (2013). Sections were stained with Alcian Blue-PAS-Giemsa (Martoja and Martoja-Pierson, 1970). Stained sections were observed under an Olympus cx41rf optic microscope (Tokyo, Japan) and evaluated by two different researchers. Micrographs were obtained with an adapted camera Olympus xc50 (Tokyo, Japan). Morphometrical analyses were conducted with Image Pro Plus software (Media Cybernetics, Silver Spring, USA) (n=30 slides /region/treatment).

4.2.5. Structural studies

Samples for Scanning Electron Microscopy (SEM) (3 samples/ 3 fish/ group) were dehydrated, dried (Hitachi HCP-2, Chiyoda, Tokyo, Japan) and washed in CO_2. The dried samples were mounted on aluminium stubs and metallization was conducted by sputter coated with argon-gold (Gibbons, 1986). SEM samples were examined and photographed with a JEOL JSM-6335F field emission scanning electron microscope (JEOL USA, Inc, USA).

For transmission electron microscopy (TEM) studies, dissected out samples (n=9 samples/ treatment) were cut in small pieces and immediately fixed in 2.5% glutaraldehyde in 0.15M HEPES buffer (pH=7.4), post fixed in 2% osmium tetroxide and 2% uranyl acetate, dehydrated in graded ethanol series, and embedded individually in an Embed 812 (Electron Microscopy Sciences (EMS), PA, USA) resin block. Semithin (1 µm) serial transverse sections were contrasted with toluidine blue and examined under light microscopy (Hoffman *et al.*, 1983). Ultrathin (50 nm) sections were contrasted with lead citrate and examined with a JEOL JEM-1011 Transmission Electron Microscope (TEM; JEOL USA, Inc, USA) equipped with a digital camera MegaView III soft imaging system CCD Camera (EMSIS GmbH, Germany).

4.3
Results

4.3.1. Parasite fixation

N. girellae adults were detected on fins, cranial, dorso-lateral and ventral skin regions, presenting a total mean length of 4.54 ± 0.31cm (n=50) (Fig. 4.2. B). As detailed in Figure 4.2. B, *N. girellae* has two clear differentiated regions: a) the anterior region, composed by the oral cavity with two circular adhesion glands (Fig. 4.2.C) and b) the posterior region which presents a single discoid, symmetrical and muscular haptor, provided with attachment structures (Fig. 4.2. D). These attachment structures are composed by a pair of curved hooks, located in the most aboral region, and a pair of knife shaped anchors (Fig. 4.2. D and E). *N. girellae* uses the haptor for fixation, allowing its pivoting around it, to obtain feed from surrounding epithelial cells and mucus layer (Fig. 4.2. F).

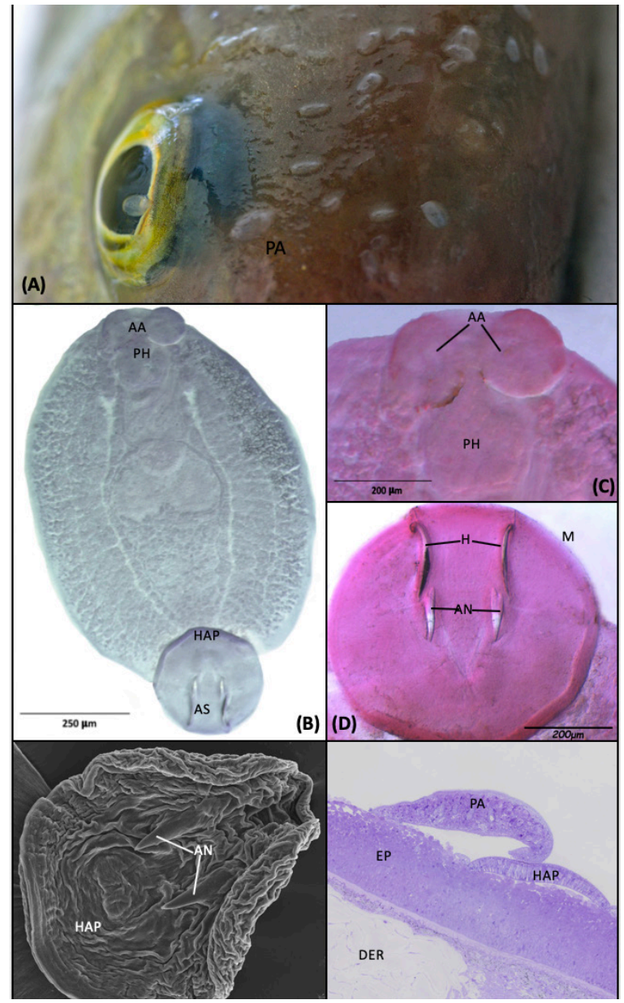

Figure 4.2.*(A) Macroscopic detail of greater amberjack (Seriola dumerilii) parasitized with Neobenedenia girellae. (B) Detailed ventral view of N. girellae and (C) its anterior attachment organs (adhesive glands) stained with Hematoxilin. Scale bars= 250 mm and 200 mm. (D) Stereomicrograph and SEM micrograph (E) of N.girellae haptor, detailing both attachment structures: hooks (hamuli) and anchors (accessory sclerites). Observe the parasite's lack of hooks due to the morphological variations among individuals in the haptor. Scale bar=200 mm and 100 mm. (F) Semithin transversal section of a greater amberjack cranial skin parasitized with N. girellae stained with toluidine blue (pH=2.5). Scale bar=50 mm. AA: anterior attachment organ (adhesive glands); PH: pharynx; HAP: haptor; AS: posterior attachment structures; M: marginal valve; H: Hooks (hamuli); AN: anchors (accessory sclerites); PA: parasite; EP: epidermis; DER: dermis.*

4.3.2. Morphological description of Seriola dumerili dorso-lateral and cranial region.

S. dumerili dorso-lateral region differs morphologically from the cranial region. In general traits, dorso-lateral region presented thinner epidermis with a higher presence of goblet cells (Fig. 4.3.A) and a dermis region containing scales inside their corresponding scale pockets, whereas cranial region presented a wider epidermis with an apparent better alignment of the basal epithelial cells along the basal membrane, and a significantly wider dermis with absence of scales (Fig. 4.3. B).

4.3.3. Effects of N. girellae attachment on Seriola dumerili epidermis

As Figure 4.3 shows, skin dorso-lateral region presents a thinner epidermis (177±37 µm; n=10) with a higher presence of goblet cells (Fig. 4.3. A) than cranial region (279±48 µm; n=10) (Fig. 4.3.B), despite this difference in width, the disposition of the epidermal cell layers followed a similar morphological pattern in both regions. Briefly, epithelial cells of *stratum basale* presented a cuboid appearance, with an oval shaped nucleus disposed centrally or slightly displaced and perpendicularly to the basal membrane. Epithelial cells of *stratum spinosum* presented ovoid to polygonal shapes with a variable nuclei size, which was disposed centrally and in transversal or parallel disposition in relation to the basal membrane. The surface layer of the epidermis, *stratum superficiale,* was constituted by 2-3 irregular rows of flattened cells containing irregular, elongated, and in some cases, pyknotic nuclei disposed parallelly to the basal membrane (Fig. 4.3. A, B).

In fact, the epidermis morphological alterations derived from *N. girellae* fixation, did not differ between cranial and dorso-lateral regions. In general terms, a slight edema and sloughing of epidermal cells were observed around the site of attachment. No signs of hyperplasia or inflammation were observed. Similarly, and regardless of the region where *N. girellae* was fixed, it produced an epithelial overpressure by its haptor attachment structures, which induced a disruption of the epidermal layer structure by over-flattening the *stratum superficiale's* epidermal cells (Fig. 4.4. A). This alteration resulted in a disruption of the normal cellular linear organization of surface epithelial cells, however it was not accompanied by cellular hypertrophy.

The parasite surrounding adhesion region was characterized by cell detachment of the first epithelial layer (Fig. 4.5. A). The TEM study revealed also a partial digestion of the *stratum superficiale* cells near the parasite adhesion area, where cells presented a less electron-dense cytoplasm appearance and signs of necrophanerosis, as disruption of microridges and intercellular junctions structures, which lead to cellular detachment and to the appearance of intercellular spaces between them (Fig. 4.5. E, F) compared to non-parasitized areas of the *stratum superficiale.* Cellular organelles showed signs of degradation and nuclei were under

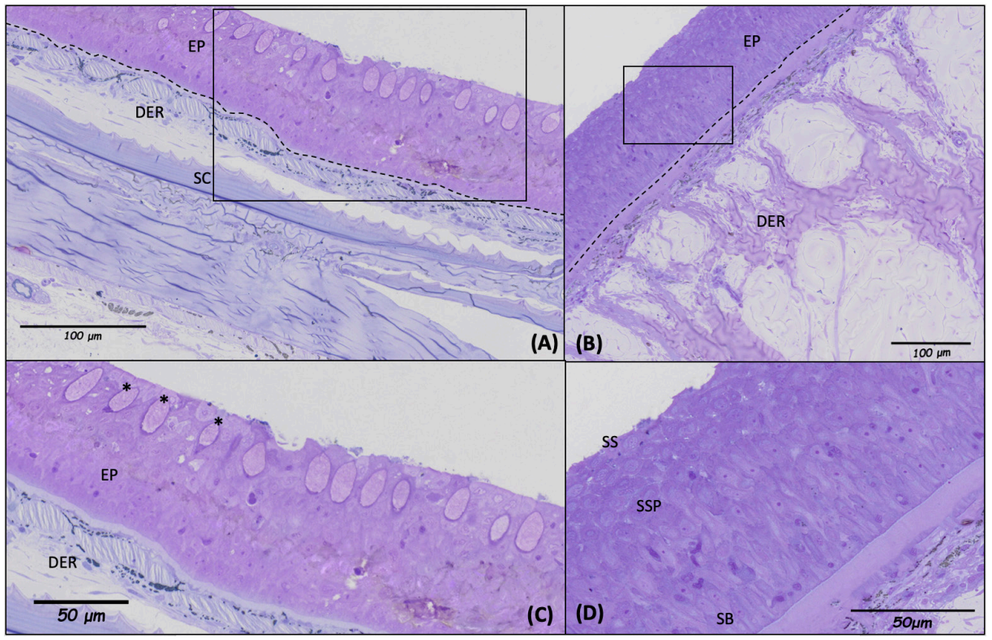

Figure 4.3. *Dorso-lateral (A) and cranial (B) semithin sections of greater amberjack (Seriola dumerili) skin stained with Alcian blue-PAS-GIEMSA and toluidine blue (pH: 2.5). Observe the evident differences in the thickness of the epidermis and dermis, the irregular stratum basal alignment pattern of dorso-lateral region (--) and the lack of imbricated scales (SC) in cranial region dermis (n=10). Scale bar= 100 µm. Detail of dorsolateral (C) and cranial (D) epidermis regions and layers disposition. Observe the variations on the epithelial cells shape and nuclei disposition. Stratum basale epithelial cells presented a central/basal nuclear position, perpendicular to the basal membrane. Stratum spinosum presented round-shaped epithelial cells with a centric and small nucleus transversal/parallel to the basal membrane. Stratum superficiale presented flat-shaped cells with irregular a centric/parallel to the basal membrane nucleus. Observe the higher density of goblet cells (*) on greater amberjack dorso-lateral region compared to cranial region (C vs D). Alcian blue-PAS-GIEMSA and toluidine blue, pH: 2.5. Scale bar= 50 µm. EP: epidermis; DER: dermis; SC: scales; SB: stratum basale; SSP: stratum spinosum; SS: stratum superficiale.*

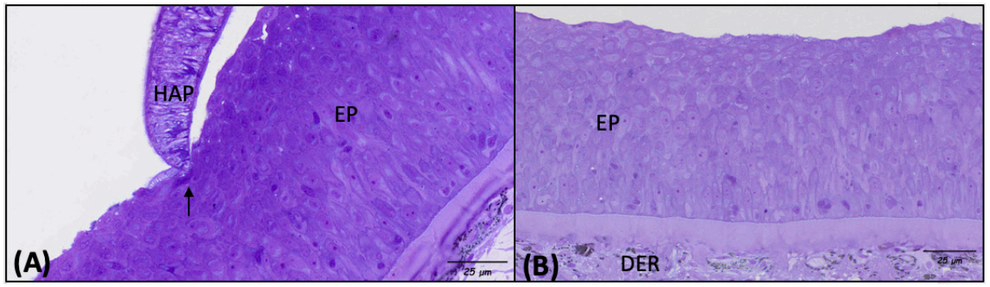

Figure 4.4. *(A) Semithin section of Neobenedenia girellae haptor fixed to greater amberjack (Seriola dumerili) skin. Observe the induced cellular disruption and disorganization of epidermal stratum superficiale (⇒) in relation to a non-parasitized greater amberjack (B). Alcian blue-PAS-GIEMSA, pH=2.5. Scale bar= 25 µm. HAP: haptor; EP: epidermis; DER: dermis*

a pyknotic or karyorrhexic process (Fig. 4.5. F).

N. girellae hooks and, specially, the anchors produced a deep perforation of epidermis (Fig. 4.6. A, B), inducing a reduction of its thickness (15849 µm and 220±67 µm for dorsal and cranial region, respectively, n=10). These punctures had different width (around 50 µm) and depth (around 130 µm) depending on the size of the parasite attachment structures (Fig. 4.6. B). The mechanical perforation altered the structure of the epithelial cells surrounding the lacerations in both, the *stratum superficiale* and *stratum spinosum*, which led to architectural changes to an ovoid and/or flattened nucleus epidermal cell shape compared to a conserved round nucleus in non-parasitized fish (Fig. 4.6. B).

The incidence of *N. girellae* caused an increase in the number of scattered goblets cells in greater amberjack epidermis compared with non-parasitized fish. Goblet cells were mainly disposed in one single layer in the *stratum spinosum* and also in the *stratum superficiale* (Fig. 4.7. B). Goblet cells showed a high variability among individuals, especially in size and composition contents (PAS positive vs Alcian blue positive stain). Mucins were packed inside the goblet cells in mucosomes of similar electron-density and different size, which fill almost the whole cytoplasm of the goblet cells and the nuclei was located basally (Fig. 4.7. C).

Oedematous areas were observed around the perforation sites, where hydropic degenerated epithelial cells could be observed mainly in the *stratum spinosum* and the *stratum*

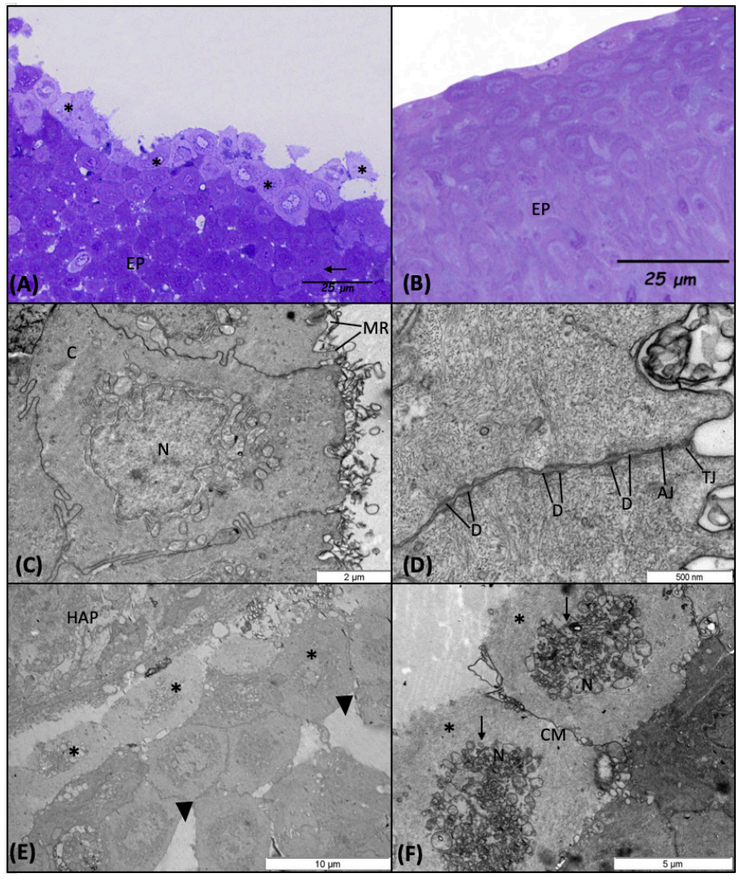

Figure 4.5. *Detailed micrographs of greater amberjack (Seriola dumerili) parasitized and non-parasitized skin. (A) surface epithelial cells of a greater amberjack parasitized skin. Observe the partially digested surface epithelial cells (*) compared to non-parasitized fish skin (B). Alcian blue-PAS-GIEMSA, pH=2.5. Scale bars= 25 µm. (C) Stratum superficiale epithelial cells of non-parasitized skin areas, observe the maintenance of the cellular structure and micro-ridges. Scale bar= 2 µm. (D) Detail of the cell structure of non-parasitized areas with tight junctions, adherens junctions and desmosomes still maintaining their structure and cells cohesion. Scale bar= 500 nm. (E) TEM micrograph of surface parasitized greater amberjack (Seriola dumerili) epithelial cells in contact with Neobenedenia girellae haptor, where focal acantholytic processes represented as intercellular spaces (D) could be observed with degraded cells (*). Scale bar= 10 µm. (F) Detailed TEM micrograph of parasitized greater amberjack degraded cells (*) due to the parasite attachment. Observe the necrophanerosis signs as digested apical membrane, pyknotic or karyorrhexic nucleus (®) and the absence of intercellular junctions. EP: epidermis; IS: intercellular spaces; N: nucleus; C: cytoplasm; AJ: adherens junctions; TJ: tight junctions; D: desmosomes; MR: micro-ridges*

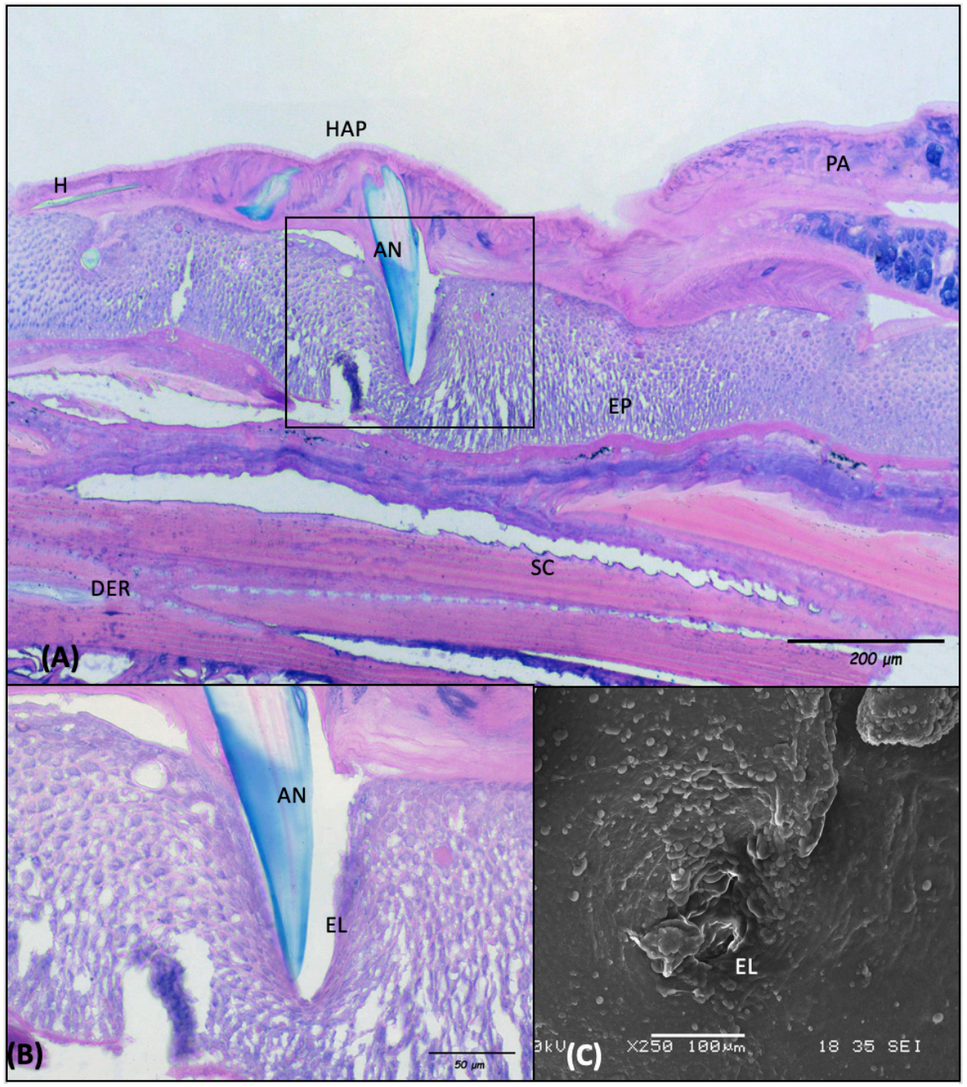

Figure 4.6. *(A, B) Transversal section of Neobenedenia girellae attached to greater amberjack (Seriola dumerili) skin. Observe how anchors almost reach the dermis, induce lacerations and pack epidermal cells while hooks are fixed more superficially. Alcian blue-PAS-GIEMSA, pH=2.5. Scale bars= 200 µm and 50 µm. (C) Detailed SEM micrograph of the laceration produced by N. girellae attachment. Scale bar= 100 µm. PA: parasite; EP: epidermis; H: hooks (hamuli); HAP: haptor; AN: anchor (accessory sclerites); DER: dermis; SC: scales; EL: epidermal lacerations*

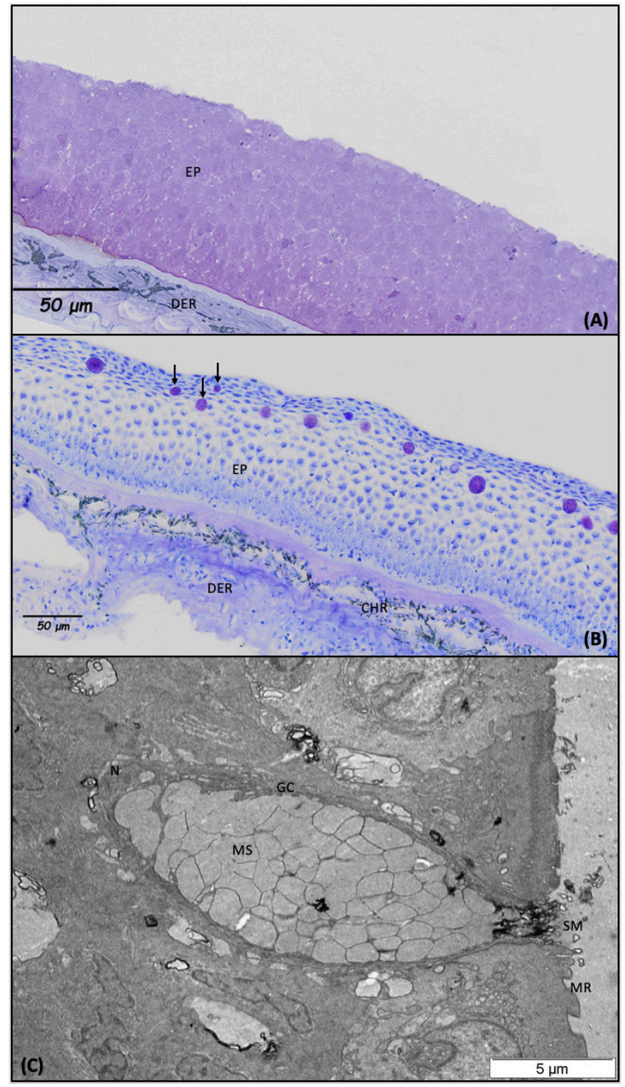

Figure 4.7. *Semithin sections micrographs detailing the differences between non-parasitized fish (A) and infected (B) greater amberjack (Seriola dumerili) epidermal goblet cells density (®). Alcian blue-PAS-GIEMSA, pH= 2.5. Scale bars= 50 µm. Observe the higher density of goblet cells in parasitized fish skin. (C) TEM micrograph of a greater amberjack skin goblet cell. Scale bar= 5 µm. EP: epidermis; DER: dermis; GC: goblet cell; CHR: chromatophores; MS: mucosomes; MR: micro-ridges; SM: secreted mucus: N: nucleus.*

basale of infected fish. In those areas, affected epithelial cells presented intracytoplasmatic hydropic vacuoles, which caused nuclei displacement (Fig. 8. A, B). These morphological alterations entailed a cellular hypertrophy pattern (diameter: 50 µm), which altered the typical *stratum basale* cell cubic appearance observed in non-parasitized areas. In this cell layer, non-parasitized skin regions presented a well conserved oval nuclei disposed in the center of the cell (diameter: 21.3± 2.2, n=10) and an organized continuous cellular layer, with some digitations in their cell membranes that increase cellular cohesion perpendicular to the plane of the basal surface (Fig. 4.3. D & 4.8. D). In consequence, epidermis of infected fish presented widening of intercellular spaces and spongiosis (Fig. 8. C).

Mobilizations of lymphocytic-like cells were observed mainly in the stratum basale, near the parasite adhesion region. In some cases, these mobilizations reached higher stratums of epidermis

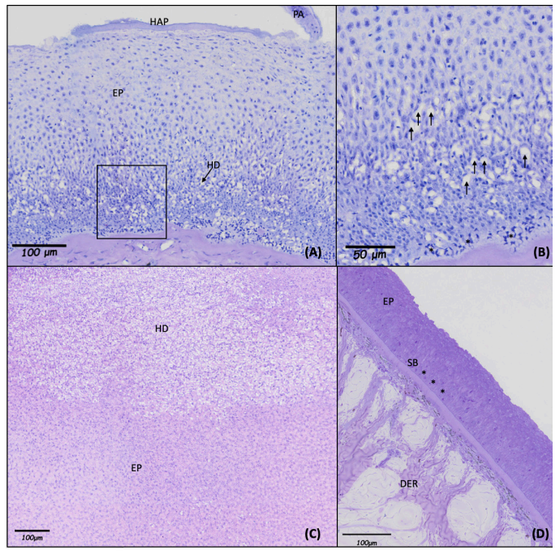

Figure 4.8. *(A) Transversal section of parasitized cranial skin of greater amberjack, Seriola dumerili. Observe, the parasite-induced water imbalance on stratum spinosum and basale cells with hydropic degeneration (→). Intraepithelial lymphocytes could be also observed (*) (B). Alcian blue-PAS-GIEMSA, pH=2.5. Scale bars= 100 and 50 µm. (C) Longitudinal section of greater amberjack cranial skin. Observe the hydropic degeneration morphological pattern covering extensive areas of the tissue (spongiosis). Alcian blue-PAS-GIEMSA, pH=2.5. Scale bar= 100 µm in relation to a transversal section of no parasitized greater amberjack cranial skin (D) where this symptomatology is absent. Observe cellular cohesion and conserved structure of stratum basale (*) with oval nuclei disposed in the center of the cell. Alcian blue-PAS-GIEMSA, pH=2.5. Scale bar= 100 µm. PA: Parasite; HAP: haptor; EP: epidermis; HD: hydropic degeneration.*

as stratum spinosum surrounding the anchors and hooks of the attached parasite (Fig. 4.9. A, B). Intraepithelial lymphocytes (IELs) could be observed near the spongiotic foci (Fig. 4.9. C, D).

4.3.4. Dermis

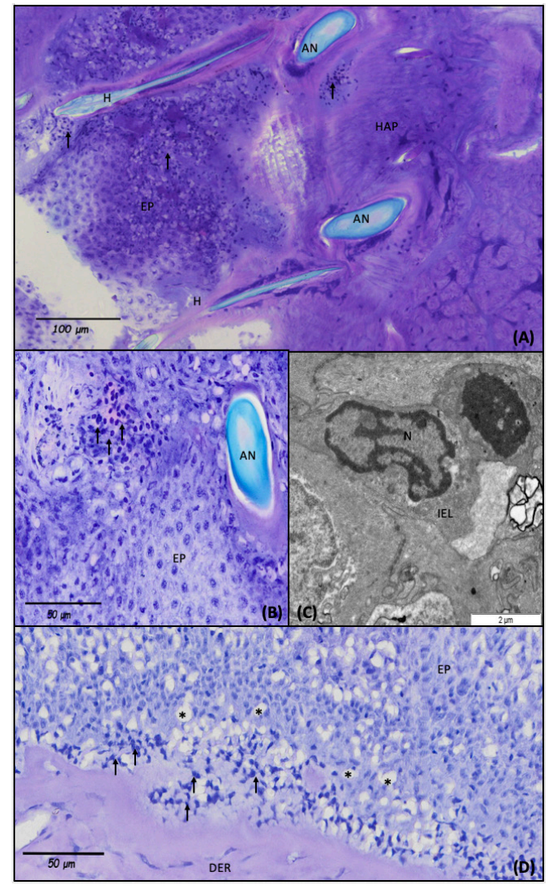

Figure 4.9. *(A, B) Longitudinal section of Neobenedenia girellae haptor fixed to greater amberjack, Seriola dumerili skin. Observe the lymphocyte infiltrations (→) near the parasite anchors and hooks. Alcian-blue-PAS-GIEMSA, pH= 2.5., Scale bars= 100 and 50 µm. (C) TEM micrograph of intraepithelial lymphocyte (IEL) in the epidermis of a parasitized fish. Scale bar= 2 µm. (D) Detail of an epidermis area presenting IEL infiltrations (→) near the basal membrane associated to hydropic degeneration processes (*), near the parasite adhesion regions and mainly in stratum basale and spinosum. Alcian-blue-PAS-GIEMSA, pH= 2.5. Scale bar= 50 µm EP: epidermis; HAP: haptor; AN: anchors (accessory sclerites); H: hooks (hamuli); IEL: intraepithelial lymphocyte; N: nucleus; DER: dermis.*

No mechanical alterations were observed in the dermis of greater amberjack after *N. girellae* fixation, since haptoral attachment structures did not reach the dermis. However, the cranial region presented a wider dermis (753±118 µm) than the dorsal region (430 ±34 µm) (Fig. 4.3). *The dermis is mainly formed by two different layers, the stratum compactum and the stratum spongiosum*. The *stratum spongiosum* is located just below the epidermis and is mainly composed by vascular connective tissue and imbricated scales, however in this fish species scales were observed only in dorso-lateral region. The S. compactum is located below the S. spongiosum and limits with the hypodermis, and it is composed by long fibres of collagen disposed in curly waves with chromatophores in apical disposition of this layer. No hydropic degeneration was found in the dermis of infected fish, but IELs foci were observed in the stratum spongiosum of infected fish in relation mainly to parasite adhesion areas. The incidence of IELs focuses was higher in the dermis than in the epidermis, and in some cases were not associated to a focal point (Fig. 4.10).

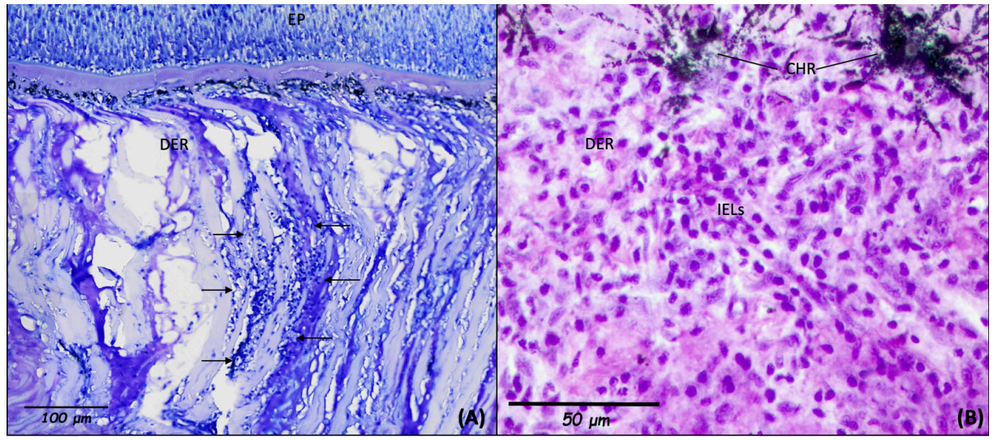

Figure 4.10. *(A) Detail of greater amberjack (seriola dumerili) cranial region transversal section. Observe the focal dermic extravasation of intraepithelial lymphocytes (IELs) (→) associated a focal point near blood vessels. Alcian blue-PAS-GIEMSA, pH= 2.5. Scale bar= 100µm. (B) Detail of IELs focuses in the dermis stratum spongiosum. Hematoxilin-eosin, pH=4.3. Scale bar=50µm.. EP: epidermis; DER: dermis; IEL: Intraephithelial lymphocytes; CHR: chromatophores.*

4.4. Discussion

To study of host-pathogen interactions is determinant in order to understand the mechanisms of infection. Particularly, for greater amberjack and due to its sensitivity to *N. girellae* infections when cultured in intensive conditions, to describe the skin-induced morphological alterations and the immunological response to its attachment is a decisive factor for the viability of greater amberjack intensive culture.

Fish skin morphology varies depending on the fish species and skin region studied (Hawkes, 1974; Ashley, 1975). In this sense, due to the proximity to cranial bones, cranial region skin has a thicker epidermis than dorso-lateral region, being also a region particularly susceptible to the appearance of blows and wounds. Previous studies based on the study of the fixation mechanism of *N. girellae* suggest that the most susceptible regions are the fins (Hirayama *et al.,* 2009) and the cranial region (Hirazawa *et al.,* 2011), similarly to Atlantic salmon infected by sea lice (Genna *et al.,* 2005). The existence of morphological and physical differences between the cranial and the dorso-lateral region in this fish species may explain partially the different incidence ratios depending on the fish skin region studied similarly as occurs in other fish species. For example, in salmonids, the dorso-lateral skin morphology is characterized by a thin epidermis layer disrupted by the insertion of the scales (Fast *et al.,* 2002 a). Despite greater amberjack scales size and morphology differ from Atlantic salmon scales (Jonsdottir et al., 1992; Mazzola *et al.,* 2000), their presence in dorso-lateral region supposes to *N. girellae* oncomiracidia an extra physical barrier to overcome in order to fix themselves to the fish skin compared to fins and cranial region. Our results, in terms of a higher density of scattered goblet cells in the epidermis of the dorso-lateral region compared to cranial region, are in agreement with previous studies in other fish species, such as Atlantic salmon (Pittman *et al.,* 2013). Moreover, the monogenean *Gyrodactylus derjavini* fixation is strongly correlated with a lower density of goblet cells per skin region, as described by Buchmann and Bresciani (1997), who highlighted the preference of this region in early infection stages, which was later supported by Yokoyama *et al* (2019) studies in greater amberjack. Additionally, greater amberjack cranial region skin has been pointed to have low acquired protection against *N. girellae* infection (Hirazawa *et al.,* 2011). Altogether, supporting our findings in relation to site/region-specific preferences for attachment of *N. girellae* in greater amberjack.

Besides the morphological differences of cranial and dorso-lateral regions, *N. girellae* fixation induced similar morphological alterations in both regions. An overpressure over epidermis caused by parasite haptor fixation has already been reported for other species such as Atlantic salmon during sea lice infection (Jones *et al.,* 1990). This process is related with a disruption of cell organization and cellular hyperplasia as a reaction to a chronic irritation, which finally uses to be related with a cellular necrotic process (Jones *et al.,* 1990; Jones, 2001). The fixation of *G. dejarvini* to rainbow trout has been associated to an up-regulation of proinflammatory cytokines as *il1-β,* which may contribute to cause hyperplasia of the surrounding epithelial cells (Buchmann and Brescianni, 1997). However, for greater amberjack and in contrast with other fish species, such as salmonids, no severe hyperplasia associated to *N. girellae* infection was observed on the epidermis. On the other hand, the observed epidermal over-flattening and crushing of *stratum superficiale* cells could derive in a medium to long-term basis in cellular necrosis and increased cell detachment (Jones *et al.,* 1990) for this fish species.

Epithelial cellular apoptosis foci in the first layer of *stratum superficiale* is a normal pattern of fish skin regeneration process (Hawkes, 1974). Nevertheless, the appearance of this process associated to more than one layer specially near the parasite's adhesion regions of the haptor and adhesive glands, and on larger surfaces compared with non-parasitized fish denotes a pathological origin. Indeed, an epidermal damage induced by sonication in goldfish *(Carassius auratus)* presented a similar cellular detachment and necrotic pattern in the first layers of epidermal cells as the one described in the present study (Frenkel *et al.,* 1999). This process has already been observed for ectoparasite infections, as sea lice infection in Atlantic salmon (Jones et al., 1990; Jonsdottir *et al.,* 1992), and *N. girellae* in barramundi (Trujillo-González *et al.,* 2015). As it was mentioned above, the process of fixation and overpressure caused near the haptor is related with cellular detachment. Particularly, in sea lice infections, it is related with ulcerative processes, which imply a complete lose and erosion of skin layers via destabilization of epithelial cells intercellular junctions (Boxshall, 1977; Roubal, 1986; Jensen *et al.,* 2015). In fact, ectoparasites produce specific proteases during the adhesion process, which act as immune disruptors and virulence factors (Esteban, 2012). In sea lice, proteases detected during the attachment to Atlantic salmon are mainly composed by serine-proteases (Ross *et al.,* 2000), which specifically digest cellular membranes and intercellular unions. Hirazawa *et al.* (2006) reported also the existence of some *N. girellae* specific serine-proteases with presumed similar functions to those described for sea lice. *N. girellae* digestion patterns of epithelial cells observed in the present study agree with the ones described for sea lice, losing the structure of intercellular junctions and micro-ridges at initial stages and disappearing during the subsequent cell degradation process (Nolan *et al.,* 1999). Sea lice feeding activity in Atlantic salmon, Coho salmon and rainbow trout has been associated with a reduction of the epidermis thickness (Fast *et al.,* 2002a,b; Holm *et al.,* 2015), as observed for greater amberjack infected with *N. girellae* in the present and previous studies (Hirayama *et al.,* 2009; Hirazawa *et al.,* 2016). This effect is highly dependent on the body site studied (Trujillo-González *et al.,* 2015), and particularly for greater amberjack, the most affected skin areas are the ventral and the dorsal region, as observed in the present study (Hirazawa *et al.,* 2011). Thus, summarizing the process of *N. girellae* fixation to greater amberjack skin, it starts by a fixation of the haptor to the epidermis, and the parasite anterior adhesion glands contribute to adhere the oral cavity to skin and produce proteases in order to facilitate *N. girellae* feeding from epithelial cells. In fact, *N. girellae* after anchoring is able to pivot around the anchor for feeding as described before by Yokoyama *et al.* (2019). Besides the morphological alterations described above, *N. girellae* attachment structures haptor penetrate at least the epidermal *stratum superficiale* and/or the *stratum spinosum,* generating mechanical damage in form of epidermal lacerations. Previous studies with blood feeding ectoparasites like polyopisthocotylea or sea lice have evaluated the mechanical damage induced on host skin (Jones *et al.,* 1990; Jonsdottir *et al.,* 1992) and gill (Montero *et al.*, 2004; Mansell *et al.,* 2005). Particularly for sea lice chalimus, which gets fixed to its host with the second antennae, associated ulcerations disrupting the basement membrane according to their blood-feeding have been observed (Jones *et al.,* 1990). Contrary for greater amberjack and since the basal membrane structure was perfectly conserved in all the sections studied, this mechanical damage was limited to first skin layer and directly conditioned by the attachment structures size. Besides, greater amberjack skin damage caused by *N. girellae* is also associated to an induced scratching behaviour, which facilitates the appearance of secondary infections (Roubal and Bullock, 1988; Svendsen and Bogwald, 1997; Sutherland *et al.,* 2011; Stien *et al.,* 2013).

Infected greater amberjack also presented a focal skin hydropic degeneration process. Hydropic degeneration caused by an ectoparasite infection has been also described for other cultured fish infected with *Amyloodinium ocellatum* and developmental stages (Paperna, 1980). In the present study, in early stages of the infection the hydropic degeneration was

mostly focused near the parasite adhesion region, whereas in advanced stages of the infection, the hydropic degeneration was massive, making unfunctional the epithelium and classified as a typical pattern of spongiosis (Paperna, 1980; Speare *et al.,* 1991). Other ectoparasites have demonstrated to produce this kind of lesions, as *Ichthyobodo necator* in salmonids (Robertson, 1985).

