UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

Contamination of farmed gilthead sea bream (*Sparus aurata*, Linnaeus, 1758) by the parasite *Amyloodinium ocellatum* – A proteomic approach

Márcio Júlio Vicente Moreira

Dissertação para obtenção do grau de Mestre em Aquacultura e Pescas

Trabalho efetuado sob a orientação de:

Doutora Florbela Soares

Professor Doutor Pedro Rodrigues

Faro 2015

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

Contamination of farmed gilthead sea bream (*Sparus aurata*, Linnaeus, 1758) by the parasite *Amyloodinium ocellatum* – A proteomic approach

Márcio Júlio Vicente Moreira

Dissertação para obtenção do grau de Mestre em Aquacultura e Pescas

Trabalho efetuado sob a orientação de:

Doutora Florbela Soares

Professor Doutor Pedro Rodrigues

Faro 2015

DECLARAÇÃO DE AUTORIA DE TRABALHO

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

Universidade do Algarve, 28 de setembro de 2015

_____

This work was published in the following formats (in Annex):

- ✓ *As poster communication, with the following title*:

  - M. MOREIRA, D. SCHRAMA, F. SOARES, P. POUSÃO-FERREIRA, P. RODRIGUES (2014). "Proteomic analysis of gilthead sea bream plasma with amyloodiniosis", presented in COST Action FA1002 Final Showcase Meeting on Farm Animal Proteomics, 17-18 November 2014, Milan (Italy).

- ✓ *As book chapter, with the following title*:

  - M. MOREIRA, D. SCHRAMA, F. SOARES, P. POUSÃO-FERREIRA, P. RODRIGUES (2014). "Proteomic analysis of gilthead sea bream plasma with amyloodiniosis", Farm Animal Proteomics 2014, pp. 188-193, DOI: 10.3920/978-90-8686-810-0.

- ✓ *As oral communication*:

  - Presentation of a seminar with the title "Estudios sobre la infección por el dinoflagelado *Amyloodinium ocellatum* en la dorada (*Sparus aurata*)", ministred on 20 March 2013, in Salón de Actos do Centro IFAPA Agua del Pino (Crta. El Rompido-Punta Umbría Km 3.8, Cartaya, Huelva, Spain).

Acknowledgment

I would like to thank my formal supervisor Dra. Florbela Soares, from the Aquaculture Research Centre, National Institute for the Sea and Atmosphere (IPMA), for bringing me to the pathology area, for all the patience, motivation, all the opportunities, scientific formation, and all the support provided on this thesis. I want also to thank to my other formal supervisor, Professor Pedro Rodrigues, from CCMAR – Centre of Marine Sciences, University of Algarve for the opportunities and scientific formation in proteomics provided under the COST Action FA1002 - Farm Animal Proteomics, and the support provided on this thesis. I thank also to project BONAQUA (0433_BONAQUA_5_E), for all the financial support to this thesis.

I also want to thank to Denise Schrama from CCMAR, for all the support and supervising on the proteomic analysis, on the structuring of this thesis and for sharing with me their expertise in a scientific manner; to Marcelino Herrera from IFAPA Agua del Pino, for all the support and supervising on the stress indicator analysis; to Tune Wulff from The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark for all the help in the spot analysis ;to Dr. Pedro Pousão from EPPO-IPMA, for the inestimable contribution by providing facilities and fishes for my thesis; and to Dr. Jose Ignacio Navas Triano from IFAPA Agua del Pino, for the three months internship in IFAPA Agua del Pino and motivation, all the opportunities and scientific formation, and all the great conversations about science, pathology, parasites and bacteria.

My special thanks to all the EPPO-IPMA staff, especially to Margarida Gamboa, Marina Cabral, Luis Magina, Paula Moura, Marta Santos, Hugo Ferreira and João Moura for the participation in the final sampling of my thesis and to João Veríssimo for all the help in the conception and assembly of the system, and also to Dra. Laura Ribeiro and Dra. Emília Cunha for all the support along this years; to all the IFAPA Agua del Pino staff, especially Monse López Sanmartín and Jose López Fernández, dear colleagues and friends, for all the support and help on my three months internship in IFAPA; and to all the AQUAGROUP staff, for all the sympathy and help during the proteomic and osmotic analysis. I also want to thank Dra. Maria Teresa Dinis, because she was the person that captivated me to the Aquaculture area with all of her passion and love for this area of work.

I dedicate this thesis to my parents and brother, for all the support along this years, and especially to a unique person, that I love very much and that has committed the crazy act of marrying me: Lília Cabral.

Abstract

Global world population is expected to reach approximately 9 billion by 2050, and the world food-producing sector must secure food for the growing population through increased production and reduced waste. Due to this, aquaculture can represent a solution to the substitution and increase of fish protein available to the populations. However, a higher development of aquaculture is constrained by different causes, being one of the most important diseases. In Southern Europe, especially in the Mediterranean area, amyloodiniosis represents a major bottleneck for semi-intensive aquaculture production in Southern Europe affecting the majority of fish farms, in different types of rearing tanks and systems, and causing extremely high mortalities. *Amyloodinium ocellatum* is the most common and important dinoflagellate parasitizing fish, and is one of the few fish parasites that can infest almost all fish, crustacean and bivalves that live within its ecological range are susceptible to infestation. In recent years there has been developed a large amount of largescale approaches in biology that promise to revolutionize systems-level understanding of cellular and organism functions. Amongst them, proteomics is getting an increasing interest, because information of DNA sequence, mRNAs and transcriptional activity provides only a static snapshot of the various ways in which a cell might use its proteins, whereas the life of the cell is a dynamic process. So in this work we have analysed the proteome and relate it with osmoregulatory and stress indicators of gilthead sea bream (*Sparus aurata*) plasma, in order to enlighten the different physiological responses when exposed to an *A. ocellatum* outbreak. After the proteomic DIGE, condition index (CI), hepatosomatic index (HSI) and spleen somatic index (SSI), and all the stress (cortisol), metabolic (glucose and lactate) and osmotic (pH, osmolarity) analysis, we have obtained statistical differences in the cortisol and lactate levels, and identified several proteins associated with immune response, acute phase response, inflammation, lipid transport, homeostasis, osmoregulation, wound healing, neoplasia, oxidative stress, and iron transport. Overall, this preliminary study revealed that amyloodiniosis can upregulate or downregulate proteins with known functions and provoke stress and metabolic responses in gilthead sea bream.