Meanwhile all these mechanical effects are surrounding the fixation of the parasite, host response presented different strategies. *N. girellae* infection in the present study induced an increase in the density of goblet cells present in the epidermis of infected fish, being an increase on mucus production one of the clearest effects in the host response to avoid parasite fixation and to trigger an specific immune response against the parasite (Paperna, 1991; Fast, 2014; Fernandez-Montero *et al.,* 2019; Hirazawa *et al.,* 2016; Yokoyama *et al.,* 2019). In contrast, Buchmann and Bresciani (1997) denoted the reduction of quantity of goblet cells during long term parasitation of rainbow trout with *G. derjavini*, probably due to a host response overload. For this reason, more studies need to be addressed to understand mucus production dynamics and its regulation along infection periods.

On the other hand, host immune response entails an adaptive and an innate immune response. *N. girellae* infection produced an acute response to the parasite infection characterized by appearance of IELs foci in the dermis. In a long-term response, IELs foci could become massive, filing the dermis. Besides that, no associated inflammatory process was observed in dermis, as a dermatitis morphological pattern. A dermatitis histopathological status has been observed in other species with pathogens causing skin diseases (Lunder *et al.,* 1995; Rizgalla *et al.,* 2016). Besides, ectoparasite infections suppose a source of acute and chronic stress, which entails a cortisol response from the host related with a limited capacity of host to carry out a successful wound healing process, but also with immunosuppression and the appearance of secondary infections by opportunistic pathogens (Krasnov *et al.,* 2012).
In summary, a higher incidence of *Neobenedenia girellae* fixation in greater amberjack cranial region could be related with the morphological characteristics of this region as less quantity of goblet cells and lack of imbricated scales. The attachment of *Neobenedenia girellae* to greater amberjack skin induced a mechanical damage characterized by focal epithelial vacuolization, epithelial cell digestion, cellular detachment and epidermis disorganization. Besides, *N. girellae* infection induces an increased density of epidermal goblet cells and a migration of IELs to the fixation area. In a long-term basis and due to the observed

skin lesions, an infection of greater amberjack with *N. girellae* may favour the appearance of secondary bacterial infections caused by opportunistic pathogens, limiting the production performance of this species in sea cages.

CHAPTER 05

PROTEOMIC PROFILE, MICROBIOTA AND PROTEASE ACTIVITY OF SKIN MUCUS IN GREATER AMBERJACK (*SERIOLA DUMERILI*) INFECTED WITH THE ECTOPARASITE *NEOBENEDENIA GIRELLAE*

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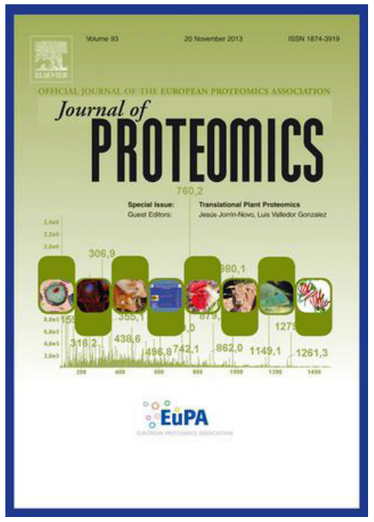
Abstract

Skin mucus is one of the first fish barriers against pathogenic diseases. Greater amberjack (*Seriola dumerili*) skin mucus protein profile and its changes due to a chronical *Neobenedenia girellae* infection were studied by combining 2-DE-MS/MS and gel-free LC-MS/MS. 2-DE-MS/MS identified 69 and 55 proteins in non-infected and infected fish, respectively, revealing that proteomes of both groups mainly differed in structural proteins as keratins, which were specifically degraded in parasitized fish. Remarkably, skin mucus of infected fish showed a higher protease activity due, at least in part, to a higher serine and metalloprotease activities. Additionally, by gel-free LC-MS/MS, 959 and 357 proteins were identified in the mucus of healthy and parasitized fish, respectively. The functional analysis of these proteins demonstrated an over-representation of proteins related to cytoskeleton in *N. girellae* infected fish. Proteins involved in stress response, central metabolism and immunity were detected in the skin mucus of both, healthy and infected fish but with different profiles. Moreover, LC-MS/MS allowed to analyze the skin mucus microbiota of *S. dumerilli,* being *Pseudomonas* the most represented genus. Despite the absence of differences in genera between parasitized and non-parasitized fish, this study represents the first approach to skin mucus associated microbiota of greater amberjack.



5.1. Introduction

The mucus of different vertebrates has been studied from diverse points of view to determine its function, composition and variations (Gladysheva *et al.,* 1986; Sepahi and Salinas, 2016). However, due to its environment, mucosal surfaces/tissues acquire a relevant role in fish, being the first barrier against a wide variety of chemical, physical and biological stressors (Ellis, 1999). Fish mucus is produced by goblet cells, which are scattered among mucosal tissues

epithelial cells (Pickering, 1974; Ellis, 1999; Salinas, 2015). Although most of the fish mucus studies have been focused in gut (Salinas and Parra, 2015), skin mucus knowledge is increasing due to its biomechanical and immunological issues (Xu *et al.,* 2013; Fudge *et al.*, 2015).

Skin mucus is mainly composed by water (95%) and mucins, a family of high molecular weight glycosylated proteins (Fast *et al.,* 2002). Mucins are structural proteins, which play a key role in mucus viscosity providing the surface of the fish body with rheological, viscoelastic or adhesion characteristics that could be modified depending on the types and the grade of mucin glycosylation (Koch *et al.*, 1991; Roberts and Powell, 2005). Keratins are also important structural proteins in fish skin mucus, although in a different way that in other vertebrates, since in aquatic species the absence of a specialized matrix and corneous cell envelope proteins avoid the cornification necessary as a barrier against loss of water in amniotes and, thus, fish require less specialized epidermal keratins with a specific mechanical role as compared with terrestrial vertebrates (Alibardi, 2002). Similar to other mucosal tissues, mechanical and physiological aspects of skin mucus are important for the associated microbiota (Merrifield and Rodiles, 2015). The microbiota is important in barrier function, although information about skin mucus microbiota interactions with host in aquaculture is still limited (Chiarello *et al.*, 2015; Lowrey *et al.*, 2015). To the date, most of the studies addressed to fish skin mucus proteins have been focused on its innate immune role (Esteban, 2012; Gómez *et al.*, 2013; Salinas, 2015), being the most studied mucus components those enzymes with biostatic or biocidal activities such as lysozyme, phosphatases, proteases, cathepsins and esterases (Esteban, 2012). Fish skin mucus proteases are secreted in response to bacterial and especially to ectoparasite infections (Firth *et al.*, 2000; Ross *et al.*, 2000). Similarly, parasites produce their own proteases during the attachment for feeding or disrupting the immune system of the host (Firth *et al.*, 2000). Parasite protease activity – host interactions have been specially studied in sea lice (*Lepeophtheirus salmonis*) and salmonids (Ross *et al.,* 2000). Nevertheless, some studies have been conducted for other ectoparasites as isopods (Manship *et al.*, 2008) and monogeneans like *Gyrodactylus sp.* (Jones, 2001).

Nowadays, monogenean infections are considered an important bottleneck for some aquaculture species, as *Seriola spp.* Indeed, the prevalence in sea farms of this infection could reach 70% of the cultured population (Ogawa *et al.*, 1995). *Neobenedenia girellae* is a monogenean ectoparasite with a wide host range distributed in warm waters worldwide (Whittington, 2012), being greater amberjack especially susceptible to its infection when reared in sea cages (Ohno

et al., 2008). A high parasite loads in greater amberjack induces fasting, stress-related changes in color appearance, erratic swim and a scratching behavior with tanks furniture, which develops in ulcerative processes with a subsequent appearance of opportunistic bacterial infections (Leong and Colorni, 2002). Some studies about the mechanical damage of *N. girellae* to greater amberjack skin (Hirayama *et al.,* 2009; Hirazawa *et al.*, 2016) and how this infection affect the mucus glycoproteins and serine proteases profile (Hirazawa *et al.,* 2006; Ohashi *et al.*, 2007) are available. However, as far as we know, this is the first work addressing the study of the proteome, the protease and the microbiota characterization of greater amberjack juveniles skin mucus before and after an experimental *N. girellae* infection.

5.2. Material and methods

5.2.1. Experimental fish and skin mucus collection

Sixteen greater amberjack juveniles (150 ± 11.4 g body weight) reared in the facilities of the University of Las Palmas de Gran Canaria were disposed in four cylindroconical 500 L tanks. Fish were fed with a commercial diet (Europe 22%, Skretting) to apparent satiety, 3 times a day. Skin mucus was obtained as described elsewhere (Fernandez-Montero *et al.*, 2019). Briefly, after 7 days of acclimation, fish were anesthetized with clove oil (5mL/L; Guinama S.L; Spain, Ref. Mg83168) and skin mucus was extracted by carefully scrapping the left dorso-lateral side of the fish with autoclaved microscopy slides, introduced in sterile 2ml Eppendorf and frozen in liquid nitrogen. Samples were pooled by tanks (4 animals per tank) in 2 pools (non-parasitized 1 (NP1) and non-parasitized 2 (NP2)). Infection of greater amberjack with *Neobenedenia girellae* was carried out as previously described (Fernandez-Montero *et al.,* 2019; Chapter 7). An experimental tank with greater amberjack previously infected with *N. girellae* was used for collecting the parasite's eggs in a 5 mm mesh for 24 hours. These eggs were introduced into a tank with uninfected greater amberjack juveniles. After 15 days all the fish were parasitized at the same level and used for the cohabitation challenge. For this purpose, two infected fish marked with a visible implanted elastomer (VIE) (Astorga *et al.,* 2005) were included in each tank. After 30 days of cohabitation, fish were sampled (4 fish per tank) to obtain parasitized greater amberjack skin mucus as described above. Samples were pooled by tanks in 2 pools (parasitized 1 (P1) and parasitized 2 (P2)). All mucus samples were stored at -80°C until analysis.

The animal experiments described here comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals.

5.2.2. Sample preparation for proteomic analyses

Mucus samples were solubilized in an equal volume of buffer (8 M urea, 2% CHAPS, 60 mM DTT and 1% protease inhibitor mixture) and centrifuged at 15000 *g* for 15 min at 4˚C. The resultant supernatant was used for determining the mucus proteome and the precipitate for the microbiota analysis. In the supernatant the protein concentration was determined using the Bradford assay (Bradford, 1976) and bovine serum albumin was used as standard. The precipitate was resuspended in 200 µl of buffer (50 mM Tris-HCl pH 7.6, 60 mM DTT and 2% SDS) and treated with three 30 s ultrasonic pulses (90W) separated by 30 s on ice. After centrifugation (15000 *g*, 15 min, 4˚C) the protein concentration was determined as described above.

For all proteomic procedures, samples from non-parasitized (NP) and parasitized (P) fish were pooled in two groups each (NP1, NP2, P1 and P2) and prepared using 2-D Clean-Up kit (GE Healthcare). After cleaning, the proteins were resuspended in 6 M urea and 200 mM ammonium bicarbonate for gel-free LC-MS/MS or, alternatively, in a rehydration buffer (8 M urea, 2% CHAPS, 12 µl/ml DeStreak reagent, 2% ampholyte solution pH 4-7, and 1% protease inhibitor mixture) for 2-DE experiments.

5.2.3. Two-dimensional gel electrophoresis and MS analysis

Skin mucus proteins suspended in the rehydration buffer (340 µλ) were firstly separated by isoelectric point in 18 cm, pH 4-7 IPG strips, and then by SDS-PAGE. Gels were stained with SYPRO Ruby dye for further scanning with Molecular Imager FX (Bio-Rad). Analytical and preparative gels were performed using 200 µg and 400 µg of proteins, respectively. The most abundant spots in preparative 2-DE gels were excised using an Investigator™ ProPic station (Genomic Solutions). For confirming that the desired protein spots were accurately obtained, the gel was rescanned after the excision. The selected spots were destained and digested with trypsin using an Investigator™Progest (Genomic solution). Peptide mixture was purified by C18 microcolumn (ZipTip, Millipore) and spotted with matrix solution (3 mg/ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile, 0.1% trifluoroacetic acid) onto an Opti-TOF 96-well

MALDI plate and analyzed using a 4800 Plus MALDI-TOF/TOF Analyzer (AB Sciex). Spectra were obtained using the reflector positive acquisition mode in the mass range of 800 to 4000 Da, precision ± 20 ppm, and 20kV of acceleration voltage. The eight strongest precursors from the MS scan were isolated and fragmented by collision-induced dissociation system. Protein identification was performed by combining MS and MS/MS spectra against public NCBInr database, subset Actinopterigii (taxid:7898; Nov2017), using MASCOT v2.0 (MatrixScience) integrated into GPS Explorer™ software (AB Sciex) and the following parameters: parent ion mass tolerance at 100 ppm, MS/MS mass tolerance of 0.2 Da, carbamidomethylation of cysteine selected as fixed modification, and methionine oxidation as variable modification. The probability score (95% confidence level) was calculated by the software and used as criteria for protein identification. Mass spectrometry procedures were performed at the Proteomics Unit, SCAI (Central Facilities for Research Support), University of Córdoba (Spain).

5.2.4. Gel-free LC-MS/MS analysis

Samples were clean up, reduced, alkylated and digested with trypsin using standard protocols. All analyses were performed at the Proteomic Unit, SCAI, University of Córdoba using a Dionex Ultimate 3000 nano UHPLC system (Thermo Fisher Scientific) connected to a mass spectrometer Orbitrap Fusion (Thermo Fisher Scientific) equipped with nanoelectrospray ionization interface. The peptide mix was previously concentrated and cleaned up on a 300 µm x 5 mm Acclaim Pepmap precolumn (Thermo Scientific) in 2% acetonitrile, 0.05% trifluoroacetic acid for 5 min at 5 µl/min. The trapping column was switched on-line with the separation column and the gradient was started at 40 °C, using mobile phase buffer A (0.1% formic acid) and mobile phase B (80% acetonitrile, 0.1% formic acid). Peptides were separated at 300 nL/min according to the following elution conditions: 4–45% buffer B for 60 min; 45–90% buffer B for 3 min followed by 8 min washing with 90% solution B, and re-equilibration during 15 min with 4% solution B. The mass spectrometer was operated in the positive mode. Survey scans of peptide precursors from 400 to 1500 m/z were performed at 120K resolution (at 200 m/z) with a 5×10^5 ion count target. For tandem MS, precursor ions were previously isolated in the quadrupole at 1.6 Da, and then CID-fragmented in the ion trap with 35% normalized collision energy. Monoisotopic precursor selection was turned on. Parameters for ion trap were automatic gain control $2x10^3$, max injection time 300 ms. Only those precursors with charge state 2–5 were sampled for a second in-tandem mass analysis. The dynamic exclusion time was set to 15 s with a 10-ppm tolerance around the selected precursor and its isotopes to avoid redundant fragmentation. For protein identification mass spectrometry raw data were processed using Proteome Discoverer v2.1.0.81 (Thermo Fisher Scientific). MS2 spectra were searched with SEQUEST HT engine against UniprotKB database restricted to Actinopterigii class (taxid:7898; Apr2017) in the study of the mucus proteome or restricted to bacteria (taxid:2; Oct2017) in the microbiome analysis. Theoretical peptides were generated from a tryptic digestion with up to two missed cleavage. Methionine oxidation was set as variable modification and carbamidomethylation of cysteines as fixed modification. A value of 10 ppm was set for mass tolerance of precursor ions, and 0.1 Da tolerance for product ions. Peptide identifications were accepted if they exceeded the filter parameter Xcorr score versus charge state with SequestNode Probability Score (+1 = 1.5, +2 = 2.0, +3 = 2.25, +4 = 2.5). Peptide spectral matches (PSM) were validated using percolator based on *q*-values at a 1% false discovery rate (FDR).

5.2.5. Protease activity analyses

Skin mucus samples were diluted in a 1:1 ratio (v:v) with a solubilization buffer (urea 8 M, CHAPS 2%, β-mercaptoethanol 10 mM) and centrifuged twice before analysis. Total protein concentration was determined using the Bradford assay (Bradford, 1976) as described above. Protease activity was quantified using the azocasein hydrolysis assay according to the method of Ross ***et al.***, (2000). All determinations were carried out at least in triplicate. For positive control skin mucus was replaced by trypsin (5 mg/ml, Sigma). Negative control was performed using 100 mM ammonium bicarbonate. Protease activity in each sample is expressed as the percentage relative to the trypsin positive control (100%). For protease characterization, azocasein hydrolysis assay was conducted with specific inhibitors of proteases: 10 mM EDTA as metalloprotease inhibitor or 5mM PMSF for serine protease inhibitor.

5.2.6. Statistical analyses

Gene ontology categories were compared with Fisher exact test (two tailed comparison) in combination with FDR (false discovery rate) correction for multiple testing by using *Blast2GO* software (v5.2.5) (Conesa *et al.*, 2005). Non-parasitized set of sequences was used as reference set and parasitized set as test set. The cutoff threshold for statistical significance was established at FDR<0.05.

Statistical analyses of protease activity followed the methods outlined by Sokal and Rolf (1995). Means and standard deviations (SD) were calculated and a *t*-Student test was conducted. Differences were considered significant when $p<0.05$. Data was analyzed with SPSS software (SPSS for Windows 10 V23).

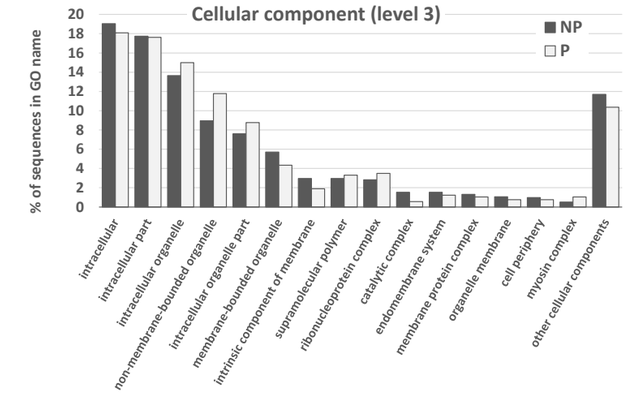
5.3. Results and discussion

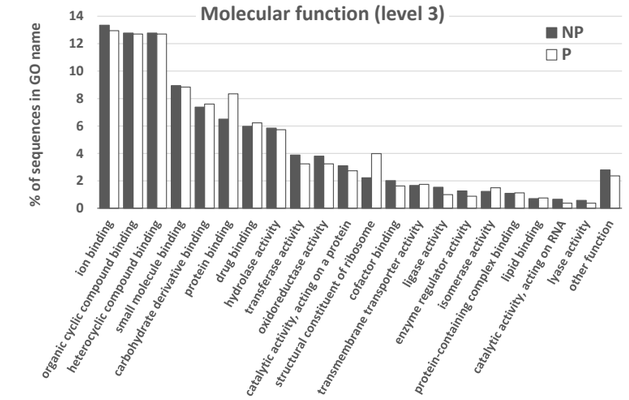
5.3.1. Two complementary approaches to study the proteomic profile of greater amberjack (Seriola dumerili) skin mucus

In this study the proteome of skin mucus of greater amberjack juveniles parasitized with *Neobenedenia girellae* (P) and non-parasitized (NP) is discussed. The proteomic profiles have been analyzed by using two methodologies. Firstly, we used a gel-free approach, in which epidermal mucus protein samples of both, healthy and parasitized fish, were digested with trypsin and the resulting unlabeled peptides were separated and analyzed by LC-MS/MS. To complete our study, a second approach involving proteins separation by 2-DE, in-gel trypsin digestion and analysis by PMF using combined MS and MS/MS was performed. To our knowledge, this study is the first report of skin mucosal proteome of greater amberjack.

5.3.1.1. Gel-free LC-MS/MS. Abundance of cytoskeletal proteins in parasitized fish

For proteomic analysis two pools of mucus samples from NP group (NP1 and NP2) and two pools of samples from P fish (P1 and P2) were investigated. An average of 1500 proteins per pool were identified at a high confidence level. A total of 2061 unique proteins were identified in NP group, 959 of them appear in both NP1 and NP2 pools. And 1955 unique proteins were identified in P group, 357 of them were common to both pools (P1 and P2). All subsequent studies were performed using these 959 and 357 set of common proteins. To obtain a global functional level view of the proteome, gene ontology (GO) annotations were obtained from *UniProt* database and the analysis was performed using *Blast2GO* (v5.2.5) (Conesa *et al.,* 2005). A summary of main distribution of proteins by molecular function, biological process, and cellular component categories at level 3 is shown in Fig. 5.1. Due to differences in absolute number of sequences between NP and P groups and in order to better compare both experimental conditions, the percentage of sequences per GO name is shown in Fig. 5.1.





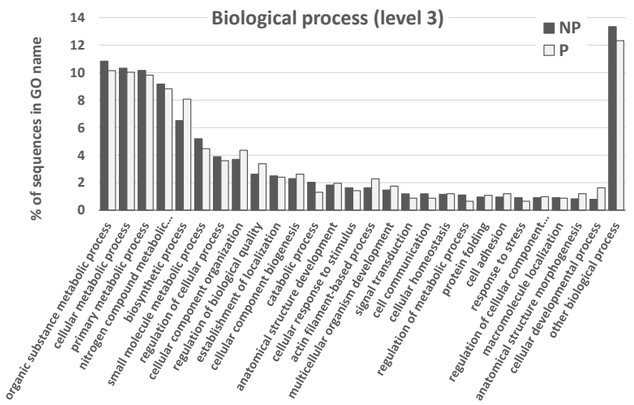


Figure 5.1. *Functional classification of identified proteins from greater amberjack (Seriola dumerili) skin mucus. Mucus proteins of non-parasitized (NP) and parasitized (P) fish are classified according to cellular component, molecular function or biological process categories at level 3 of the gene ontology system (GO) using Blast2GO software (Conesa et al., 2005). For comparison of NP and P groups the percentage of sequences in each GO term is represented in Y axis. Only GO categories having more than 1 % of the sequences have been individually considered.*

The annotation of cellular component showed that the most represented GO terms were intracellular and intracellular part (>18%), and intracellular organelle and non-membrane-bounded organelle (> 9%). All these four categories together account for more than 60% of the sequences. A statistical assessment of GO term enrichment was performed, including all levels, by a Fisher's exact test in combination with a false discovery rate (FDR) correction using *Blast2GO*. The test displayed significant differences (FDR<0.05) between NP and P groups in five GO terms: (1) intracellular organelle, (2) organelle, (3) intracellular non-membrane-bounded organelle, (4) non-membrane-bounded organelle and (5) cytoskeleton (Fig. 5.2). All these GO terms were statistically over-represented in the parasitized fish. Moreover, these five GO terms are parent-child related and represent the same concept but at different levels of specificity, being the most specific GO term GO: 0005856, cytoskeleton (Fig. 5.2). Ectoparasites feed from mucus, cellular components and blood. Because of attachment and feeding behavior, ectoparasites induce tissular damages to the host, which are related to the higher presence of intracellular proteins (Easy and Ross, 2009). Moreover, the presence of the parasite in the skin triggers modifications on the structure, composition and properties of fish skin mucus (Aranishi *et al.,* 1998; Bosi *et al.,* 2005; Easy and Ross, 2009). A higher presence of cytoskeletal proteins is needed for a proper skin wound healing process in skin (Jensen *et al.,* 2015; Cordero *et al.,* 2017), which also is in accordance with the results obtained in the present study.

The annotation of molecular function showed a dominance of the ion binding, the organic cyclic compound binding and the heterocyclic compound binding categories (together, around 40% of sequences in both, NP and P groups). Although protein binding and structural constituent of ribosome GO terms shown a remarkable higher percentage in parasitized fish (Fig. 5.1) a statistical difference between the experimental groups was not found when all levels of GO terms were included.

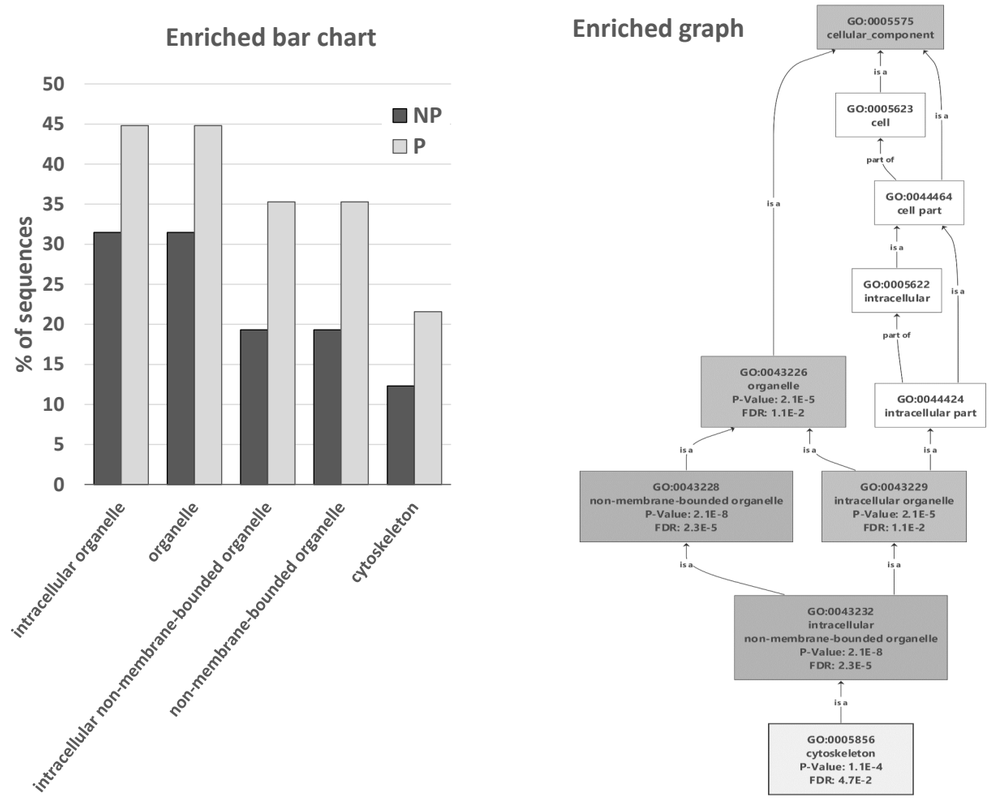


Figure 5.2. *GO terms statistically enriched in the comparison between the skin mucus proteins identified in parasitized (P) and non-parasitized (NP) greater amberjack (Seriola dumerili). Bar-graph (left) and enriched graph (right). A two-tailed Fisher exact test (FDR < 0.05) were carried out using Blast-2GO software (Conesa et al., 2005). In the enriched graph nodes are color highlighted proportionally to their significance value.*

The annotation of biological process indicated that organic substance , cellular , primary and nitrogen compound metabolic processes were the most relevant GO terms in both experimental conditions (more than 8% of sequences each term). In contrast, biosynthetic process category reached this threshold only in P group, but again, the difference although pronounced, was not statistically significant.

Besides the global analysis reported above, some biologically relevant proteins related to structural function, stress response, metabolism and immune response, are shown in Table 4.1.

Table 5.1. *Proteins obtained from LC-MS/MS biologically relevant for discussion.*

Structural proteins			
Actin, adductor muscle	B5XFZ3	x	x
Actin, beta	A0A1A7X2B6	x	
Actin, beta 1	H2LZV3	x	
Actin, cytoplasmic 2	A0A146UVU6	x	x
Actin-related protein 2	A0A146VYT2	x	
Actin-related protein 2/3 complex subunit 2 (Fragment)	A0A146QTS5		x
Actin-related protein 2/3 complex subunit 3 (Arp2/3 complex 21 kDa subunit)	A0A1A7WF24	x	
Actin-related protein 2/3 complex subunit 4	A0A0P7YAY7	x	
Actin-related protein 2/3 complex subunit 4	W5K3Y7	x	x
Actin-related protein 2/3 complex subunit 5 (Fragment)	A0A147AK30	x	
Actin-related protein 3	A0A0F8AHR7	x	x
Alpha-actin (Fragment)	Q8JIZ5		x
Alpha-actinin-4	A0A0F8C435	x	
Beta-actin (Fragment)	Q1WMK7	x	
Beta-actin (Fragment)	A0A0S2VH97		x
Beta-actin (Fragment)	A0A140ZR28		x
Beta-actin (Fragment)	A7IZV4		x
Capping protein (Actin filament) muscle Z-line, alpha 1b	A0A1A8LYW3	x	
Capping protein (actin filament) muscle Z-line, beta	I3KRT7	x	
Capping protein (actin filament), gelsolin-like b	I3KRY6	x	x
Capping protein (actin filament), gelsolin-like b	M4AMP9		x
Cofilin 1 (non-muscle), like	M3ZVB0	x	
Cofilin-2	C3KHN8	x	
Cytokeratin (Fragment)	A0A0U2JSR7	x	
Cytokeratin (Fragment)	B9V3W0		x
Cytoplasmic 1 actin	W5ZMG9	x	
F-actin-capping protein subunit alpha-1	C3KJN6	x	
Keratin 1	A2SXI5	x	
Keratin 15	A0A096LY91	x	x
Keratin 15 (Fragment)	B9V3U1	x	x
Keratin 17	W5K0S8	x	x
Keratin 18	H2L7L7	x	
Keratin 18 (Fragment)	B3GPH2	x	
Keratin 4	M4AW48	x	
Keratin 4	W5K1N1	x	
Keratin 5	G3NI10	x	x
Keratin 5	A0A1A8IH58		x
Keratin 5 (Fragment)	A0A1A8NJ47		x
Keratin 8	I3JRI8	x	x
Keratin 8	H2L7B5		x
Keratin 97	G3Q222	x	x
Keratin 97	I3JR12	x	x
Keratin 97	M4AUU8		x
Keratin 97 (Zgc:92061)	Q6DHU3	x	x
Keratin K10 (Fragment)	A1KQZ8	x	
Keratin type II (Fragment)	S5UM11	x	x
Keratin type II E3	G8G8Y2	x	
Keratin, type I cuticular Ha6-like (Fragment)	A0A0P7V0L9	x	x
Keratin, type I cytoskeletal 13	C3KHW5	x	x
Keratin, type I cytoskeletal 13 (Cytokeratin-13) (CK-13) (Keratin-13) (K13)	Q8JFQ6		x
Keratin, type I cytoskeletal 16	A0A147ALS7	x	
Keratin, type I cytoskeletal 18	A0A0F8AXL8	x	
Type I cytokeratin (Fragment)	A0A0K0NJB6	x	
Type I cytokeratin, enveloping layer	H2RQ00	x	
Type I cytokeratin, enveloping layer	M4AX75	x	x
Type I keratin isoform 1	A4UYK3	x	
Type I keratin-like protein (Fragment)	C0LMQ3	x	
Type II keratin	Q6QD65	x	
Type II keratin	C0SW46		x
Type II keratin E1	Q90W76	x	x
Type II keratin E3	E6ZFH2	x	x
Type II keratin E3	Q8JFG4	x	
Type II keratin E3-like protein	Q4QY72		x
Stress response			
Cathepsin Ba	G3PUU3	x	x
Cathepsin E	A0A146QSG3	x	
Glutathione peroxidase 4b	I3JVM7	x	x
Glutathione reductase, mitochondrial	A0A147B140	x	
Glutathione S-transferase mu, tandem duplicate 1	B8JIS8	x	
Glutathione S-transferase omega class	F1AQ77	x	x
Glutathione S-transferase rho	I3JAS8	x	
Glutathione S-transferase(Rho)	D3TJL0	x	
Glutathione S-transferase, alpha tandem duplicate 2	G3NTD1	x	
Heat shock cognate 70	A0A0E4G1U1	x	
Heat shock cognate 70 kDa	A0A1B2G2Q0	x	
Heat shock cognate 71 kDa protein	P47773	x	x
Heat shock cognate protein 70	C9WE63	x	x
Heat shock protein 105 kDa	A0A146VLT2	x	
Heat shock protein 60	A0A097BVP4		x
Heat shock protein 60	A0A0B6VWS4		x
Heat shock protein 60 kDa	A0ELV8	x	
Heat shock protein 70	A0A0B6VWU0		x
Heat shock protein 8	I3JAU8	x	
Heat shock protein 90, beta (Grp94), member 1	A0A1A7XB59	x	
Heat shock protein 90beta	A0A0U1Z3U1	x	
Heat shock protein 90-beta, cytosolic	F8SLH0	x	x
Heat shock protein 94c	A0A146T8N9	x	
Peroxiredoxin 2	H2SYI2	x	
Peroxiredoxin 2	G3Q5U7	x	
Peroxiredoxin 3	A0A0B4UEZ5	x	
Peroxiredoxin 4	H2LQC7	x	
Peroxiredoxin 5	A0A0N7AV88	x	
Peroxiredoxin-6	A0A0F8ARY3	x	
Warm temperature acclimation protein (hemopexin)	H2UMC6	x	x
Warm temperature acclimation protein (hemopexin)	H3CMK6	x	x
Warm temperature acclimation protein (hemopexin)	M3ZG42	x	x
Warm temperature acclimation protein (hemopexin)	A0A161FU20	x	x
Warm temperature acclimation protein (hemopexin)	A1YTM9	x	x
Warm temperature acclimation protein (hemopexin)	D5A7I1	x	
Warm temperature acclimation protein (hemopexin)	F2QKY4	x	
Metabolism			
Iron metabolism			
Serotransferrin	A0A0S2SWP8	x	x
Serotransferrin	A0A0U1Z6E4	x	x
Serotransferrin	A0A146MRY3	x	
Serotransferrin	G9I0G6	x	x
Serotransferrin	G9I8A5	x	x

40S ribosomal protein S17 (Fragment)	I1SRV1	x	
40S ribosomal protein S18	A0A147AP54	x	
40S ribosomal protein S19	A0A146ZKR0	x	
40S ribosomal protein S26 (Fragment)	A0A1A8LNV4		x
40S ribosomal protein S28-like	A0A0P7VAS6	x	x
40S ribosomal protein S3	C1BKU2	x	
40S ribosomal protein S30 (Fragment)	A0A147A525		x
40S ribosomal protein S3a	A0A0F8AGM8	x	x
40S ribosomal protein s3-like	A0A0P7WNH2	x	
40S ribosomal protein S5 (Fragment)	A0A147A028	x	x
40S ribosomal protein S8	A2Q0S3	x	
40S ribosomal protein S8	H2LDM6	x	
40S ribosomal protein S8	Q90YR6	x	
40S ribosomal protein S9 (Fragment)	A0A146MSQ0	x	x
40S ribosomal protein SA	A0A146WIH6	x	x
40S ribosomal protein SA (37 kDa laminin receptor precursor) (37LRP) (3	A0A1A7WLK5	x	
60S acidic ribosomal protein P0	A0A060W7L0		x
60S acidic ribosomal protein P0	A9ZTC3	x	
60S acidic ribosomal protein P0	C3KGY0	x	
60S ribosomal protein L13 (Fragment)	F5BZL2		x
60S ribosomal protein L14	A0A0F8BL99	x	x
60S ribosomal protein L17 (Fragment)	D6PVQ9		x
60S ribosomal protein L18	A0A0F8B7U1	x	
60S ribosomal protein L18	P69090	x	x
60S ribosomal protein L21	D6PVQ4		x
60S ribosomal protein L21 (Fragment)	A0A146NT48	x	
60S ribosomal protein L23 (Fragment)	A0A147B0T5	x	x
60S ribosomal protein L28 (Fragment)	A0A146SHE2	x	x
60S ribosomal protein L30	A0A147B2V7		x
60S ribosomal protein L34	C0H7M9	x	
60S ribosomal protein L36	C0H7I0	x	
60S ribosomal protein L37a	A0A147A3T6	x	
60S ribosomal protein L38	C0H7L4	x	x
60S ribosomal protein L6 (Fragment)	F6KMN0	x	
60S ribosomal protein l8-1	E3TDI8		x
Acidic ribosomal protein P0 (Fragment)	I1SRY1	x	
Putative ribosomal protein L7 protein (Fragment)	Q90YH2		x
Ribosomal protein L11 (Fragment)	A8HTH7	x	
Ribosomal protein L12	F6KMH2	x	x
Ribosomal protein L15	A0A146P902	x	
Ribosomal protein L19	A0A1A7XM72	x	
Ribosomal protein L21	I3K9H1		x
Ribosomal protein L22-like 1	A0A1A8HNV6		x
Ribosomal protein L24	A0A1A8A9H5	x	x
Ribosomal protein L26	A0A087X490		x
Ribosomal protein L27a	Q8JJ03	x	x
Ribosomal protein L3	G3PNT5		x
Ribosomal protein L3	H2L7K6	x	
Ribosomal protein L30	A0A087Y9S0	x	
Ribosomal protein L4	M3ZMU3	x	
Ribosomal protein L4 (Fragment)	Q6Y213	x	x
Ribosomal protein L7a	A0A1A8SI27	x	
Ribosomal protein L7a	A9ZT81	x	x
Ribosomal protein L9	A0A1A8PF43	x	
Ribosomal protein S11 (Fragment)	A0A1A7WYR6		x

Ribosomal protein S6 kinase (EC 2.7.11.1)	I3J6B5	x	
Ribosomal protein S6 kinase b, polypeptide 1b	A0A1A8UAE1	x	
Ribosomal protein, large, P1	A0A1A7WMD1	x	
S11 ribosomal protein (Fragment)	A6MWV7		x
Ubiquitin-40S ribosomal protein S27a	A0A0F8C7T1	x	
Immune response			
Adenylyl cyclase-associated protein	A0A087X5K0	x	x
Adenylyl cyclase-associated protein	G3NDS5	x	x
Adenylyl cyclase-associated protein	H2TFR0	x	
Adenylyl cyclase-associated protein	Q6YBS2	x	
Adenylyl cyclase-associated protein	W5UMC9	x	
Alpha-2-macroglobulin	A0A097IQZ2	x	
Alpha-2-macroglobulin	A0A0F8BZW0		x
Aminopeptidase W07G4.4	A0A146MX13		x
ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)	A0A146NGZ5	x	
Calpain 12	I3J735	x	x
Calpain 12	G3P5S8	x	
Calpain 2, (m/II) large subunit a	I3JJX0		x
Calpain 2, (m/II) large subunit b	G3Q932	x	x
Calpain 8	A0A096MDV5	x	
Calpain 9	H2MZK5	x	
Calpain 9	I3ITW3	x	
Calpain, small subunit 1 a	A0A1A8EP70	x	
Calpain-1 catalytic subunit	M1FSS0	x	
Calpain-2 catalytic subunit	A0A0F8CQK2	x	
Calpain-2 catalytic subunit	A0A0F8AL09	x	
Calpain-9	A0A0F8AU23	x	x
Carnosine dipeptidase 2	H3DJI6	x	
CNDP dipeptidase 2 (Metallopeptidase M20 family)	A0A1A8HY19		x
Complement C3	A0A0F8AMU3	x	x
Complement component 1 Q subcomponent-binding protein, mitochondrial	A0A0F8AE02	x	
Copine I	H2N199	x	x
Copine-1	A0A0F8CIS2	x	x
Copine-1	W5UM43	x	
Coronin	A0A087XY51	x	
Coronin	A0A146YZP6	x	
Coronin	A0A0F8CYM1	x	
Coronin	I3K6Y0	x	
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit (Oligosaccharyl transferase 48 kDa subunit)	A0A147ABN2	x	x
H2A histone family, member Y	A0A1A7Y5J7	x	
Histone deacetylase complex subunit SAP18-like	A0A0P7WMM5	x	
Histone H2A	H2T5P7	x	
Histone H2B	H2L6L3	x	
Histone H2B	M4A7C6	x	
Histone H3 (Fragment)	Q5SET8	x	
Histone H4	Q4SKJ3	x	x
Histone-binding protein RBBP4	A0A146YG32	x	
Interleukin enhancer-binding factor 2	A0A146XDZ9	x	
Malectin (Fragment)	A0A146NV17	x	
MHC class Ia antigen	G9BBU1	x	
Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	A0A060XG86	x	
Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	A0A0F6QH59	x	

Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	I3IW09	x	x
Platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit	G3P458	x	x
Polypeptide N-acetylgalactosaminyltransferase (EC 2.4.1.-) (Protein-UDP acetylgalactosaminyltransferase)	A0A0F8CHM6	x	
UDP-glucose glycoprotein glucosyltransferase 1	H2UQ25	x	
UDP-glucose:glycoprotein glucosyltransferase 1	A0A146V2J7	x	x
Xaa-Pro aminopeptidase 1	A0A146NNX6	x	

5.3.1.2. Two-dimensional gel electrophoresis. Specific fragmentation of keratins in parasitized fish

A 2-DE study was performed in order to, qualitatively and quantitatively, compare the proteomic profiles of skin mucus of parasitized and non-parasitized fish. We run three gels per pool of NP and P samples (a total of 12 gels). The 12 gel images obtained are shown in Fig. 5.3. The evident differences between gels from NP and P samples made impossible a quantitative analysis of spot intensity differences mainly because of the difficulty of matching the spots among the 12 gels. Instead, the most abundant spots were selected and identified using LC-MS/MS and database retrieval. A representative gel of each experimental condition, including the position of identified spots, symbol of protein and a reference number, is shown in Fig. 5.4.

The most relevant observation is the presence in the gels of the NP group of up to four trains of acidic proteins with p*I* 4.6-5.6 and Mw between 40 and 60 kDa, corresponding to different species of keratins. This group of proteins, which quantitatively accounts for more than a 40% of the total fluorescence signal of the sample, was not present in the P group. Instead, in the gels from parasitized fish, numerous spots corresponding to proteins with lower Mw, between 20 and 30 kDa, were also identified as keratins. Proteolytic fragmentation of keratins would explain the arrangement of these proteins in the P group. Keratins seems to be a major component of the skin mucus of healthy greater amberjack, given its abundance in NP samples. It should be noted that the fragmentation of the keratins does not correspond to a general degradation of P samples but to a specific fragmentation of this type of proteins. Evidence of this is that other proteins such as Trfe, Enoa, Capg, Hsc70 or Grp78, appear in gels from both groups of samples, NP and P, at the same coordinates and, apparently, in a similar amount. Both types of keratin components of intermediate filaments were found: (i) Type I, keratins 13 and 17 in NP samples, and keratins 9, 13, 91, 97 in P samples. And (ii) Type II, keratins 4, 8 and 73 in NP samples, and keratins 4 and 5 in P samples (Table 5. 1).

Keratins were also found by gel-free LC-MS/MS, representing 34 out of 959 unique proteins in NP samples and 25 out of the 357 unique proteins in P group. The presence of keratins in fish skin mucus has been repeatedly reported by other authors (Easy and Ross, 2009; Rajan *et al.,* 2011; Rajan *et al.,* 2013; Jun *et al.,* 2013; Jurado *et al.,* 2015; Perez-Sanchez *et al.,* 2017). It is well known the structural role of keratins, but a protective function of this protein has also been described. Thus, a pore-forming activity against bacteria of a glycosylated

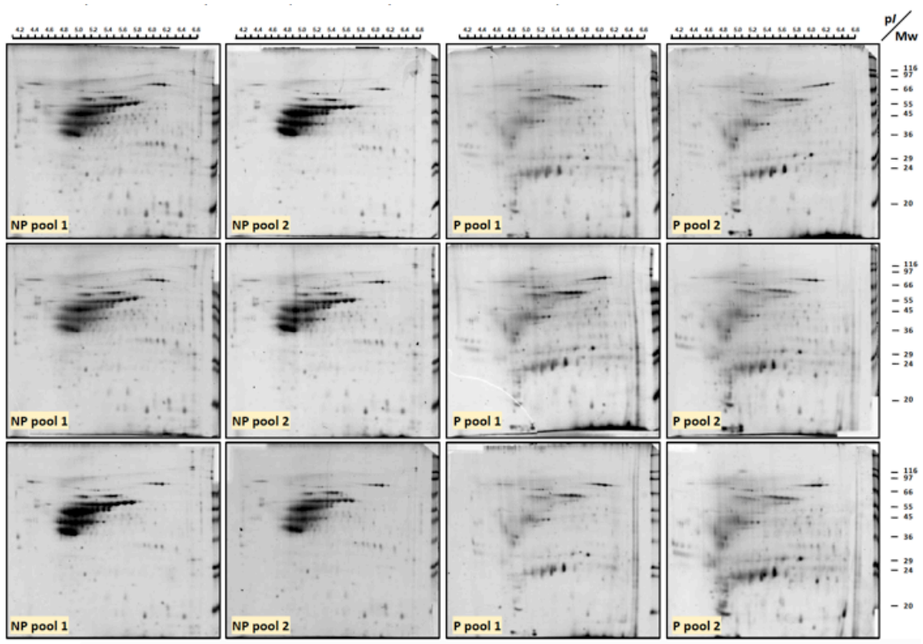


Figure 5.3. Experimental dataset of 12 gels images using 18 cm IPG 4-7 strips. Four pools of samples (non-parasitized, NP, pools 1 and 2, and parasitized fish, P, pools 1 and

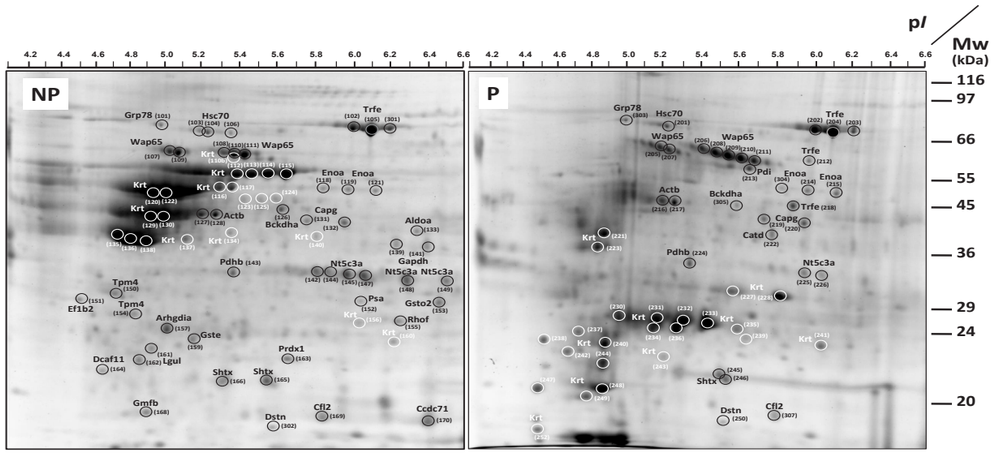


Figure 5.4. Representative 2-DE gel images of skin mucus proteins from non-parasitized (NP) and parasitized (P) greater amberjack (Seriola dumerili). Identified proteins are indicated by their UniProt entry names and reference spot number. Spot identified as keratins are labeled in white.

protein, similar to type II cytokeratin, has been described in rainbow trout (*Onchorrynchus mykiss*) skin mucus (Molle *et al.,* 2008). Moreover, an increase of epidermal keratin after sea lice infection in Atlantic salmon (*Salmo salar)* skin mucus has been associated not only to cellular damage and tissue regeneration, but also to a specific response against the pathogen (Easy and Ross, 2009). Additionally, in mammals, it has been described that cytokeratin produced antimicrobial peptides (AMP) after proteolysis by extracellular proteases (Qi and Boyao, 1999; Tam *et al.,* 2012). According to this, the fragmentation of keratins we have found in parasitized greater amberjack could be a response of the host to prevent bacterial infections. In the other hand, it is known that ectoparasites use their own proteases to facilitate its feeding and to evade host immune responses (Firth *et al.,* 2000). In this line, we can also hypothesize that proteases from the ectoparasite could be responsible of the keratin's cleavage observed in P samples. This is an important issue that needs to be clarified in order to design new therapies against *N. girellae* infections.

5.3.2. Other structural proteins

The main changes observed in 2-DE gels concern structural proteins as keratins, as discussed above. Moreover, other structural proteins common to both NP and P experimental groups were found in the present study: beta-actin (Actb), capping protein (Capg), destrin (Dstn) and cofilin-2 (Cfl2) (Table 5. 1). Accordingly, chronically-ulcered gilthead seabream skin mucus proteome showed a high quantity of structural proteins. These results, supported by transcriptomics complementary studies, determined that the structural genes category was the most affected by ulceration (Cordero *et al.,* 2017). More specifically, beta-actin species are consistently found in fish skin mucus [41, 43, 48] (Rajan *et al.,* 2011; Cordero *et al.,* 2015; Jurado *et al.,* 2015). In fact, the higher presence of actin in Atlantic salmon infected with sea lice has been pointed by other authors to have a role in mucus structure and organization (Easy and Ross, 2009), and it has also been reported that actin fragments produce an effect on immune stimulation (Easy and Ross, 2010). Nevertheless, the two spots identified as beta-actin in gels from NP and P samples are not apparently altered by the proteolytic process affecting keratins (Fig. 5.4).

5.3.3. Stress response proteins

A total of 18 spots related to stress response were found by 2-DE MS/MS in healthy fish skin mucus compared with the 13 spots found in parasitized fish (Table 5. 1). Some of these spots were oxidative stress-related proteins such as glutathione *S*-transferases (Gsto2, Gste) and peroxiredoxin 1 (Prdx1), only found in NP samples, and protein disulfide-isomerase (Pdi), only identified in gels from P samples. Gel-free LC-MS/MS also revealed glutathione-S-transferases species as Gstm, Gstr and Gsta and peroxiredoxins species Prdx2, Prdx3, Prdx4, Prdx5 and Prdx6 just in NP samples (Table 5. 1). By contrast, Gsto was found in both NP and P samples. Gel-free LC-MS/MS also identified other enzymes implied in glutathione metabolism as glutathione reductase (Gsr) in NP samples, and glutathione peroxidase 4 (Gpx4) in NP and P fish. Oxidative stress-related proteins in mucus are involved in the neutralization of reactive oxygen species produced by pathogens as bacteria (Canesi *et al.,* 2010). In fact, increased activity of glutathione peroxidase and glutathione S- transferase after challenging Nile tilapia (*Oreochromis niloticus*) with fungal pathogens as *Aphanomyces* or *Phoma* has been reported (Ali *et al*., 2011). In contrast, a reduction of these proteins in the skin mucus was reported for Atlantic cod *(Gadus morhua*) challenged against *Vibrio sp* (Rajan *et al.,* 2013). Similar results were previously found in gilthead seabream in response to a chronic infection by the intestinal parasite *Enteromyxum leei* (Sitja-Bobadilla *et al*., 2008). Prdx1, 2, 4, 5 and 6 have been previously found in skin mucus of several fish species but Prdx3 did not (reviewed in Brinchmann, 2016). In mammals, in addition to their antioxidative activity, the function of Prdxs has been linked to inflammation, tissue repair and modulation of the host immune response against parasites (Ishii *et al.,* 2012), but its role in skin mucus teleost has not yet been defined.

Warm temperature acclimation protein (Wap) and the chaperons heat-shock proteins (Hsp and Hsc) are involved in the response to temperature changes and they were identified by both proteomic techniques in NP and P samples (Table 5. 1). Wap protein, also called hemopexin, presents a high affinity for heme-iron. Given that iron is a key element in bacterial infections and because of the presence of enhancer motifs including cytokine responsive elements on its gene sequence, Wap has also been associated to innate immunity (Kikuchi *et al.,* 1997). Hsp and Hsc proteins have been related to a wide variety of stressors. In fish, these chaperons have been differentially expressed after infection in Atlantic salmon skin

mucus and catfish skin (reviewed in Brinchmann, 2016). In the present study, cathepsin D was observed only in gels from parasitized fish skin mucus. Nevertheless, other cathepsin species were found by gel-free approach: cathepsin E in NP samples and cathepsin Ba in both, NP and P samples. Cathepsins are usually activated by low pH in lysosomes, but they can be also activated by metalloproteases in fish skin mucus cleaving histone H2A to produce the antimicrobial peptide (AMP) parasin I (Cho *et al.,* 2002).

5.3.4. Metabolic proteins

Intermediate enzymes of glycolysis, nucleotide and amino acid metabolism were found by 2-DE approach in parasitized and no parasitized fish skin mucus (Table 5. 1). These proteins catalyze basic cellular processes and their presence in the mucus has been related to cell damage or alternative extracellular functions (Henderson and Martin, 2002). In this category, gel-free-LC-MS/MS methodology identified 50 ribosomal proteins (RSP) in NP fish and 32 in P samples (Table 5.1.), although any RSP was obtained by using 2-DE. This kind of proteins has previously been identified in rainbow trout, Atlantic cod and gilthead seabream skin mucus (Fernandes and Smith, 2002; Rajan *et al.,* 2013; Jurado *et al*., 2015). Besides their key role in protein synthesis, secondary immune functions have been attributed to these proteins or fragment thereof, which could act as AMP in fish skin mucus (Fernandes and Smith, 2002; Esteban, 2012). The presence of a higher number of RSPs in parasitized fish skin mucus (Table 5. 1.) could be triggered by the cell damage caused by the parasite, and once in the mucus, their secondary function as AMP would facilitate the onset of the innate immune response.

Serotransferrin (Trfe) was detected in skin mucus of NP and P groups of greater amberjacks. Transferrins are iron binding transport proteins which can bind two Fe^{3+} ions. Several studies have found Trfe in fish skin mucus and due to its high chelating affinity, a role in the innate defense system against pathogenic microorganism have been attributed to this protein (reviewed in Brinchmann, 2016). In this line, liver Trfe gene expression was upregulated in grass carp (*Ctenopharyngodon Idella*) after a copepod infection (Chang *et al*., 2005). In addition, it has been reported an increase in Trfe fragmentation in Atlantic salmon skin mucus infected with sea lice (Easy and Ross, 2009), and according to this, we have found fragments of low molecular mass (~57 and ~45kDa, spots 212 and 218, respectively) only in gels from P group. By contrast, non-cleaved forms of Trfe (~70-75kDa) appeared in both, NP and P samples in the upper right corner of the gels (Fig. 5.4).

5.3.5. Immune-related proteins

Some proteins related to immune response pathways were differentially detected in parasitized and non-parasitized fish. These proteins were only identified by using gel-free LC- MS/MS (Table 5.1.), demonstrating the effectiveness and convenience of combining the two proteomic techniques used here. Toll-like receptor cascades are one of the first signaling pathways of the innate immune system (Palti, 2011). Coronin (Cor) participates in the NCoR co-repressor complexes removal of Toll-like receptor and in the subsequent de-repression of inflammatory response genes (Huang *et al*., 2011). Moreover, catfish skin coronin was down regulated after 5 days of exposure to *Aeromonas sp* (Lee *et al.,* 2013), in agreement with the present work where coronin was only found in NP samples.

A central pathway of the fish innate immune response is the complement system. Activation of the complement cascade enhances the proinflammatory response caused by parasite infections, with C3a and C5a proteins having a direct killing effect on oncomiracidia by binding C3 to the parasite surface (reviewed in Boshra *et al.,* 2006). Complement component 1 Q subcomponent-binding protein (C1qbp) is considered an endothelial receptor of complement with an important role in inflammation and infection processes, especially involved in viral infections (Yang, 2013) .Innate immune parameters in fish suffer an upregulation during the acute phase of stress response or infection process (Tort, 2011) but this response tends to decrease after 3 days, being the complement one of the first parameters affected in chronical processes (Sunyer and Tort, 1995). In this line, it has been reported that chronical and parasitic wound process could entail down-regulation of skin complement in seabream (Cordero *et al.,* 2017). In consonance with this behavior of complement proteins in fish, in the present study, complement C3 component (Co3) was found in NP and P samples while C1qbp was only detected in NP fish (Table 5.1.).

Proteins related to proinflammatory interleukins were found by gel-free LC-MS/MS in the skin mucus of non-parasitized and parasitized greater amberjack. Adenylyl cyclase-associated protein (Cap1) is considered as a receptor of resistin, which is involved on interleukin 1, 6 and 12 pathways and macrophage and monocyte activation (Lee *et al.,* 2014). Cap1 was detected in NP and P greater amberjack skin mucus. In contrast, interleukin enhancer-binding factor 2 (Ilf2), which participates in IL-2 production by activated T-cells (Zhao

et al., 2005) was only found in NP samples (Table 5.1.). Another protein related to IL-2 pathway found in both experimental conditions was peptidyl-prolyl cis-trans isomerase (PPIase). This protein, whose upregulation in humans has been associated to dermatitis, inhibits calcineurin and consequently affects T-cells activity and IL-2 production (Liu *et al.,* 1991). Other group of proteins related to proinflammatory cytokines found in the present study is linked with the Tnfα pathway. Copine 1 (Cpne1) that in mammals has been related to the activation of the transcription factor NF-κB by Tnfα (Tomsig *et al.,* 2004; Ramsey *et al.*, 2008), was represented in NP and P groups (Table 5. 1.). Although inflammatory response associated with cytokines is typically elicited in fish under parasite infections (González *et al.,* 2007; Covello *et al.,* 2009) the referred proteins related to proinflammatory cytokines were more represented in the skin mucus of healthy fish than in the mucus of parasitized animals (Table 5. 1.). An anti-inflammatory response usually appears in the chronical phase of a parasitosis as a regulatory mechanism to safeguard cytokine homeostasis (Perez-Cordon *et al.,* 2014). Therefore, the observed under-representation of these proinflammatory proteins in parasitized fish could be the consequence of a delayed response, or the result of a down-regulation of the cytokine pathways in greater amberjack skin. Furthermore, some cytokines as IL-1β, and IL-6 could participate in the activation of other non-specific immune processes like antiprotease activity by stimulation of alpha-2-macroglobulin (A2mg) (Strauss *et al.,* 1992). We have found A2mg protein in NP and P skin mucus samples (Table 5. 1.). The presence of this protein in the skin mucus of healthy fish could be related to the inhibition of a wide range of proteases in their body fluids (Starkey and Barret, 1982) in order to maintain homeostasis and to regulate physiological pathways as coagulation and complement cascades (Dalmo *et al.,* 1997). In infected fish, this host response via antiproteases also would counteract the action of the proteases produced by pathogens in general and specifically by ectoparasites, as virulence factors for extra-cellular digestion of host tissues to facilitate anchoring (Dalmo *et al.,* 1997).

Proteins involved in the major histocompatibility complex (MHC) have been also detected, amongst them, MHC class Ia antigen (Hla) that was found in NP group (Table 5. 1.). In fish, MHC genes are important in the prevention of parasite attachment (Glover *et al.,* 2007; Simkova *et al.,* 2013) and for vaccine development against ectoparasites (Raynard *et al.,*2002). Glycosylation pathways play an important role in immunity considering that proteins in mucus, including components of MHC and mucins, have a high level of glycosyla-

tion. We have found UDP-glucose:glycoprotein glucosyltransferase 1 (Uggg1) and oligosaccharyl transferase 48 kDa subunit (Ost48) in both, NP and P groups, and protein-UDP acetylgalactosaminyltransferase (Galt) and malectin (Mlec) only in NP samples. Mucins are structural highly glycosylated proteins present in fish mucus, and their glycosylation is related to lectin recognition of pathogens ectoparasites (Buchmann, 2001). Different glycosylation profiles of mucus have been observed in Atlantic salmon and brown trout (*Salmo trutta*) under different stressful situations, as smoltification, fasting or sea lice infection (Bosi ***et al.***, 2005; Landeira-Dabarca *et al.,* 2014). On the other hand, it has been shown that the specific pattern of glycosylation of some proteins, as Wap65, in skin mucus of tiger puffer fish (*Takifugu rubripes*) induce *N. girellae* oncomiracidia attachment, playing and important role in the mechanism of host specificity in this parasite (Ohasi *et al.*, 2007).

5.3.6. Proteases

Proteases in fish skin mucus are implicated in the resistance to infection, directly by cleaving proteins of pathogens, or modifying properties of mucus to avoid parasite attachment and facilitating its removal. As discussed above, 2-DE analysis showed a specific degradation of some structural proteins (such as keratins) in parasitized fish skin mucus (Fig. 5. 4). In accordance, several proteases have been identified in the skin mucus of greater amberjack including cathepsin D (Table 5. 1), aminopeptidase W07G4.4 and CNDP dipeptidase 2 only in P samples, cathepsin E, calpains 1 and 8, and Xaa-Pro aminopeptidase 1, carnosine dipeptidase 2 in NP samples, and cathepsins Ba, calpains 2, 9, and 12, and ATP-dependent Clp protease proteolytic subunit, in both, NP and P samples. Azocasein assay reported here (Fig. 5. 5) showed that protease activity was more than 2-fold higher in P samples than in healthy fish samples ($p < 0.01$). This result agrees with that reported for Atlantic salmon skin mucus parasitized with sea lice (Ross *et al.*, 2000). The addition of EDTA (metalloprotease inhibitor) to the protease assay triggered a decrease of about a 60% of the protease activity in parasitized samples, while only of a 30 % in non-parasitized samples. Similar results were obtained when using PMSF, a serine protease inhibitor (Fig. 5. 5). These data demonstrate that metalloproteases and serine proteases are responsible, at least in part, for the increase in protease activity in parasitized fish. It has been previously described that parasites use the strategy of producing its own serine proteases for facilitating its fixation and feeding and for interfering on the immune response of the host (Firth *et al.*, 2000). Nonetheless, it is also known that host's serine proteases modify structural proteins in skin mucus, as mucins, in order to change mucus consistency or viscosity to facilitate the removal of pathogens (Aranishi *et al.*, 1998). Besides, host serine proteases production is mainly related with a defensive process as part of the innate immunity against pathogens (Hjelmeland *et al.*, 1983). Serine proteases are also related to the enhancement and activation of several innate immune components present in fish mucus such as complement, immunoglobulins, or AMP (Cho *et al.*, 2002 a, b). Moreover, serine proteases also activate metalloproteases (Knauper *et al.*, 1990). In mammals, metalloproteases are known for its role in the wound healing process for re-epithelialization and in leukocyte infiltrations (Caley *et al.*, 2015). In this line, the higher metalloprotease activity observed in greater amberjack parasitized with *N.girellae* would be in accordance with the feeding behavior of these ectoparasites and the attachment damage produced in the epidermis of the infected fish (Hirazawa *et al.*, 2016).

5.3.7. A proteomic approach to the microbiota composition of greater amberjack skin mucus

Proteomic methodology has already been used in gilthead seabream as a tool to obtain preliminary information about the skin mucus microbiota (Jurado *et al.,* 2015). Samples of mucus of healthy and parasitized greater amberjack, processed specifically as described in the material and methods section, were analyzed by gel-free LC-MS/MS against bacteria database. Selected peptides from the proteins retrieved were individually used to perform a BLAST search against full database, without any organism restriction. One hundred and eight of these peptides exclusively matched bacterial sequences (Fig. 5. 3). Some peptides matched only one genus, this was the case of the genera *Pseudomonas, Paracoccus, Acinetobacter, Serratia, Clostridium, Bartonella, Escherichia, Streptomyces* and *Thermotoga* (Fig. 5. 6), indicating that species in these genera are most likely living in greater amberjack skin mucus. The most abundant genera found were *Pseudomonas* (12 and 14 peptides matched no more than this genus in NP and P samples, respectively), followed by the genus *Paracoccus* (5 and 7 peptides for NP and P samples, respectively). Parasitosis processes in fish have been associated with skin mucus dysbiosis status (Llewellyn *et al.*, 2014), however, no remarkable differences were observed in bacterial genera distribution between parasitized and non-parasitized fish. Quantitative differences in the presence of some species and genera of bacteria between parasitized and non-parasitized greater amberjack skin mucus need further investigations.

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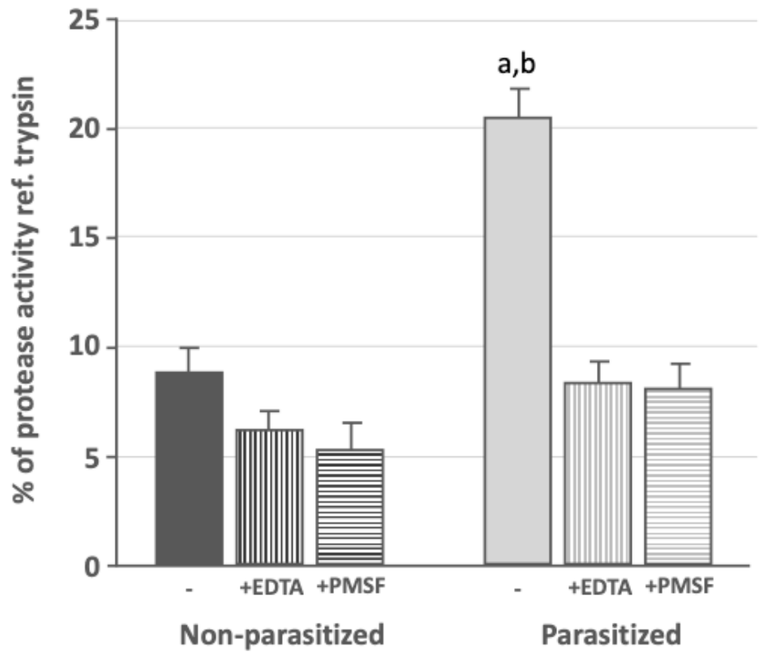

Figure 5.5. *Protease activity of skin mucus of non-parasitized (NP) and parasitized (P) greater amberjack (Seriola dumerili). Solid filled bars: protease activity without inhibitors. Vertical striped bars (+EDTA): protease activity in presence of 10mM EDTA (metalloprotease inhibitor). Horizontal striped bars (+PMSF): protease activity in presence of 5mM PMSF (serine protease inhibitor). Data are mean ± SD of protease activity expressed as percentage of positive control (trypsin 5mg/ml) from at least three determinations in each group. Comparisons were made by the Students's-test. Statistical significance: p< 0.01. (a) Comparison of fish parasitized vs non-parasitized. (b) Comparison of activity levels in presence of EDTA or PMSF vs absence of inhibitors.*

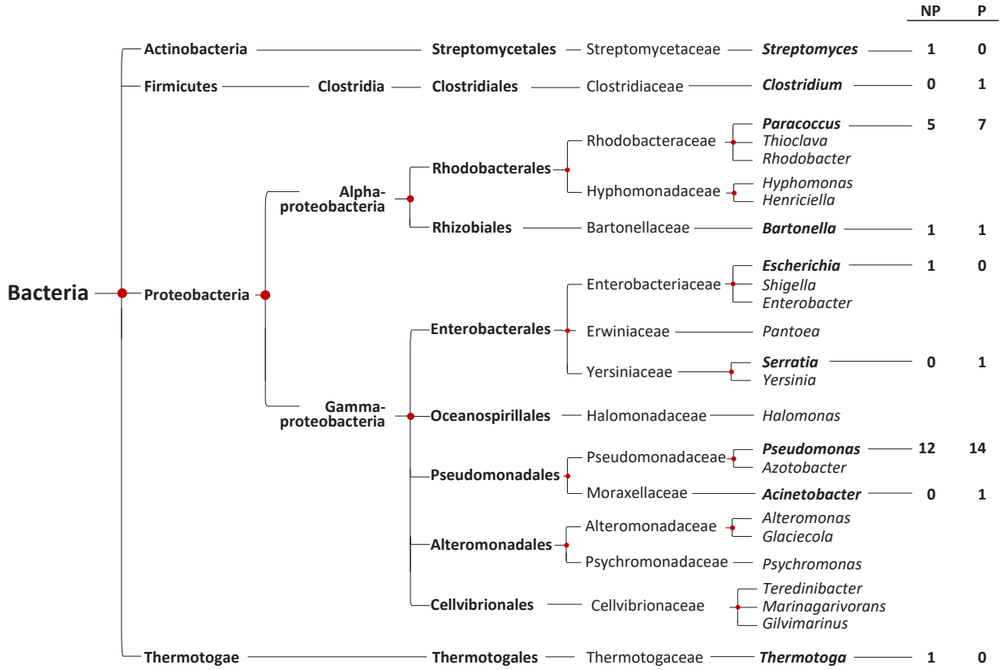

Figure 5.6. *Clade organization of the bacterial genera identified using gel-free LC-MS/MS in skin mucus of non-parasitized (NP) and parasitized (P) greater amberjack (Seriola dumerilii). Genus name are highlighted in bold when at least one peptide matched exclusively this genus. Numbers of these peptides are indicated for NP and P samples.*

5.4. Conclusions

Ectoparasite infections are one of the most important challenge for aquaculture sustainability, and particularly for the culture of greater amberjack, since information about skin structure, skin-associated lymphoid tissue (SALT) and proteins associated to skin mucus is still scarce. In this study, the skin mucus proteome of greater amberjack was characterized for the first time. Cytoskeleton proteins were statistically overrepresented in the skin mucus *N. girellae* parasitized fish , as inferred from gel-free LC-MS/MS data. Nevertheless, the

most remarkable difference between parasitized and non-parasitized fish was the specific cleavage of keratins in the mucus of infected fish, as revealed by 2-DE approach. This fact implies the presence of a specific set of proteases in mucus of parasitized fish. The higher protease activity in the mucus of parasitized fish was due, at least in part, to an increment of the metalloprotease and serine protease percentages, as confirmed by enzymatic assays. The differences observed could be the result of a response from the host to the infection, or a strategy of the ectoparasite to feed or to evade the host immune system. The characterization of this specific protease activity should be investigated in order to better understand parasitosis by *N. girellae.* Besides structural proteins and proteases, the map of the proteome of *S. dumerili* presented here include stress response proteins, metabolic proteins, and immune related proteins as relevant biological functions. Moreover, proteomic data have provided information to design a map of microbial communities associated to skin mucus of greater amberjack. However, no differences were observed in genera distribution when healthy and parasitized fish were compared. This study is the first proteomic approach to define the greater amberjack skin mucus associated microbiota and shows new insights for understanding the interaction between *N. girellae* and greater amberjack.

CHAPTER 06

AN INSIGHT INTO PISCIDINS: THE DISCOVERY, MODULATION AND BIOACTIVITY OF GREATER AMBERJACK, *SERIOLA DUMERILI*, PISCIDIN

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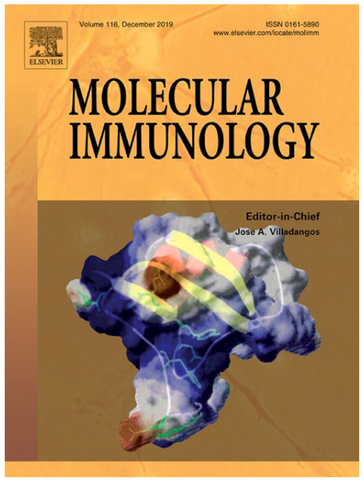
D.J.M. Conducted the piscidin clonation, the PAMPs stimulation trial, the recombinant piscidin challenge with bacteria, helped in the nutritional challenge with the PCRs and wrote the manuscript. A.F.M Helped with the PAMPs stimulation trial, conducted the nutritional challenge and made the PCRs associated to that challenge. M.K.G. Helped with the blasts of the piscidin sequence from the newly discovered greater amberjack genome. T.W. Supervised and participated with the molecular biology work and the experimental design. F. A. participated in the experimental design and with the PAMPs stimulation trial. S.T. Participated in the experimental design and the nutritional trial. D.M. Participated in the experimental design, the nutritional trial and the review of the manuscript. J.Z. Supervised and participated with the molecular biology work and the experimental design. J.S. Participated in the experimental design. C.J.S. Coordinated the whole study and experimental design and participated in the manuscript elaboration and review.

Abstract



Antimicrobial peptides (AMPs) play an important role in the innate immune response of vertebrates by creating a hostile environment for any invading pathogens. Piscidins are potent teleost specific AMPs, which have a broad spectrum activity. We have identified a novel piscidin active peptide, in the greater amberjack, *Seriola dumerili,* that consists of 25 aa, which forms an amphipathic helix with distinct hydrophobic and positively charged regions. Following homology and phylogenetic analysis the greater amberjack piscidin was deemed to belong to the group 3 family of piscidins. Piscidin was expressed constitutively at immune sites, with transcript level highest in the spleen and gut, at an intermediate level in the gills and lowest in the head kidney. Following in vivo stimulation with PAMPs (poly I:C, LPS and flagellin) piscidin transcript level increased in gills in response to flagellin, in gut and spleen in response to poly I:C, and in head kidney in response to poly I:C, LPS and flagellin. Head kidney and spleen cells were then isolated from greater amberjack and incubated with each of the PAMPs for 4, 12 and 24 h. Piscidin expression was unchanged at 4 and 12 h post PAMP stimulation in head kidney cells but at 24 h transcript level was markedly upregulated compared to control (unstimulated) cells, especially with the bacterial PAMPs. In contrast, spleen cells upregulated piscidin expression by 4 h post stimulation with poly I:C and flagellin, and remained upregulated to 24 h with flagellin exposure, but had returned to baseline levels by 12 h using poly I:C. To determine if piscidin expression could be modulated by diet, greater amberjack were fed diets supplemented with MOS and cMOS for 30 days when transcript level was determined. It was found that MOS supplemented diets increased expression in the spleen, cMOS supplemented diets upregulated transcript levels in the gills and head kidney, whilst a diet containing both MOS and cMOS upregulated transcript in the gut, when compared to fish fed the control diet. Finally, a synthetic greater amberjack piscidin was produced and showed bacteriostatic activity against a number of bacterial strains, including both Gram positive and Gram negative fish pathogens.

6.1. Introduction

Amberjack species (genus Seriola) are emerging aquaculture candidates world-wide. The yellowtail amberjack, *Seriola lalandi* is currently actively cultured throughout Australia and New Zealand (Stuart and Drawbridge, 2011), the Almaco jack, *Seriola rivoliana,* in Hawaii (Verner-Jeffreys *et al.*, 2006) and the Japanese amberjack, *Seriola quinqueradiata*, is cultured throughout Asia on a large scale, with 160,000 tonnes produced in 2015 (FAO, 2016; Nakada, 2000). These species are highly coveted due to their excellent flesh quality, which is used in high value products such as sushi and sashimi, and are ideal for aquaculture due to their rapid growth, amenability to culture, high consumer acceptability and worldwide market availability and demand (Nakada, 2000, 2002; Symonds *et al.*, 2014). The European union has recently started to invest in the aquaculture of *Seriola sp.*, but have instead opted for the greater amberjack, *Seriola dumerili,* which is the largest of the amberjack species and is endemic to the Mediterranean sea and Atlantic Ocean making it suited to aquaculture along European coasts (Cummings *et al.*, 1999). Despite the world wide culture of a variety of amberjack little is known about the immune response of these fish, which needs to be addressed if the sustainable culture of amber- jack is to be achieved (Fernández-Montero *et al.* 2019). As such, this study aims to better understand the importance of the antimicrobial peptide (AMP) piscidin in the greater amberjack immune response, as a key component of the innate defences.

AMPs are small, highly charged molecules, of low molecular weight, capable of inhibiting the growth and/or reducing the viability of microorganisms. The most commonly studied AMPs are the defensins, hepcidins and cathelicidins due to their abundance and importance in the mammalian innate immune system (Maroti *et al.*, 2011). Many of these AMPs are cysteine rich and require specific conformational folding, stabilised by disulphide bonds that form between cysteine residues, if they are to become/remain biologically active. For example β-defensins require disulphide bond formation between cysteines 1–5, 2–4 and 3–6 otherwise the functional activity of the AMP is greatly reduced (Dhople *et al.*, 2006). Once folded antimicrobial peptides have their propeptide region cleaved off and are stored in the secretory vesicles of immune cells, such as neutrophils, and non-immune cells, such as Paneth and epithelial cells. However there are a few exceptions that are stored with the propeptide region intact, as with human defensin-5 (Ghosh *et al.*, 2002). These AMPs are secreted consti-

tutively at low levels to protect against environmental microbes but are markedly and rapidly upregulated when infection occurs and in response to inflammatory cytokines such as IL-1β, IL-6 and TNF-α (Nemeth *et al.,* 2004; Voss *et al.*, 2005). Once secreted these molecules can act in a number of ways ranging from direct interaction with microbes (such as pore forming lytic effects), resource partitioning, by creating environments devoid of molecules essential for microbial survival (such as the binding of free iron/ heme by hepcidins) or by stimulating and activating cells of the immune system which in turn reduce microbe viability (Niyonsaba et al., 2010; Hazlett and Wu, 2011; Drakesmith and Prentice, 2012).

While AMPs such as the defensins and hepcidins are present and fairly conserved throughout vertebrates, piscidins are unique in that they are only found in teleost fish. As such, piscidin expression and bioactivity is not as well documented as other AMPs, although a number of studies into the expression and bioactivity of piscidin activity in a variety of fish species have been undertaken. From these studies it can be seen that piscidins are structured very differently when compared to the more commonly studied AMPs, and have a relatively simple tertiary structure. These AMPs lack cysteine residues and therefore do not form disulphide bonds, instead the mature peptide forms an amphipathic α-helix, which generally consist of ˜26 aa (Fernandes *et al.,* 2010; Niu *et al.*, 2013) and appear homologous to AMPs such as cecropins found in the Cecropia moth, Hyalophora cecropia (Masso-Silva and Diamond, 2014). However, the size of these AMPs does vary as larger active peptides have been predicted for piscidin 4 and 5 in hybrid striped bass (*Morone chrysops × Morone saxatilis*), with the amphipathic α-helical structure predicted to remain even in these extended molecules (Salger *et al.*, 2011). Piscidins can be separated into three distinct groups with each possessing a broad spectrum antimicrobial activity, demonstrating anti- viral, anti-bac- terial, anti-fungal and anti-parasitic activity (Mulero *et al.*, 2008; Sung *et al.*, 2008; Colorni *et al.*, 2008; Falco *et al.,* 2009; Muncaster *et al.*, 2018). Hence, an understanding of the anti-microbial activity of piscidin and how expression can be modulated would aid in the defence against pathogens, helping to facilitate the sustainable culture of the greater amberjack. Therefore, this study aims to provide an insight into the expression and bioactivity of piscidin in greater amberjack.

6.2. Material and methods

6.2.1. Fish Husbandry

Healthy greater amberjack (*Seriola dumerili)* were supplied by the marine biosecurity station (MBS) of the Scientific and Technologic Park of the University of Las Palmas de Gran Canaria, Las Palmas, Canary Islands, Spain. Ninety fish (105 g mean body weight) were acclimatized to nine cylindrical-conical 500 l tanks (10 fish per tank) at a temperature of 23 °C ± 0.7 °C, an oxygen saturation of 6 ± 1 ppm, a salinity of 37 ppt and photoperiod of 14 h light: 10 h dark. Fish were fed a commercial Seriola base diet (Skretting, Stavanger, Norway) to apparent satiety three times a day.

6.2.2. Molecular cloning of piscidin

Total RNA was extracted from a pool of greater amberjack gill, mid- gut, head kidney and spleen homogenate in TRI reagent (Sigma Aldrich), following the manufacturer's protocol. The total RNA was then reverse transcribed using SuperScript III (ThermoFisher) and Oligo dt (T26VN). Partial sequences were obtained by PCR using MyTaq DNA polymerase (Bioline) and consensus primers (Table 6. 1) designed to conserved regions of piscidin in closely related species. The amplicon produced by the PCR reaction was ligated into pGEM-T easy vector (Promega) and transformed into RapidTrans TAM1 competent cells (Active motif), which were plated onto MacConkey agar plates (Sigma Aldrich) and incubated overnight at 37 °C. Plasmid DNA was extracted from positive colonies using a QIAprep Spin Miniprep Kit (Qiagen) and sent to Eurofins Genomics for sequencing. Next, 5′ and 3′ RACE was performed using primers designed from the partial sequence (Table 6. 1), as described by Milne *et al.* (2017), with the resulting amplicons undergoing the same cloning and sequencing protocol. Finally, full coding sequence was confirmed by sequencing of the amplicon generated from a PCR reaction using Pfu DNA polymerase (Promega) and specific consensus primers to the 5′ and 3′ ends (Table 6. 1) of greater amberjack piscidin.

Table 6.1. *Primers used for gene Discovery and qPCR. This table gives the primer names, sequence (5´to 3´) and primer function*

Ann. temp. (°C)*	Forward Sequence	Function
GA piscidin F	ATCGCCCTGTTTCTTGTGTT	Partial cloning
GA piscidin R	GTCAAACTGCCCCTGCTCTA	Partial cloning
GA piscidin 5N1	CTCCTCCTGTTGATCGCGCTGTGGATC	5´RACE nested PCR
GA piscidin 5N2	AATGAAACCTTCCCCAGGTTCAGCC	5´RACE nested PCR
GA piscidin 3N1	CCACAGCGCGATCAACAGGAGGAG	3´RACE nested PCR
GA piscidin 3N2	GGAATGACAGAGCTAGAGCAGG	3´RACE nested PCR
GA piscidin full F	GGGCGGGTCTGGGCGATACACATC	Full sequence cloning
GA piscidin full R	TTTGTCATTTTGAAAGACAAGAAGCATTTCTCTTC	Full sequence cloning
GA piscidin qPCR F	ATCGTCCTGTTTCTTGTGTTGTCAC	Gene expression
GA piscidin qPCR R	CGCTGTGGATCATTTTTCCAATGTGAAA	Gene expression
GA EF1α qPCR F	TGCCATACTGCTCACATCGCCTG	Gene expression
GA EF1α qPCR R	ATTACAGCGAAACGACCAAGAGGAG	Gene expression

6.2.3. Sequence analyses of piscidin

Amino acid sequences were determined by the translation of cDNA sequence using the ExPASy translate tool (http://web.expasy.org/ translate) and then subjected to BLAST analysis (http://www.ncbi. nlm.nih.gov). Protein similarity and identity were calculated using MatGAT 2.0 software (Campanella *et al*., 2003). An amino acid alignment of all the selected piscidin sequences was generated using MAFFT v.7 (Katoh and Standley, 2013). Bayesian phylogenetic analysis was performed on the finished alignment using Beast v.1.8 (Drummond *et al*., 2012), incorporating an uncorrelated lognormal relaxed molecular clock model (Drummond *et al*., 2006), a yule speciation tree prior (Gernhard, 2008) and a best fitting substitution model inferred by maximum likelihood using MEGA v.6 (Tamura *et al*., 2013). The Beast MCMC chain was run twice for 10 million generations sampling every 1000th generation. Adequate mixing and convergence of the MCMC chain was confirmed on TRACER v.1.6 (Rambaut*et al*., 2014) after removing 10% of the samples; effective sample sizes were > 200 for all the parameters. TreeAnnotator v.1.8 (Drummond *et al*., 2012) was used to generate a maximum clade credibility tree from one run. The properties of the protein were then determined using various software programs; Compute pI/Mw tool (http://www.expasy.ch/) for the isoelectric point and molecular mass, TMHMM tool (http://www.cbs.dtu. dk/services/TMHMM/) for determining transmembrane domains and ExPASy Prosite (http://prosite.expasy.org) for identifying conserved domains and signatures.