Keywords: Amyloodinium, gilthead sea bream, proteomics, DIGE, physiological responses

Resumo

É esperado que a população mundial atinja 9 biliões de pessoas em 2050, sendo necessário que o sector alimentar assegure comida para esta população em crescimento, através de um aumento de produção e de uma redução de desperdícios alimentares. A aquacultura apresenta-se como uma área que pode representar uma solução para o aumento de pescado disponível para as populações. No entanto, um maior desenvolvimento desta indústria está limitada por diferentes causas, sendo uma das mais importantes as doenças que afetam as espécies cultivadas. No sul da Europa, uma das doenças que se apresenta como limitativa para o desenvolvimento da aquicultura é a amilodiniose. Esta doença afeta grande parte das pisciculturas (com diferentes sistemas de produção), provocando mortalidades muito elevadas. Esta doença é causada pelo parasita dinoflagelado *Amyloodinium ocellatum*, que é um dos poucos parasitas capazes de infetar todos os peixes, crustáceos e bivalves no seu alcance ecológico. Têm-se vindo a desenvolver desde há alguns anos uma série de abordagens de larga escala em biologia, que prometem revolucionar o nosso conhecimento a nível do funcionamento celular e do organismo. Entre as mais promissoras encontra-se a proteómica, porque providencia infirmações sobre os processos dinâmicos da vida de uma célula ou órgão, ao contrário de outras técnicas como a sequenciação de ADN, ARN mensageiro ou da transcriptómica. Dados estes factos, decidimos analisar o proteoma do plasma de dourada (*Sparus aurata*), assim como diversos indicadores metabólicos, osmorregulatórios e de stress, em resposta a um surto de *A. ocellatum*. Após a análise proteómica por intermédio de géis DIGE, com posterior sequenciação dos *spots* selecionados por LC-MS/MS, análise do índice de condição (IC), do índice hepatossomático (HSI), índice somático do baço (SSI) e de todas as análises de stress (cortisol), metabólicas (glucose, lactato) e de índices osmorregulatórios (pH, osmolaridade), obtivemos diferenças nos níveis de cortisol e lactato, assim como a identificações de diversas proteínas com funções a nível da resposta imunitária, de resposta de fase aguda, de resposta inflamatória, a nível de homeostasia e osmorregulação, de stress oxidativo, de cicatrização e neoplasia, e de transporte lipídico e de iões de ferro. Podemos assim verificar que este estudo revelou que o parasita *A. ocellatum* pode provocar uma super ou sub expressão de proteínas com funções conhecidas, assim como respostas metabólicas e de stress em dourada.

Palavras-chave: Amyloodinium, dourada, proteómica, DIGE, respostas fisiológicas

Abbreviations

µl – microliters

µl/min – microliters per minute

µm – micrometers

2D PAGE - two-dimensional polyacrylamide gel electrophoresis

2DE - two-dimensional gel electrophoresis

ADN – ácido desoxirribonucleico

ARN – ácido ribonucleico

AMP – antimicrobial peptides

amp. – ampliation

ANOVA - Analysis of variance

ApoAI - Apolipoprotein A-I

BLAST - Basic Local Alignment Search Tool

C3 complex – Complement mediator

CHAPS - 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate

cm – centimeters

CO_2 – carbon dioxide

Cy2 – bright, green fluorescent dye that can be excited using the 492 nm laser line

Cy3 - bright, orange-fluorescent dye that can be excited using the 532 nm laser line

Cy5 - bright, far-red-fluorescent dye with excitation ideally suited for the 633 nm or 647 nm laser lines.

Da – dalton

DIGE - Differential in Gel Electrophoresis

DNA – deoxirribonucleic acid

DTT - DL-Dithiothreitol

EPPO – IPMA -

FAO – Food and Agriculture Organization

FCR – feed conversion ratio

FDR – frequency discovery rate

g – grams

HPI - hypothalamic-pituitary-interrenal axis

hr – hour

HSI - hepatosomatic index

IC - condition index

ID – identification

IPG buffer – rehydration buffer

kDa – kilodalton

L – liters

LC-MS/MS - Liquid chromatography-tandem mass spectrometry

LPS – lipoproteins

m – meters

M – molar

mA/gel – milliamperes per gel

mg/L – miligrams per liter

mm – milimeters

mM - millimolar

mRNA – Messenger ribonucleic acid

MS/MS - tandem mass spectrometry

Mw - molecular weight

Na^+/H^+ - Sodium/Hidrogen ratio

NaOH – sodium hydroxide

ng/ml – nanograms per mililiter

nmol – nanomole

ºC – degrees Celsius

pI – isoelectric point

pmol – picomole

ppm – parts per million

psu – pratical salinity units

rpm – rotations per minute

SDS – sodium dodecyl sulphate

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEI - ice-cold sucrose-EDTA-imidazole buffer

SSI - spleen somatic index

T/C – Theoretical vs obtained

T1 – treatment 1

T2 - treatment 2

Tf – Transferrin

Tris-HCl - Tris(hydroxymethyl)aminomethane hydrochloride buffer

Tris-HCl SDS-PAGE - Tris(hydroxymethyl)aminomethane hydrochloride sodium dodecyl sulfate polyacrylamide gel electrophoresis

USD – United States Dollar

UV – ultraviolet

V – volts

v/v – volume per volume

Vhr – volts per hour

w/v – weight per volume

Wap65 - warm temperature acclimation related 65 kDa protein

Contents

List of Figures

List of Tables

I. Introduction

1.1. General Perspective of Aquaculture

Global world population is expected to reach approximately 9 billion by 2050, and the world food-producing sector must secure food for the growing population through increased production and reduced waste (The World Bank, 2013; FAO, 2014a). Due to this, aquaculture can represent a solution to the substitution and increase of fish protein available to the populations, as a response to a stagnation and decline in fisheries.