6.2.4. PAMP stimulation and sample collection

Four groups of 10 healthy greater amberjack received either a 100 µl intraperitoneal (ip) injection of phosphate buffered saline (PBS, Sigma Aldrich) or 100µl ip injection of PBS containing 100µg of poly I:C (Sigma Aldrich), 400 µg of LPS (Sigma Aldrich) or 100 ng recombinant *Yersinia ruckeri* flagellin, the latter produced as described by Wangkahart *et al.* (2016). 24 h later the gills, gut, head kidney and spleen were harvested for piscidin expression analysis. For in vitro PAMP stimulation, the head kidney and spleen were taken from 8 healthy greater amberjack, pressed through a 70 µm nylon mesh (Greiner) with 10ml L15 medium (ThermoFisher) containing penicillin (1000 units/ml), streptomycin (1000 µg/ml) (P/S, ThermoFisher) and 2% fetal calf serum (FCS, Sigma Aldrich). The cell suspensions were then centrifuged for 10 min at 400 g, the supernatant discarded, the pellet suspended in 10 ml of L15 media plus 2% FCS and P/S, and centrifuged for 10 min at 400 g. The supernatant was again discarded and the pellet re-suspended in 30ml of fresh media. 5ml aliquots of the cell suspension where then transferred to the wells of a 12 well plate (Greiner). 250 µl of PBS (control) or PBS containing poly I:C, LPS or flagellin was added to the appropriate wells giving final concentrations of 100 µg/ml, 50 µg/ml and 100 ng/ml respectively. After 4, 12 and 24 h cells were collected, centrifuged for 10 min at 400 g, the supernatant removed and the pellet re-suspended in 1.5 ml of RNA later (Sigma Aldrich). All samples were stored at –20 °C until use.

6.2.5. Diet trial

Greater amberjack were separated into 4 groups with each group consisting of fish housed as described previously (Fernandez-Montero *et al.,* 2019). Each group was fed a different diet over a period of 30 days prior to tissue sampling for piscidin expression analysis. Diet A was the control diet and consisted of a commercial Seriola base diet (Skretting, Stavanger, Norway). Diet B contained 5 g· kg^{-1} of MOS (Bio-Mos®), diet C contained 2 g· kg^{-1} of cMOS (Actigen®), and diet D was a combination of diets B and C as it contained both 5 g· kg^{-1} of MOS and 2 g· kg^{-1} of cMOS. Each diet was randomly assigned to triplicate groups of fish (n= 3 x 3).

6.2.6. Real time quantitative PCR

Total RNA was extracted from each sample using TRI reagent, following the manufacturer's standard protocol. The samples were then treated with TURBO DNase (ThermoFisher) to remove genomic DNA contamination prior to reverse transcription with SuperScript III and Oligo dT (T26VN). The transcript level of piscidin was then quantified using a Light Cycler 480 (Roche) and normalised to EF1α, as described by Wang *et al.* (2011).

6.2.7. Synthesis and bioactivity of greater amberjack piscidin active peptide

The active peptide amino acid sequence was synthesized by BIOMATIK (US) (http://www.biomatik.com/services/peptide-synthesis. html) using a solid phase multiple peptide system. The resulting product was purified to > 85% purity by high performance liquid chromatography. Once received the peptide was reconstituted in a solution of 3 parts acetic acid (Sigma Aldrich) to 7 parts molecular grade water (Sigma Aldrich) and stored at −20 °C until use.

Bacterial strains MT004 (*Aeromonas salmonicida* - avirulent), MT423 (*Aeromonas salmonicida* - virulent), MT252 (*Yersinia ruckeri*), NCIMB 12260 (*Escherichia coli*), MT2055 (*Lactococcus garvieae* - noncapsulated) and MT2291 (*Lactococcus garvieae* - capsulated) were grown in tryptic soy broth (TSB, Sigma Aldrich) at 22 °C. In addition, bacterial strains MT1741 *(Vibrio anguillarum* –serogroup 01), MT1742 (*Vibrio anguillarum* –serogroup 02) and MT1415 (*Photobacterium damselae* - capsulated) were grown in TSB containing 1% NaCl (Sigma Aldrich) at 22 °C.

Bacteria were taken during the exponential growth phase and diluted to 1 × 107 cells/ml. 270 µl of bacteria in tryptic soy broth (Sigma Aldrich) were added to wells of a clear flat bottomed 96 well plate (Griener), with 8 replicates for each treatment group. 30 µl of media was added to the control group, 30 µl of media containing 1 µl acetic acid/molecular grade water at a ratio of 3:7 to the buffer only group and 30 µl containing 1.1, 2.2, 4.4, 8.8, 16 or 35 µM piscidin peptide was added to the 270 µl bacterial cell suspension. As an additional control 300µl of media was added to further wells to ensure media optical density did not change over time. The OD650 of the bacterial suspensions, as a measure of bacterial number/growth, was determined using a Spectra Max Plus Spectrophotometer (Molecular devices) before

the addition of the synthetic piscidin, immediately after addition and then every 30 min for 3 h. The cells were then left for an additional 21 h and measured again.

6.2.8. Data transformation and statistical analyses

QPCR data were initially calculated as arbitrary units. These values were then used to show the relative constitutive expression between tissues. Data from in vivo and in vitro PAMP stimulation were transformed to a fold change relative to the respective PBS control samples, while data from the diet trial were transformed to a fold change relative to fish on the control diet. Differences in constitutive expression between tissues were determined by One-way ANOVA followed by the Tukey post hoc test. This was also the case for the in vivo and in vitro experiments with PAMPs, as well as groups differing in diet. All statistical analysis was carried out using Statistical Product and Service Solutions (SPSS) software, and were deemed significant when $p \leq 0.05$.

6.3. Results

6.3.1. Molecular cloning of greater amberjack piscidin

In this study, we identified a novel piscidin sequence in the greater amberjack. The full length cDNA of this pro-peptide consists of 402bp (GenBank: MG831182), containing a 96 bp 5′ untranslated region (UTR), a 198 bp open reading frame (ORF) with a putative propeptide of 65 aa and a 108 bp 3′ UTR including a 17 bp poly (A) tail (Fig. 6. 1A). The ORF encodes an N-terminal signal peptide consisting of Met^{1} to Gly^{22} and a C-terminal pro-domain consisting of Gly^{48} to Val^{65} which is immediately preceded by a furin-like cleavage site (RRRH). Removal of the signal peptide and pro-peptide domain results in the formation of a 25 aa active peptide from Phe^{23} to His^{47}. The active peptide has a molecular weight of 3.015 kDa, an isoelectric point of 12.31 and is rich with cationic residues resulting in a net charge of +5. The active peptide was then modelled in I-TASSER (Zhang, 2009) and was shown to form a tightly packed, amphoteric alpha helix, which consists of Ile^{24} to Arg^{44},while the remaining Arg^{45}, Arg^{46} and His^{47} form a highly flexible coiled tail (Fig. 6. 1B). Furthermore, the Schiffer–Edmundson helical wheel projection (Fig. 6. 1C) showed a distinct, isoleucine rich, clustering of hydro-

phobic residues on one side of the helix consisting of Phe[1], Ile[2], Ile[5], Ile [6], Ile[9], Ile[12], Met[15], Ile[16], and Ile [20], with the region opposite primarily consisting of potentially charged and hydro- phic residues. This highly organised division of hydrophobic and hy- drophilic/charged regions suggests that greater amberjack piscidin is highly amphipathic in nature and hints at a possible interaction site between peptide and pathogen (Niu *et al.*, 2013).

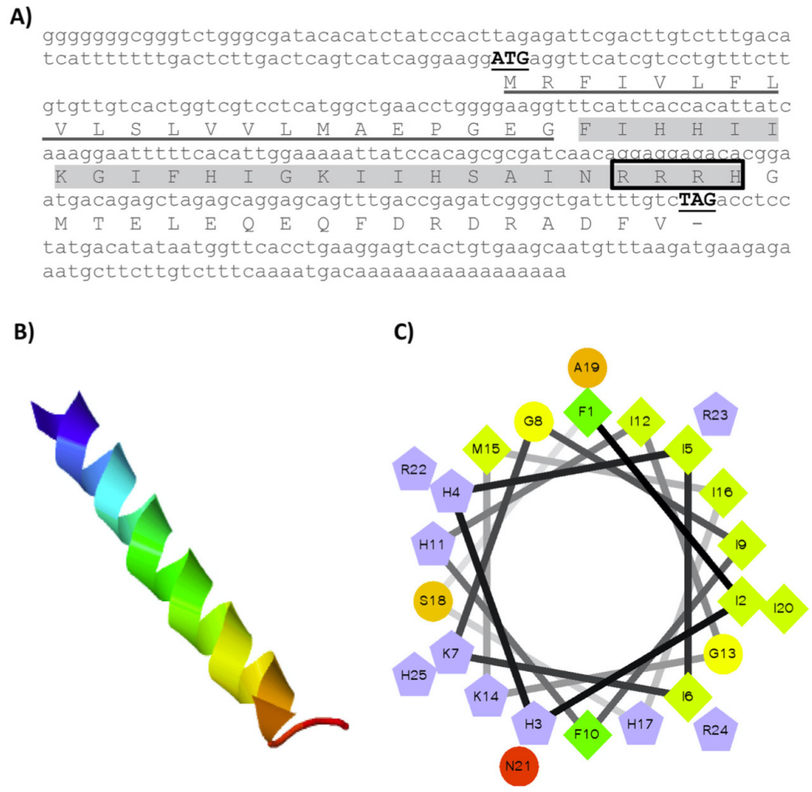


Figure 6.1. *A) Nucleotide and deduced amino acid (aa) sequence of greater amberjack piscidin 3. The putative aa sequence is shown under its respective triplet codon. Start and stop codons are in bold, the signal peptide has been underlined, the active peptide has been highlighted in grey, the furin-like cut site has been boxed. B) A predictive model of the greater amberjack active peptide produced by I- TASSER https://zhanglab.ccmb.med.umich. edu/I-TASSER/. C) A Schiffer-Edmundson diagram of greater amberjack piscidin 3 produced using RZLAB software http://rzlab.ucr. edu/scripts/wheel/wheel.cgi. Circles = hydrophilic; Diamonds = hydrophobic; Pentagons = positively charged. The degree of each property is colour coded, with dark colours indicating an intense property and light colours indicating a weak property. Green to Yellow = Hydrophobic; Red = Hydrophilic and Blue=potentially charged residues (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).*

6.3.2. Homology of greater amberjack piscidin

The greater amberjack putative protein sequence shared the most similarity and identity with group 3 piscidins found in perciform fish (Table 6. 2). Greater amberjack piscidin showed both a high similarity (58.1–88.4 %) and high identity (48.9–80.2 %) with group 3 piscidins, while showing considerably lower similarity and identity with group 2 (44.3–51.2 % and 29.1–36.2 %) and group 3 (48.8–57 % and 33.7–41.4%) piscidins. Alignment of fish piscidin sequences (Fig. 6. 2) highlighted the conserved nature of the signal peptide for all piscidins, and the notable differences in the predicted active peptide regions and propetide regions between the three groups. The cleavage sites were predicted using ProP software http://www.cbs.dtu.dk/services/ProP/ (Duckert *et al.*, 2004), which identified a conserved 'KR' duplet pro- protein convertase cleavage site present in Group 1 piscidins, which in turn indicates that this group has the largest active peptides (35–39 aa). Group 2 and 3 piscidins have smaller and similarly sized active peptides of 24-27aa if the prediction of the Group 2 protein cleavage site, consisting of a charged H R/K duplet, and the Group 3 cleavage site, consisting of a conserved RRRH motif, is correct. Interestingly, this indicates that Group 2 and 3 piscidin active peptides contain few to no hydrophobic residues and primarily consist of polar and charged residues while Group 1 piscidins consist of the same polar/charged region with an additional C-terminal region that is densely packed with hydrophobic residues. The presence/absence of this densely hydrophobic region will likely have a significant effect on the bioactivity and membrane interaction capability of these molecules. Given the similarities in sequence homology, predicted proprotein convertase cleavage site and size it appears that greater amberjack piscidin belongs to the Group 3 piscidins found in fish. This is further confirmed by phylogenetic analysis, which places greater amberjack piscidin with Group 3 piscidins, as seen in Fig. 6. 3. Furthermore, greater amberjack piscidin strongly groups with piscidins found in other *Seriola sp.* identified as group 3 piscidins.

Table 6.2. *Amino acid (aa) similarity (bottom, left) and identity (top, right) of greater amberjack piscidin with other known aa sequences of perciform fish piscidins*

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Group3	Greater amberjack	MG831182.1		48.9	50.5	80.2	79.1	29.1	36.2	34.1	32.6	31.4	38.4	33.7	41.4	36	39.5
	Sablefish	ACQ58110.1	58.1		66.7	52.2	52.9	38	33.3	40.3	35.1	35.1	51.4	42.9	43.1	43.8	61.8
	Duskytail	ADY86110.1	61.6	76.8		50.5	56.8	32.9	31.5	36.4	34.7	38.7	50	40	45.3	42.1	51.4
	Yellowtail amberjack	ARK85994.1	86	61.6	64		94.2	30.2	38.3	36	36	34.9	38.4	36.8	43	36	41.9
	Japanese amberjack	DC609456.1	88.4	58.1	64	97.7		31.4	39.4	35.6	36	34.9	41.9	40.2	47.7	39.5	44.2
Group2	Mummichog	JAR66906.1	44.2	54.9	59.2	45.3	48.8		39.1	42.1	45.8	43.1	32.5	29.7	29.7	32.5	33.8
	Stickleback	EG588124.1	51.1	46.7	50	51.1	51.1	58.7		45.8	41.3	41.3	32.6	28.3	32.6	30.4	33
	Grouper	AKA60777.2	45.3	53.9	51.3	43	47.7	67.1	65.2		61	57.1	27.6	28.9	32.9	32.5	36.4
	Seabass	APQ32049.1	50	57.1	61.4	51.2	51.2	69	63	73.7		91.4	32	32.9	26.8	30.6	41.3
	White bass	APQ32050.1	51.2	57.1	62.9	50	52.3	67.6	62	72.4	95.7		31.6	32.9	28.8	32.9	41.3
Group1	Seahorse	XP_019738230.1	54.7	66.7	71	53.5	54.7	56.3	46.7	46.1	50	55.7		54.8	53.5	53.4	52.9
	Drum	AFV40526.1	48.8	64.7	63.8	50	52.3	47.9	43.5	43.4	48.6	45.7	72.5		77.3	73.7	46.5
	Meagre	ASW20416.1	57	62	64.8	54.7	55.8	52.1	45.7	48.7	38	45.1	71.8	87.3		87.5	42.3
	Croaker	AGN52988.1	54.7	65.3	63.9	51.2	52.3	55.6	43.5	48.7	45.8	50	69.4	81.9	90.3		44.4
	Knifejaw	BAM99885.1	55.8	79.1	69.6	53.5	53.5	53.5	51.1	51.3	62.9	61.4	66.7	64.7	59.2	61.1	


```
Seahorse - XP_019738230.1        Group1   MKWTAAFLVLVIVVLMAQPGECFLGLI---FHGLVHA------GKLIHGLIHR---NRG-
Drum - AFV40526.1                         MKCTAVFLVLFMVVLMAEPGECIWGLI---AHGVAHV------GSLIHGLVNG---NHGG
Meagre - ASW20416.1                       MKFTAVFLVLFMVVLMAEPGECIWGLI---AHGVAHV------GSMIHGLIH----NHG-
Croaker - AGN52988.1                      MKCTAVFLVLFMVVLMAEPGECIWGLI---AHGVGHV------GRLIHGLIR----GHG-
Knifejaw - BAM99885.1                     MKCITLFLVLSMVVLMAEPGEAFFHHI---FNGLVGV------GKTIHRLITG---GRN-
Mummichog - JAR66906.1           Group2   MKLVAIFLVASLVVLMAEPGDCFLRKL---WKGIKAVYKGAKQGYNAFKSQQQN--IQ--
Stickleback - EG588124.1                  MKYVTIFLVLSLVVLMADPGDCFFKNI---WRGAKAIFKGARRGWKEHRAIARN--HRG-
Grouper - AKA60777.2                      MKFVMVFLVLSLVVLMAEPGEGFFRHIKSFWKGAKAIFRGARQGWREHRALSKQ--RKM-
Seabass - APQ32049.1                      MKCVMIFLVLTLVVLMAEPGEGFFRHL---FRGAKAIFRGARQGWRAHKVVSRY--RNR-
White bass - APQ32044.1                   MKCVMIFLVLTLVVLMAEPGEGFFRHL---FRGAKVLFQGARQGWRAHKAVSRY--RNR-
Greater amberjack - MG831182     Group3   MRFIVLFLVLSLVVLMAEPGEGFIHHI---IKGIFHI------GKMIHSAINRRRH--G-
Sablefish - ACQ58110.1                    MKCITLFLVLSLVVLMAEPGECFIHHI---FNGLVKV------GKSIHGLI-RRRH--G-
Duskytail - ADY86110.1                    MRCIALFFVLSLVVLMAEPGEGFFFHI---IKGLFHA------GKMIHGLIHRRRHRHG-
Yellowtail amberjack - ARK85994.1         MKFIGLFLVLSLVVLMAEPGEGFFHHI---LSGIFHV------GKMIHGAIQRRRH--G-
Japanese amberjack - DC609456.1           MKFIALFLVLSLVVLMAEPGEGFFHHI---LSGIFHV------GKMIHGAIHRRRH--G-
                                          *:    *:*  :*****:**: :   :    .*         *   .

Seahorse - XP_019738230.1        Group1   --FEE-QEELNKRSVEFVADQQAMN-------------------
Drum - AFV40526.1                         NQAEEQQEQLNKRSLSYDHP-----------------------
Meagre - ASW20416.1                       --AEEQQEQLDKRSLSYDPPKKLQWRD-----------------
Croaker - AGN52988.1                      --AEEQHVQLDKRSLSYDPPKKLQWRE----------------D
Knifejaw - BAM99885.1                     ---QQDQKELDKRFLNQQQAAFN---------------------
Mummichog - JAR66906.1           Group2   ------QDMQDQQAPDNPPPPYKR--------------------
Stickleback - EG588124.1                  ------QEQQGQQADNDEGQPYWQDISPRRHRALAFYFARQEAN
Grouper - AKA60777.2                      ------DQGGGGNEVDNGTPPYWQK-------------------
Seabass - APQ32049.1                      ---------DVPETDNNQEEPYNQR-------------------
White bass - APQ32044.1                   ---------YVPETDNNQEQPYNQR-------------------
Greater amberjack - MG831182     Group3   ---MT---ELEQEQFDRDRADFVTSYDIWFTRSHCEAMFKMKRN
Sablefish - ACQ58110.1                    --AMTEQQEMEQRAFDREQA-FA---------------------
Duskytail - ADY86110.1                    ---MEELQDLDQRAFEREKA-FA---------------------
Yellowtail amberjack - ARK85994.1         ---MT---ELEQEQFDRDRADFATSYDLCFTRSHCEAIHTQLSN
Japanese amberjack - DC609456.1           ---MT---ELQQEQLDRDRADFATSYDLWFTRSHCEAIHTQLSN
```

Figure 6.2. *A multiple alignment with aa sequence from the three groups of piscidin found in fish. The multiple alignment was produced using MAFFT alignment software https://mafft.cbrc.jp/alignment/server/index.html. The signal peptide has been highlighted with a double headed arrow above the alignment; amino acids consisting of the predicted proprotein convertase cleavage site are in bold and predicted using ProP software http://www.cbs.dtu.dk/services/ProP/ (Duckert et al., 2004). Predicted active peptides have been highlighted in grey and hydrophobic amino acids outwith the signal peptide have been highlighted in blue. Identity (*), strong similarity (:) and weak similarity (.) are also indicated (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).*

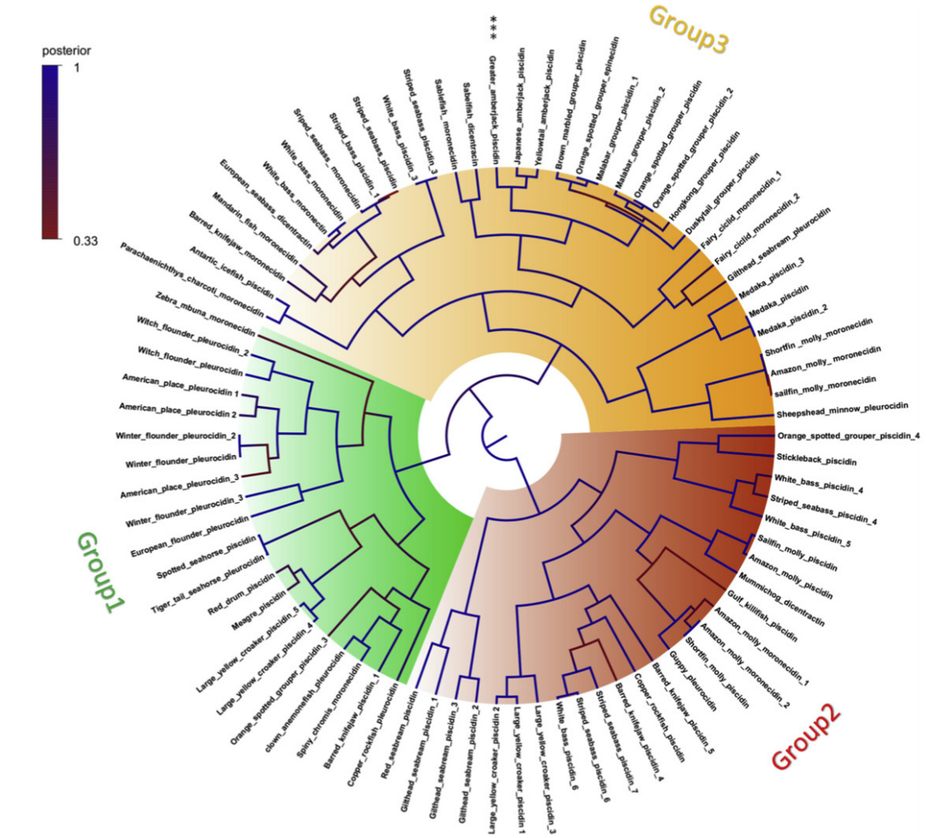


Figure 6.3. *Maximum likelihood topology of currently known teleost piscidin sequences. This tree was constructed using sequence alignment of 111 amino acids. Colours on the branches indicate the posterior probability support value for every reconstructed node from very likely (1, Blue,) to very unlikely (0.33, Red). Piscidin groups have been highlighted as Group 1 - Green, Group 2 – Red and Group 3 - yellow. The accession numbers for the piscidin sequences incorporated into the tree are as follows : Amazon molly moronecidin (XP_014834599.1), Amazon molly moronecidin 1 (XP_016518673.1), Amazon molly moronecidin 2 (XP_014834599.1), Amazon molly piscidin (XP_007572108.1), American plaice pleurocidin 1 (AAP55793.1), American plaice pleurocidin 2 (AAP55794.1), American plaice pleurocidin 3 (AAP55795.1), Antarctic icefish piscidin (CBX55949.1), Barred knifejaw moronecidin (BAM99885.1), Barred knifejaw piscidin 1 (AMB38762.1), Barred knifejaw piscidin 4 (ATU75059.1), Barred knifejaw piscidin 5 (ATU75060.1), Brown marbled grouper piscidin (ADE06665.1), Clown anemonefish pleurocidin (XP_ 023120021.1), Copper rockfish piscidin (GE811382.1), Copper rockfish pleurocidin (GE814250.1), Duskytail grouper piscidin (ADY86110.1), European flounder pleurocidin (DV566089.1), European seabass dicentractin (P59906.1), Fairy ciclid mononecidin (XP_006805732.1), Fairy ciclid moronecidin (XP_006805731.1), Gilthead seabream piscidin 1 (FM145418.1), Gilthead seabream piscidin 2 (FM149199.1), Gilthead seabream piscidin 3 (FM154367.1), Gilthead seabream pleur- ocidin (AM973057.1), Gulf killifish piscidin (JW547320.1), Guppy pleurocidin (XP_017157770.1), Hongkong grouper piscidin (ACE78290.1), Japanese amberjack piscidin (DC609456.1), Large yellow croaker piscidin 1 (AIL82388.1), Large yellow croaker piscidin 2 (AIL82389.1), Large yellow croaker piscidin 3 (AQS27931.1), Large yellow croaker piscidin 4 (ACE78289.1), Large yellow croaker piscidin 5 (AGN52988.1), Malabar grouper piscidin 1 (AFS68802.1), Malabar grouper piscidin 2 (AFS68801.1), Mandarin fish moronecidin (AAV65044.1), Meagre piscidin (ASW20416.1), Medaka piscidin (DK151805.1), Medaka piscidin 2 (DK161574.1), Medaka piscidin 3 (DK192306.1), Mummichog dicentractin (JAR66906.1), Orange spotted grouper epinecidin (AAQ57624.1), Orange spotted grouper piscidin (AFM37317.1), Orange spotted grouper piscidin 2 (ADY86111.1), Orange spotted grouper piscidin 3 (AKA60776.1), Orange spotted grouper piscidin 4 (AKA60777. 2), Antarctic dragonfish moronecidin (AOW44479.1), Red drum piscidin (AFV40526.1), Red seabream piscidin (DC607430.1), Sabelfish dicentracin (ACQ58110.1), Sablefish moronecidin (ACQ57928.1), Sailfin molly moronecidin (XM_015038741.1), Sailfin molly piscidin (XP_014878007.1), Sheepshead minnow pleurocidin (GE334746.1), Shortfin molly moronecidin (XM_014977986), Shortfin molly piscidin (XP_014834599.1), Spiny chromis moronecidin (XP_022059843.1), Spotted seahorse piscidin (AAX58115.1), Stickleback piscidin (EG588124.1), Striped seabass moronecidin (Q8UUG0.1), Striped seabass piscidin (Q8UUG0.1), Striped seabass piscidin 3 (APQ32046.1), Striped seabass piscidin 4 (APQ32049.1), Striped seabass piscidin 6 (APQ32043.1), Striped seabass piscidin 7 (APQ32054.1), Tiger tail seahorse pleurocidin (XP_019738230.1), White bass moronecidin (Q8UUG2.1), White bass moronectin (AAL57318.1), White bass piscidin 3 (APQ32047.1), White bass piscidin 4 (APQ32050.1), White bass piscidin 5 (APQ32052.1), White bass piscidin 6 (APQ32044.1), Winter flounder pleurocidin (P81941.2), Winter flounder pleurocidin 2 (AAG10397.1), Winter flounder pleurocidin 3 (Q90VW7.1), Witch flounder pleurocidin (AAP55799.1), Witch flounder pleurocidin 2 (AAP55800.1), Yellowtail amberjack piscidin (ARK85994.1) and Zebra mbuna moronecidin (XP_004550684.1).*

6.3.3. Immune regulation of piscidin

To establish the baseline expression of piscidin, samples were taken from greater amberjack gills, gut, head kidney and spleen, from which transcript expression was determined. Of these organs the highest piscidin expression occurred in the gut and spleen, followed by the gills and the lowest in the head kidney (Fig. 6. 4A). Fish were then challenged with poly I:C, LPS and recombinant *Yersinia ruckeri* flagellin and samples collected after 24 h to better understand how piscidin is regulated in response to PAMPs. As seen in Fig. 6. 4B, each organ responded differently to PAMP stimulation. The gills responded only to flagellin stimulation, resulting in the doubling of piscidin expression in response to this PAMP, whilst the gut and spleen only responded to poly I:C stimulation, which again led to a doubling of transcript levels after 24 h. Interestingly, the head kidney was the most responsive, with piscidin expression increasing in response to each of the PAMPs used, with transcript levels tripling or

quadrupling depending on the PAMP. This brought stimulated head kidney transcript levels to values similar to hose in the unstimulated gut and spleen. To better understand the temporal regulation of piscidin in response to PAMP exposure primary cell cultures were taken from the spleen, which has the highest constitutive expression, and the head kidney, which has the lowest constitutive expression, and piscidin transcript levels monitored 4, 12 and 24h post PAMP stimulation. PAMP stimulated head kidney cells (Fig. 6. 4C) did not modulate their expression of piscidin until 24 h post stimulation, at which point there was a significant increase in transcript levels in response to poly I:C (10 fold), LPS (60 fold) and flagellin (70 fold) stimulation. In contrast, spleen cells showed a quicker but less intense upregulation in piscidin expression following PAMP stimulation (Fig. 6. 4D). After 4 h both poly I:C and flagellin induced an increase in piscidin expression but by 12 h only poly I:C induced piscidin upregulation was seen that continued to 24 h.

6.3.4. Dietary modulation of piscidin

Functional feeds are often used to modulate the immune response in fish. Therefore to determine if piscidin expression could be modulated through changes in diet greater amberjack were split into four groups with each fed a different diet (Diet A–D) for 30 days, after which piscidin expression was determined in the gills, gut, head kidney and spleen (Fig. 6. 5). Piscidin expression remained very similar to control diet A in gills when amberjack were fed diets B and D, however a dramatic increase in expression was seen in fish fed diet C, that reached levels 3 to 4 times higher than in gills of fish on the other diets. Piscidin expression in the gut remained fairly consistent when fish were fed diets A, B and C, while fish fed diet D increased piscidin expression ~4 fold. Head kidney expression of piscidin did not differ from the control diet when fish were fed diets B or D, but a significant increase was observed in fish fed diet C. Lastly, spleen expression of piscidin was increased 2.5 fold in fish fed diet B compared to those fed the control diet, however no effect was seen when fish were fed diets C and D (Fig. 6. 5).

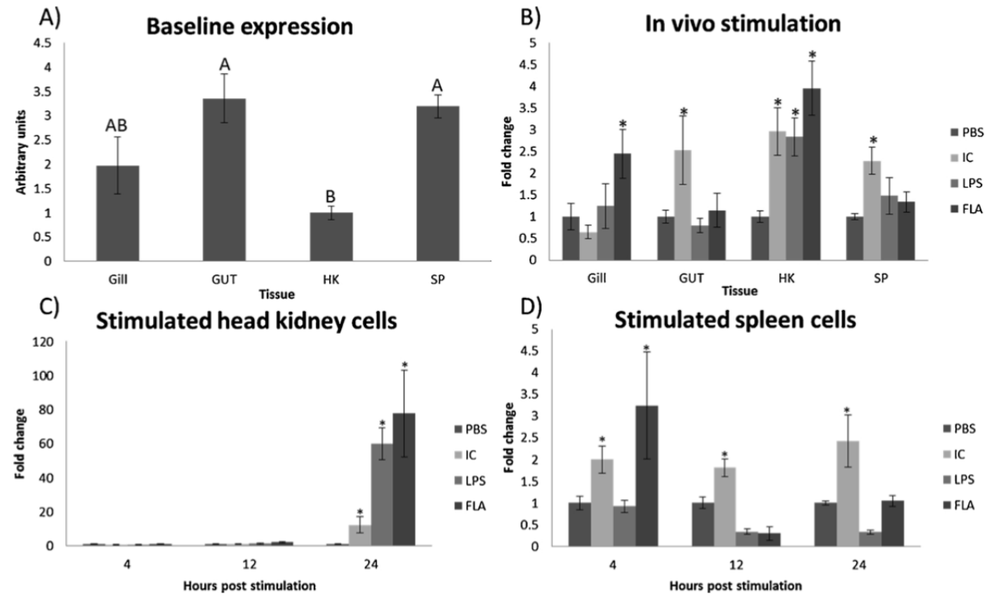


Figure 6.4. *Transcript expression of greater amberjack piscidin. A) shows the relative constitutive expression of piscidin between gill, gut, head kidney (HK) and spleen (SP) tissues. B) shows the fold change in piscidin expression relative to values from PBS control fish for each tissue, for fish stimulated with poly I:C (IC), LPS or recombinant Yersinia ruckeri flagellin (FLA) for 24 h. C) shows the fold change in piscidin expression after 4, 12 and 24 h following in vitro stimulation of HK cells with poly I:C, LPS and flagellin, compared to PBS treated cells at each time point. D) shows the fold change in piscidin expression after 4, 12 and 24 h following in vitro stimulation of spleen cells with poly I:C, LPS and flagellin, compared to PBS treated cells. Piscidin transcripts were detected by qPCR and normalised to EF1α. Bars are either mean arbitrary units ± SEM, or mean fold-change ± SEM. N = 10. Letters denote significant differences ((p ≤ 0.05) between tissues, whilst asterisks denote significant differences relative to PBS controls.*

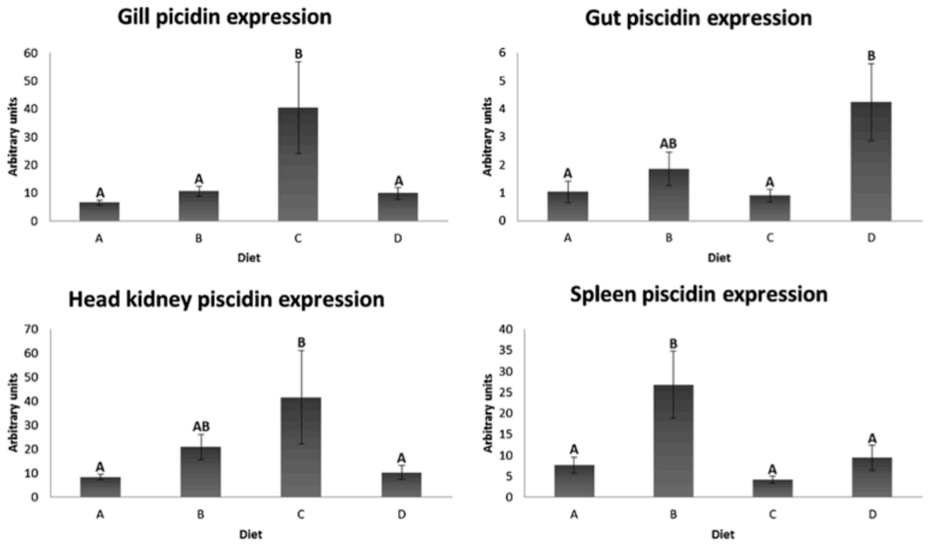
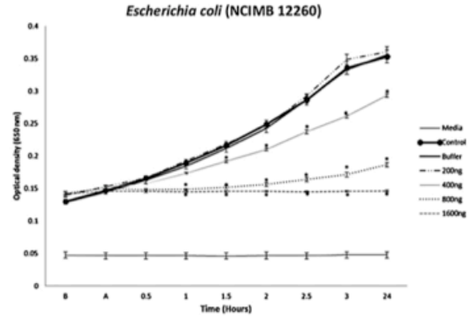


Figure 6.5. *Piscidin expression of greater amberjack on different diets. Greater amberjack were fed diets A) Skretting Seriola base diet (control), B) MOS enhanced diet, C) cMOS enhanced diet, and D) MOS and cMOS enhanced diet for 30 days. Piscidin expression was then quantified by qPCR and normalised to EF1α in the gills, gut, head kidney and spleen. Bars are mean arbitrary units ± SEM, N = 6. Letters denote significant differences ((p ≤ 0.05) in piscidin expression between diets, within an organ.*

6.3.5. Synthetic piscidin inhibits bacterial growth

To study the bioactivity of greater amberjack piscidin the predicted active peptide was synthesised by BIOMATIK and the ability of this synthetic piscidin to inhibit the growth of known fish bacterial pathogens and *E. coli* was tested. During the first 3 h of bacterial growth the higher concentrations (800 and 1600 ng/ml) of synthetic piscidin significantly reduced the growth rate of all the bacterial strains tested and for most strains growth completely ceased during this period (Fig. 6. 6). These results were largely mirrored after 24 h. The majority of bacterial strains incubated with 800 ng/ml of piscidin show a ≥85% growth inhibition except for *Lactococcus garvieae,* strain MT2055, which saw a 70% reduction in growth and *Photobacterium damselae*, strain MT1415, which saw a 55% reduction in growth. However, when incubated with a concentration of 1600ng/ml all bacterial strains showed ≥95% growth inhibition. A medium dose of piscidin (400ng/ml) led to a 20–60% reduction in growth at both early and later time points. Most notably, *Vibrio anguillarum*, strain MT1742, showed a 50% reduction in growth, *Yersinia ruckeri,* strain MT242, a 40% reduction in growth and *Aeromonas salmonicida,* strain MT423, a 55% reduction in growth after 24 h incubation with this dose. Low doses of piscidin (200 ng/ml) were largely ineffective at reducing the growth rate of the tested bacterial strains. Only a slight reduction in growth ≤15% was observed in *V. anguillarum,* strains MT1741 and MT1742, *A. salmonicida,* strain MT004, and *L. garvieae,* strain MT2055, while no change was observed in the growth of the other bacterial strains at this dose.



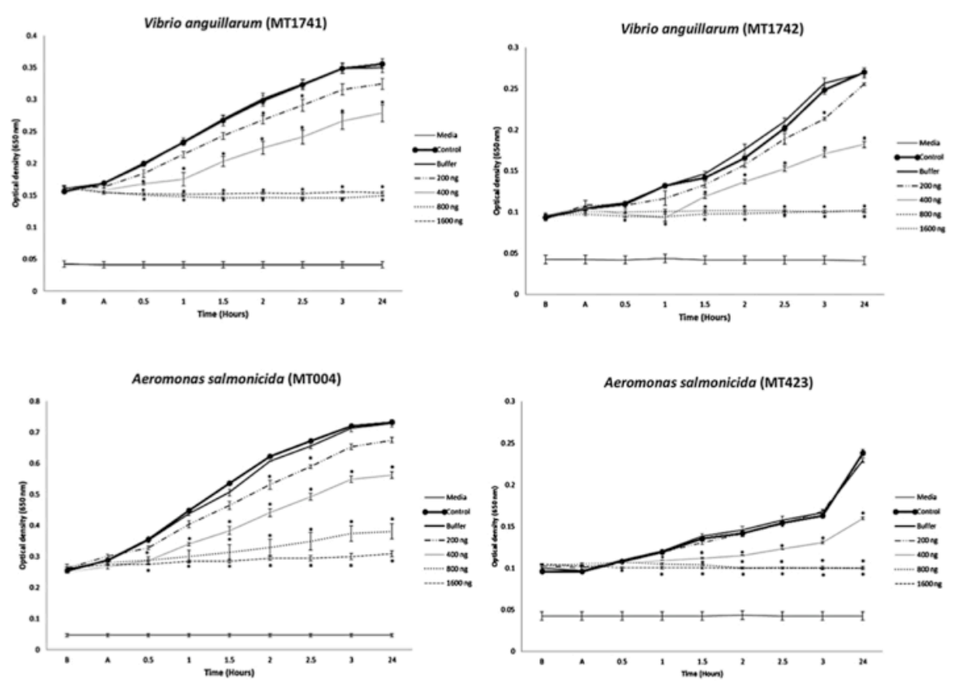


Figure 6.6. *The effects of synthetic greater amberjack piscidin on bacterial growth. The growth of bacterial strains NCIMB 12260 (Escherichia coli), MT423 (Aeromonas salmonicida - virulent), MT004 (Aeromonas salmonicida - avirulent), MT1741 (Vibrio anguillarum –serogroup 1), MT1742 (Vibrio anguillarum –serogroup 2), MT252 (Yersinia ruckeri), MT2055 (Lactococcus garvieae - noncapsulated), MT2291 (Lactococcus garvieae - capsulated) and MT1415 (Photobacterium damselae) were monitored at 650 nm before (B), after addition of piscidin (A) and then every 30 min for 3 h, with a final reading taken at 24 h. The optical densities of the media (media), untreated bacteria (control) and bac- terial samples treated with the piscidin buffer solution (buffer) were measured at each time point as controls, in comparison to bacterial samples incubated with piscidin at doses of 200, 400, 800 and 1600 ng/ml. Data are presented as mean optical density ± SEM, N = 10. Asterisks denote a significant difference (P≤0.05) from the control group at the same time point.*

6.3. Discussion

Antimicrobial peptides (AMPs) are a critical component of the innate immune response in jawed vertebrates. Multiple types of AMPs have been identified within vertebrate species, with many unique to particular lineages, as seen with piscidins that are restricted to teleost fish (Masso-Silva and Diamond, 2014). Whilst piscidins are highly variable in sequence between fish species, they can be allocated to one of three groups (ie group 1–3) (Muncaster *et al.,* 2018). A single fish species may have all three groups of piscidin (eg Orange spotted grouper, *Epinephelus coioides*), and even multiple copies of each, or may be missing entire groups, as seen in Large yellow croaker, *Larimichthys crocea* (Fig. 6. 3) that lacks group 3 piscidins. However, with the continuation of gene discovery elusive piscidin sequences may still be obtained, perhaps revealing further isoforms within species. Interestingly, the active peptides of group 2 and group 3 piscidins appear to be of a similar size and consist of amino acids with similar properties. Apart from their differing cleavage motifs, a notable difference between them is that group 2 piscidins have 10 aa between the conserved glycines (G) with only 3 aa after the second conserved glycine (before the cleavage site), while group 3 piscidins have only 4 aa between the conserved glycines and 7–8 aa after the second conserved glycine/ before the cleavage site (Fig. 6. 2). In contrast, the active peptide of group 1 piscidins is much larger, encoding an additional C-terminal region, which is rich in hydrophobic aa. This hydrophobic region of group 1 piscidins likely plays an important role in the initial embedding of the molecule into microorganism cell membranes and the eventual lysis of the microorganism (Park *et al.,* 2011).