Nowadays, aquaculture is the food industry with the highest annual growth rate (6.3% average growth rate in 2000-2012, 4.9% in 2014) (FAO 2014b; FAO 2014c) with a contribution to total food fish supply that grew from 9% in 1980 to 48 % in 2011 (FAO, 2013), with a forecast for 2015 of 54% of the total food fish supply, a 5% increase in relation to 2014 (FAO, 2015b). The estimated number of fish farmers also grew from 3.9 million in 1990 to 16.6 million in 2010 (Bank 2013). The World Bank (2013) and FAO (2014a) predicts a growth of more than 50% until 2030 (in a conservative/baseline scenario), supplying over 60% of fish destined for direct human consumption. However, a higher development of aquaculture is constrained by different causes, like fishmeal and fish oil prices, global warming and diseases (The World Bank, 2013; FAO, 2014a).

1.2. Impact of Diseases in Aquaculture

Diseases are one of the most important constraints in aquaculture, especially in intensive fish farming, representing severe annual costs to producers (Murray and Peeler, 2005). The open design of many of the aquaculture systems also allows the transmission of infectious pathogens, where they find ideal conditions to cause a disease outbreak (Balcázar, 2006; Mladineo, 2006). Global warming tends to increase the frequency of the outbreaks and increase the ecological range of the diseases (Diez and Perlado, 2014; FAO, 2015). Parasitic diseases caused by obligate or opportunistic eukaryotic pathogens have a major impact on global finfish and shellfish aquaculture, and in many regions they represent a key constraint to production, sustainability and economic viability of aquaculture facilities (Shinn *et al.*, 2014).

In Southern Europe, especially in the Mediterranean area, amyloodiniosis is a parasitic disease that represents a major threat for semi-intensive aquaculture, and is one of the most serious impediments for the production of several warm water aquaculture species, with severe economic costs associated (Noga, 1996). For example, according to Shinn *et al.* (2014), in 2004 a reported case of *Amyloodinium ocellatum* in a milkfish (*Chanos chanos*) hatchery had a total loss cost of 20,000 USD, or an *A. ocellatum* outbreak reported by Soares *et al.* (2012) in an earthen pond in Portugal with gilthead sea bream (*Sparus aurata*) and meagre (*Argyrosomus regius*), in which Shinn *et al.* (2014) calculated a total cost loss of 4170 USD. This aetiological agent is one of the most common and important parasitic dinoflagellate in fish.

1.2.1. Dinoflagellates as Aetological Agents

Dinoflagellates are commonly found in aquatic ecosystems. They are important primary producers and consumers, as well as endosymbionts in many invertebrates (Taylor, 1987; Fensome *et al.*, 1993). Many dinoflagellates produce ichthyotoxins, which have caused mass mortalities in wild and cultured fish (Rensel and Whyte, 2003). About 140 of the approximately 2000 known living species are parasites, most being parasites of invertebrates (Drebes, 1984). Five genera have been reported as fish parasites: *Amyloodinium*, *Piscinoodinium*, *Crepidoodinium*, *Ichthyodinium* and *Oodinioides* (Buckland-Nicks & Reimchen, 1995). Only *Amyloodinium*, *Piscinoodium* and *Ichthyodinium* are of any known or potential economic significance.

A. ocellatum is the most common and important dinoflagellate parasitizing fish (Becker, 1977; Lawler, 1980; Lauckner, 1984; Noga, 1996), and is one of the few fish parasites that can infest both elasmobranchs and teleosts (Lawler, 1980), and almost all fish that live within its ecological range are susceptible to infestation. This parasite can also hyperparasite some parasites of fish like *Neobenedenia melleni* (Monogenea: Capsalidae) in gilthead sea bream (Colorni, 1994), and inflict moderate to intense tissue reaction in several commercially important shrimp species (Aravindan *et al.*, 2007) and bivalves like the pacific oyster (*Crassostrea gigas*) (Sousa, 2015). Some aquaculture reared species affected by *A. ocellatum* are present in Table 1.1.

Table 1.1 - Some aquaculture species affected by *Amyloodinium ocellatum*

Fish species	Occurence	Reference
Gilthead sea bream *(Sparus aurata)*	Mediterranean Sea, Israel, Red Sea	Paperna and Baudin Laurencin (1979); Colorni (1989); Woo *et al*. (2002); Rigos and Troisi (2005); Pereira *et al.* (2010)
Mahimahi/dolphinfish *(Coryphaena hippurus)*		The Ocean Institute (1996)
Groupers	Asia, North America	Chong and Chao (1986); Tseng and Ho (1988)
Red drum *(Sciaenops ocellatus)*	North Carolina,U.S.A	Johnson (1988); Gallet de Saint Aurin (1989); Noga *et al.* (1991)
Tilapia (Oreochromis sp.)	U.S.A	Plumb (1997a); Tonguthai and Chinabut (1997); Shoemaker *et al*. (2006)
Barramundi *(Lates calcarifer)*	Pacific Ocean	Owens (2003); Rimmer (2003)
Striped Bass *(Morone saxatilis)* and Striped Bass hybrids	U.S.A	Paperna and Zwerner (1976); Noga *et al*. (1991); Plumb (1997b)
Sea bass *(Dicentrarchus labrax)*	Caribbean Sea, Mediterranean Sea and Spain	Paperna and Baudin Laurencin (1979); Gallet de Saint Aurin (1987); Alvarez-Pellitero *et al*. (1993); Woo *et al.* (2002); Rigos and Troisi (2005)
Guppies *(Poecilia reticulata)*	U.S.A	Lawler (1980); Noga and Bower (1987); Kuperman and Matey (1999)
Turbot *(Scophthalmus maximus)*	Portugal	Saraiva *et al.* (2011)
White seabream *(Diplodus sargus)*	Mediterranean Sea	Soares *et al.* (2011)
Two-banded sea bream (*Diplodus vulgaris)*	Mediterranean Sea	Soares *et al.* (2011)
Sharpsnout sea bream *(Diplodus puntazzo)*	Mediterranean Sea	Rigos *et al*. (1998); Soares *et al.* (2011)
Black sea bream (*Spondyliosoma cantharus*)	Adriatic Sea	Ivona (2006)
Western Atlantic seabream (*Archosargus rhomboidalis*)	Venezuela	Zambrano and Villalobos (2000)
Senegalese Sole (*Solea senegalensis)*	Mediterranean Sea	Soares *et al.* (2011)
Milkfish *(Chanos chanos)*	Philippines	Cruz-Lacierda *et al.* (2004)
Mangrove red snapper *(Lutjanus argentimaculatus)*	Philippines	Cruz-Lacierda *et al.* (2004)
Brazilian Flounder *(Paralichthys orbignyanus)*	Brasil	Lawler (1980); Landsberg *et al.* (1994); Abreu (2005)
Bullseye puffers (*Sphoeroides annulatus)*	Caribbean Sea	Gaspar (1987); Fajer-Ávila *et al.* (2003)
Meagre (*Argyrosomus regius)*	Portugal	Soares *et al.* (2012)
Snappers		Chong and Chao (1986); Tseng and Ho (1988)
Mullet *(Mugil cephalus)*	Atlantic Ocean	Baticados and Quinitio (1984); Noga *et al.* (1991)
Pacific threadfin *(Polydactylus sexfilis)*	Phillipines	Ostrowski and Molnar (1998); Montgomery-Brock *et al.* (2001)
Yellowtail (*Seriola dumerili*)	Sicilia, Mediterranean Sea	Aiello and D'Alba (1986)
Cobia (Rachycentron *canadum*)		Reed and Francis-Floyd (1994)
Ayu (*Plecoglossus altivelis*)	Australia	Fielder and Bardsley (1999)
Mulloway (*Argyrosomus japonicus*)	Thailand	Chonchuenchob *et al*, (1987)
Pompano (*Trachinotus carol*)	U.S.A	Lawler (1977); Noga *et al.* (1991)
Spotted Seatrout (*Cynoscion nebulosus)*	U.S.A	Masson *et al.* (2011)
Spotted Scat (*Scatophagus argus)*	Phillipines	Cruz (1998); Lio-Po and Barry (1988)
Australian snapper (*Pagrus auratus*)	Australia	Booth *et al.* (2012)
Red Snapper (*Lutjanus campechanus*)	U.S.A	Masson *et al.* (2011)
Slender seahorse (*Hippocampus erectus*)	U.S.A	Gomezjurado (2009)
Indian halibut (*Psettodes erumei*)	India	Kizhakudan *et al.* (2014)
Silver pompano (*Trachinotus blochii*)	India	Kumar *et al.* (2015)
Fenneropenaeus indicus	India	Aravindan et al. (2007)
Macrobrachium rosenbergii	India	Aravindan et al. (2007)
Penaeus monodon	India	Aravindan et al. (2007)
Penaeus semisulcatus	India	Aravindan et al. (2007)
Macrobrachium malcomsonii	India	Aravindan et al. (2007)