The lack of di-sulphide bonds and helical structure of piscidins offer unique opportunities that are not present in other AMPS. They are considerably easier to synthesise using a solid phase multiple peptide system, due to their linear nature, as they do not require any further steps to produce a biologically active peptide, unlike defensins and hepcidins which would require additional processing, ie di-sulphide stapling, to ensure the correct secondary structure (Lau *et al.,* 2015). In addition, piscidins are able to retain antimicrobial activity at high salt concentrations (Noga *et al*., 2009), are highly thermostable (Sun *et al.,* 2012) and have a low cytotoxicity against mammalian cells (Kim *et al*., 2010), highlighting pharmaceutical prospects in modern medicine as well as in aquaculture.

Initially greater amberjack piscidin was cloned and the sequence analysed to verify its identity. Greater amberjack piscidin showed a reasonable similarity with piscidins from other perciform species but showed the highest similarity and identity with the group 3 piscidins. This was further confirmed by phylogenetic analysis which placed greater amberjack piscidin with other known group 3 piscidins and very closely with piscidins identified in other Seriola species. Furthermore, the predicted active peptide was of a similar size, charge and residue homology as other group 3 piscidins and also contained the conserved ‘RRRH’ sequence

unique to group 3 piscidins, which acts as the cleavage site of a furin-like proprotein convertase (Duckert *et al.,* 2004). The signal peptide of greater amberjack piscidin is highly homologous to all the other piscidins analysed and hints at a conserved piscidin transport network and storage, the latter suggested to occur in the granules of fish phagocytic granulocytes (Mulero *et al.,* 2008). In contrast, the active peptide and pro domain of greater amberjack piscidin differs dramatically from group 1 and 2 piscidins, as with other group 3 piscidins. Such variation between active peptides has been suggested to be the result of a continual positive selection process focused on these regions driving changes in order to adapt the peptides to combat new and constantly evolving pathogens, which would explain why there is such variation in piscidin sequence even between closely related species (Tennessen, 2005; Fernandes *et al.,* 2010). Interestingly, two glycine residues are conserved within all groups of piscidin, although their location differs between piscidins, and are located 8 and 13 aa into the active peptide of greater amberjack piscidin. Due to the small size of the glycine side chain (a single hydrogen atom) it is possible that these residues are conserved to reduce steric hindrance during the formation of the peptides helical structure.

Baseline expression of piscidin is highest in the gut and the spleen followed by the gills, with lowest expression in the head kidney (HK). This is in agreement with constitutive expression of piscidin 3 in other Seriola species, as seen with yellowtail kingfish, *Seriola lalandi,* where piscidin 3 expression is highest in spleen followed by gills and lowest in the liver, a profile also mirrored in other perciform fish (eg Hybrid striped bass) (Lauth *et al.,* 2002). Following immune stimulation with poly I:C greater amberjack piscidin could be upregulated in the gut, HK and spleen, indicating a possible role in viral responses at both mucosal and systemic immune sites. Upregulation of piscidin in response to viral PAMPs in both mucosal and systemic sites has also been observed in other perciforms such as meagre, *Argyrosomus regius,* (Campoverde *et al.,* 2017) and the effectiveness of piscidins in inhibiting infection from channel catfish virus has been demonstrated (Chinchar *et al.,* 2004). LPS and flagellin are both bacterial PAMPs, however the greater amberjack response to these PAMPs, in terms of piscidin transcript regulation, differed. LPS stimulation upregulated expression in the head kidney whilst flagellin induced upregulation in both the gills and HK. Piscidin upregulation in systemic organs in response to LPS stimulation is to be expected as piscidins have potent anti-bacterial properties and are capable of binding and neutralising LPS in a dose dependant manner (Kumar *et al.,* 2017). Flagellin from *Yersinia ruckeri* is a highly potent PAMP in rainbow trout, eliciting very strong immune responses (Wangkahart *et al.,* 2016) and *Salmonella typhimurium*

flagellin has been shown to highly upregulate piscidin expression in the HK of rock bream, *Oplegnathus fasciatus* (Umasuthan *et al.,* 2016). It may be that the potency of flagellin as an immune stimulator led to the mucosal effect seen. However, it is also important to note that the receptor by which flagellin is detected, Toll-like receptor 5, and the intracellular signalling pathway triggered differ from those used to detect LPS, and as such can lead to different outcomes which may explain why flagellin and LPS produce different piscidin expression profiles despite both being bacterial PAMPs (Gonzalez-Stegmaier *et al.,* 2015).

Next the kinetics of piscidin induction was studied in vitro using HK and spleen cells. Upon stimulating greater amberjack HK cells with the immune stimulants it was apparent that the cells do not respond immediately, and no effect was seen until 24 h post stimulation. At this time a strong upregulation was seen to all of the PAMPs tested, similar to the in vivo situation with the HK. The only notable difference was the dramatic increase to the bacterial stimulants (70 fold increase) relative to the in vivo HK result. This behaviour was also observed in other perciforms such as crocodile icefish, *Chionodraco hamatus,* and is likely due to lack of negative feedback loops present for cells in vitro and/or the dilution of immune stimulant concentration when travelling to the HK in vivo (Buonocore *et al*., 2012). The splenocytes also responded in a similar manner to the spleen of PAMP stimulated amberjack, in up- regulating piscidin in response to poly I:C. However, an early (4 h) upregulation of piscidin occurred to poly I:C and flagellin, and this could explain the lack of response to flagellin (and perhaps LPS) in vivo, where only a single timing (24 h) was studied.

While the injection of immune stimulants may be an effective method of piscidin modulation, it would be time consuming, labour intensive and expensive to do on a large scale. In contrast the modulation of piscidin expression through managed feed alteration would be relatively quick, easy and inexpensive. Thus, the successful modulation of piscidin with the functional feeds used in this trial offers exciting prospects. The ability to upregulate piscidin expression may be used to protect against various pathogens by preventing initial infection, by boosting piscidin expression in organs targeted by microbes. The cMOS diet (diet C) containing 2g· kg^{-1} of Actigen had the most pronounced effect, increasing piscidin expression in the gills and head kidney, known to be sites of invasion by bacterial species such as *Flavobacterium spartansii* (Rangdale *et al*., 1997). Furthermore, MOS containing diets (diet B) could be used to boost piscidin expression in the spleen, to help prevent and mitigate systemic infections with pathogens like *Lactococcus garvieae (*Young *et al.,* 2012) and diet

D, that upregulates piscidin in the intestine, could be used to protect against pathogens where the intestine is the primary infection site, as with *Aeromonas salmonicida* (McCarthy, 1980). cMOS has already been shown to have value as a dietary supplement, and is able to increase expression of a range of immune molecules and proteins in greater amberjack, and to increase parasite resistance (Fernández-Montero *et al.,* 2019).

Lastly, the bioactivity of greater amberjack piscidin was studied. When incubated with a range of bacterial species the synthetic greater amberjack piscidin demonstrated strong bacteriostatic activity on all tested strains of bacteria, at concentrations of 800 ng/ml or higher, over short (3h) and long (24h) bacterial incubation times. This broad spectrum activity of piscidin is to be expected, and is a common feature of AMPs (Iijima *et al.,* 2003; Chinchar*et al.*, 2004). That said, the most prominent bacteriostatic activity of greater amberjack piscidin was observed against *Vibrio anguillarum* strains, *Y. ruckeri* and *A. salmonicida*, where the minimum inhibitory dose to reduce bacterial growth by 50% after 24h (MIC) was 0.88–1.6μM. This reveals that there is a degree of increased efficacy to certain pathogens over others.

In conclusion, we have cloned and characterised a group 3 piscidin from greater amberjack, an upcoming species for European aquaculture. We show it is expressed in a range of organs, and that this expression can be modulated by PAMPs and diet. The bioactivity of greater amberjack piscidin was verified and potential applications of these findings have been discussed.

CHAPTER 07

INCREASED PARASITE RESISTANCE OF GREATER AMBERJACK (*SERIOLA DUMERILI* RISSO 1810) JUVENILES FED A CMOS SUPPLEMENTED DIET IS ASSOCIATED WITH UPREGULATION OF A DISCRETE SET OF IMMUNE GENES IN MUCOSAL TISSUES.

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Abstract

The main objective of this study was to determine the effect of two forms of mannan oligosaccharides (MOS: Bio-Mos® and cMOS: Actigen®, Alltech Inc, USA) and their combination on greater amberjack (*Seriola dumerili)* growth performance and feed efficiency, immune parameters and resistance against ectoparasite (*Neobenedenia girellae*) infection. Fish were fed for 90 days with 5 g kg^{-1} MOS, 2 g kg^{-1} cMOS or a combination of both prebiotics, in a *Seriola* commercial base diet (Skretting, Norway). At the end of the feeding period, no differences were found in growth performance or feed efficiency. Inclusion of MOS also had no effect on lysozyme activity in skin mucus and serum, but the supplementation of diets with cMOS induced a significant increase of serum bactericidal activity. Dietary cMOS also reduced significantly greater amberjack skin parasite levels, parasite total length and the number of parasites detected per unit of fish surface following a cohabitation challenge with *N. girellae*, whereas no effect of MOS was detected on these parameters. Of 17 immune genes studied cMOS dietary inclusion up-regulated hepcidin, defensin, Mx protein, interferon-γ (IFNγ), mucin-2 (MUC-2), interleukin-1β (IL-1B), IL-10 and immunoglobulin-T (IgT) gene expression in gills and/or skin. MOS supplementation had a larger impact on spleen and head kidney gene expression, where piscidin, defensin, iNOS, Mx protein, interferons, IL-1β, IL-10, IL-17 and IL-22 were all upregulated. In posterior gut dietary MOS and cMOS both induced IL-10, IgM and IgT, but with MOS also increasing piscidin, MUC-2, and IL-1β whilst cMOS induced hepcidin, defensin and IFNγ. In general, the combination of MOS and cMOS resulted in fewer or lower increases in all tissues, possibly due to an overstimulation effect. The utilization of cMOS at the dose used here has clear benefits on parasite resistance in greater amberjack, linked to upregulation of a discrete set of immune genes in mucosal tissues.

7.1. Introduction

Seriola aquaculture has traditionally been focused on yellowtail kingfish (*S. lalandi)* and Japanese amberjack *(S. quinqueradiata*) (Nakada, 2000). In Europe, greater amberjack (*Seriola dumerili,* Risso 1810) is considered an emerging aquaculture species due to its high commercial value and fast-growth (Takakuwa *et al.,* 2006), where under appropriate culture conditions they can reach 6 kg in 2.5 years (Mazzola *et al.,* 2000). Nevertheless, greater amberjack production in sea cages is limited by several bottlenecks, with monogenean ectoparasite outbreaks a key concern (Ogawa *et al.,* 1995; Hirayama *et al.,* 2009; Repullés-Albelda *et al.,* 2013).

Neobenedenia girellae is a monogenean ectoparasite that has become one of the main causes of greater amberjack parasitic infections. It is characterised by a broad host range and wide distribution in warm waters, with an important prevalence in aquaculture farms (Ogawa *et al.,* 1995; Ogawa and Yokoyama, 1998). Its lifecycle is highly dependent of seasonal temperature (Ernst *et al.,* 2005;Hirazawa *et al.,* 2010; Fernandez-Montero *et al.,* 2016; Fernandez-Montero *et al.,* 2017) which promotes the parasite attachment to the host. Furthermore, parasite attachment to fish skin produces important alterations (Hirayama *et al.,* 2009; Hirazawa *et al.,* 2016) such as wounds and ulcers, promoting secondary infections (Hagiwara *et al.,* 2011), thereby increasing mortality. To fight secondary infections, especially those caused by fungi and bacteria, several different strategies have been adopted, mainly based on the use of antibiotics and topical treatments that have some risks (Cabello, 2006). Nowadays, one of the most common strategies to avoid the use of antibiotics is to boost the immune system to enable fish to overcome pathogen infections (Ringø *et al.,* 2014; Guerreiro *et al.,* 2017). These strategies include dietary inclusion of prebiotics and use of functional feeds, some of which have been shown to affect ectoparasite prevalence (Buentello *et al.,* 2010; Dimitroglou *et al.,* 2011).

Prebiotics are commonly used in the animal production industry due to their effects on the immune system leading to pathogen protection (Lomax and Calder, 2008). It has been well established that the by-products produced when beneficial commensal bacteria ferment prebiotics play a key role in improving host health (Choque-Delgado *et al*., 2011). New pre-

biotics have been showing successful results (Song *et al.,* 2014), including mannan oligosaccharide (MOS) by-products (Torrecillas *et al.,* 2007, 2011a,b; Eryalcin *et al.,* 2017). Studies of MOS beneficial effects have focused on growth performance and health, especially the modulation of intestinal microbiota and promotion of gut integrity in adult and juvenile fish (Torrecillas *et al.,* 2011a, 2013). However, MOS effects are known to be highly dependent upon the biotic parameters of the cultured fish, including the species, culture conditions, duration of the supplementation, age and size (Sweetman *et al.,* 2010; Song *et al.,* 2014).

Previous studies have shown that an inclusion level of 4g MOS kg^{-1} in diets increases growth performance, feed efficiency and feed intake in salmonids and seabass after 67 days of supplementation (Yilmaz *et al.,* 2007; Torrecillas *et al.,* 2007). In contrast, in gilthead seabream and channel catfish no effect was observed on these parameters using this inclusion level during 63 and 42 days respectively (Dimitroglou *et al.*, 2010; Peterson *et al.,* 2010) but changes of the immune system were found. Similarly, in rainbow trout (Staykov *et al.,* 2007) fed a functional diet with 2 g MOS kg^{-1} during 42 and 90 days improved antibody production and lysozyme activity were found, and in Japanese flounder, after 56 days of dietary inclusion of 5 g MOS kg^{-1} gave higher lysozyme activity, although no differences were observed in the numbers of cells undergoing phagocytosis or the phagocytic index (Ye *et al.,* 2011).

However, in Atlantic salmon (200g) fed a diet supplemented with 10 g MOS kg^{-1} for 4 months no effects on the innate immune system were seen (Grisdale-Helland *et al.,* 2008). Such studies suggest that the effects are not consistent between species or that there is a limited duration of the MOS effect on the host immune response. Recently the study of key regulatory cytokines as markers has also become a useful indicator of the immune system status in fish. For instance, previous studies with Atlantic cod showed that MOS dietary inclusion produces changes in gut cytokine expression levels after 35 days of supplementation (Lokesh *et al.,* 2012). Clearly future studies on cytokines are warranted to shed light on MOS effects. Little information is available about the immune system of greater amberjack (Fernandez-Montero *et al.,* 2016; Milne *et al.,* 2016) and few studies have investigated the use of immunostimulants with this species (Itami *et al.,* 1996; Militz *et al.,* 2013; Hossain *et al.,* 2017), with none using MOS or concentrated MOS (cMOS) inclusion. For this reason, the objective of the present work was to determine the effect of MOS and cMOS (Bio-Mos® and Actigen®) and their combination on greater amberjack juveniles, focusing on immune parameters, protective effects against a *N. girellae* and any impact on growth/feed efficiency.

7.2. Materials and methods

The present study was conducted at the Scientific and Technologic Park of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain). The animal experiments described comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals. For the whole trial, a tank is considered as an experimental unit.

7.2.1. Experimental fish and conditions

Two hundred and sixteen fish (mean weight 331.4 ± 30 g) were distributed in twelve cylindroconical 1,000 L tanks with an open circulation (18 fish/tank). Water conditions were monitored daily, maintaining salinity at 37 mg L^{-1}, oxygen values at 6.0 ± 1 ppm O_2 and temperature at 23°C ± 0.3 during July, August and September. Fish were fed by hand 3 times per day to apparent satiety. Uneaten pellets were recovered, dried and weighed.

7.2.2. Diets

The diets used combined a *Seriola* base diet designed by Skretting (Stavanger, Norway) and containing 55% protein, 55% fish meal and 10% fish oil, with two different prebiotics, namely MOS and cMOS (Bio-Mos® and Actigen® developed by Alltech, Inc.). Diet C (control) was composed exclusively of the *Seriola* base diet, the MOS diet included 5 g Bio-Mos® kg^{-1}, the cMOS diet 2 g Actigen ® kg^{-1}, and the MOS + cMOS diet had 5 g Bio-Mos ® kg^{-1} and 2 g Actigen ® kg^{-1}. Each diet was randomly assigned to triplicate groups of fish (n=3x3).

7.2.3. Sampling procedures

Sampling was conducted after 0, 30 days, 60 days and 90 days of feeding, where growth and feed utilization parameters were evaluated. Additionally, at the end of the feeding trial head kidney, spleen, gills, posterior gut and skin of 3 fish per tank were sampled for immune gene expression analysis. Skin mucus and blood (serum) were also collected from 3 fish per tank. Finally, a parasite challenge against *N. girellae* was performed (as outlined below).

7.2.4. Fish performance parameters

Specific growth rate (SGR) and feed efficiency were calculated as follows:

SGR = (Ln (final weight) – Ln (initial weight))*100/feeding time (days)

Feed efficiency= 1/(feed intake/ weight gain)

7.2.5. Gene expression analyses

Samples for gene expression analyses were collected in RNAlater and stored for 48 h at 6oC. Total RNA was subsequently extracted using the Trizol reagent method (Invitrogen) according to the manufacturer's instructions. RNA concentration and purity were determined by spectrophotometry measuring the absorbance at 260 and 280 nm (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain). Electrophoresis in agarose gels was conducted to check extracted RNA quality by visualization of RNA bands. DNase treatment was applied to the extracted RNA, according to the manufacturer's instructions, to remove possible contaminating genomic DNA (AMPD1–1KT, Sigma–Aldrich, Broendby, Denmark). Total RNA was reverse transcribed in a 20 µL reaction volume containing 2 µg total RNA, using a ThermoScript ™ Reverse Transcriptase (Invitrogen) kit, until cDNA was obtained in a thermocycler (Mastercycle ® nexus GSX1, Eppendorf AG, Hamburg, Germany) run according to the manufacturer's instructions. The samples were then diluted 1:20 in miliQ water and stored at -20oC.

Specific primers were designed to target genes found in genbank from species phylogenetically related with *S. dumerili* (Table 7. 1), following the methodology described by Milne (2018). The primers were used to amplify products using amberjack cDNA obtained from a pool of gill, posterior-gut, head kidney and spleen tissue, and the products cloned and sequenced. At least a partial sequence was obtained for all the target genes and these partials were sufficient in length to determine gene identity and develop qPCR primers. qPCR was conducted with SYBRgreen and truestar taq following a programme of: 1 cycle of 6 min denaturalization at 95°C, 45 cycles of amplification (25 s at 95°C, 30 s at the annealing temperature, 25 s at 70°C for the extension, and 5 s at 82°C), 1 cycle for the melting curve of 5 s at 95°C and 1 min at 75°C, ending with 1 cycle of cooling for 1 min at 40°C. MUC-2 was only analysed in the mucosal tissues and not head kidney and spleen.

Table 7.1. *Primers used for gene expression analysis by RT-qPCR in skin, gill, posterior gut, head kidney and spleen of greater amberjack juveniles (Seriola dumerili) fed MOS and cMOS (t = 90 days). *Ann. temp: anneling temperature*

Gene	Name	Ann. temp. (°C)*	Product size (bp)	Forward Sequence	Reverse Sequence
Hep	Hepcidin	61	99	GATGATGCCGAATCCCGTCAGG	CAGAAACCGCAGCCCTTGTTGGC
Pis	Piscidin	58	112	ATCGTCCTGTTTCTTGTGTTGTCAC	CGCTGTGGATCATTTTTCCAATGTGAAA
Def	Defensin	60	133	ATGAGGCTGCATCCTTTCCATG	AGAAAATGAGATACGCAACACAAGAAGCC
iNOS	Inducible Nitric oxyde synthase	60	151	TGTTTGGCCTTGGCTCCAGGG	GCCCAAGTTCTGAATGACTCCTCCTG
TNFα	Tumor necrosis factor α	62	212	GAAAACGCTTCATGCCTCTC	GTTGGTTTCCGTCCACAGTT
MX Prot	Interferon-inducible Mx protein	61	211	GGCTACATGATTGTGAAGTGCAGGG	CTTCCAGTCGAGGCAGAGATTTCTCAATGT
IFN ɣ	Interferon γ	59	163	AACTTGGTTTCACGGTGCAG	TCACAACACCGAGAAAGTCCT
IFN d	Interferon type I	59	111	GTCAGGGTGCAGCTGAGTTA	ACAGAAACGGCAGCTCAAAC
MUC-2	Mucin-2	62	342	ATTGAGTTTGGCAACAAACAGAAAGCCC	TAC AGC ACA GAA CTG AGG TGT CCT C
IL-1β	Interleukin 1β	62	205	TGATGGAGAACATGGTGGAA	GTCGACATGGTCAGATGCAC
IL-8	Interleukin 8	58	164	GAAGCCTGGGAGTAGAGCTG	GGGGTCTAGGCAGACCTCTT
IL-10	Interleukin 10	58	134	CTCAAGAGTGATGTCACCAAATGTAGA AACT	AGC AAA TCC AGC TCG CCC ATT
IL-17F	Interleukin 17F	62	120	GGTGGCCCCAGAGGATCTCC	GGAGGACCAAAACCTGGTAGTAGATGG
IL-17D	Interleukin 17D	62	111	CGGTCTACGCTCCCTCCGTG	GCGGCACACAGGTGCATCCC
IL-22	Interleukin 22	61	146	GCCAACATCCTCGACTTCTACCTGAAC	TGG TCG TGG TAG TGA GTC ACA TTG C
IgM	Immunoglobulin M	58	148	CTCTTTGATAGGAATACCGGAGGAGAG	CAACTAGCCAAGACACGAAAACCC
IgT	Immunoglobulin T	59	196	TGGACCAGTCGCCATCTGAG	GGGAAACGGCTTTGAAAGGA
β-Actin	β-Actin	61	212	TCT GGT GGG GCA ATG ATC TTG ATC TT	CCT TCC TTC CTC GGT ATG GAG TCC
EF1 α	Elongation factor 1α	60	194	TGC CAT ACT GCT CAC ATC GCC TG	ATT ACA GCG AAA CGA CCA AGA GGA G

7.2.6. Blood and mucus immunological parameters

Serum was obtained by centrifuging the collected blood after clotting overnight at 4°C. Skin mucus was obtained following the methodology described by Guardiola *et al.* (2014) with some modifications. Skin mucus was collected by gently scrapping the surface of the fish skin with autoclaved microscopy slides and diluted 1:1 with filtered and autoclaved salt water. Lysozyme activity was determined as described by Ellis (1990). Lysozyme activity was expressed in units ml^{-1}, were one unit of lysozyme was considered as the quantity of enzyme needed for reducing absorbance by 0.001 per millilitre of serum and mucus per minute. Bactericidal activity was measured with a modification of the method described by Sunyer and Tort (1995), using *Photobacterium damselae.*

7.2.7. Parasite infection

The parasite source was a tank (10,000 L) of *S. dumerili* naturally infested with *Neobenedenia girellae* at high parasite density. Nets (0.14 mm pore diameter) were placed into the tank to entangle the eggs and collect them. After 24 h eggs were introduced into a 1,000 L tank with 200 uninfected *S. dumerili* juveniles. After 10 days, all the fish were infected to the same degree. Then, 96 infected animals from the source tank were placed into twelve $0.03m^3$ cages (8 infected fish per cage and one cage per experimental tank) for 15 days, to enable a cohabitation challenge after 100 days of prebiotic inclusion. After 15 days of cohabitation, the remaining one hundred eighty experimental fish were sampled, and a visual evaluation of infection level for each fish was carried out by 3 different trained researchers. The levels were scored between 0 (no parasites observed), 1 (between 1 and 5 parasites), 2 (between 6 and 15) and 3 (more than 15). After that, the fish were introduced into freshwater to release all of the attached parasites, and the parasites counted and measured. The number of parasites per fish was converted into the number of parasites per square centimetre of fish surface area, calculated following the method described in Ohno *et al.* (2008). Total length of 50 adult parasites per tank was recorded using a profile projector (Mitutoyo, PJ-A3000).

7.2.8. Statistical analyses

The statistical analyses followed the methods outlined by Sokal and Rolf (1995), with means and standard deviations (SD) calculated for each parameter measured. All data were tested

for normality and homogeneity of variance. Data were subjected to one-way ANOVA and differences were considered significant when $p< 0.05$. Two-way ANOVA was conducted for MOS, cMOS and the interaction among treatments. If the variances were not normally distributed, data were transformed (log_{10}) and the Kruskall-Wallis non-parametric test applied. Kruskall-Wallis analysis was also used for range-comparison statistical analyses. Analyses were performed using SPSS software (SPSS for windows 10).

Multivariant analyses and their plots were performed using PRIMER 7 and PERMANOVA. The number of permutations was established at 999. PERMANOVA analysis considered differences significant when the permutation p-value (p perm.) was below 0.05.

7.3. Results

7.3.1. Growth performance

No effect of MOS, cMOS or their combination was observed in final weight, SGR or feed efficiency among fish fed the different dietary treatments ($p>0.05$), although fish fed the cMOS diet tended to perform better (+4% SGR) (Table 7.2).

7.3.2. Serum and skin mucus immunological parameters

After 90 days of feeding, two way-ANOVA analysis revealed a significant increase in serum bactericidal activity in fish fed MOS ($F=6.68$, $P=0.04$) and cMOS ($F=17.56$, $P=0.02$), whereas no effect was detected when measured in mucus (Table 7. 2). Lysozyme activity in mucus and serum was not affected by MOS or cMOS dietary supplementation. No interaction between MOS and cMOS was detected for the mucus and serum immune parameters evaluated (Table 7. 2).

Table 7.2. *Growth performance, serum and skin mucus immunological parameters (lysozyme activity and bactericidal activity) and parasite data of greater amberjack juveniles (Seriola dumerili) after 90 days on the feeding trial.*

	DIETARY TREATMENTS				TWO WAY ANOVA		
	C	MOS	cMOS	MOS + cMOS	MOS	cMOS	MOS*CMOS
Growth performance							
Final Weight (g)	1046.75 ±129.61	1024 ±161.17	1090.37 ±135.49	1036.55 ±126.88	NS	NS	NS
SGR (%)	1.09 ±0.04	1.09 ±0.06	1.13 ±0.09	1.08 ±0.07	NS	NS	NS
Feed efficiency	0.654 ±0.06	0.656 ±0.01	0.698 ±0.04	0.704 ±0.08	NS	NS	NS
Skin mucus							
Lysozyme activity (U/ml)	103.92 ±17.64	114.25 ±28.1	124.55 ±31.64	121.9 ±11.97	NS	NS	NS
Bactericidal activity (%)	3.72 ±1.86	5.03 ±1.21	6.54 ±0.89	5.22 ±2.61	NS	NS	NS
Serum							
Lysozyme activity (U/ml)	301.61 ±42	348.76 ±52.1	253.88 ±25.86	287.69 ±39.04	NS	NS	NS
Bactericidal activity (%)	4.89 ±1.06	5.91 ±1.70	8.27 ±1.05	9.51 ±1.27	P=0.04 F=6.68	P=0.02 F=17.56	NS
Parasite challenge							
Parasitation level (range)	2-3	2	1-2	1-2	NS	P=0.01	NS
Parasite total length (mm)	4.44 ±0.31	3.9 ±0.43	3.32 ±0.40	3.56 ±0.43	NS	F=6.17	NS
N° parasites / fish surface (cm2)	0.101 ±0.01	0.087 ±0.02	0.015 ±0.01	0.042 ±0.01	NS	P=0.01 F=15.47 P=0.01 F=52.36	NS

Diet C (control diet, non-supplemented), MOS (MOS supplemented diet), cMOS (cMOS supplemented diet), MOS + cMOS (combined MOS and cMOS supplemented diet). Values expressed in mean ± SD (n = 3 tanks/diet). Two-way ANOVA comparation (p<0.05). SGR: Specific growth rate; parasitation level: ranged among 1 (lower) to 3 (higher).

7.3.3. Parasite challenge

Greater amberjack given dietary supplementation of cMOS for 90 days had significantly reduced skin parasite levels (F=6.17, P=0.01), parasite total length (F=15.47, P=0.01) and the number of parasites by unit of fish surface (F=52.36, P=0.01) following challenge with *N. girellae*. No specific effect of MOS was found on these parameters (Table 7.2) and no interaction between MOS and cMOS was detected.

7.3.4. Gene expression

At the end of the feeding trial (90 days), two way-ANOVA analyses showed that dietary cMOS up-regulated skin hepcidin, MUC-2, IL-1ß, IL-10 and IgT (Table 7. 3). On the other hand, a down-regulation of skin iNOS gene expression was detected after dietary MOS supplementation, and supplementation with both products resulted in a down-regulation of skin IL-10, IL-17D and IgT and a reduced impact on IFN expression vs the single supplements (Table 7. 3).

In gills, dietary cMOS up-regulated hepcidin, defensin, Mx protein and IFNγ transcript levels (Table 7. 4). No effects of dietary MOS were found. However, supplementation with both products resulted in down-regulation of gill IgT and reduced the cMOS effect on defensin and Mx protein gene expression in gills (Table 7. 4).

Regarding fish posterior gut, two way-ANOVA analysis showed that dietary cMOS up-regulated expression of hepcidin, defensin, IFNγ, IL-10, IgM and IgT. Additionally, dietary MOS up-regulated piscidin, MUC-2, IL-1ß, IL-10, IgM and IgT gene expression. However, supplementation with both products down-regulated IFNγ (F= 1.09, P= 0.02) and IgM (F=2.41, P= 0.02) gene expression and lost the effects on IL-10 and IgT (Table 7. 5).

Table 7.3. *RT-qPCR gene expression in skin of Seriola dumerili juveniles after 90 days on the feeding trial*

DIETARY TREATMENTS					TWO WAY ANOVA		
Gene	**Diet C**	**MOS**	**cMOS**	**MOS+cMOS**	**MOS**	**cMOS**	**MOS*cMOS**
Hep	3.05 ±1.16	3.67 ±1.26	6.11 ±2.25	2.01 ±0.34	NS	P= 0.04, F= 2.13	NS
Pis	507.47 ±184.49	1825.94 ± 992.81	2961.56 ±969.37	3448.44 ±657.29	NS	NS	NS
Def	181.69 ±85.59	422.65 ±179.34	472.89 ±215.85	285.93 ±79.93	NS	NS	NS
iNOS	354.02±132.51	56.34±15.48	514.35±208.57	83.7±31.8	P=0.01, F=9.34	NS	NS
TNFα	10.78±2.50	10.88±0.97	18.65±4.72	8.21±2.67	NS	NS	NS
MX prot	571.15±279.59	362±272.29	805.57±460.93	112.64±38.44	NS	NS	NS
IFN ɣ	31.25 ±5.57	100.13 ±46.91	130.18 ±66.74	41.97 ±16.39	NS	NS	P= 0.01, F= 3.89
IFN d	9.83 ±2.01	29.20 ±10.77	44.04 ±21.61	12.71 ±3.11	NS	NS	P= 0.01, F= 2.35
MUC-2	9.96 ±4.18	8.48 ±2.85	24.74 ±6.08	8.94 ±5.51	NS	P= 0.04, F= 3.27	NS
IL-1β	4.32 ±0.87	4.48 ±1.34	9.52 ±5.36	2.08 ±0.39	NS	P= 0.02, F= 5.52	NS
IL-8	10.50 ±2.73	9.85 ±3.16	21.04 ±2.50	11.99 ±4.48	NS	NS	NS
IL-10	1468.17 ±398.19	1521.55 ±364.18	2724.73 ±812.56	231.37 ±167.44	NS	P= 0.01. F=9.52	P= 0.01, F= 4.81
IL-17F	25.37 ±7.1	13.36 ±2.58	29.66 ±9.83	10.69 ±2.78	NS	NS	NS
IL-17D	6.19 ±1.52	10.65 ±3.77	65.30 ±36.34	4.63 ±1.50	NS	NS	P= 0.01, F= 5.23
IL-22	2.26 ±0.9	4.72 ±3.98	3.29 ±2.57	1.56 ±0.75	NS	NS	NS
IgM	1008.43 ±246.86	1074.15 ±502.02	921.66 ±545.95	2155.97 ±835.60	NS	NS	NS
IgT	5.25 ±0.97	6.32 ±1.40	12.14 ±3.51	3.05 ±0.87	NS	P= 0.04, F= 3.27	P= 0.01, F= 2.23

Diets: C (control diet), MOS (5 g kg^{-1}), cMOS (2 g kg^{-1}), MOS+cMOS (5 g kg^{-1} of MOS and 2 g kg^{-1}of cMOS). Data are presented as means ± SD. N=3 tanks/diet. Two-way ANOVA analyses are presented when p<0.05. NS= Not significant.

Table 7.4. *RT-qPCR gene expression in gills of Seriola dumerili juveniles after 90 days on the feeding trial*

DIETARY TREATMENTS					TWO WAY ANOVA		
Gene	**Diet C**	**MOS**	**cMOS**	**MOS+cMO**	**MOS**	**cMOS**	**MOS*cMOS**
Hep	3.83 ±1.09	8.07 ±3.58	20.74 ±6.40	3.05 ±0.63	NS	P= 0.01, F= 3.22	NS
Pis	123559.34 ±57885.9	49101.56 ±20481.84	145655.85 ±39802.1	220796.87 ±115335.48	NS	NS	NS
Def	154.83 ±57.91	269.99 ±117.38	1538.51 ±560.83	352.82 ±181.47	NS	P= 0.01, F=7.48	P= 0.03, F= 2.59
iNOS	22.57 ±13.59	287.96 ±200.29	591.18 ±261.70	41.52 ±26.58	NS	NS	NS
TNFα	30.65 ±12.60	68.12 ±20.59	91.23 ±32.13	32.95 ±4.90	NS	NS	NS
MX prot	24.50 ±5.55	366.77 ±244.42	1125.22 ±336.94	49.10 ±33.21	NS	P= 0.02, F= 2.27	P= 0.03, F= 4.37
IFN ɣ	57.11 ±9.1	118.43 ±54.38	220.41 ±52.02	69.04 ±18.96	NS	P= 0.03, F= 3.86	NS
IFN d	18.63 ±8.95	43.70 ±19.93	102.95 ±21.84	20.83 ±3.73	NS	NS	NS
MUC-2	1138.31 ±250.05	1149.28 ±633.21	522.40 ±212.30	592.53 ±226.38	NS	NS	NS
IL-1β	12.90 ±6.21	16.20 ±6.23	15.92 ±4.82	5.92 ±1.79	NS	NS	NS
IL-8	17 ±2.92	27.21 ±5.20	74.62 ±28.04	31.26 ±12.15	NS	NS	NS
IL-10	2347.06 ±824.85	2373.74 ±203.84	5078.92 ±2726	4073.95 ±2180.10	NS	NS	NS
IL-17F	7.47 ±3.96	11.69 ±4.62	21.20 ±8.31	4.78 ±1.64	NS	NS	NS
IL-17D	18.19 ±4.07	69.54 ±28.57	63.85 ±15.36	16.28 ±5.56	NS	NS	NS
IL-22	15.51 ±5.79	19.75 ±6.01	21.95 ±4.96	10.18 ±2.65	NS	NS	NS
IgM	65941.75 ±43329.10	16665.56 ±5287.38	1575.48 ±1071.64	144185.46 ±56001.67	NS	NS	NS
IgT	8.72 ±2.17	14.04 ±4.26	20.80 ±5.31	7.98 ±2.12	NS	NS	P= 0.01, F= 9.88

Diets: C (control diet), MOS (5 g kg^{-1}), cMOS (2 g kg^{-1}), MOS+cMOS (5 g kg^{-1} of MOS and 2 g kg^{-1}of cMOS). Data are presented as means ± SD. N=3 tanks/diet. Two-way ANOVA analyses are presented when p<0.05 NS= Not significant.

Table 7.5. *RT-qPCR gene expression in posterior gut of Seriola dumerili juveniles after 90 days on the feeding trial.*

DIETARY TREATMENTS					TWO WAY ANOVA		
Gene	**Diet C**	**MOS**	**cMOS**	**MOS+cMO**	**MOS**	**cMOS**	**MOS*cMOS**
Hep	3.77 ±1.05	9.43 ±0.86	103.78 ±44.61	12.18 ±2.88	NS	P= 0.02, F= 6.23	NS
Pis	18159.99 ±6184.25	70552.71 ±20631.15	16733.78 ±2690.02	100257.10 ±47228.58	P= 0.03, F= 7.35	NS	NS
Def	103.35 ±60.43	526.85 ±272.52	5909.07 ±2592.71	1721.73 ±483.36	NS	P= 0.03, F=2.98	NS
iNOS	352.72 ±56.94	699.99 ±361.29	615.43 ±278.11	1183.26 ±418.29	NS	NS	NS
TNFα	32.27 ±12.53	64.93 ±19.44	108.79 ±43.23	71.82 ±17.04	NS	NS	NS
MX prot	410.19 ±47.28	831.96 ±175.50	955.28 ±867.09	1962.94 ±909.99	NS	NS	NS
IFN ɣ	34.21 ±16.26	427.99 ±193.55	1241.66 ±542.39	284.67 ±41.87	NS	P= 0.04, F= 3.32	P= 0.02, F= 1.09
IFN d	7.49 ±3.48	57.75 ±19.91	81.64 ±30.78	38.93 ±9.07	NS	NS	NS
MUC-2	2800.02 ±511.50	6819 ±1350.56	3375.84 ±993.78	2701.72 ±810.98	P= 0.04, F= 17.72	NS	NS
IL-1β	6.73 ±1.08	66.97 ±28.67	14.06 ±8.09	18.65 ±6.65	P= 0.02, F= 3.52	NS	NS
IL-8	20.75 ±6.67	53.22 ±12.95	191.49 ±99.69	75.67 ±14.98	NS	NS	NS
IL-10	1578.98 ±194.29	9495.37 ±4244.02	107128.07 ±45885.12	17241.35 ±6641.88	P=0.03, F=9.48	P= 0.02, F=2.79	NS
IL-17F	8.53 ±5.53	21.20 ±8.05	11.73 ±4.99	7.20 ±1.49	NS	NS	NS
IL-17D	31.43 ±10.75	71.53 ±15.36	74.54 ±28.76	64.69 ±23.07	NS	NS	P= 0.01, F= 5.23
IL-22	19.61 ±7.73	22.75 ±9.25	13.19 ±3.38	13.42 ±3.26	NS	NS	NS
IgM	73788.21 ±41586.91	$29.2x10^5$ ±$14.2x10^5$	$22.3x10^5$ ±$80.3x10^4$	573173.42 ±319410.84	P= 0.01, F=8.24	P= 0.02, F= 6.14	P= 0.02, F=2.41
IgT	18.47 ±13.64	70.28 ±16.82	56.15 ±19.18	34.52 ±13.48	P= 0.01, F=2.78	P= 0.02, F= 3.11	NS

Diets: C (control diet), MOS (5 g kg^{-1}), cMOS (2 g kg^{-1}), MOS+cMOS (5 g kg^{-1} of MOS and 2 g kg^{-1} of cMOS). Data are presented as means ± SD. N=3tanks/diet. Two-way ANOVA analyses are presented when p<0.05. NS= Not significant.

Table 7.6. *RT-qPCR gene expression in head kidney of Seriola dumerili juveniles after 90 days on the feeding trial*

DIETARY TREATMENTS					TWO WAY ANOVA		
Gene	**Diet C**	**MOS**	**cMOS**	**MOS+cMO**	**MOS**	**cMOS**	**MOS*cMOS**
Hep	8.38 ±3.65	11.90 ±3.86	23.23 ±5.85	31.40 ±6.99	NS	P= 0.01, F= 10.96	NS
Pis	113233.81	107678.83	224992.61±92470.72	102237.60 ±19950.65	NS	NS	NS
Def	±40305.31	±50906.83	2407.93 ±1279.35	1518.66 ±793.53	NS	NS	P= 0.03, F= 2.19
iNOS	187.37 ±71.37	3198.20 ±1666.94	1164.43 ±150.86	4101.22 ±791.51	P= 0.02, F= 8.32	NS	NS
TNFα	1809.69 ±689.22	12437.49 ±2634.66	115.74 ±60	45.05 ±8.84	NS	NS	NS
MX prot	57.46 ±30.26	84.34 ±22.70	1252.17 ±62.92	4399.24 ±1623.50	P= 0.01, F= 8.48	NS	P= 0.03, F= 3.65
IFN ɣ	727.39 ±183.65	10088.19 ±1439.72	294.81 ±213.67	352.18 ±102.91	NS	NS	NS
IFN d	216.77 ±108.53	355.11 ±101.34	62.30 ±15.11	55.16 ±18.84	P= 0.02, F= 4.23	P= 0.02, F= 7.15	NS
MUC-2	29.62 ±6.46	71.28 ±10.82	121.57 ±63.93	62.41 ±9.84	NS	NS	NS
IL-1β	52.81 ±29.24	262.49 ±117.76	22.41 ±9.72	105.56 ±48.61	NS	NS	NS
IL-8	8.05 ±1.99	32.80 ±9.16	3090.39±1025.95	577.80 ±117.01	P=0.02, F=5.28	P= 0.02, F=7.68	P= 0.01, F= 9.51
IL-10	852.98 ±203.37	5077.33 ±2249.74	25.56 ±8.95	14.47 ±3.03	NS	NS	NS
IL-17F	7.22 ±2.41	21.74 ±2.96	15.47 ±2.31	58.98 ±16.09	P=0.04, F=1.67	NS	NS
IL-17D	36 ±18.68	139.82 ±32.31	36.33 ±10.45	24.68 ±8.25	P=0.03, F=4.89	P=0.02, F=9.93	NS
IL-22	4.79 ±0.79	43.93 ±9.44	298249.40±112084.4	99464.83 ±24438.90	NS	NS	NS
IgM	18166.59 ±386.67	44482.19±18652.10	80.64±38.20	62.13 ±15.32	NS	NS	NS
IgT	48.93 ±27.35	60.15 ±32.54					

Diets: C (control diet), MOS (5 g kg^{-1}), cMOS (2 g kg^{-1}), MOS+cMOS (5 g $kg^{-1}$of MOS and 2 g kg^{-1} of cMOS). Data are presented as means ± SD. N=3 tanks/diet. Two-way ANOVA analyses are presented when p<0.05. NS= Not significant.

Table 7.7. *RT-qPCR gene expression in spleen of Seriola dumerili juveniles after 90 days on the feeding trial.*

	DIETARY TREATMENTS				TWO WAY ANOVA		
Gene	Diet C	MOS	cMOS	MOS+cMO	MOS	cMOS	MOS*cMOS
Hep	61.72 ±19.67	64.87 ±13.07	33.35 ±6.53	147.64 ±48.89	NS	P= 0.01, F= 7.39	NS
Pis	165899.47±86928.35	326203.01±92385.94	38108.33 ±27208.51	313455 ±159603.02	P= 0.01, F= 9.95	NS	NS
Def	276.29 ±82.03	1416.65 ±289.94	450.51 ±207.30	2815.82 ±1277.64	P= 0.01, F=8.61	NS	P= 0.01, F=7.35
iNOS	1830.64 ±504.84	2494.56 ±945.27	1089.47 ±407.30	778.71 ±279.99	NS	NS	NS
TNFα	411.98 ±82.99	925.46 ±257.80	203.52 ±63.54	429.05 ±146.70	NS	NS	NS
MX prot	1021.71 ±307.35	2182.75 ±821.69	860.82 ±350.61	439.08 ±155.54	NS	NS	NS
IFN ɣ	405.34 ±107.19	870 ±130.56	300.82 ±62.79	434.63 ±140.21	P= 0.02, F=3.29	NS	NS
IFN d	30.54 ±3.73	75.70 ±21.16	27.95 ±6.56	84.80 ±27.22	NS	NS	NS
MUC-2	18.91 ±5.84	43.14 ±7.19	12 ±1.45	19.54 ±2.35	P= 0.01, F=14.36	NS	NS
IL-1β	23.68 ±5.68	61.37 ±23.18	19.51 ±7.61	44.89 ±16.51	NS	NS	NS
IL-8	2268.78 ±944.39	5478.43 ±2040.92	2305.29 ±1080.20	6791.58 ±2267.62	NS	NS	NS
IL-10	9.75 ±2.30	15.45 ±4.67	3.76 ±0.88	11.38 ±6.08	NS	NS	NS
IL-17F	14.42 ±4.08	40.39 ±12.23	14.12 ±3.22	43.94 ±22.28	P= 0.04, F=1.36	NS	NS
IL-17D	6.15 ±1.53	26.11 ±10.74	6.46 ±1.91	19.35 ±7.50	NS	NS	NS
IL-22	152198.50±42526.77	28665.69 ±6833.63	04560.74 ±35002.44	51173.12 ±15474.28	P= 0.028	NS	NS
IgM	26.17 ±10.84	63.59 ±14.76	16.13 ±3.46	38.16 ±10.21	NS	NS	NS
IgT							

Diets: C (control diet), MOS (5 g kg^{-1}), cMOS (2 g kg^{-1}), MOS+cMOS (5 g kg^{-1} of MOS and 2 g kg^{-1} of cMOS). Data are presented as means ± SD. N=3tanks/diet. Two-way ANOVA analyses are presented when p<0.05. NS= Not significant.

Head kidney gene expression analyses showed that dietary cMOS up-regulated hepcidin, IFNd, IL-10 and IL-22, while MOS up-regulated iNOS, Mx protein, IFNd, IL-10, IL-17D and IL-22. Supplementation with both products resulted in up-regulation of defensin and Mx protein but down-regulated IL-10 transcript levels relative to single supplementation (Table 7. 6). In addition, the effects on IFNd and IL-22 were lost.

Lastly, cMOS down-regulated spleen hepcidin gene expression whilst dietary MOS induced expression of piscidin, defensin, IFNγ, IL-1β and IL-17D in this tissue. Supplementation with both products further increased defensin expression (Table 7. 7).

Multivariant analyses comparing gene expression data presented different responses for each tissue and are presented in figures 7.1 to 7.6. Principal coordinates analysis (PCO) of skin clearly separated responses in fish fed the cMOS diet from fish fed the other dietary treatments, with the main sources of variation due to anti-microbial peptides (AMPs) (piscidin and defensin), MUC-2, iNOS, TNFα, Mx Protein, IL-8, IL-10, IL-17 and IFN genes. PERMANOVA analysis indicated differences in gene expression between MOS and cMOS, with an interaction effect more related to PC1 (p-perm. <0.05) (fig. 7. 1).

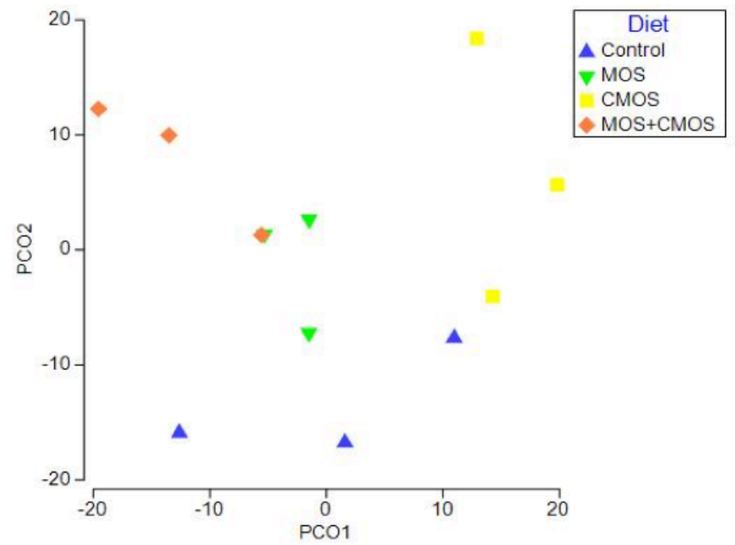

Figure 7.1. *Principal coordinate analysis (PCO) of greater amberjack skin gene expression*

PCO analysis in gill partially separated the MOS and cMOS effects due to AMPs and IFNs. Nonetheless, PERMANOVA analysis showed no difference between MOS and cMOS in this tissue (p-perm. >0.05) (fig. 7.2).

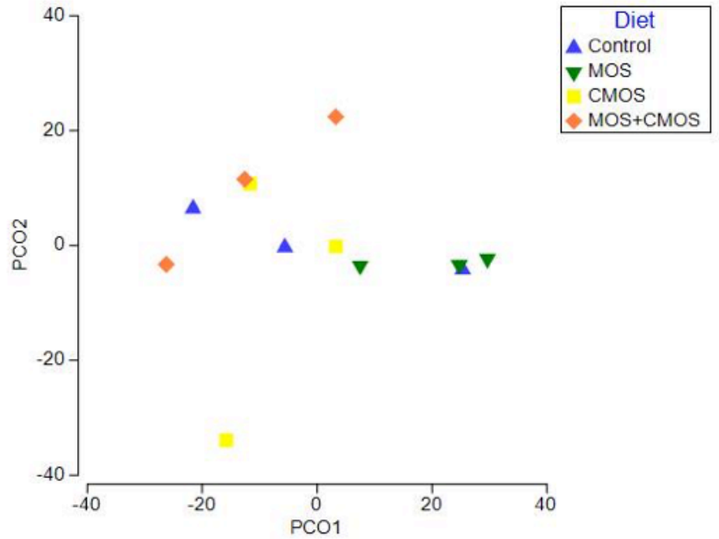

Figure 7.2. *Principal coordinate analysis (PCO) of greater amberjack gills gene expression.*

PCO analysis of posterior gut clearly separated dietary treatments into three different groups: control, MOS and cMOS, and MOS+cMOS. This variation was due to the effect on AMPs, IL-10, IFNs and iNOS gene expression. Hence, the posterior gut PCO PERMANOVA analysis found differences between MOS, cMOS and an interaction effect more related to PC2 (p-perm. <0.05) (Fig. 7. 3).

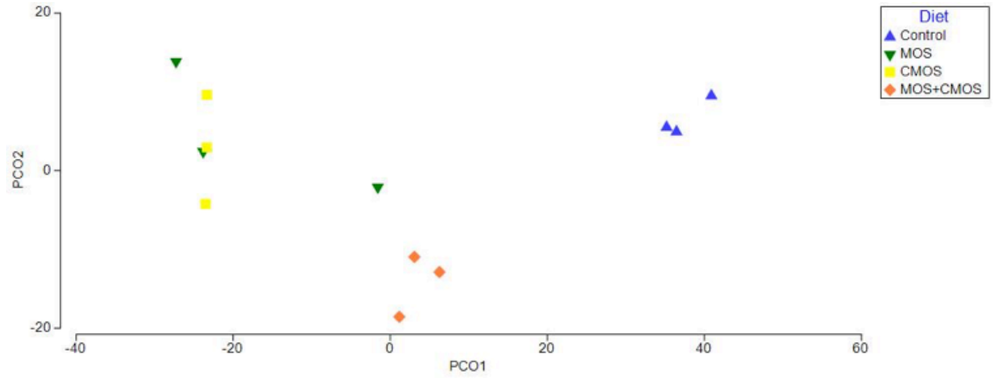

Figure 7.3. *Principal coordinate analysis (PCO) of greater amberjack gills gene expression.*

PCO analysis of head kidney discriminated cMOS from the other treatments due to the effect of this prebiotic on Igs and AMP gene expression. MOS treatment was also differentiated from the other treatments in the spatial distribution by PCO analysis due to effects on IFNs, ILs, defensin and TNFα gene expression. PERMANOVA comparisons showed differences in the MOS and cMOS dietary effects and also on interaction (p-perm. <0.05) (Fig. 7. 4). In spleen

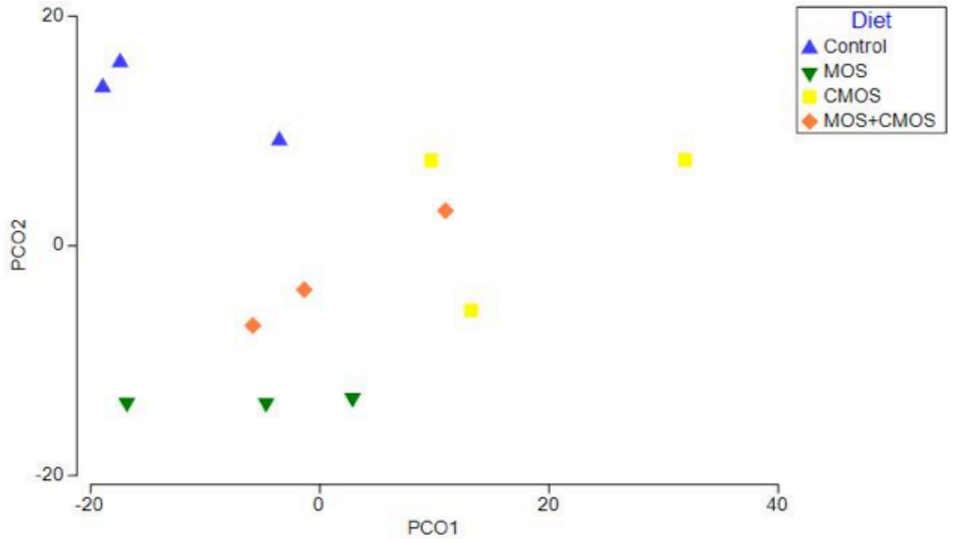

Figure 7.4. *Principal coordinate analysis (PCO) of greater amberjack head kidney gene expression.*

PCO analysis discriminated MOS from the other treatments mainly due to its effect on piscidin and IgM gene expression. PERMANOVA analysis only showed a difference for the MOS treatment (p-perm. <0.05) (Fig. 7. 5).

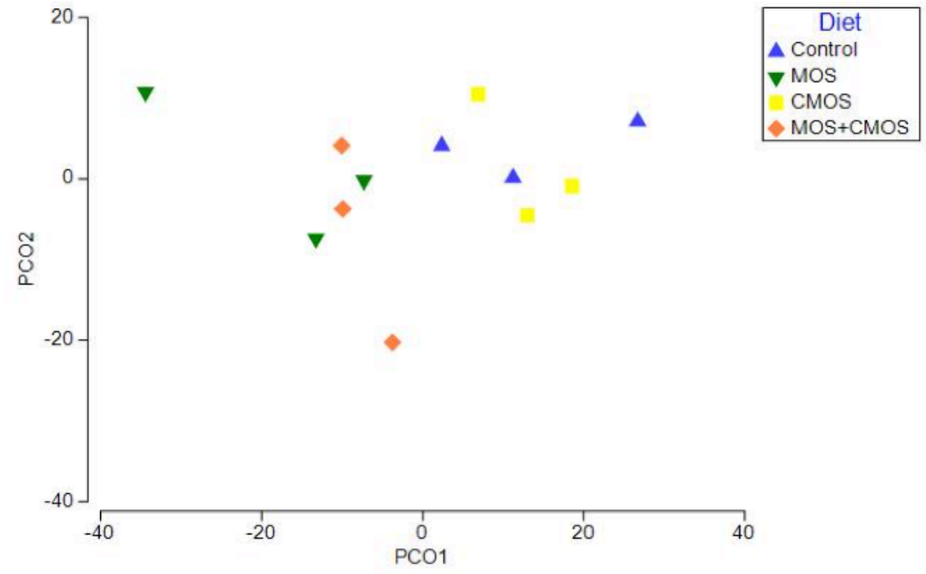

Figure 7.5. *Principal coordinate analysis (PCO) of greater amberjack spleen gene expression.*

Fish fed cMOS were differentiated from other groups in skin and posterior gut, together with MOS in this last tissue, with differences found using PERMANOVA (p-perm. <0.05) in terms of increasing immune parameters compared with control fish. Fish fed dietary MOS showed an up-regulation in immune parameters in spleen and head kidney (p-perm. <0.05), with cMOS responsible for increased Ig levels.

7.4. Discussion

The present study examined the effects of dietary supplementation with MOS and cMOS on greater amberjack growth, immunity and disease resistance. No effects on growth performance were found, in agreement with previous studies on hybrid tilapia (*Oreochromis niloticus x O. aureus) or* channel catfish *(Ictalurus punctatus*) (Genc *et al.,* 2007; Peterson *et al.,* 2010). In contrast, in studies conducted with European sea bass (Dicentrarchus labrax), MOS and cMOS enhanced fish growth performance and improved FCR (Torrecillas *et al.,* 2007, 2011a). Similarly, in fresh water species such as rainbow trout (Oncorhynchus mykiss), MOS dietary inclusion increases growth performance and reduces FCR (Staykov *et al.,* 2007). These effects are likely related with the enhanced nutrient availability due to changes in digestive enzyme activity or in gut morphology, that subsequently increase absorption efficiency (Torrecillas *et al.,* 2014). However, such differences in the impact of MOS on growth parameters among species suggest that these effects are highly dependent on the supplementation level, fish species and age, rearing conditions and diet composition (Sweetman *et al.,* 2010).

An increase in mucus production has been shown to be a key factor for reducing ectoparasite adhesion in fish species such as Atlantic salmon (*Salmo salar*) (Fast, 2014). MOS promotes both the enhancement of the innate immune system and mucus production (for reviews see Sweetmann *et al.,* 2010; Torrecillas *et al.,* 2014; Guerreiro *et al.,* 2017), reducing bacterial and parasite adherence to the host. In the present study, cMOS induced an up-regulation of skin MUC-2 compared with fish fed the other dietary treatments, suggesting it promotes mucus production. Dietary MOS showed a similar effect on the gut, in agreement with previous results in European sea bass (Torrecillas *et al.,* 2011a). Whilst the impact of prebiotics on ectoparasite resistance is poorly studied (Dimitroglou *et al.,* 2011), cMOS showed a clear effect on parasite adhesion in the present work. cMOS not only prevented parasite attachment but also reduced the growth and development of the parasites concomitant with increased immune responses (see below). A mobilization of fish defences to the skin mucus has been described as an effect of prebiotics (Sheikhzadeh *et al.,* 2012), and could prevent the correct development of parasites as they attempt to overcome the first physical and chemical barriers of the host. In line with this, red drum (*Sciaenops ocellatus*) show a reduced mortality and parasite level after challenge with *Amyloodinium ocellatum*, when receiving a diet supplemented with MOS at 10 g kg^{-1} for 30 days (Buentello *et al.,* 2010). Similarly, Atlantic salmon fed for 98 days with 4 g MOS kg^{-1} had a significantly reduced parasite load (Dimitroglou *et al.*, 2011).

MOS has shown a more consistent effect on the immune system, improving parameters such as lysozyme activity in fish species including channel catfish, Japanese flounder (*Paralichthys olivaceus)*, rainbow trout or European sea bass when supplemented at similar doses (Torrecillas *et al.,* 2014). Whilst skin mucus and serum lysozyme activity were unaffected by

dietary MOS in the present study, serum bactericidal activity was increased in fish fed the supplemented diets. This indicates that other molecules within the innate immune system that effect antimicrobial responses are affected by these prebiotics (Ellis, 1999). Indeed, the results of the present study show there is upregulation of antimicrobial peptide (AMP) gene expression in all of the tissue studied, and these molecules are an important part of the innate immune system in fish. AMPs are stored in cells so that they are readily available after an infection (Oren and Shai, 1996; Terova *et al.,* 2009). That MOS mainly increased piscidin whilst cMOS mainly increased hepcidin and defensin is curious. It is known that different cytokines can have unique specificity regarding AMP gene induction (Costa *et al.*, 2011; Hong *et al.,* 2013; Wang et al., 2018) and may be a factor here. The kinetics of AMP induction can also vary, as seen in rainbow trout after dietary inclusion of peptidoglycans (Casadei *et al.,* 2013). Adaptive immunity also plays a key role in the host response against ectoparasites (Raynard *et al.,* 2002; Fast, 2014). IgT is considered a mucosal associated immunoglobulin in fish (Zhang *et al.,* 2010; Salinas *et al.,* 2011; Xu *et al.,* 2013). The increase of IgT transcript levels in skin after feeding cMOS in the present study supports the key role of this immunoglobulin at mucosal surfaces, and could be related with the reduction of the parasite load induced by cMOS. The mode of action of this immunoglobulin is not completely understood, although an up-regulation in IgT expression in skin has been observed as a response to sea lice infection in Atlantic salmon (Tadiso *et al.,* 2011), as well as to parasites in the gills and gut (Zhang *et al.,* 2010; Xu *et al.,* 2016).

Key genes of the immune system have traditionally been selected as markers of immune system activation by prebiotics, including TNFα, IL-1β, IL-8, IL-10, iNOS, IFNs, IgM, TLRs and MHC (Hoseinifar *et al.,* 2015). As discussed above, there is a direct linkage between MOS administration and innate immune system modulation (Torrecillas *et al.,* 2013; Ringø *et al.,* 2014), with the skin a key point of entry of potential pathogens in fish (Zapata and Cooper, 1990). In humans an increase of TNFα expression with no IL-10 response is associated with an increase of mucosal IL-17 (Sfikakis and Petros, 2010; Koch and Nusrat, 2012; Torrecillas *et al.,* 2014), similar to the results obtained in the present study. A balanced pro and anti-inflammatory response in the skin is linked to an increased inflammatory response at the moment of parasite attachment, and gives lower parasite levels in Atlantic salmon infected with sea lice (Poley *et al.,* 2013). Indeed, our PCO analysis showed a higher effect of cMOS in skin, relative to MOS, mainly due to upregulation of AMPs (hepcidin, defensin, piscidin), MUC-2, TNFα, Mx Protein, IL-8, IL-10, IL-17 and IFNs as revealed by PERMANOVA.

In studies of prebiotics, especially MOS, the gut is the main tissue where the effects of the prebiotic take place. Although cMOS induced higher hepcidin, defensin, IFNγ, IL-10, IgM and IgT, the stimulatory effect of MOS was equal to or even higher for IL-10, IgM and IgT and also impacted piscidin, MUC-2 and IL-1β unlike cMOS. This modulation of the expression of these selected genes reveals an increased cytokine response and enhanced mucus production (Rogers, 2001; Torrecillas *et al.,* 2013; Torrecillas *et al.,* 2014). Hence both MOS and cMOS could potentially have positive effects on resistance to gut parasites and this should be explored in future studies.

The impact of dietary MOS was also assessed in head kidney and spleen, two important systemic immune tissues in fish that play a key role in the maturation of B-cells and phagocytic cells (Secombes *et al.,* 2001). The importance of the head kidney and spleen response during parasite infections has been described in many studies where systemic responses help coordinate the fight against secondary infections and participate in the wound healing process (Fast, 2014). Furthermore, upregulation of proinflammatory cytokines such as IL-1β, IL-17 and TNFα in head kidney and spleen has been associated with reductions in sea lice load in pink salmon (Jones *et al.,* 2007, 2008), akin to the results found in spleen in the present study where IL-1β, IFNγ and IL-17D were increased. cMOS is a more purified product than MOS, and some components of the outer cell wall of Saccharomyces cerevisiae strains (probably β-glucans) could have been removed during the production process, as suggested by Torrecillas *et al.* (2015). Since β-glucans are potent PAMPs able to trigger innate immunity (Ringø *et al.,* 2012), this would explain the higher stimulation of innate immune parameters with MOS but not cMOS. On the other hand, B-cell stimulation will lead to increased adaptive immunity, with Ig transcripts notably increased by dietary cMOS in the present study. Indeed, the dispersion patterns seen in the head kidney PCO analysis in the cMOS dietary group were explained by the increased number of Ig transcripts, which separated cMOS from the other dietary groups. Tadiso *et al.* (2011) found that immunological changes in spleen affected the skin response, strengthening the relationship between systemic and mucosal immune responses.

The combination of MOS and cMOS showed similar results to the control diet group for most of the genes analysed. PCO and PERMANOVA analyses typically showed an interaction be-

tween MOS and cMOS, probably related to a loss of effect by overstimulation. It has been reported previously that the combination of two different prebiotics, like MOS and peptidoglycans, can have positive synergic effects in the immune system when suitable doses are used (Yousefian and Amir, 2009). In the case of cMOS, it is a second generation MOS, therefore the pathways of action of these two prebiotics should be similar. Thus, the combination of both prebiotics likely induces effects similar to using a high dietary inclusion of these prebiotics alone, and may result in receptor overload or immune fatigue related to a high energy cost of continued immunostimulation (Sakai, 1999; Moret and Schmid-Hempel, 2000; Solidum *et al.,* 2016).

In conclusion, the utilization of dietary cMOS at 2 g kg^{-1} increased protection against *N. girellae* after 90 days of feeding, by reducing the parasite level and parasite total length. This protection was associated with up-regulation of several proinflammatory cytokines, AMPs, MUC- 2 and IgT genes in skin and enhanced serum bactericidal activity. In contrast, dietary MOS at 5 g kg^{-1} stimulated AMPs, IFNs and proinflammatory cytokines in head kidney and spleen, but had little effect in skin and these fish had a higher parasite level compared with fish fed the cMOS diet. The posterior gut also showed immune stimulation with dietary MOS and cMOS, in terms of effects on expression of AMPs, proinflammatory cytokines, IgM and IgT. However, the combination of MOS and cMOS appears to have delivered an over stimulation of the immune system, resulting in a lack of effect.

CHAPTER 08

IMPROVING GREATER AMBERJACK (*SERIOLA DUMERILI*) DEFENCES AGAINST *NEOBENEDENIA GIRELLAE* INFECTION THROUGH FUNCTIONAL DIETARY ADDITIVES

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Abstract

The main objective of this study was to determine de effect of two feeding additives (cMOS: Actigen®, Alltech Inc, USA) and a phytogenic specifically designed for reducing ectoparasite incidence combining garlic and *labiatae* herbal extracts (HERB; Delacon, Austria) on growth performance, stress and immune response of greater amberjack (*Seriola dumerili)* before and after an experimental parasitization with *Neobenedenia girellae.* Two parallel trials were conducted in order to determine the effect of 70 days of functional diets supplementation (0.2% cMOS and 200ppm HERB) on greater amberjack: a) Growth performance and stress resistance and b) disease resistance against *N. girellae*. Additionally, and in order to evaluate the effects of the dietary supplements pre and post parasitization challenge against *Neobenedenia girellae* ,skin *ef1α, Il-1β, piscidin, hepcidin, IgT, tnf-α, muc-2, cathelicidin, complement c3, cd8, StAR* and *caspase 3* gene expression, skin mucus and serum protease and lysozyme activity were evaluated, as well as parasite load and parasite total size. The use of both functional additives did not affect fish growth performance. However, the dietary supplementation of HERB reduced circulating cortisol levels after the stress challenge, whereas dietary cMOS reduced the parasite density of infection and total parasite size. When comparing pre and post parasitization challenge, skin mucus protease activity was higher in all the treatments before parasitization, while the use of both functional additives increased skin mucus lysozyme activity after challenge. From the eleven immune genes studied, only piscidin was up regulated by HERB supplementation before challenge, whereas after parasitization, *muc-2* and piscidin were upregulated by HERB and *tnf-α, il1-β, hep, c3, cd8* and *casp3* were up regulated by both functional additives. Moreover, *hep, IgT, cath, c3, cd8* and *casp3* expression were negatively correlated with the parasite load.

In general, greater amberjack supplementation with cMOS and HERB at the dose used in the present study is effective for reducing stress and for increasing the SALT immune response against *Neobenedenia girellae* during the infection process.

8.1. Introduction

The study of natural functional ingredients as boosters of the fish health, welfare and growth is a trend in the current aquaculture sector. Among them, prebiotics and phytogenics are promising candidates which has been demonstrated to improve disease resistance in aquatic animals (Torrecillas *et al.,* 2014; Reverter *et al.,* 2014; Guerreiro *et al.,* 2017; Dawood *et al.,* 2018).

Mannan oligosaccharides (MOS) are prebiotics that have been evaluated as dietary supplements in several fish species (For review see: Dimitroglou *et al.,* 2010; Torrecillas *et al.,* 2014; Guerreiro *et al.,* 2017). Some of the recognized effects of dietary MOS are improved growth performance, gut integrity, and boost of the systemic and the mucosal immune system (Ye *et al.,* 2011; Mansour *et al.,* 2012; Anguiano *et al.,* 2013; Torrecillas *et al.,* 2014; Fernández-Montero *et al.,* 2019). However, their beneficial effects are highly dependent on the MOS source, time and dose supplemented, as well as culture conditions or fish species and fish age (Torrecillas *et al.,* 2014). A second concentrated generation of MOS (cMOS), has been described to enhance fish immune system response capacity through the stimulation of gut associated lymphoid tissue (GALT) functioning, modulating its inflammatory response, blocking unfavorable organisms in gut, stimulating intraepithelial lymphocytes (IELs) recruitment, lymphocyte activity and increasing serum immune parameters activity, such as lysozyme and complement (Spring *et al.,* 2015). Furthermore, dietary cMOS supplementation induces changes on the gene expression level of certain European sea bass (*Dicentrarchus labrax*) immune-related genes, particularly by down regulating transforming growth factor b (*TGFb*), while up-regulating immunoglobulin (*Ig*), major histocompatibility complex class II (*MHCII)*, T cell receptor b (*TCRb*) and caspase 3 (*Casp-3*) (Torrecillas *et al.,* 2015), which, in turn, has been also associated to a general innate immune system and other mucosal tissues stimulation in other fish species (Fernández-Montero *et al.,* 2019; Milne *et al.,* 2019).

Additionally, the use of phytogenics has been related with enhanced fish growth performance and survival rates, as well as with a direct antimicrobial effect on some potential aquaculture related pathogens (Immanuel *et al.,* 2004; Reverter *et al.,* 2014), profiling themselves as promising candidates to be used in aquaculture (Dawood *et al.,* 2018). Particularly, in terms of reducing the incidence of parasitic pathologies, extracts of plant origin are less likely to

produce drug resistance in parasites due to the high diversity of plant extract molecules (Olusola *et al.,* 2013; Reverter *et al.*, 2014).

The effects of essential oils and herb extracts supplementation on fish and shellfish disease resistance have been associated with a migration of the product to mucosal tissues and to a direct effect of the active compounds to the pathogen an to their anti-inflammatory role, increasing pathogen resistance by antimicrobial peptides (AMPs) production (Chakraborty and Hancz, 2011). Besides, some essential oils from plant origin have been related with an improved stress response pattern by favoring the homeostatic recovery during the acute and the adaptive phase of cortisol response (Ji *et al.,* 2007, 2009; Souza *et al.,* 2019).

Despite the different pathways of action, both functional additives (cMOS and phytogenics) have shown to improve resistance against ectoparasites in different species, including in Atlantic salmon (*Salmo salar)*, barramundi (*Lates calcarifer*), gilthead seabream (*Sparus aurata*) and greater amberjack (*Seriola dumerili)* (Jensen *et al.,* 2015; Hutson *et al.,* 2012; Militz *et al.,* 2013; Rigos *et al.,* 2016; Fernandez-Montero *et al.,* 2019). In fact, these infections are considered an important bottleneck for some aquaculture species, in particular for greater amberjack due to the difficulties in controlling ectoparasites outbreaks (Shirakashi *et al.*, 2013).

Greater amberjack is considered a new emerging aquaculture species in Mediterranean region due to its fast-growing condition, flesh quality and highly acceptance in worldwide market (Mylonas, 2019). However, the on-growing phase of this species in sea cages faces important ectoparasites infections, especially by monogeneans, such as *Neobenedenia girellae* (Shirakashi *et al.,* 2013). Indeed, the prevalence of this infection in sea farms could reach a 70% of the cultured population (Ogawa *et al.,* 1995), producing important wounds and ulcers in fish (Hirazawa *et al.,* 2016), which are usually associated with skin secondary bacterial infections (Alcaide *et al.,* 2000) and high mortalities. Consequently, strategies for increasing greater amberjack parasite resistance have been focused on chemical treatments (Sharp *et al.,* 2004) or/and breeding selection programs (Ozaki *et al.,* 2013). However, little is known about the effect of dietary concentrated mannan oligosaccharides and phytogenics inclusion in greater amberjack diets, and particularly, its effects on the welfare and the immune response pre and post-parasitization with *N. girellae* in this fish species, being the main objective of the present study.

8.2. Material and methods

This study was conducted at the marine biosecurity station (MBS) of the Scientific and Technologic Park of ECOAQUA university institute of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain). The animal experiments described comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals.

8.2.1. Experimental diets, fish and conditions

Two experimental diets were formulated using a commercial greater amberjack diet as base diet (55% protein, 21% lipids, 55% fish meal and 10% fish oil). The diet cMOS was supplemented with cMOS at 0.2% (Alltech, USA) while the diet HERB was supplemented with a blend of herbal derived essential oils (EOs) based on a mixture of garlic and different *labiateae* plants at 200ppm (Delacon, Austria). The base diet without supplementation was used as a control diet. The experimental diets were fed 3 times per day to greater amberjack juveniles in two different trials: a first trial was conducted to assess the effect of the different diets on growth performance, feed efficiency and stress response (Trial 1), while the second trial determined the effect on immune parameters and expression of related genes before and after a parasitization challenge against *N. girellae.*

8.2.1.1. Trial 1

8.2.1.1.1 Feeding trial

One hundred and eighty greater amberjack juveniles (255.4± 15.5 g) were distributed in nine cylindroconical 500 liters tanks (20 fish per tank, three tanks per treatment), each three tanks in independent recirculating system. Temperature (22ºC ± 0.4), salinity (37.1 mg L^{-1} ±1.2) and oxygen (7.7 mg L^{-1} ± 0.6) were continuously monitored. After being fish acclimated to the new experimental conditions, fish were fed the experimental diets three times per day, 6 days per week until apparent satiation for 70 days. Feed intake was daily recorded, and growth parameters were assessed at 0, 30 and 70 day of feeding. For individual biometry sampling, fish were anesthetized with clove oil diluted 1:1 with 96% ethanol (clove oil, 5mL/L; Guinama S.L; Spain, Ref. Mg83168). Specific growth rate (SGR) and feed efficiency were calculated as follows:

SGR = (ln (final weight) – ln (initial weight)) * 100/days of feeding

Feed efficiency= 1/ (feed intake/ weight gain)

8.2.1.1.2. Stress challenge

After 70 days of feeding, a confinement stress challenge was conducted, which consisted on the confinement of fish in 0.03m^3 submerged cages at a stocking density of 40 kg m^3 (3 animals per cage, 3 cages per dietary treatment and 9 fish per dietary treatment) for 24h. Plasma was obtained from three fish per tank to assess basal cortisol levels (time 0, pre-confinement) and 3h and 24h post-confinement to evaluate the fish acute response and recovery pattern. All samples at each specific sampling point were collected in less than 5 min to avoid changes in stress parameters between individuals.

8.2.1.2. Trial 2

8.2.1.2.1 Feeding trial

Two hundred and twenty-five greater amberjack juveniles (75± 10 g) were distributed in nine cylindroconical 500 liters tanks (25 fish per tank, three tanks per treatment, each three tanks in independent recirculating system). Temperature (22ºC ± 0.8), salinity (37.4 mg L^{-1} ± 1.4) and oxygen (8.1 mg L^{-1} ±0.7) were continuously monitored. Feeding protocol and diets were the same as for trial 1. At the end of the feeding period (70 days), skin samples of three fish per tank were obtained from the dorso-lateral region and conserved in RNAlater (Sigma-Aldrich, Sant Louis, MO, USA) for gene expression studies. Serum and skin mucus samples were collected for immune parameters analyses (3 fish/tank).

8.2.1.2.2 Parasitization challenge

At the end of feeding trial, an experimental infection with the ectoparasite *N. girellae* was conducted following the methodology described by Fernandez-Montero *et al.* (2019). Briefly, *N. girellae* eggs were collected from a parasitized greater amberjack tank in 0.5 mm nets and were placed in a tank with non-parasitized fish. Eggs were collected and introduced in the tank at the same day to guarantee that all fish were parasitized at the same time and during the consecutive following 15 days, all greater amberjacks were checked to be parasitized in the same level. The objective was to obtain a prevalence of the 100%. Parasitized greater amberjacks were then introduced in cages of 0.03 m^3 (3 parasitized fish per cage) and one cage was placed on each triplicate tank from each dietary treatment for 15 days to challenge by cohabitation greater amberjacks after 70 days of functional diets feeding. After that, experimental fish from each tank were subjected to a freshwater bath to release all the attached parasites, which were introduced in 96% ethanol for counting (total number of parasites/ tank) and measuring the parasite total length.

8.2.2. Samplings and analysis

8.2.2.1 Blood collection and sampling preparation

For trial 1, blood was collected from the caudal vein of 3 fishes per tank per sampling point (0, 3, 24 hours) using heparinized syringes and then plasma was obtained by centrifugation at 4ºC for 5 minutes, at 5000 g, and kept at -80°C until analysis. For trial 2, serum was obtained by clotting overnight at 4°C, centrifuged 5000g for 5 minutes and the resultant supernatant conserved at -80°C until analysis. For trial 2, the same protocol was used for obtaining blood and skin mucus of three fish per tank after 70 days of feeding (before parasitization) and after 85 days (after parasitization).

8.2.2.2. Plasma cortisol determination

Plasma from trial 1 was used for cortisol determination by immunoassay (Access Immunoassays system, Cortisol ref: 33600, Beckman Coulter, Inc., USA).

8.2.2.3. Parasite load and measurements

Parasites detached from parasitization challenge after trial 2 were counted and measured for each experimental tank. The number of parasites per fish was calculated assuming the same number of parasites by fish and converted into the number of parasites per square centimeter of fish surface area (mean by tank), calculated following the method described in Ohno *et al.* (2008). Total length of 50 adult parasites per tank was recorded using a profile projector (Mitutoyo, PJ-A3000, Kawasaki, Japan).

8.2.2.4. Gene expression analysis

Total RNA was extracted from trial 2 greater amberjack skin (samples pooled of 3 fish/tank) using TRI Reagent (Sigma-Aldrich, Sant Louis, MO, USA) and RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was quantified with NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and integrity evaluated on a 1.4% agarose gel with Gel Red™ staining (Biotium Inc., Hayward, CA). Total RNA was reverse transcribed in a 20 µL reaction volume containing 2 µg total RNA, using a ThermoScript TM Reverse Transcriptase

(Invitrogen, California, United States) kit, until cDNA was obtained in a thermocycler (Mastercycle ® nexus GSX1, Eppendorf AG, Hamburg, Germany) run according to the manufacturer's instructions. The samples were then diluted 1:10 in miliQ water and stored at -20°C. Selected primers of immune genes were analyzed in iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, California, United States) using *ef1-α* as housekeeping (Milne et al., 2019). All reactions were carried out in a final volume of 20 µl, with 7.5 µl of Brillant SYBR Green QPCR Master Mix (Bio-Rad, Hercules, California, USA), 0.6 µl of each primer (10 mM), 5 µl of cDNA (1:10 dilution) and 1.3 µl of MiliQ water. MiliQ water also replaced cDNA in blank control reactions.