Amyloodinium has continued to be one of the most serious impediments to warmwater mariculture, with well over one hundred species known to be susceptible. It can cause serious morbidity and mortality in brackish and marine warmwater food fish at aquaculture facilities worldwide and is often considered the most consequential pathogen of marine fish (Paperna *et al.*, 1981). Outbreaks can occur extremely rapidly, resulting in 100% mortality within a few days. Even freshwater fish are highly susceptible to infestation when they´re reared in saline waters (Lawler, 1980; Noga and Bower, 1987; Kuperman and Matey, 1999).

A. ocellatum can appear in different types of rearing tanks and systems, like salt pans, semi-intensive earthen ponds, cement tanks, glass fibre tanks and even in cages (gilthead sea bream, sharpsnout sea bream (*Diplodus puntazzo*), black sea bream (*Spondyliosoma cantharus*) and sea bass (*Dicentrarchus labrax*) outbreaks in sea cages in Adriatic sea) (Rigos *et al*., 1998; Rigos and Troisi, 2005; Ivona, 2006; Abowei *et al*., 2011). Additionally, dinospores of *A. ocellatum* could travel in aerosol droplets (up to 440 mm in a static system and up to 3 m in adynamic one). This is the first record of this transmission pathway for a marine protozoan parasite (Roberts-Thomson *et al.*, 2006).

The disease, amyloodiniosis, is a major bottleneck for semi-intensive aquaculture production in Southern Europe because it affects the majority of fish farms, causing extremely high mortalities. It's a "quiet" disease because usually when the fish-farmer finds out that the fish is contaminated it is already too late and they do not respond to treatment (Soares *et al.*, 2011).

1.3. Characterization of the Pathogen

1.3.1 Taxonomy

As previously written, *A. ocellatum* is a dinoflagellate that parasites fish (Becker, 1977; Lawler, 1980; Lauckner, 1984; Noga, 1996). A study made by Murray *et al.* (2005), using rRNA (ribosomal RNA), indicates that *A. ocellatum* is closely related with *Pfiesteria piscicida*. Saldarriaga *et al.* (2004) and Ki *et al.* (2012) (Figure 1.1), puts *A. ocellatum* in the Blastodiniales order, but phenotypical characteristics puts him near the order Peridiniales, which is in agreement with Landsberg *et al.* (1994), Steidinger *et al*. (1996) and Fensome *et al.* (1999). Some studies have suggested that there may be more than one species or strain of *Amyloodinium* (Lom and Lawler, 1973, Lawler, 1980, Noga *et al.*, 1991).

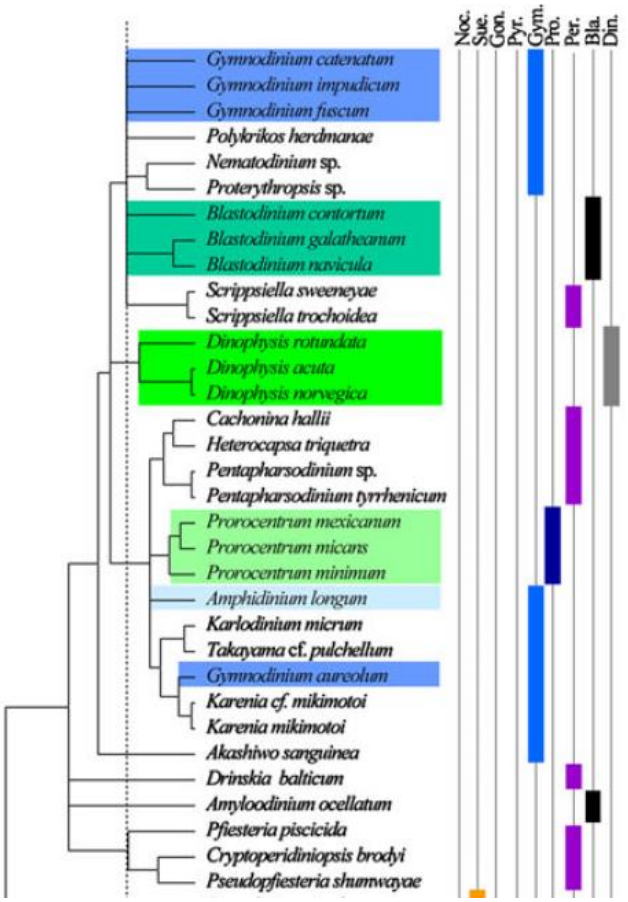

Figure 1.1 - Phylogenetic tree representing the order and phylogenetic proximity of *Amyloodinium ocellatum* (Adapted from Ki *et al.*, 2012)

1.3.2 Life Cycle

Biologically *A. ocellatum* is a dinoflagellate with a life cycle that comprises three stages: trophont (parasitic state); tomont (encapsulated state, a cyst that develops after the trophont leaves the fish); and dinospores (free living state, released from the tomont) with 6.1±0.8 microns length 11.7±0.5 microns in width (Landsberg *et al.*, 1994) The actively feeding parasitic trophont is attached to fish gills and skin; the reproductive encysted tomont is inserted into sediments; and the free-swimming infective dinospores develop after the tomonts divide (Kuperman and Matey, 1999; Woo, 2007) (Figure 1.2). Each tomont can produce up to 256 dinospores in three days at 25 ºC, each one capable to infect a new host and produce a trophont

(Brown and Hovasse, 1946). Dinospore production and infectivity occur over a broad range of temperatures (16 to 30°C) and salinities (10 to 60 psu) (Paperna, 1984). The reproductive rate is very fast completing its life cycle in less than a week in optimal conditions.

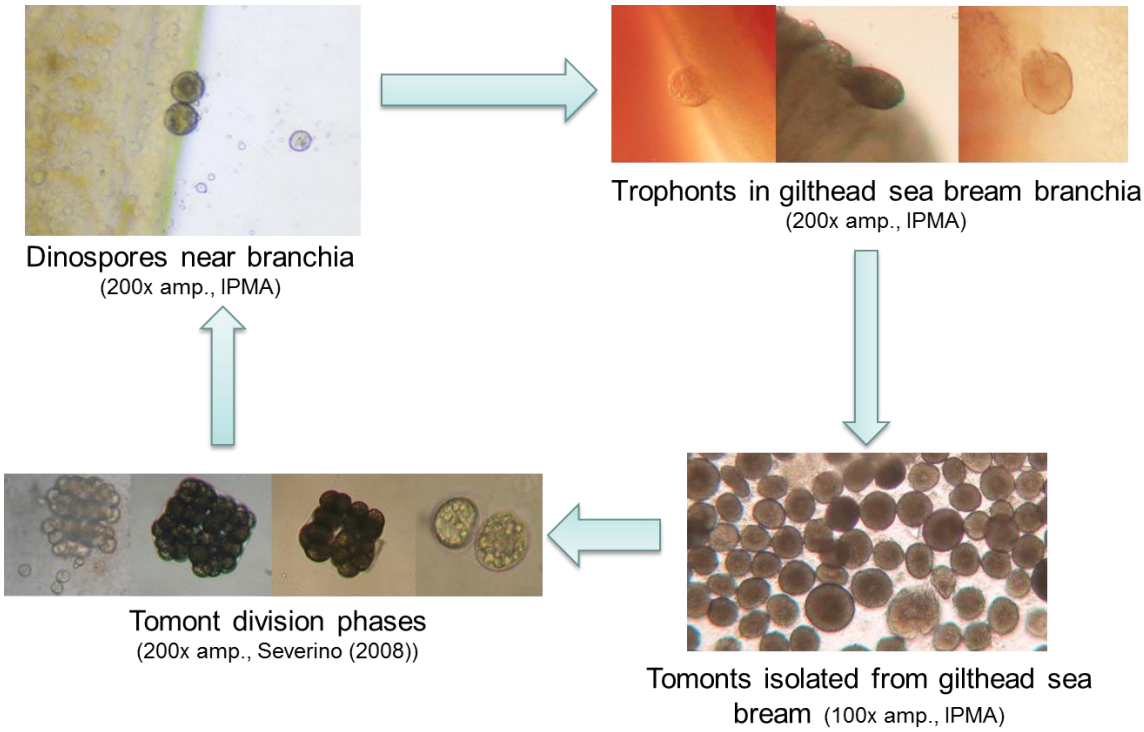

Figure 1.2 - Life cycle phases of *Amyloodinium ocellatum*

1.3.3 Clinical Signs and Diagnostics

The symptomatology is characterized by changes in fish behaviour, with jerky movements, swimming at the water surface and decreased appetite (Soares *et al*., 2011). These may include increased respiratory rate (rapid gilling and movement of the opercula), "piping," and gathering at the surface or in areas with higher dissolved oxygen concentrations, as well as reduced appetite. If the primary site of infection is skin, infected fish sometimes develop a white or brown coloration ("velvet") or cloudy appearance, which is most visible when viewed with indirect lighting such as a flashlight (Levy *et al.*, 2007). Such fish may display signs of "flashing" or rubbing on tank walls, the substrate, or other structures in their environment.

Again, feeding behaviour likely will be poor and some fish may appear emaciated (Francis-Floyd and Floyd, 2011). The method of diagnostic is the identification of the trophont in the skin and gills of infected fish, which can be done through microscope observation of these tissues (Figure 1.3).