Primers for *ef1α, Il-1β, pis, hep, IgT, tnf-α* and *muc-2* were obtained from Fernandez-Montero *et al.* (2019). However, new primers for greater amberjack were designed, including *cath, c3, cd8, StAR* and *caspase 3,* following the methodology described by Milne *et al.* (2018) from the genome of greater amberjack (Araki *et al.,* 2018). All primers used and melting temperatures are described in Table 8.1

8.2.2.5. Serum and mucus protease and lysozyme activities

Skin mucus of fish from trial 2 was obtained following the methodology described by Guardiola *et al.*(2014) with some modifications described in Fernandez-Montero *et al.* (2019). Lysozyme activity was determined as described by Ellis (1990) and was expressed in units x ml^{-1} considering one unit of lysozyme as the quantity of enzyme needed for reducing absorbance by 0.001 per milliliter and per minute. Samples protein content were determined by the methodology described by Bradford (1976) using BSA as a standard. For protease activity determination, skin mucus samples were diluted 1:1 with a solubilization buffer (urea 8 M, CHAPS 2%, β-mercaptoethanol 10 mM) and azocasein hydrolysis assay was conducted as described by Ross *et al.* (2000). All data was normalized by mg·ml^{-1} of protein and by the protease activity of trypsin.

8.2.2.6. Statistical analysis

Statistical analyses were conducted following the methodology of Sokal and Rolf (1995).

Table 8.1. *Primers used for gene expression analysis by real-time PCR in skin of greater amberjack juveniles (Seriola dumerili) fed cMOS and phytogenic (t = 70-85 days).*
Primers used and melting temperatures are described in Table 8.1

Name	Efficiency (%)	Ann. temp. (°C)*	Forward Sequence	Reverse Sequence
Hepcidin	93.4	61	GATGATGCCGAATCCCGTCAG	CAGAAACCGCAGCCCTTGTTGGC
Piscidin	99.7	58	ATCGTCCTGTTTCTTGTGTTGTC	CGCTGTGGATCATTTTTCCAATGTGAAA
Tumor necrosis factor α	99.1	62	GAAAACGCTTCATGCCTCTC	GTTGGTTTCCGTCCACAGTT
Mucin-2	96.4	62	ATTGAGTTTGGCAACAAACAGAAA	TACAGCACAGAACTGAGGTGTCCTC
Interleukin 1β	99.5	62	TGATGGAGAACATGGTGGA	GTCGACATGGTCAGATGCAC
Immunoglobulin T	98.8	59	TGGACCAGTCGCCATCTGA	GGGAAACGGCTTTGAAAGGA
Elongation factor 1α	99	60	TGCCATACTGCTCACATCGC	ATTACAGCGAAACGACCAAGAGGAG
Cathelicidin	90.6	58	TGAAGATGAAGGTCCAGGT	GAATTGGACGGAAAGCCTGC
Complement C3	99.8	59	ATAGGTGATGTGCGTGGCA	AAGGGTTGATTAGGCTGGGC
Cluster of differentiation 8	90.6	59	AAAATGTCGCCACCACTTC	GTCCTTCATGCTCCAACGGA
Stereidogenic acute regulatory protein	99.9	59	GGAACATGGAGCAAATGGG	ACAAAATCCCTCGGTCCCAC
Caspase 3	98.2	60	GCTCGGCCTCATTCATCTG	AGAGTTTGGGCTTTCCCACC

Means and standard deviations (SD) were calculated for each parameter. All parameters were tested for normality and homoscedasticity.

Data was compared using one-way ANOVA and T-Student, considering significant differences when P<0.05. If the variances were not normally distributed, data were transformed (log10) and the Kruskall-Wallis non-parametric test was applied. Analyses were conducted with SPSS software (SPSS 25 for MAC, New York, US). Gene expression Pearson's correlations and linear regression models were performed with SPSS software (SPSS 25 for MAC, New York, US).

For multivariant analysis, Primer 7 with PERMANOVA package (Massey University, Albany Auckland, New Zealand) was used, considering differences when permutational p- value < 0.05 and number of permutations set in 999.

8.3. Results

8.3.1. Trial 1

8.3.1.2. Growth performance

After 70 days of trial, fish fed the control diet, cMOS or HERB presented similar (p>0.05) final weight (417.89 ±59.34 g, 396.17 ±29.39 g and 397.51 ±36.36 g, respectively) and SGR (0.72 ±0.12, 0.66 ±0.03 and 0.63 ±0.1, respectively). Similarly, no significant differences (p>0.05) were obtained on feed efficiency (control 0.58±0.02, cMOS supplemented diet 0.51 ±0.01 and HERB 0.60 ±0.11).

8.3.1.3. Stress response

Circulating basal plasma cortisol concentration (unstressed) was not affected by the different treatments, with a mean plasma cortisol concentration of 27.8 mg·ml^{-1}. However, 3 hours after the confinement stress, HERB dietary supplementation reduced (p<0.05) plasma cortisol levels compared to fish fed the control diet (Fig. 8.1) . Twenty-four hours after confinement, plasma cortisol values almost returned to basal levels (mean value of 51.18 ng·ml^{-1}) and no differences among fish fed the different experimental diets were found (p>0.05) (Fig. 8.1).

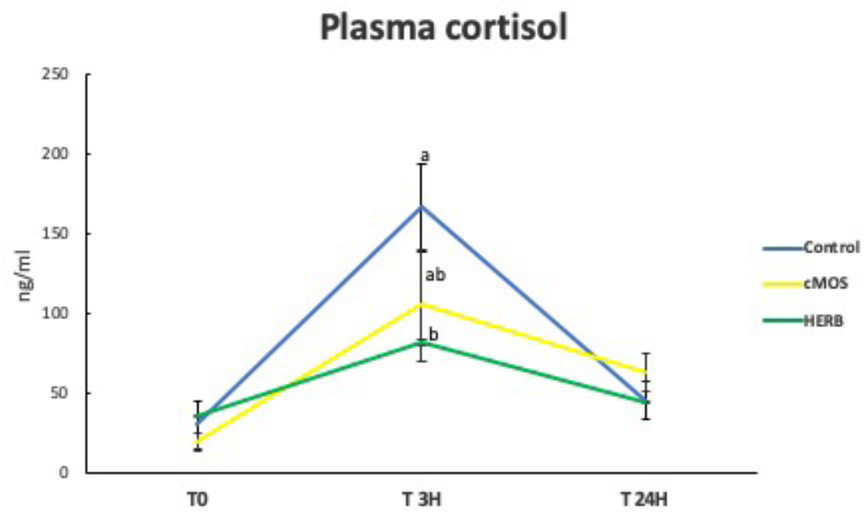

Figure 8.1. *Plasmatic cortisol levels (ng/ml) of greater amberjack (**Seriola dumerili**) under cage-confinement stress challenge at different sampling points (t0= before stress, T 3 and 24 H= 3 and 24 hours after the confinement in cages). Different letters denote significant differences (p<0.05)*

8.3.2. Trial 2

8.3.2.1. Parasite challenge

Greater amberjack fed cMOS diet showed a significant reduction on the number of parasites per unit of fish surface compared to those fed the control diet or the HERB diet (p<0.05) (Table 8.2). Despite no statistical differences were observed in parasitization level between fish fed HERB and control diet, fish fed HERB tended to present lower values than control fish. Similarly, *N.girellae* obtained from fish fed cMOS diet were significantly smaller (p<0.05) than those fed the control diet.

Table 8.2. *Parasitization data of greater amberjack juveniles (Seriola dumerili) after 15 days of co-habitation trial with Neobenedenia girellae at the end of trial 2.*

Parasitization challenge	C	cMOS	HERB
Parasite total length (µm)	920.83 ±52.2^{a}	749.07 ±61.16^{b}	884.98 ±40.58^{ab}
N^{o} parasites / fish surface (cm^{2})	2.41± 0.24^{a}	1.40 ±0.21^{b}	1.99 ±0.29^{a}

Diet C (Control diet, non-supplemented), cMOS (0.2% cMOS supplemented diet), HERB (phytogenic supplemented diet). Values expressed in mean ± SD. Different letters denote significant differences (p<0.05) among dietary treatments. N=3/diet

8.3.2.2. Immunological parameters of skin mucus and serum

No differences (p>0.05) were observed in skin mucus and serum protease and lysozyme activities before parasitization among fish fed the different diets (Table 8.3). However, fish fed HERB and cMOS diet obtained a significantly higher (p<0.05) skin lysozyme activity than fish fed the control fish after parasitization.

In addition, parasitization induced a significant (p<0.05) increase in skin mucus and serum protease activities compared with pre-parasitization values, irrespectively of the diet fed (Table 8.3).

Table 8.3. *Serum and skin mucus immunological parameters (lysozyme activity and protease activity data of greater amberjack juveniles (Seriola dumerili) after pre and post-parasitization challenge.*

		PRE-PARASITIZATION (70 DAYS)			POST-PARASITIZATION (85 DAYS)		
		C	cMOS	HERB	C		
Skin mucus							
Protease activity (% ref. trypsin)		13.34 ±0.79^{A}	16.13 ±2.41^{A}	15.08 ±1.71^{A}	49.09 ±10.27^{B}	38.35 ±5.7^{B}	40.28 ±9.29^{B}
Lysozyme activity (µg/ml)		16.17 ±2.56	12.05 ±1.08^{A}	13.63 ±3.36A	18.41 ±6.6^{a}	29.77 ±3.73^{bB}	33.44 ±6.43^{bB}
Serum							
Protease activity (% ref. trypsin)		10.49 ±1.98^{A}	13.16 ±1.63^{A}	13.21 ±2.03^{A}	17.22 ±1.64^{B}	20.06 ±3.9^{B}	17.32 ±4.84^{B}
Lysozyme activity (µg/ml)		122.01 ±43.54	137.42 ±15.99	116.54 ±37.64	136.92 ±27.2	155.73 ±46.2	169.68 ±39.56

Diet C (control diet, non-supplemented), cMOS (0,2% cMOS supplemented diet), HERB (Phytogenic supplemented diet). Values are expressed in mean ± SD. Lower case letters indicate differences within pre-parasitization or post-parasitization groups (p<0.05). Uppercase letters denote differences between pre-parasitization and post-parasitization groups (p<0.05)

8.3.2.3. Gene expression

At the end of the 70 days feeding trial, and before parasitization, no differences were recorded in the relative expression of the selected immune genes, except for piscidin, fish fed HERB diet showing an up regulation ($p<0.05$) of compared to fish fed the control diet (Table 8.4.). This was also clearly showed by the multivariant PCA analyisis, where PC1 accumulated the 90 % of the total variation in skin gene expression, while PC2 only the 7.6 %, however no significant differences in the dispersion of fish fed the different experimental diets was found by PERMANOVA analysis (p-perm.>0.05) (Fig.8.2).

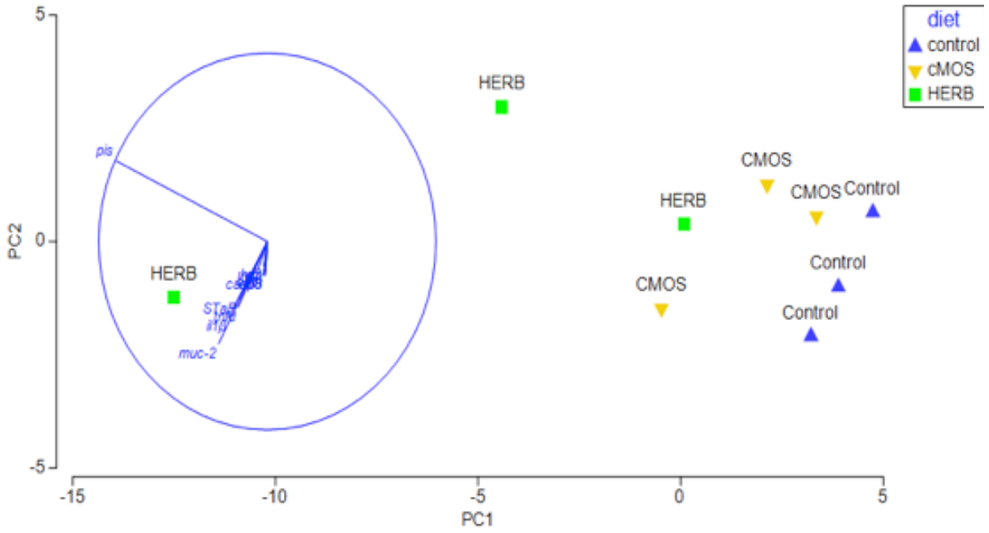

Figure 8.2. *Skin pre-parasitization gene expression principal components analysis (PCA). PERMANOVA analysis showed no significant differences (p>0.05) in diets spatial distribution.*

Table 8.4. *RT-qPCR skin gene expression and correlation with number of parasites per surface unit in skin of Seriola dumerili juveniles after pre and post parasitization*

	Pre-parasitization			Post-parasitization			
	Control	cMOS	HERB	Control	cMOS	HERB	Correlation
muc-2	1.97 ±0.42	2.04 ±0.56	3.89 ±1.37	0.94 ±0.31^{a}	3.84 ±1.10^{ab}	5.81 ±1.9^{b}	P>0.05
tnf-α	1.08 ±0.22	1.17 ±0.37	2.12 ±1.78	0.45 ±0.22^{a}	2.85 ±0.27^{b}	3.08 ±0.91^{b}	P>0.05
Pis	2.34 ±0.12^{a}	4.81 ±1.97^{ab}	11.59 ±3.98^{b}	1.93 ±0.31^{a}	1.02 ±0.07^{a}	4.09 ±0.19^{b}	P>0.05
il1-β	1.11 ±0.29	1.20 ±0.31	1.33 ±0.13	0.33 ±0.04^{a}	2.36 ±0.33^{b}	2.15 ±0.30^{b}	P>0.05
Hep	1.08 ±0.23	1.15 ±0.27	1.15 ±0.13	0.58 ±0.29^{a}	2.32 ±0.27^{b}	1.89 ±0.06^{b}	P=0.02;R=0.74
IgT	1.07 ±0.22	1.03 ±0.17	1.09 ±0.21	0.44 ±0.13^{a}	3.81 ±0.19^{b}	1.93 ±0.21^{c}	P=0.01;R=0.79
cath	1.04 ±0.17	1.16 ±0.42	1.05 ±0.05	0.38 ±0.18^{a}	2.99 ±0.12^{b}	2.10 ±0.15^{b}	P= 0.003 ;R=0.85
c3	1.08 ±0.22	1.14 ±0.29	1.23 ±0.08	0.42 ±0.13^{a}	2.66 ±0.21^{b}	1.97 ±0.43^{b}	P=0.01;R=0.79
cd8	1.17 ±0.40	0.99 ±0.28	1.31 ±0.17	0.48 ±0.14^{a}	3.91 ±0.85^{b}	3.82 ±0.21v	P=0.02;R=0.74
STaR	1.11 ±0.27	1.20 ±0.33	1.18 ±0.21	1.21 ±0.46	1.19 ±0.24	1.24 ±0.13	P>0.05
casp3	1.15 ±0.31	1.13 ±0.34	1.10 ±0.12	3.32 ±0.19^{a}	0.91 ±0.28^{b}	1.61 ±0.54^{b}	P=0.01;R=0.77

Diet C (Control diet, non-supplemented), cMOS (0.2% cMOS supplemented diet), HERB (phytogenic supplemented diet). Values expressed in mean ± SD. Different letters denote significant differences ($p<0.05$). Expression is relative to control diet in pre-parasitization and post-parasitization

Moreover, after parasitization, both functional additives induced a significant ($p<0.05$) up-regulation of *tnf-α*, *il1-β*, *hep, IgT, cath, c3* and *cd8* gene expression. Additionally, fish fed HERB diet presented an up-regulation ($p<0.05$) of *muc-2* compared to fish fed fed the control diet and of *piscidin* compared to the rest of the dietary treatments (Table 8.4). Besides, fish fed cMOS diet presented a significantly higher expression of *IgT* than fish fed HERB diet. In addition, *hep, cath, c3* and *cd8* highest values were obtained by fish fed cMOS. Both dietary functional additives induced a significant ($p<0.05$) down-regulation of *casp3* when compared with fish fed the control diet. The correlation matrix applied showed significant correlations between *hepcidin* (p=0.02;r=0.74), *IgT* (p=0.01; r=0.79), *cathelicidin* (p=0.003; r=0.85), *C3* (p=0.01; r=0.79), *cd8* (p=0.02; r=0.74) and *casp3* (p=0.01; r=0.77) expression levels and the number of parasites per cm^2 (Table 8. 4) found in the present study. These differences were clearly demonstrated by the PCA analysis of skin immune related genes expression, where fish fed cMOS and HERB diets clearly detached from those fed the control diet, and in which PC1 accumulated 68.7% of the total variation and PC2 accumulated 14.8% (Fig.8.3). The correlation circle showed that this variation was mainly influenced by the up regulation of *hep, tnfα, cd3, cd8, cath* and *IgT*. Furthermore, PERMANOVA showed

significant differences in the variation of skin gene expression among fish fed the different diets between control and supplemented diets (p-perm. <0.01).

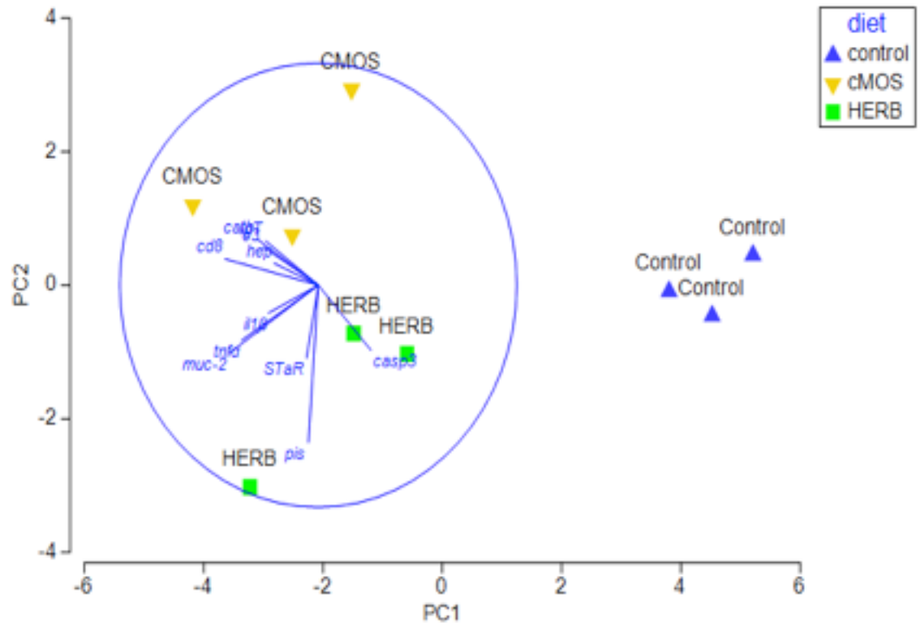

Figure 8.3. *Skin post-parasitization gene expression principal components analysis (PCA). PERMANOVA analysis showed significant (p<0.05) differences in diets spatial distribution.*

Consequently, PCA analysis clustering pre-parasitization and post-parasitization showed pre-parasitization group clearly detached from post-parasitization, showing significant differences in the distribution by PERMANOVA (p-perm. <0.05), where PC1 accumulated 58.6% of the total variations and PC2 accumulated 30.3% (Fig. 8.4). However, this difference between pre and post-parasitization gene expression response seemed to be clearer in those fish fed cMOS or HERB than in those fed the control diet, reflected by the proximity of both pre and post-parasitization control fed fish.

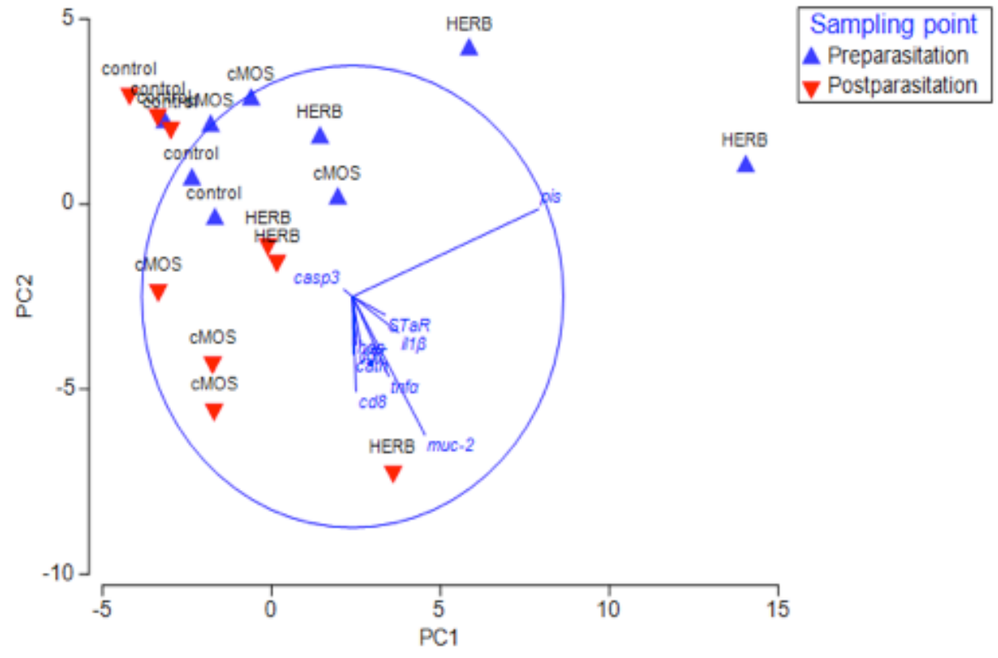

Figure 8.4. *Skin pre and post-parasitization comparison of gene expression principal components analysis (PCA). PERMANOVA analysis showed significant (p<0.05) differences among pre and post-parasitation gene expression spatial distribution*

8.4. Discussion

The dietary supplementation of functional ingredients, either cMOS or phytogenic HERB, did not induce deleterious effects on growth performance of greater amberjack juveniles after 70 days of feeding, in agreement with previous studies on greater amberjack or yellowtail kingfish (*Seriola lalandi)* juveniles fed with 0.2% and 0.1% cMOS in the diet, respectively (Stefanetti, 2016; Fernandez-Montero *et al.,* 2019). The use of both generations of MOS (MOS for first generation and cMOS for the second one) did not induce changes on growth performance in other fish species, including Atlantic salmon (0.4% MOS during 98 days), channel catfish (*Ictalarus punctatus*) (0.2% MOS during 28 days), gilthead seabream (0.2-0.4% MOS during

63 days) or channel catfish (0.1% cMOS during 63 days) (Dimitroglou *et al.,* 2010; Peterson *et al.,* 2010; Dimitroglou *et al.,* 2011; Zhao *et al.,* 2015). In contrast, MOS inclusion in the diet has been also related with a better performance in Atlantic salmon (1-2% during 70 days), Nile tilapia (*Oreochromis niloticus)* (0.4-0.6% during 12 days), Japanese flounder (*Paralichthys olivaceus*) (0.5% during 56 days) or European seabass (*Dicentrarchus labrax*) (0.4-0.6% during 60 days) (Samrongpan *et al.,* 2008; Refstie *et al.,* 2010; Torrecillas *et al.,* 2011; Ye *et al.,* 2011; Torrecillas *et al.,* 2012). Most of those studies have been done using MOS, but less information is available on the use of cMOS, as a concentrated product. For instance, 0.12% of cMOS inclusion in diet significantly increased growth performance of catfish *(Pangasianodon hypopthalmus)* (Hung, 2015), as well as for European sea bass when fed at 0.16% (Torrecillas *et al.,* 2015). These beneficial effects in growth are related with an enhanced nutrient availability due to changes in digestive enzyme activity, in gut morphology or to favored LC-PUFA accumulation and promoted b-oxidation in muscle and liver (Torrecillas *et al.,* 2014; 2015). However, different results obtained in the growth performance among all these studies could be due to fish species, culture conditions, additive source, and/or dose dependent reasons (Torrecillas *et al.,* 2014).

On the other hand, the use of herb extracts seems not to play an important role on promoting fish growth performance (recently reviewed by Dawood *et al.,* 2018), although some studies evidence a positive effect in different fish species, including barramundi (*Lates calcarifer)* (1-2% of *Allinum sativum* during 14 days), Nile tilapia (1-4% of *A. sativum* powder during 14 days), rainbow trout (*Oncorhynchus mykiss*) (0.5-1% of *A. sativum* powder during 58 days) or Japanese flounder (0.3-1% of herbal mix powder during 84 days) (Shalaby *et al.,* 2005; Ji *et al.,* 2007; Nya and Austin 2009; Talpur and Ikhwanuddin, 2012; Hung, 2015).

A proper fish growth performance is many often closely associated to fish welfare (Schreck and Tort, 2016). Circulating plasma cortisol is considered as one of the most important welfare indicators (Scherck and Tort, 2016) and the basal or post-stress plasma concentration may be modulated by changes on the diet (Montero and Izquierdo, 2010). In the present study, the use of functional ingredients did not alter basal plasma cortisol concentration, but the dietary HERB supplementation for 70 days induced a significantly lower post-stress plasma cortisol level (3h), suggesting a modulation of the stress response pattern by the dietary supplementation with this additive. Active compounds extracted from *labiatae* plants, such

as thyme or oregano, have lipophilic properties and liposolubility, contributing to the rapid dispersion through biological membranes, including the blood-brain barrier in the central nervous system (Zahl *et al.,* 2012). Those active compounds have been proposed to exert an effect in the modulation of brain function by regulating the gamma-aminobutyric acid receptor complex (GABA), which, in turn, is associated to a reduction in stress response and an induction of sedative effects (Zahl *et al.,* 2012; Manayi *et al.,* 2016). Other additives, such as garlic have been also reported to have beneficial effects on circulating plasma corticosteroids in other vertebrates. For example, the dietary supplementation of garlic powder (0.2-0.4% of the diet) for 35 days reduced plasma cortisol levels in broilers (Ao *et al.,* 2011). Besides, in the present study the use of functional additives induced a down-regulation of *caspase 3* after parasitization when compared to fish fed the control diet, which maybe related with both, the lower parasite density found and a potential improved skin health status after feeding these functional additives.

The stress response is a modulator factor of fish immune response with a wide variety of immune changes described after applying different kinds of stressors in fish (Tort, 2011). The parasite-associated increase in skin mucus protease activity found in the present study was in agreement with previous studies in Atlantic salmon (Ross *et al.,* 2000), and was not affected by functional diets supplementation. Protease activity in skin mucus is a common indicator of fish response to pathogens, including parasites (Hjelmeland *et al.*, 1983). Thus, some proteases present in fish skin mucus are also related with the activation of innate immune components, such as complement, immunoglobulins or AMPs (Cho *et al.,* 2002).

On the other hand, lysozyme has a direct lytic effect against gram positive bacteria being constitutively secreted in skin mucus for avoiding secondary bacterial infections (Jung*et al.,* 2012). In the present study, no significant differences were observed in serum and skin mucus lysozyme activity after 70 days of HERB and cMOS supplementation, in accordance with previous studies after 120 and 64 days of supplementation with cMOS (0.2% and 0.1%) in greater amberjack and yellowtail kingfish respectively (Stefanetti, 2016; Fernandez-Montero *et al.,* 2019). In contrast, significantly higher lysozyme activity was observed in skin mucus of cMOS and HERB supplemented diets after parasitization. As it has been observed in snakehead (*Channa striata*) after 84 days of MOS 0.2% supplementation and in rohu (*Labeo rohita*) after 60 days of garlic extract 0.1-1% supplementation challenged with *Aeromonas*

hydrophila, both additives increased lysozyme activity after a bacterial challenge, leading in a higher response against the pathogen similarly to the results found in the present study (Sahu *et al.,* 2007; Talpur *et al.,* 2014)

In addition, skin mucus plays a fundamental role against ectoparasite infections as skin physical and immunological barrier. In the present study, dietary cMOS reduced *N. girellae* load and parasite size. These results corroborate those found in our previous study with the same species fed also on 0.2% cMOS for 120 days and challenged against *N. girellae* (Fernandez-Montero *et al.,* 2019). A similar effect on parasite load has been described for Atlantic salmon fed for 98 days with 0.4% MOS (Dimitroglou *et al.,* 2011). Red drum (*Sciaenops ocellatus)* fed 0.1% MOS for 30 days showed a reduced fish mortality and parasitization level after *Amyloodinium ocellatum* challenge (Buentello *et al.,* 2010). Regarding the use of HERB, despite no significant differences were observed in the parasite load and total parasite size, fish fed HERB diet and challenged against parasites showed a tendency to had lower values of parasites per cm^2 of fish surface together with a lower parasite total size. In agreement, other natural extracts and compounds, for instance ginger, *Ulva sp. and Asparagopsis taxiformis* extracts, showed a direct antiparasitic effect *in vitro* on *Neobenedenia sp. (*Hutson *et al.,* 2012; Trasviña-Moreno *et al.,* 2017)*.* In particular, the dietary inclusion of garlic extract (50 ml L^{-1}) induced a reduction of 70% of *Neobenedenia* infection in farmed barramundi *(Lates calcarifer)* after 30 days of feeding (Militz *et al.,* 2013). Similarly, the use of oregano oil 0.02% had a prevention effect on the flagellate *Ichthyobodo salmonis* and the ciliate *Trichodina truttae* ectoparasites infection in chum salmon (*Oncorhynchus keta)* (Mizuno *et al.,* 2018). Active compounds from EOs, for instance thymol or carvacrol are characterized by a high permeability through epithelial cell membrane (Cristani *et al.,* 2007; Di Pasqua *et al.,* 2007), which may contribute to their migration and distribution to skin and mucus, as suggested by Mizuno *et al.* (2018) who detected carvacrol on the skin of cultured juvenile chum salmons fed a diet supplemented with oregano oil 0.02%.

The anti-parasitic effect of prebiotics and phytogenic in diet have been related not only with the direct effect on the parasite, but also with an stimulation of the skin associated lymphoid tissue (SALT), which contributes to generate an hostile microenvironment for parasite development (Esteban 2012; Fernandez-Montero *et al.,* 2019). In the present study, functional additives did not induce differences in the basal relative expression of skin immune-related

genes assayed except for the expression of piscidin. In contrast with our results, a previous study with the juveniles of the same species fed 0.2 % of dietary cMOS for 120 days, reported an up-regulation of a wide set genes related with mucus production, inflammatory process modulation, AMPs and immunoglobulins (Fernández-Montero *et al.,* 2019). The differences between these studies point out the optimal cMOS and HERB supplementation doses and time of supplementation in greater amberjack to produce an immune stimulation, which seems be longer than for other fish species (Torrecillas *et al.,* 2014; Reverter *et al.,* 2014). However, both functional additives induced evident changes on the pattern of the immune skin response against the parasite. Mannan oligosaccharides and phytobiotics have been related with an up-regulation of *tnf*-α in accordance with the present study (Torrecillas *et al.,* 2015; Abu-Elala *et al.,* 2016). Indeed, *tnf*-α is known to respond directly to cellular damage induced by the ectoparasite fixation (Buchmann, 1999) and triggers the inflammatory response (Secombes *et al.,* 2001). Moreover, *tnf*-α also stimulates *il 1*-β production by leukocytes (Secombes *et al.,* 2009), in agreement with the functional additive-associated up-regulation of *il 1*-β after parasitization found in the present study. The up-regulation of *il 1*-β triggers an increase of skin mucus production (Buchmann, 1999) and has been described to occur in rainbow trout, infected by the monogenean *Gyrodactylus salaris* (Lindenstrøm *et al.,* 2006). Increases in skin mucus production are associated with a reduction of parasite fixation and with the appearance of secondary infections (Buchmann, 1999; Esteban, 2012; Saleh *et al.,* 2019). Therefore, during first stages of ectoparasite infections, an enhanced in skin mucus production is commonly observed in parasitized fish (Urawa, 1992, Buchmann, 1992, Vagianou *et al.,* 2006; Jensen *et al.,* 2015). In the present study, HERB diet up-regulated *muc-2* after parasitization, suggesting a higher potential mucus production in those fish in agreement with previous studies using functional additives such as the use of MOS (0.2-0.4 %) or garlic extracts (1.5%), which have shown an increasing effect in skin mucus production in response to parasite infections (Jensen *et al.,* 2015; Ghehdarijani *et al.,* 2016; Fernandez-Montero *et al.,* 2019).

tnf-α also participates in the recruitment of complement and complement participates in the activation of *tnf*-α (Sunyer *et al.,* 2005). The use of dietary MOS (0.2% for 90 days) has been described to increase the complement activity in rainbow trout (Staykov *et al.,* 2007), as well as plant EOs administration (5 days baths at 100 μg ml^{-1}) have been related with an activation of the classical and alternative complement pathway (Sutili *et al.,* 2015). The functional

additive-induced up-regulation of *c3* observed in the present study after parasitization in fish fed the supplemented diets could be related with the response of complement against monogeneans (Buchmann, 1999). Some proteins of the complement system, like C3a and C5a have a direct killing effect on monogenean oncomiracidia by binding C3 to the parasite surface (reviewed in Boshra and Sunyer, 2006).

tnf-α and *il 1*-β modulation is also related with the increase of AMPs (Rajanbabu and Chen, 2011). AMPs have shown antiparasitic activity in fish (Nuñez-Acuña *et al.,* 2018) as well as in humans (Liu *et al.,* 2019). In particular, in Atlantic salmon, hepcidin is up-regulated during sea lice infection, highlighting the importance of the iron-metabolism in ectoparasite infections (Easy and Ross, 2009; Valenzuela-Muñoz *et al.,* 2017). The functional additives act as a reinforcement of those natural defenses against skin parasites, enhancing AMPs levels in skin of parasitized fish, in agreement with previous studies done in greater amberjack and Nile tilapia (Sutili *et al.,* 2018; Fernandez-Montero *et al.,* 2019; Milne *et al.,* 2019).

Although prebiotics and phytogenics seemed to modulate the innate immune system by increasing the expression of different genes after parasite challenge, some effects on the adaptive immune system have also been observed. In the present study, both functional additives induced an up-regulation of *IgT* after parasite challenge. *IgT* is mainly produced on the mucosal tissues (Zhang *et al.,* 2010) and therefore, plays a key role during ectoparasite fixation (Wang *et al.,* 2019). Similarly, other studies focused in dietary prebiotics, such as cMOS (0.2%) or MOS (1%) were associated with an *IgM* and *IgT* up-regulation in greater amberjack (Fernandez-Montero *et al.,* 2019) or giant sturgeon (*Huso huso*) (Ta'ati *et al.,* 2011). The stimulating effect of functional additives on Igs levels has been also described in broilers (IgA and IgM) supplemented during 36 days with thymol and cinnamaldehyde at 0.01% (Li *et al.,* 2012). In addition, in the present study both functional additives up-regulated the expression of *cd8* in the skin of parasitized fish, denoting the effects of these additives on the reinforcement of cytotoxic pathways controlled by T-cells after parasitization. CD8 has been observed to be up-regulated in the skin of grouper (*Ephinephelus coioides*) infected with *Cryptocaryon irritans* (Josepriya *et al.,* 2015).

The strong negative correlation detected among the gene expression of *hep, cath, c3, IgT* and *cd8* and the number of parasites observed in greater amberjack skin surface in the present study highlights the importance of those components of the humoral immune system during *N. girellae* infection, and demonstrate the role of functional additives as anti-parasitic tools by enhancing the skin immune response and subsequently contributing to reduce the parasite load in the skin. The expression of *casp3* showed a positive correlation with parasite load, in opposite manner when compared with the other studied genes. During parasite attachment, skin suffers important wounds that activate pathways related with apoptosis for wound healing, as it has been observed in humans (Rai *et al.,* 2005; Li *et al.,* 2010). Whether the functional additives are directly decreasing the expression of *casp3* or indirectly *via* the reduction of parasite load by the enhancement of skin immune barrier cannot be explained in the present study and specific experiments must be taking into consideration to elucidate the specific role of cMOS and HERB.

In general prebiotics, such as cMOS, present PAMPs (pathogen-associated molecular patterns) that are recognized by immune cells of the GALT (gut associated lymphoid tissue) with specific PRRs (pattern recognition receptors), leading to the activation of the immune response and prepare the organism to fight against pathogens (Ringø *et al.,* 2010; Torrecillas *et al.,* 2014; Hoseinifar *et al.,* 2015). In contrast, the immune stimulation by phytogenics seems to be partially explained by a direct effect of their active compounds which have a potent antimicrobial and a protective effect against oxidative stress as well was modulators of the microbiota populations- (Chakraborty and Hancz, 2011). The supplementation of dietary cMOS at 0.2% or HERB at 200ppm for 70 days were effective in facilitating the immunological response of skin once the parasite was fixed, generating a hostile microenvironment in skin and lowering the parasite load. This protective effect was associated with an up-regulation of some immune genes during the parasite fixation related with inflammation, AMPs, complement, lymphocytes and IgT in the skin. Furthermore, the phytogenic HERB also showed potential to reduce cortisol response during acute stress. Both functional additives can be considered as an effective tool to reduce the consequences of the parasitic infection that greater amberjack suffers in sea cages, as this is considered a highly sensible species to *N. girellae* parasitization.

CHAPTER 09

GENERAL DISCUSSION

9.1.
Greater amberjack: Which are the main bottlenecks for its intensive production?

The greater amberjack (*Seriola dumerili)* can be considered as a fast-growing species with a high commercial value and acceptance by the consumers (Siccuro and Luzzana, 2016). The initial stages of greater amberjack culture started in Asia and Europe in the decade of 80s and were limited to the fattering of wild juveniles harvested or caught for this purpose (Nakada, 2002; Ottolenghi *et al.*, 2004; Lovatelli and Holthus, 2008). However, since the knowledge of greater amberjack reproductive cycle under controlled conditions has increased during the last years (Sarih *et al.*, 2018, 2019), captive breeding and adaptation to controlled conditions of this species has supposed an important step forward in its intensive culture. Notwithstanding the mentioned advances, still exist some bottlenecks that limit an optimal success for this species along the whole life cycle, including reproduction, larval rearing and the on-growing period in sea cages.

The production of high-quality eggs is one of the main limitations to obtain enough amounts of fish for a sustainable and continuous production in farms. The use of proper husbandry conditions, in relation to an adequate tank size, environmental conditions such as rearing temperature, and broodstock nutrition (Sarih *et al.*, 2019) have shown that it is possible to obtain a very high-quality spontaneous spawn in this fish species. Indeed, a GnRHa (Gonadotropin releasing hormone agonist) weekly injections protocol leads to a similar egg viability and hatching rates than spontaneous spawn (Sarih *et al.*, 2018). Moreover, adjusting the nutritional balance of greater amberjack broodstock diets in combination with GnRHa injections have shown improved spawn quality parameters compared to spontaneous spawns (Sarih *et al.*, personal communication).

The elevated mortality during early stages and the high incidence of skeletal anomalies also contribute to limit the production of greater amberjack juveniles. Nowadays still exists a limited knowledge on this fish species larval nutritional requirements, although during the last years several studies have addressed this obejectiva, such as stablishing the recommended n-3 HUFA dietary level during *Artemia* feeding between 12 and 17% of total fatty acids in order to improve skeletal abnormalities, growth and survival (Roo *et al.*, 2019).

Similarly, during the on-growing period several limitations are still noticeable which may be partially reduced by the establishment of optimized culture conditions practices, by a proper determination of this species nutritional requirements and by reducing the incidence of monogenean ectoparasite infections along its production cycle (Ogawa *et al.*, 1995; Hirayama *et al.*, 2009; Repullés-Albelda *et al.*, 2013).

Regarding the establishment of optimized culture conditions for this fish species in terms of greater amberjack production performance, temperature is decisive. An optimum rearing temperature for greater amberjack juveniles has been established at 26°C, similarly to Yellowtail kingfish *(Seriola lalandi)* (Abbink *et al.*, 2012; Fernandez-Montero *et al.*, 2018). Temperature is a key factor in maintaining optimal fish growth and welfare conditions. In consequence, ectoparasites lifecycle is strongly related with seasonal temperature, producing large outbreaks when fish are immunosuppressed after relative long periods of low water temperature, such as occurs in the Mediterranean during winter months (i.e. winter syndrome). Indeed, some ectoparasites, such as *Neobenedenia girellae* or *Zeuxapta seriolae* take advantage of this situation in greater amberjack, producing massive mortalities (Grau *et al.*, 2003; Repullés-Albelda *et al.*, 2013). For other *Seriola* species, as yellowtail kingfish *(Seriola lalandi)*, its experimental and commercial rearing has been adapted to recirculating aquaculture systems (RAS) in order to control rearing conditions and thus, reduce the risk of ectoparasites infection (Abbink *et al.*, 2012).

Similarly, fish culture density is also an important culture condition to determine when rearing a new aquaculture species. The optimal stocking density for pelagic fish has been established in offshore cages at 5 $kg \cdot m^{-3}$ (Mylonas *et al.,* 2019). Particularly for greater amberjack juveniles in Chapter 3 has been determined that fish reared at a low density of 4 kgm^{-3} performed better and recover faster from an acute stress by shallow water than those reared at 8 $kg \cdot m^{-3}$ (which showed a final density after 90 days of rearing of 11.5 $kg \cdot m^{-3)}$, which could have important implications in disease resistance against potential pathogens (Tort, 2011), as could be ectoparasites.