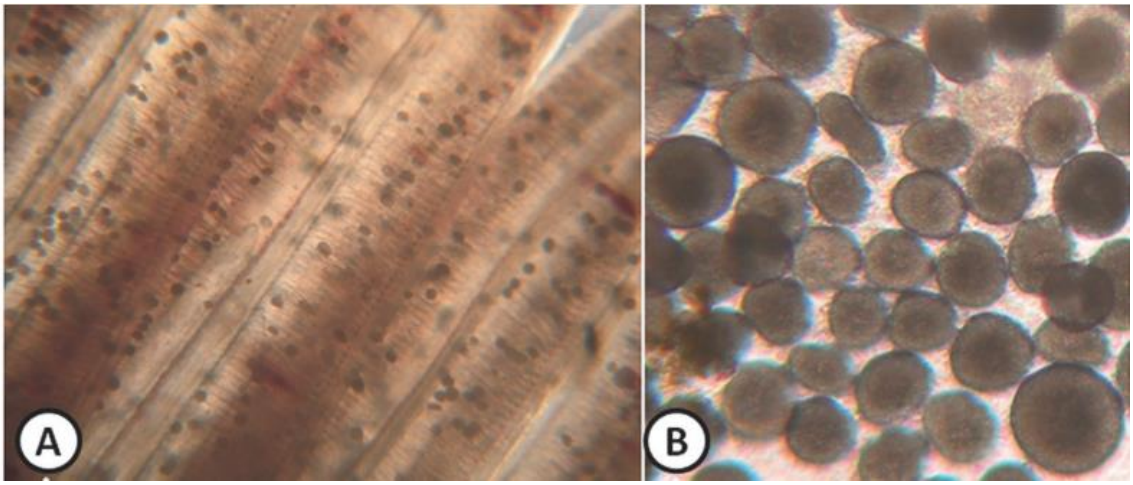

Figure 1.3 – A - *Amyloodinium ocellatum* trophonts in meagre *(Argyrosomus regius)* gills (40x) B- *Amyloodinium ocellatum* tomonts detach from meagre *(Argyrosomus regius)* gills (100x) (Adapted from Soares *et al.*, 2012)

Fish mortalities are normally attributed to anoxia (associated with serious gill hyperplasia, inflammation, haemorrhage and necrosis) in heavy infestations (Lawler, 1980). However, there are high mortalities associated with mild infestations that, according to Noga (2012), could be associated with osmoregulatory impairment and secondary microbial infections due to severe epithelial damage. Physiological information about the responses from the host to *A. ocellatum* infestation are limited, and are mainly available in some publications about antimicrobial protein expression in gills and skin (Colorni *et al.*, 2008; Noga *et al.*, 2009; Corrales *et al.*, 2010; Noga *et al.*, 2011) and general immunological responses of the host to *A. ocellatum* (Smith *et al.*, 1992; Smith *et al.*, 1994., Noga *et al.*, 2002; Woo, 2007; Alvarez-Pellitero, 2008; Woo & Ardelli, 2014). However, information regarding other physiological responses are very rare.

Trophonts are ovoid with 150-350 microns (Lom and Diková, 1992). Gills are first to be infected. Tomonts are occasionally observed in the intestinal tract. Subclinical infestations by phase-free dinospores can go unnoticed since they are not yet identified by expedite methods, although some studies have been done in this field (Levy *et al.*, 2007). Fish that are recovering from spontaneous *Amyloodinium* infestation or that have been experimentally exposed to

parasite antigen may produce serum antibody that is detectable by ELISA or dinospore agglutination assay (Smith *et al.*, 1992; Cobb *et al.*, 1998 a,b; Cecchini *et al*., 2001). Such assays might be useful for monitoring levels of protection in susceptible populations and detect subclinical infestations, since elevated antibody titres have been associated with resistance (Cobb *et al.*, 1998 a, b).

As one stage of its life cycle is free (dinospores), its observation under a microscope to assess the presence in the water column is impractical in fish farms. In this sense the technique for detection of dinospores (free phase) in water based on the PCR technique is a sensitive and specific method for detecting a low number of individuals (Levy *et al.*, 2007) and can be used in an environmental monitoring program in aquaculture, as an effective control of this disease involves the ability to detect an infestation in a primordial state to take effective control measures (Soares *et al.*, 2011).

1.4 Treatments, Prevention, Control and Prophylaxis

A. ocellatum is a virulent parasite and outbreaks must be treated as quickly as possible to avoid high mortalities. Attempts to eliminate *A. ocellatum* at its different life states using different methods and therapies show some progress.

A. ocellatum tolerate a wide range of temperature and salinity, which make the control of this disease by physical and chemical parameters manipulation very difficult. Inhibition of growth of the parasite can be achieved in temperatures below 15°C (Paperna, 1984) and reduced salinities. Lowering the temperature to 15°C arrests the disease process, but this is almost never feasible. Lowering salinity delays but does not prevent infestations (Barbaro and Francescon, 1985), unless fish are placed in fresh water. A short freshwater bath of up to 5 min dislodges most but not all trophonts (Kingsford, 1975; Lawler, 1977b).These are measures that cannot be used in cropping systems that use earthen ponds (tanks of large dimensions, 0.4 to 1 hectare and bigger) and species that do not tolerate wide variations in salinity, as is the reality of most southern European aquaculture. A recent study by Pereira *et al.* (2011) indicates that the water quality kept within production ponds with a defined fish stock density and a good water renewal rate can avoid the *A. ocellatum* infestation in seabream.

The free living state (dinospores) are susceptible to chemotherapy (Lawler, 1980; Paperna, 1984), but the parasitic and encapsulated state of trophont and tomont are difficult to eradicate

or treat (Soares *et al.*, 2011). The most common treatment in controlling and eliminating this parasite is copper in the form of copper sulfate, a chemical widely used in agriculture. Tomonts tolerate levels of copper at concentrations 10 times higher than levels that are toxic to dinospores (Paperna, 1984). A further option is a mixture of 5-hydrate copper sulphate with citric acid monohydrate, to yield 0.15 ppm copper ion concentration in the water (Kabata, 1985). During copper treatments the active component is the copper ion that must be kept in water at 0.15 to 0.2 mg/L for 10-14 days to control the epidemic. High concentrations of copper should be avoided since it is toxic to fish. The free copper is unstable in water and levels should be monitored in order to be maintained at the desirable concentration. It should be noticed that at high water temperatures (25 to 30ºC) the diseases and consequently the treatment with copper sulphate may last for more than a month which, linked to the high cost of the product, may jeopardize the commercial viability of a fish farm (Soares *et al.*, 2011).

On the other hand treatments with formaldehyde in concentrations of 100-200 mg/L during 6 to 9 hours, act over the trophont stage that detached from the fish. The most successful medical approaches have involved repeated treatments, often followed by removal of the fish to an uncontaminated tank. For example, treatment of juvenile bullseye puffers (*Sphoeroides annulatus*) in sea water with 51 mg/L formalin for 1 hr or 4 mg/L formalin for 7 hr significantly reduced *A. ocellatum* load on the skin and gills. Reinfestation occurred after 15 days but was controlled by repeating the treatment (Fajer-Ávila *et al.*, 2003).

Treatment with hydrogen peroxide to 75-150 mg/L was effective in eliminating trophonts in pacific threadfin (*Polydactylus sexfilis*) (Montgomery-Brock *et al.*, 2001). All treatments must be continued long enough to be effective, but is still necessary to have a very tight control by testing the fish for potential re-infestation.

Many other chemicals, such as chloroquine diphosphate, have been tested in the treatment of this parasite, but without satisfactory results. But tests done with, N-methylglucamine lasalocid, completely inhibited dinospore emergence at concentrations as low as 0.01 mg/L, *in vitro*. Treatment of infestations on red drum fry for 24 hr with 0.10 mg/L reduced gill infestation intensity by 80% and none of the fish treated with 1 mg/L had any signs of infestation (Oestmann and Lewis, 1996).

For biological control of this disease, a biocontrol strategy was proposed by Oestmann *et al.* (1995), based upon the observation that larval brine shrimp (*Artemia salina*) readily prey on

dinospores. All dinospores were eliminated after 8 hr in an *in vitro* assay at a ratio of 8 *nauplii*/ml and 10,000 dinospores/ml (1 nauplius/1250 dinospores). Adding *nauplii* prior to experimental challenge of fish also reduced, but did not eliminate, the infestation. There are also new experiments with plant products (Harikrishnan *et al.,* 2011).

Another route that is getting some results is the immunostimulation of fishes (Noga *et al*. 2001; Ewart & Tsoi (2004); Ullal *et al.*, 2008; Noga *et al*., 2009; Corrales *et al*., 2010; Noga *et al*., 2011), with special attention to H2B histones and other antimicrobial peptides (AMP) that are present in most of the reared species. For example, the use of *Debaryomyces hansenii* live yeast has improved the resistance of juvenile leopard grouper *Mycteroperca rosacea* to *Amyloodinium ocellatum* (Reyes-Becerril *et al.*, 2008). There is also investigation to get a vaccine for this parasite. The grouper *Epinephelus coioides* was immunized with a low dose of *Cryptocaryon irritans* theronts and manifested acquire protective immunity (Yambot and Song, 2006), suggesting the promise of immunization against *A. ocellatum*.

In terms of risk prevention the introduction of dinospores in aquaculture systems can be prevented by treating the intake water with ultraviolet (UV) (Lawler, 1977). However this method is only possible in closed systems such, and for maintenance of ornamental fish in aquaria. In the case of the Portuguese aquaculture production which is mostly done in open systems in tanks ranging 0.4 to 1 hectare, the water treatment with UV is impractical. Moreover the use of UV does not sterilize the pond sediment where tomonts remain. In open systems prevention is only possible by the close monitoring of the presence of the parasite on fish during optimal periods of growth and by avoiding stressful situations in fish. When fish are subjected to stress the parasite can quickly increase and cause heavy mortalities. Routine hygiene procedures, like the elimination of dead fish from culture tanks should be followed (Soares *et al.*, 2011).

1.5 Stress and Osmotic Indicators

Marine animals including fish are very sensitive to stressors in the nature and artificial aquaculture conditions (Iwama, 2006). Some aquaculture studies describes that chronic and acute stress is associated with many aspects regarding to biochemical or physical perturbations

and have physiological effects on the organisms (Kubilay and Ulukoy, 2002; Vijayan *et al.*, 2009).

There are different definitions for stress. Cannon (1935) defined for the first time the "homeostasis" theory. Selye (1950) postulated the theory of non-specific physiological response to stressors, where he divided all reactions of stressors in 2 parts: reaction in terms of special stressors or reaction associated with general stressors with no special focus on the stressor in advance. Later on, McCarty *et al.* (1996) said that stress is a non-specificity biological reaction or no specificity response of animals to any stressors in emergency periods.

The origin of stressors can be divided into:

1. Biological stressors due to parasites (Landsberg *et al.*, 1998), and high organism density;
2. Chemical stressors created by spilling contaminants into the water, abiotic environmental stressors (Van Weerd and Komen, 1998), nitrogen, and faeces (Askarian and Kousha, 2006).
3. Physical stressors such as capture (Bolasina, 2011), thermal fluctuations, crowding or density (McCormick *et al.*, 1998; Bolasina, 2011), low water quality (Santos *et al.*, 2010), water-soluble gases, light, water pressure, feed intake in relation to feed conversion ratio (FCR) and noisiness (Askarian and Kousha, 2006). The input water quality monitoring has an important role to prevent physical stressors such as periodic decrease of dissolve oxygen or increasing of CO_2 (Person-Le Ruyet *et al.*, 2002).

Numerous studies have been made on stress and biological relationships in marine animals. Most of the studies states that cortisol and glucose are the most prevalent stress indicators (Einarsdóttir and Nilssen, 1996; Einarsdóttir *et al.*, 2000; Olsen *et al.*, 2008; Fernandes de Castilho *et al.*, 2008; Martinez-Porchas *et al.*, 2009) that can affect the fish physiological activities as well as increasing of cardiac output and gill vascularity along with glycogenolysis with activation of "*brain-sympathetic-chromaffin cell axis*" (Nolan *et al.*, 1999; Olsen *et al.*, 2008).

Activation of hypothalamus-pituitary gland-gonad axis (Carragher *et al.*, 1989; Pottinger and Mosuwe, 1994) with release of catecholamines (Einarsdóttir and Nilssen, 1996) such as epinephrine, adrenaline (Martinez-Porchas *et al.*, 2009), norepinephrine (Askarian and Kousha, 2006), glucocorticoid steroid hormones (Vijayan *et al.*, 2009) and corticosteroid hormones within bloodstream (Weirich, 1997; Martinez-Porchas *et al.*, 2009) have been the subject of different studies.

If stress occurs, the activation of the stress system is characterized by different stages of responses:

1. *Alarm*, primarily with an increase in plasma cortisol and catecholamines via neuro-physiological axis that are called the hypothalamic-pituitary-interrenal (HPI) axis (Vijayan *et al.*, 2009);
2. *Resistance*, secondarily with the enhancement of plasma metabolites (i.e. glucose and lactate) and a reduction of the tissue reserves (i.e. glycogen);
3. *Exhaustion*, with reductions in several physiological and immunological processes (growth, reproduction and others) (Wedemeyer *et al.*, 1990; McCarty *et al.*, 1996; Wendelaar Bonga, 1997; Askarian and Kousha, 2006).