In terms of determining the nutritional requirements of this fish species, although an increasing effort to develop balanced diets for juveniles is being done during the last decades (García-Gómez, 2000; Takakuwa *et al.*, 2006; Mylonas *et al.*, 2019). An inappropriate fee-

ding regime or fasting, could lead to a diminished capacity of reaction of the fish immune system (Chapter 3) and skin mucus production (Landeira-Dabarca *et al.,* 2013), which in turn is related with the appearance of opportunistic pathogens as ectoparasites (Barber, 2007). Furthermore, *N. girellae* supposes an important bottleneck for greater amberjack production worldwide, and an strategy of adaptation of the culture conditions for reducing stressful situations and ectoparasite infestations, potentiation of skin integrity and the systemic and mucosal immune status must be conducted for reducing ectoparasite impact on greater amberjack culture.

9.2.
Among these limitations, which are the consequences of *Neobenedenia girellae* outbreaks in greater amberjack culture? Do they have implications in other associated pathologies?

Due to the still limited aquaculture production of this species, there are no available public data about mortalities and economic cost of *N. girellae* infection to the greater amberjack industry. However, Ogawa *et al.* (1995) highlighted that the mortality associated to *N. girellae* in Japan could reach the 70%.

Similarly, to other ectoparasites, for instance sea lice *(Lepeophtheirus salmonis), Neobenedenia girellae* shows a fixation pattern preference in greater amberjack, being more susceptible the cranial region than the dorso-lateral region (Genna *et al*., 2005; Hirazawa *et al*., 2011), as described in Chapter 4. This difference could be related with the morphological characteristics of the cranial region, since it contains less quantity of goblet cells and lacks imbricated scales, which may affect parasite feeding patterns and fixation as described in Chapter 4.

As described in Chapter 4 the attachment of *N. girellae* to greater amberjack skin induces a mechanical damage characterized by an epithelial cell digestion and detachment. In agreement to what has been proposed for sea lice attachment in Atlantic salmon, monogeneas produce serine-proteases that contribute to destabilize the mucus barrier and digest epithelial cells for feeding (Ross *et al.,* 2000). The higher protease activity in parasited greater amberjack skin mucus with *N. girellae*, suggests a similar role of those proteases during the infection process in this species, as detailed in Chapter 5. Moreover, the selective degradation of skin mucus structural proteins, including keratins, as described in Chapter 5 further support this specific role of the serine-proteases in agreement with previous studies with *N. girellae* (Hirazawa *et al*., 2006). Due to the loss of skin integrity after a severe *N. girellae* infection, it has been suggested that an osmotic disruption could reduce hematocrit and impair hepatic and kidney functions of greater amberjack (Hirazawa *et al*., 2016).

In addition, *N. girellae* fixation produces an overpressure of epidermis, entailing a disruption of cell organization (detailed in Chapter 4) as described for Atlantic salmon after sea lice infection. However the associated cellular hyperplasia as a reaction to a chronic irritation and cellular necrotic process found in Atlantic salmon infested with sea lice (Jones *et al*., 1990; Jones, 2001), was not observed in the present study with *N. girellae* and greater amberjack (Chapter 4).

N. girellae attachment is also related with an increase on the density of mucous cells in the epidermis, as described in Chapter 4. Increases on skin mucus production has been related with a host response to avoid parasite fixation and to trigger a specific immune response against ectoparasites (Paperna, 1991; Fast, 2014; Hirazawa *et al.*,2016; Yokoyama *et al.,* 2019), as could also be observed in the results detailed in Chapter 7. Despite, as described in Chapter 4 and contrary to what occurs in other species infected with ectoparasites, the area of parasite attachment was not characterized by a high inflammatory response, however in selected areas several foci of intraepithelial lymphocytes (IELs) in the epidermis and the dermis were observed. This weak immune response is in agreement with the low quantity of immune related proteins found in infected greater amberjack skin mucus (Chapter 5). Besides, the immune gene expression pattern observed in the skin of parasited greater amberjack juveniles after 15 days of infection (Chapter 8) suggest an acute inflammatory response in skin against the parasite attachment, which is reduced in long term infections, being particularly *hepcidin* and *piscidin, IgT, c3* and *cd8* gene expression especially upregulated during *N. girellae* fixation.

Consequently, physical injuries caused by *N. girellae* infection could be favoring the appearance of secondary bacterial infections caused by opportunistic pathogens, which are related with high mortalities on this species (Whittington, 2011). Moreover, the susceptibility of greater amberjack to bacterial pathogens has already been observed (Alcaide *et al*., 2000), especially to *Vibrio sp* during *N. girellae* infections (Hirazawa *et al.,* 2016; Fernandez-Montero personal observation).

Thus, to maintain greater amberjack skin epithelial integrity and to potentiate a proper immune response against ectoparasite infections seems to be decisive in terms of achieving a proper development of this species culture.

9.3.
Are also culture conditions and the subsequent fish welfare status decisive for reducing greater amberjack *N. girellae* infection?

Culture conditions, fish welfare and pathogen infections are closely related concepts that must be taken into account for a successful development of the aquaculture sector, especially when dealing with a relatively new emerging species. As it has been previously mentioned, rearing temperature in greater amberjack is an important factor related with the appearance of *N. girellae* outbreaks (Hirazawa *et al.,* 2010). However, as described in Chapter 3 the long-term adaption to temperature within the biological range of this species did not affect plasma cortisol (~7.5 ng·ml^{-1}) (Fernandez-Montero *et al.*, 2018). As it has been previously mentioned, Mylonas (2019) established the optimal culture density for pelagic species as greater amberjack in 5 kg·m^{-3}. Accordingly, the results obtained in Chapter 3 showed that the stocking density must be lower than 11.5 kg·m^{-3} to avoid deleterious effects on growth performance and to increase basal plasma cortisol levels, since density is considered a chronic stressor in fish (Montero *et al.*, 1999), which could be related with immune depletion of fish defenses (Tort *et al.,* 2011). Additionally, high stocking densities have been related with higher *Neobenedenia sp.* parasitosis in other species as cobia (*Rachycentron canadum*), Nile tilapia (*Oreochromis niloticus*), red hybrid tilapia (*O. mossambicus* x *O. niloticus)* and ornamental aquarium fish (Robinson *et al.*, 1992; Bassleer, 2000; Nowak, 2007; Kerber *et al.*, 2011; Whittington, 2011).

Greater amberjack seems to have a good capacity to adapt to standardized handling protocols, without affecting growth or stress parameters as detailed in Chapter 3, contrary to that reported for other pelagic species like Pacific bluefin tuna (*Thunnus orientalis*), which has a high susceptibility to handling procedures (Tsuda *et al.,* 2012), and therefore, increasing its susceptibility to *N. girellae* outbreaks.

To follow an adequate feeding protocol is of high importance especially for fast-growing species. A short-term fasting to greater amberjack juveniles induced a slower recovery of basal cortisol levels after shallow-water stress, as described in Chapter 3. Moreover, fasting reduced the responsiveness of greater amberjack skin mucus production in terms of the expression of *muc-2* gene, being skin mucus production a key point for the reduction of ectoparasite fixation in fish (Buchmann, 1999; Buchmann and Lindenstrøm, 2002; Esteban, 2012). Short-term fasting has been related with alterations on skin mucus glycoconjugates composition patterns and lectins related with ectoparasite defense (Buchmann and Lindenstrøm, 2002; Landeira-Dabarca *et al.*, 2013). Moreover, fasting is a factor affecting stress response in terms of circulating cortisol levels (Davis and Gaylord, 2011).

9.4.
Is it possible to reduce *N. girellae* incidence through the use of functional diets? Which mechanisms are implicated?

Thus, to properly increase the resistance to parasitic infection in greater amberjack, it is necessary both, to reinforce the skin physical barrier and to improve the general immune status of the animal. Besides, and as described in question 3, it is decisive also to maintain fish welfare through an optimization of the husbandry practices or/and using other complementary tools such as functional diets.

The use of functional diets to reduce ectoparasite infections have been studied in depth in salmonids because of the high costs that sea lice and amoebic gill disease are producing in aquaculture industry (Bridle *et al.*, 2005; Costello, 2009; Provan *et al.,* 2013; Jensen *et al.,* 2015). Furthermore, supplementation with functional diets for fast-growing species such as meagre or other *Seriola* species has been demonstrated to improve health, welfare and parasite resistance (Mata *et al.,* 2017; Peixoto *et al.,* 2017). The use of functional diets to improve greater amberjack health has been previously studied by the addition of nucleosides and inosine (Hossain *et al.,* 2017a,b), in contrast, no information about functional diets, SALT and ectoparasite resistance in greater amberjack has been conducted before (Chapters 7 and 8). In general, prebiotics by-products, such as mannan oligosaccharides (MOS) and concentrated mannan oligosaccharides (cMOS), present PAMPs (pathogen-associated molecular patterns), that are recognized by immune cells of the GALT (gut associated lymphoid tissue) with specific PRRs (pattern recognition receptors), leading to the activation of

the immune response and preparing the organism to fight against pathogens (Ringø *et al.,* 2010; Torrecillas *et al.*, 2014; Hoseinifar *et al.,* 2015), as described for greater amberjack in Chapter 7. In contrast, the immune stimulation by other type of functional ingredients, as phytogenics, is strongly dependent of the composition and dosage of the active components, which most of the times are related with an oxidative stress protective role along with microbiota modifications (Chakraborty and Hancz, 2011), as described in Chapter 8.

However, the antimicrobial effect of phytogenics is also related with the capacity of diffusion of active components of the essential oils through fish skin, modifying bacterial membranes (Chakraorty and Hancz, 2011; Nazzaro *et al.,* 2013). The effects of functional feeds are different depending of the type of ingredient used, the strategy (time of supplementation and/or use of blends of functional ingredients), each of those functional ingredients acting in different manner, as discussed in Chapters 6, 7 and 8. Despite of the enhancement effect of some immune parameters in other species after dietary supplementation of those additives, including serum and skin mucus lysozyme, no effects were obtained on basal levels of those immune parameters after 70 and 120 days of the phytogenic, MOS or cMOS supplementation in greater amberjack (Chapters 7 and 8). However, as described in Chapter 7, dietary MOS at 0.5% up regulated AMPs, IFNs and proinflammatory cytokines gene expression in head kidney and spleen, pointing to a direct effect on the systemic immune potential of greater amberjack. However, MOS seems to have less potential as functional ingredient improving local immunity of skin (Chapter 7), which could be related with gut stimulation role of MOS and systemic immunity. Indeed, the beneficial effects of the use of MOS (0.5%) as a dietary functional ingredient did not improve the resistance to ectoparasite infection as no effects on *N. girellae* load were found in Chapter 7. Moreover, the strategy to use a combination of MOS and cMOS (0.5 and 0.1% respectively) appeared to be non-effective as it resulted in a general over-stimulation of the immune system, with a discrete reduction in parasite load. However, MOS and cMOS dietary supplementation for 30 days up-regulated AMPs gene expression, including piscidin, in spleen, gills, head kidney and gut (Chapter 6), which could be suggesting also an administration dose/time dependent effect. Piscidins are AMPs exclusively from teleost and are less studied than other vertebrates AMPs despite of its relationship with ectoparasite infections (Colorni *et al.,* 2008; Niu *et al.,* 2013). However, as detailed in Chapter 6, greater amberjack skin *piscidin* was up-regulated when an ***in vivo*** PAMPs inoculation was conducted. In addition, synthetic greater amberjack piscidin showed an *in vitro* bacteriostatic effect. In addition, the dietary supplementation of cMOS at 0.2%

had a positive effect on the immunity of greater amberjack by a direct effect not only in intestine but also on other mucosal tissues including skin, as described in Chapters 6, 7 and 8. An up-regulation of several proinflammatory cytokines, AMPs, *muc-2* and *IgT* related genes in skin and enhanced serum bactericidal activity was detected after the supplementation of cMOS as described in Chapters 7 and 8. This general up-regulation by cMOS supplementation of immune-related genes in skin seems to generate a hostile microenvironment for *N. girellae,* reducing its growth and attachment capacity, triggering SALT response against the parasite once fixed by up-regulating genes related with inflammation, AMPs, complement system, cellular signaling and IgT, and thus reducing the parasite load and the parasite size (Chapter 8). Those effects seemed to be dependent on the time of additive administration, being more effective when cMOS is fed for 120 days (chapter 6) compared to 70 days of supplementation (Chapter 8).

The dietary supplementation of the phytogenic blend HERB at 200ppm for 70 days induced a higher basal expression of *piscidin* and this functional ingredient seemed to be also effective after *N. girellae* infection since, as detected with cMOS an up-regulation of genes related with mucus production, AMPs, inflammation, complement system, cellular signaling and IgT was observed after parasite challenge (Chapter 8), denoting an stimulation of SALT potential of reaction by dietary HERB, which induced a a trend to a lower parasite load in the skin. The difference found in parasite load between cMOS and HERB in spite of the immune stimulation induced by both products could be linked with a higher up-regulation of *hepcidin, cathelicidin, IgT, c3* and *cd8*, of those fish fed the cMOS diets, which is highly correlated with the reduction of the parasite load, highlighting the importance of those genes within parasite infections as detailed in Chapter 8. Moreover, further studies must be conducted to determine other factors affecting *N. girellae* fixation and development in greater amberjack skin, as a nutritional modification of glycoproteins in skin mucus or the regulation of the production and release of antiparasitic peptides (APPs) to skin mucus.

The poor response against *N. girellae* infection showed by the greater amberjack fed the non-supplemented diet could be related with a natural low response against this type of parasites. The immune response involves a high energy expenditure as it has been observed for other vertebrates (Martin *et al.,* 2003), and therefore requires using resources that could otherwise be allocated to other physiological processes, like growth performance. Additionally, fast growing species have been related with higher prevalence, parasite load

and bigger total size parasite than non-fast growing species (Barber, 2005. Thus, is possible that greater amberjack has generated an immunological evolutionary tolerance to the parasite which denotes the importance of functional diets supplementation to trigger an immune response that helps to overcome ectoparasites, bacterial or secondary infections when cultured in intensive conditions.

9.5.
Are there any effective tools to ameliorate the problems associated to greater amberjack aquaculture in an offshore culture system?

Nowadays, the commercial production of greater amberjack in offshore cages in Europe is not possible without the use of hazardous and expensive chemical treatments, such as hydrogen peroxide baths in order to avoid or treat monogeneans infections. Some of these monogeneans including *N. girellae,* present a global distribution around warm waters and have a wide range of potential species to parasitize, including the whole genus *Seriola,* causing a direct impact on seriola production. However, in other countries like Japan or Australia, greater amberjack (*Seriola dumerili*), kingfish yellowtail (*Seriola lalandi*) and Japanese amberjack *(Seriola quinqueradiatta)* commercial production is being produced mainly because of oral supplementation with praziquantel, an antihelminth that is forbidden in Europe for animal production (Williams *et al*., 2007; Hirazawa *et al.,* 2013). Besides, despite of the existence of studies for obtaining effective vaccines against sea lice (Raynard *et al*., 2002), no studies have still been conducted for developing vaccines against *N. girellae*. Thus, in an European context, other strategies must be taken into account, focusing on those based on culture conditions, functional feeds (Chapters 6, 7 and 8), and of course, the disruption of the parasite lifecycle, that should be studied in depth for commercial applications.

In this sense, *N. girellae* lifecycle has shown to be highly dependent of photoperiod and sunlight for the oncomiracidia to find its host (Shirakashi *et al*., 2013b). In fact, monogeneans have piriform eggs with long filaments to get entangled in nets (Shirakashi and Hirano, 2015). When eggs hatch, oncomiracidia gets attracted to the surface by the light intensity and quality (Ishida *et al*., 2007). Therefore, the use of classical non-submergible sea cages highly exposed to sunlight further facilitates oncomiracidia host attachment. Some studies have been conducted with greater amberjack and submerged cages obtaining a reduction

of 95% of the parasite load (Shirakashi *et al.,* 2013a). In addition, monogenean eggs are part of the fouling of net cages, which are traditionally made from nylon. Copper nets have been used successfully in the Atlantic salmon industry in Chile for reducing fouling in nets (Braithwait *et al.,* 2007). Accordingly, reductions of a 99% of *N. girellae* eggs have been detected when using copper nets in almaco jack (*Seriola rivoliana*) culture in sea cages based in Hawaii (Lowell, 2012).

Other strategies such as the use of cleaner fish in polyculture strategies have been studied for removing ectoparasites of intensive cultures, mirroring the reefs ecological strategies. However, the selected cleaner fish must share the temperature range with the cultured species and do not induce negative effects in performance or act as a vector of other pathologies. Ballan wrasse *(Labrus bergylta)* and lumpsucker *(Cyclopterus lumpus)* have been successfully used by Atlantic salmon aquaculture industry in Norway and seems to reduce sea lice infections, without affecting Atlantic salmon growth (Gonzalez and de Boer, 2017; Imsland *et al.,* 2018), but to the date no studies have been conducted with greater amberjack cultured together with a cleaner fish. Due to the cleaning behavior that amberjacks show in the wild approaching to reefs, the selection of a local labrid species as cleaners for intensive greater amberjack culture could be a solution, or at least could help to reduce the load of monogenean infection, and further research must be conducted.

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Some strategies have been applied for other new and fast-growing species as southern bluefin tuna (*Thunnus maccoyii)* in Australia in order to reduce parasites as *Caligus sp* and *Cardicola forsteri*. For these parasites, the presence of an intermediate host and fouling is important to complete its lifecycle. Thus, temporary translocation of cages 25 nautical miles offshore reduced the loads of sea lice and blood flukes (100% and 90% respectively), but also increased fish performance and health status due to the increased oxygen level and water current (Kirchhoff *et al.,* 2011; Sitjá-Bobadilla and Oidtmann, 2017). Similarly, *N. girellae* eggs are transported with shore currents and get entangled to the nets and fouling, reducing this risk if sea cages are disposed far away from coast.

Within the present study, the effectiveness of functional diets together with proper husbandry protocols has been demonstrated. The use of functional feeds in greater amberjack for reducing *N. girellae* infection has been discussed in depth in chapters 6 and 8. However, those only are preliminary studies that represent the first step to develop functional dietary

supplementation strategies for this species. The first, subsequent step and as previously discussed (chapter 8), must be to stablish the optimal dose/time of dietary cMOS or HERB supplementation and to study if higher concentrations follow a dose dependent pattern in terms of boosting the immune system and reducing the parasite load. As it has been observed that *N. girellae* outbreaks increase in spring and autumn due to changes in the photoperiod and the water temperature, to design feeding strategies for supplementing greater amberjacks prior to these periods could be interesting in terms of reducing parasite load and secondary infections at the most critical seasons.

Besides, in terms of fish welfare, the supplementation with the phytogenic HERB has been proved to be effective to reduce cortisol levels of greater amberjack during the acute response phase, pointing out the importance of this functional ingredient when preparing the fish for transport, parcel out fish stock or net changes to reduce stress and mortalities associated to this process. As described in chapters 3 and 8, a modulation of the stress response leads to a reduction in mortality associated to the routine-associated stressors in aquaculture facilities. Additionally, as it has previously mentioned, during chronic stress fish are more susceptible to bacterial disease outbreaks in relation to an immunosuppression fish status (Snieszko, 1974; Tort, 2011). Moreover, greater amberjack has shown to be especially susceptible to *Photobacterium damselae* and *Vibrio sp*. in accordance with their opportunistic status.

Studies about *N. girellae* proteases have been important for understanding the first steps of the parasite adhesion to the host and its effect in skin mucus, as detailed in chapter 5. As proposed by other authors, the use of recombinant proteins from cathepsin-L protease of *N. girellae* could lead in the development of a specific product for inhibiting these proteases and the fixation of the parasite (Rao and Yang, 2007). As part of the new bioengineering solutions, other strategies, such as the development early and accurate detection tools by qPCR could be interesting for evaluating the presence of oncomiracidia in farms in order to start with the parasite control protocol (Agawa *et al.,* 2016).

Other strategies that have reduced the presence of ectoparasites in amberjacks are the specific breeding selection programs. Indeed, specific quantitative trait loci (QTLs) related with growth performance and with resistance to the monogenean *Benedenia seriolae* have been detected in Japanese amberjack (Ozaki *et al.,* 2013), but more extended studies must be conducted to check the effectiveness of this strategies in the greater amberjack.

Although there are several options for reducing *N. girellae* infection rates in greater amberjack intensive culture without resorting to chemical treatments, the solution is probably not restricted to the use of one single method. Therefore, the combination of the proposed solutions, for instance the use of submerged cages with non-hazardous anti-fouling nets, along with supplementation strategies of functional ingredients applied during specific periods in order to reduce stress and immunomodulate greater amberjack, altogether within a proper a breeding selection program could be part of the solution for the high susceptibility of greater amberjack to monogenean outbreaks.

CHAPTER 10

CONCLUSIONS

1. The stocking density for greater amberjack juveniles must be lower than 11.5 kg·m^{-3} to keep the growth performance and to improve the recovery after a stressful situation.
2. 7.5 ng·ml^{-1} can be considered as the basal plasma cortisol concentration for greater amberjack juveniles, being circulating plasma cortisol correlated to mucus cortisol concentration under the different culture conditions assayed.
3. A short-term starvation induces deleterious effects on skin mucin production and circulating cortisol after a stressful situation.
4. *The fixation of Neobenedenia girellae* induces epidermis thickening, increase of goblet cells density, and alteration of the *stratum superficiale* structure, favoring the presence of hydropic degeneration and leucocyte-type cells foci in *stratum basale*.
5. The *Neobenedenia girellae* infection produces an increase of the number of cytoskeletal proteins in skin mucus, whereas skin mucus microbiome profile is not altered at a taxonomic level of genus.
6. *Neobenedenia girellae* degrades greater amberjack skin mucus structural proteins, mainly keratins, and increases the activities of mucus metallo- and serine-proteases.
7. The dietary cMOS at 2 g·kg^{-1} during 90 days reduces the incidence of *Neobenedenia girellae* in relation with an up-regulation of biomarkers in skin, including proinflammatory cytokines, *hepcidin, muc-2* and *IgT* gene expression.
8. The dietary MOS at 5 g·kg^{-1} during 90 days up-regulates the expression of selected biomarkers in head kidney, spleen and posterior gut, including piscidin and proinflammatory cytokines genes, with no remarkable effects on other mucosal tissues such as skin or gill.
9. The booster capacity of the immune system induced by the single dietary use of either cMOS or MOS is limited by the combination of these two functional additives in greater amberjack diets.
10. Dietary administration of cMOS reduces *Neobenedenia girellae* load correlated with an up-regulation of hepcidin, IgT, cathelicidin, cd8 and c3 gene expression in skin, validating them as the best biomarkers for greater amberjack against *N. girellae* infection.
11. The dietary supplementation of HERB at 200 ppm during 70 days ameliorates the acute stress response of greater amberjack after stress in terms of concentration of plasma cortisol.
12. HERB and cMOS boost the SALT immune response of greater amberjack against *Neobenedenia girellae* experimental infection, through the up-regulation of AMPs, ***muc-2***, *IgT*, and pro-inflammatory cytokines genes.

CHAPTER 11

RESUMEN EN ESPAÑOL

Hoy en día la acuicultura está considerada como uno de los sectores de producción animal con mayor crecimiento. Así mismo, la acuicultura se desarrolla siguiendo las demandas de los consumidores y de un mercado creciente mientras que se adapta a las circunstancias climáticas, tratando de realizarse de manera sostenible y en armonía con la naturaleza y la sociedad. No obstante, la acuicultura en Europa precisa aumentar y diversificar las especies cultivadas debido al reducido número disponible para los consumidores. Por ello es necesario encontrar nuevas especies candidatas para la industria de la acuicultura europea y realizar estudios para mejorar el cultivo, bienestar, cuidado y manejo de dichas especies seleccionadas.

El medregal *(Seriola dumerili)* esta considerado como uno de los mejores candidatos para incrementar el número de especies cultivadas en Europa debido a su condición de especie de rápido crecimiento, calidad de su filete y su gran aceptación en el mercado local y mundial. No obstante su cultivo pasa por un importante cuello de botella durante su fase de engorde relacionado con infecciones por ectoparásitos, como *Neobenedenia girellae,* considerada la infección mas frecuente en esta especie. Además, la infección de este ectoparásito suele venir acompañada de infecciones secundarias por patógenos oportunistas, que consecuentemente acaban produciendo grandes perdidas en la producción asociadas a una gran mortalidad.

De esta manera, el objetivo de la presente tesis es investigar los efectos de la infección de *N. girellae* y su relación con la capacidad para resistir esta infección por parte de juveniles de medregal mediante el uso de dietas funcionales como herramienta para incrementar sus condiciones de salud, resistencia a estrés y reducir la incidencia de este parásito.
Por este motivo, se propusieron los siguientes objetivos específicos: i) evaluar la respuesta a estrés del medregal para afrontar variaciones en diferentes condiciones de cultivo (Capítulo 3); ii) determinar los efectos de la infección por *N. girellae* en las características estructurales de la piel (Capítulo 4) y en el perfil de las proteínas del mucus de la piel (Capítulo 5) como primeras barreras del medregal; iii) evaluar el potencial de determinados ingredientes funcionales: a) para potenciar el sistema inmune e incrementar la respuesta a estrés a través de la validación de biomarcadores específicos y b) mejorar la resistencia del medregal a las infecciones por *N. girellae* (Capítulos 6, 7 y 8).

El ciclo de vida e infección de *N. girellae* en condiciones de acuicultura están regulados por condiciones ambientales y de cultivo, como la temperatura, la densidad de cultivo y el estado nutricional de los peces. Para determinar como afecta la variación en condiciones de cultivo a la robustez y capacidad de sobreponerse a dichas condiciones adversas del medregal, se realizó un primer estudio realizando tres experimento diferentes (Capítulo 3). El primero de ellos consistió en aclimatar a juveniles de medregal durante 120 días a 3 temperaturas diferentes (17°C, 22°C y 26°C) para determinar si la aclimatación a largo plazo a estas temperaturas de cultivo afecta a los niveles circulantes de cortisol en plasma y mucus y a la expresión del gen *muc-2* como indicador de la producción de mucus. El segundo experimento se diseñó para determinar la respuesta a estrés y los niveles de cortisol en plasma, mucus y expresión de *muc-2* en la piel por medio de un desafío basado en la reducción del nivel de agua en los tanques de cultivo del medregal después de ser mantenidos durante 90 días en diferentes condiciones de densidad de cultivo (4 $kg \cdot m^{-3}$ u 8 $kg \cdot m^{-3}$) y siendo sometidos a diferentes protocolos de manejo (un grupo fue muestreado para obtener peso y talla cada 7 días y otro grupo cada 30 días). El tercer estudio se realizó para determinar el efecto del ayuno en un periodo corto de tiempo (10 días) en la respuesta a estrés del medregal, de forma que tras este periodo se realizó un desafío de reducción del nivel de agua en los tanques de cultivo para evaluar el nivel de cortisol circulante en plasma y mucus y la expresión de *muc-2* en la piel. La aclimatación a cualquiera de las tres temperaturas seleccionadas no afectó ni a los niveles de cortisol en mucus y en plasma ni a la expresión de *muc-2* en la piel, estableciendo el nivel basal de cortisol en plasma en 7.5 $ng \cdot ml^{-1}$ para los juveniles de esta especie, encontrando una correlación positiva entre los niveles de cortisol plasmático y en mucus cutáneo, señalando el potencial del nivel de cortisol en mucus cutáneo como un indicador no invasivo de estrés para esta especie. Además, el cultivo a alta densidad al final del experimento (11.5 $kg \cdot m^{-3}$) produjo un aumento ($p<0.05$) del cortisol plasmático, con una lenta recuperación después de 24 horas del estímulo estresante y una reducción del crecimiento del medregal que no afectaron significativamente a los niveles de expresión de *muc-2,* a pesar de que existe una tendencia a incrementar en peces sometidos a una alta densidad de cultivo. El ayuno produjo un incremento significativo ($p<0.05$) de los niveles de cortisol en plasma y en mucus durante la fase aguda de la respuesta de estrés asociado a una menor expresión y respuesta del gen *muc-2* en piel.

Para determinar los efectos de la infección por *N. girellae* en la piel del medregal, se realizó

un experimento de parasitación en el que se estudiaron las alteraciones morfológicas producidas en las regiones craneal y dorso-lateral de la piel antes y después de su infección (Capítulo 4). Los análisis morfológicos se realizaron por medio de técnicas histológicas de microscopía óptica, electrónica de barrido y de transmisión. Los estudios realizados demostraron diferencias claras entre la región craneal y dorso-lateral, siendo la región craneal la más susceptible a ser parasitada por la ausencia de escamas, el grosor de la epidermis y la menor cantidad de células productoras de mucus.

La fijación de *N. girellae* produjo cambios en la estructura de la piel, como una desorganización por un aumento de presión del haptor del parásito en la estructura típica del *Stratum superficiale* de la epidermis del medregal. Además, las células epiteliales de la superficie del *stratum superficiale* localizadas cerca de la zona de fijación del parásito mostraron una clara degradación celular, asociada en muchos casos con procesos de separación de esas células de la epidermis. También se pudieron observar procesos focales de degeneración hidrópica en las células epiteliales asociados a la zona de fijación, que en los casos más severos degeneran en procesos espongióticos. Puntualmente se pudieron observar movilizaciones focales de leucocitos a las zonas de fijación del parásito.

Para determinar los cambios inducidos por la infección de *N. girellae* en el mucus de la piel del medregal, se realizó un estudio de proteómica combinando las técnicas 2-DE-MS/MS y LC-MS/MS en peces no parasitados y parasitados (Capítulo 5). También se determinó la actividad proteasa del mucus, así como la caracterización de dichas proteasas. También se obtuvo el microbioma del mucus de la piel mediante la búsqueda y comparación específica de las proteínas obtenidas en el LC-MS/MS frente a una base de datos de proteínas bacterianas. El 2-DE-MS/MS identificó 69 y 55 proteínas en peces no parasitados y en parasitados respectivamente, mostrando un incremento de las proteínas estructurales en peces parasitados, de las cuales muchas de ellas fueron queratinas, y fueron degradadas selectivamente en peces parasitados. De forma complementaria, y de acuerdo con esta degradación selectiva, el mucus de los peces parasitados mostró una mayor actividad proteasa que el de los peces no parasitados, tanto por parte de serinas proteasas como metaloproteasas. Los resultados del LC-MS/MS identificaron 959 proteínas en los peces no parasitados y 357 en peces parasitados. Los análisis funcionales de estas proteínas demostraron una mayor representación por parte de las proteínas del citoesqueleto en el mucus de

medregales parasitados por *N. girellae*. Se detectaron tanto en medregales no parasitados como en parasitados otra serie de proteínas relacionadas con procesos de apoptosis, algunas rutas metabólicas relacionadas con metabolismo proteico, de hidratos de carbono o lípidos, respuesta a estrés e inmunidad. No obstante, no hubo diferencias en la distribución y abundancia taxonómica de los géneros de bacterias encontrados en animales infectados y no infectados, siendo el género *Pseudomonas* el género más representativo.

Una vez descritas las alteraciones producidas sobre la piel del medregal por *N. girellae*, se elaboraron tres estudios diferentes cuyo objetivo fue potenciar el sistema inmune y reducir la incidencia de *N. girellae* por medio de dietas funcionales.

En primer lugar, tras la clonación y caracterización de la piscidina del medregal como parte de las piscidinas del grupo 3 (Capítulo 6), se analizó la expresión basal de la piscidina en diferentes tejidos, siendo el bazo y el intestino los tejidos que presentaron mayor expresión de este parámetro. Así mismo, se generó una piscidina recombinante de medregal que fue usada para testar su efecto frente a *Aeromonas salmonicida, Vibrio anguillarum, Lactococcus garvieae, Photobacterium damselae, Yersinia ruckeri* y *Escherichia coli,* obteniendo un marcado efecto bacteriostático (estudio realizado en colaboración con la Universidad de Aberdeen en el contexto del proyecto europeo DIVERSIFY). Así mismo, se realizó una estimulación *in vivo* por medio de patrones moleculares asociados a patógenos como poli I:C, lipopolisacáridos (LPS) y flagelina. La flagelina indujo un incremento en la expresión de piscidina en branquia y riñon anterior, mientras que poli I:C incrementó la expresión de piscidina en intestino, riñón anterior y bazo. El LPS solo incremento la expresión de piscidina en riñón anterior. Por otro lado, los ensayos *in vitro* realizados con leucocitos del riñón anterior estimulados mediante los anteriores patrones moleculares asociados a patógenos mostraron un aumento de la expresión de piscidina a partir de las 24 horas, especialmente por la adición de la flagelina y LPS. Por otro lado, la estimulación de las células extraídas del bazo produjo un aumento en la expresión de piscidina a partir de las 4 horas con poli I:C y flagelina, manteniéndose la expresión hasta las 24 horas de la estimulación, retornando a niveles basales después de las 12 horas de estimulación en el caso del polo I:C, lo cual demuestra los diferentes patrones de estimulación y producción de la piscidina en los tejidos seleccionados (Capítulo 6). La validación del uso como biomarcador de la expresión génica de la piscidina fue realizado a través del uso de aditivos funcionales en la dieta. Para

ello, las dietas de medregal fueron suplementadas con MOS (5g·kg^{-1}), cMOS (2g·kg^{-1}) o una combinación de ambos aditivos (5g·kg^{-1} y 2g·kg^{-1} respectivamente) durante 30 días. El uso de MOS incrementó la expresión de piscidina en el bazo, mientras que el cMOS aumentó la expresión de piscidina en branquia y en riñón anterior, mientras que la combinación de ambos suplementos indujo un aumento de la expresión en el intestino comparado con la dieta no suplementada.

Teniendo en cuenta los efectos de las dietas funcionales en la modulación de la expresión de piscidina en el medregal, y con el fin de estimular el sistema inmune sistémico y asociado a las mucosas para reducir la infección por *N. girellae* , se realizó un estudio de 90 días de suplementación con MOS (5g·kg^{-1}), cMOS (2g·kg^{-1}) y una mezcla de ambos aditivos (5g·kg^{-1} y 2g·kg^{-1} respectivamente) (Capítulo 7). Para este estudio, se realizó el análisis de expresión génica de una serie de genes relacionados con la respuesta inmune en varios tejidos como la piel, branquias, intestino anterior, bazo y riñón anterior junto con un análisis para determinar la actividad de lisozima y bactericida del suero y del mucus. Además, se realizó un test de parasitación por cohabitación durante 15 días con *N. girellae* después de los 90 días de suplementación con las dietas funcionales para determinar la carga parasitaria y el tamaño de los parásitos en cada dieta. A pesar de que las dietas funcionales no produjeron efectos en el crecimiento después de 90 días de suplementación, cMOS aumentó significativamente la actividad bactericida del suero y redujo específicamente la carga parasitaria, el tamaño de los parásitos asociados y el número de parásitos por unidad de superficie del pez, mientras que no se detectaron efectos por parte de la suplementación con MOS. Con respecto a los 17 genes del sistema inmune estudiados, el uso de cMOS incrementó la respuesta inmunológica de tejidos con inmunidad asociada a las mucosas como la piel y branquias, aumentando la expresión de hepcidina, defensina, proteína Mx, interferon-γ (*ifn*γ), *muc-2*, interleuquina-1β *(il-1*β), *il-10* e immunoglobulina-T (*IgT*). Por otro lado, el uso de MOS produjo un incremento en la expresión de genes como piscidina, defensina, *iNOS*, proteína *Mx*, interferones, *il-1*β, *il-10, il-17* e *il-22* en riñón anterior y en bazo. En el intestino posterior, ambos compuestos (MOS y cMOS) indujeron un incremento de la expresión génica de *il-10, IgM* e *IgT*, incrementando MOS la expresión de piscidina, *muc-2* e *il-1*β*,* mientras que cMOS incrementó la expresión de hepcidina, defensina e *IFN*γ. Los peces alimentados con la mezcla de ambos suplementos mostraron niveles de expresión génica similares a los peces no suplementados en todos los tejidos, probablemente debido a un efecto de sobreestimulación.

Para el último estudio, se probó un ingrediente funcional distinto. Se realizaron dos experimentos diferentes para determinar el efecto de un fitogénico (HERB, 200ppm) o de cMOS (2g·kg^{-1}) suplementados durante 70 días en dietas para juveniles de medregal para determinar el efecto en el crecimiento, en la respuesta a estrés, en la carga parasitaria y respuesta inmune asociada (Capítulo 8). En el primer experimento, se realizó un test de estrés después de 70 días, mientras que en el segundo experimento se realizó una cohabitación de 15 días con medregales parasitados con *N. girellae* , obteniendo los parásitos de la superficie de los medregales para obtener el número de parásitos por unidad de superficie de pez y el tamaño total de estos. Paralelamente se tomaron muestras de la piel de medregales antes de ser parasitados y después para realizar análisis de expresión génica y suero y mucus para realizar otros ensayos inmunológicos. Los aditivos funcionales no mostraron efectos en el crecimiento del medregal. No obstante, HERB redujo significativamente el cortisol plasmático después de 3 horas de confinamiento en una jaula comparado con los otros tratamientos, donde cMOS redujo la carga parasitaria y la talla total de los parásitos. Además, ambos aditivos incrementaron la actividad proteasa del mucus después de la parasitación y la actividad de la lisozima después de la parasitación. En relación con los genes del sistema inmune estudiados, HERB incrementó la expresión de piscidina antes de realizar el test de parasitación. Los resultados de expresión génica después de la parasitación mostraron que HERB indujo un aumento en la expresión de *muc-2* y piscidina, mientras que ambos suplementos incrementaron la expresión de *tnf-*α*, il1-* β*, hep, c3, cd8* y *casp3* . También se encontró una fuerte correlación negativa entre la expresión de hep*, IgT, cath, c3, cd8* y *casp 3* con la carga parasitaria.

En conclusión y de acuerdo con los datos obtenidos en la presente tesis, la densidad para el cultivo de juveniles de medregal debe estar por debajo de 11.5 kg·m^{-3} para no ver afectado el crecimiento y mejorar la capacidad de recuperación ante situaciones estresantes. Una vez aclimatado a las condiciones de cultivo, puede considerarse 7.5 ng·ml^{-1} como el nivel basal de cortisol plasmático para los juveniles de medregal, estando este correlacionado con los niveles de cortisol en mucus bajo las diferentes condiciones de cultivo probadas. Un ayuno por un corto periodo de tiempo produjo efectos negativos en la producción de mucina en la piel y en los niveles de cortisol en plasma después de una situación estresante.

La fijación de *N. girellae* en la piel del medregal indujo una reducción del grosor de la

epidermis, aumento de la densidad de células productoras de mucus, alteración de la estructura del *stratum superficiale* favoreciendo la presencia de degeneraciones hidrópicas y migración de células tipo leucocitarias en el *stratum basale*. Además, debido a esta fijación, se produjo un incremento en el numero de proteínas del citoesqueleto del mucus de la piel del medregal, mientras que el microbioma no fue alterado al menos al nivel taxonómico de género. La fijación del parásito también produjo una degradación selectiva de las proteínas estructurales del mucus, principalmente queratinas, incrementando la actividad de las metaloproteasas y serinas proteasas.

El uso de cMOS en la dieta a una concentración de 2 $g \cdot kg^{-1}$ durante 90 días redujo la incidencia de *N. girellae* relacionado con un aumento en la expresión de determinados biomarcadores en la piel, incluyendo citoquinas proinflamarorias, *hep, muc-2* e *IgT*. Por otro lado, el uso de MOS a 5 $g \cdot kg^{-1}$ durante 90 días aumento la expresión de biomarcadores como la piscidina y citoquinas proinflamatorias en riñón anterior, bazo e intestino posterior, sin presentar un efecto marcado en los tejidos asociados a mucosas como la piel y las branquias. No obstante, la capacidad de potenciar el sistema inmune inducido por estos aditivos se ve limitada al añadirlos en conjunto en la dieta. Así mismo, el uso de cMOS en la dieta también redujo la carga parasitaria de *N. girellae* , correlacionado con el aumento de la expresión de *hep, IgT, cathelicidina, cd8* y *c3* en la piel, validándolos como biomarcadores para el medregal frente a esta infección parasitaria. Otros aditivos como el fitogénico “HERB” a 200 ppm suplementado durante 70 días redujo la respuesta de cortisol durante la fase aguda de respuesta al estrés. Además, en respuesta a la infección por *N. girellae* , HERB y cMOS incrementaros la respuesta del tejido linfoide asociado a la piel a través del aumento de la expresión de péptidos antimicrobianos, *muc-2, IgT* y citoquinas proinflamatorias.

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