Vital functions of animals, including fish, depend on the income of oxygen from the environment to the organism. Fish possess mechanisms to cope with the demands for oxygen and adapt to variations in the environment, in terms of its availability (Martemyanov, 2013; Martemyanov, 2015). Elevation of sodium in erythrocytes is observed at the adaptation of fish to oxygen deficiency, which occurs owing to the decrease in its solubility in water when water temperature rise (Martemyanov, 2009). During the initial stage of stress, the levels of catecholamines in blood increase considerably (Mazeaud *et al.*, 1977; Mazeaud and Mazeaud, 1981); increasing the demands for oxygen (Klyashtorin and Smirnov, 1981; Aardt and Booysen, 2004; Fu *et al.*, 2007).

There is an hypothesis that the functions of Na^+/H^+ countertransport enhances in stress conditions, due to catecholamines that increase the oxygen carrying capacity of fish erythrocytes. This could be measured with different analysis, like pH and osmolarity (Thomas and Perry, 1992; Wendelaar Bonga, 1997; Perry and Bernier, 1999).

1.6 Proteomic Analysis

In recent years there has been developed a large amount of largescale approaches in biology that promise to revolutionize systems-level understanding of cellular and organism functions. These fields, referred to as ‘‘omics’’ (Joyce and Palsson 2006), can assess the structure and functioning of organisms with a comprehensive and unprecedented level of detail. Amongst these one of the most promising as a potentially new approach to conventional methods is the field of Proteomics (Alves *et al.*, 2010).

Proteomics is the global study of protein abundance and modification patterns (Dowd, 2012), and can represent an alternative strategy to investigate the protein expression pattern of an organism, using a cross-species database for mass spectrometry data interpretation (Cui *et al.*, 2013). The increasing interest in proteomics technologies is because information of DNA sequence, mRNAs and transcriptional activity provides only a static snapshot of the various ways in which a cell might use its proteins, whereas the life of the cell is a dynamic process (Kvasnička, 2003).

Protein profiling investigations involve the separation of complex protein mixtures followed by the analysis of those proteins that exhibit altered expression, and may continue with the identification of the proteins of interest. This involves the use of peptide mass and isoelectric point (pI) information in bioinformatic databases (UniProt, Mascot, and others), often in combination with additional techniques such as N-terminal sequencing, posttranslational modifications (phosphorylation, glycosylation, ubiquitination, and others), and peptide mass fingerprinting. Once identified, protein expression changes can be confirmed using additional techniques, like immunohistochemistry, western blotting or DNA and mRNA expression analysis (Jenkins and Pennington, 2001; Wetmore and Merrick, 2002).

Proteomics has an ability to potentially provide a qualitative and quantitative snapshot of the functional entities of a cell or body fluid under a particular set of conditions (Hogstrand *et al.*, 2006; Cox and Mann 2011; Tomanek, 2011). This field can potentially have two main outcomes: to assess the abundances, modifications, and interactions of all proteins present in a sample and determine how these can change through the time of the experiment, and as

consequence, to reveal novel and integrative functional responses of organisms to diverse environmental challenges, over a variety of time scales and at different levels of organization. However, they are yet largely unrealized, since the first of these has been unfulfilled mainly by technological factors, such as mass spectrometer scanning rates (Mulvey *et al.*, 2010) or techniques for separation of native proteins (Monti *et al.*, 2009). The second is frequently limited by excessive costs or limited access to instruments, shortages of genomic sequence information, incomplete knowledge of protein structure and function, or insufficient analytical methods to interpret the large data sets that are produced (Joyce and Palsson, 2006). Even so, many scientists postulate that the proteome best represents the functional molecular phenotype, because proteins perform most of the molecular work of the cell and constitute a substantial and dynamic component of cellular structures (Dowd, 2012).

The integration of traditional two-dimensional polyacrylamide gel electrophoresis (2D PAGE) with sensitive mass-spectrometric analysis methods and modern bioinformatics tools has greatly fostered the development and application of a new field of research called proteomics (Brunt *et al.*, 2001).

Two-dimensional gel electrophoresis (2DE) is still one of the most important techniques in proteomic analysis, mostly due to its high performance regarding the separation of complex mixtures of full-length proteins. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has been used since the late 1960s as a powerful tool to separate proteins and to compare protein expression between controls and diseased or chemically-exposed biological samples (Patton, 2002). The method depends on an optimal separation of proteins by pI in the first dimension, followed by mass in the second dimension, and can be able to resolve up to 2000 protein spots per gel (Herbert *et al.*, 2001; Lilley, 2001; Beranova-Giorgianni, 2003;). It is a gel-based proteomic approach that provides a top-down, comprehensive picture of the total protein expression pattern of a tissue, and enables its comparison among different physiological and pathological states. This is especially valuable when scarce information is available about an organism or tissue proteome (Addis *et al.*, 2010). A typical 2DE-based workflow is composed of several steps, with the final purpose being the identification of proteins that display abundance variations in response to some experimental factor. In order for biologically meaningful results to be obtained, importance must be given to a correct undertaking of all stages of the process (Silva *et al.*, 2014). However, and even if 2DE is a powerful technique, it

has several drawbacks, including difficulties of resolving proteins with low (<10 kDa) or high (>4200 kDa) molecular weight, as well as those with an extreme isoelectric point (pI<4 or >10), and low throughput capacity (Beranova-Giorgianni, 2003). There are many proteins that are missed, including very basic or acidic proteins, very small or large proteins, and proteins that are hydrophobic or of very low abundance (Lilley, 2001; Beranova-Giorgianni, 2003).

With the introduction of fluorescent dyes to label samples, three samples could be run on a single gel, eliminating the gel-to-gel variation (Eykmans et al., 2012). The Differential in Gel Electrophoresis (DIGE) methods has increased the standard for this proteomics approach, allowing co-separation of a control set of proteins with proteins isolated from a treatment or disease (Tonge et al., 2001). They use the same principles of 2D gels, but because of fluorescent dyes to label samples, three samples could be run on a single gel, eliminating the gel-to-gel variation. On the other hand, and like the normal 2D gels certain groups of proteins are poorly represented on a 2D gel (membrane proteins, proteins with high molecular weights, hydrophobic membrane proteins, with very low abundance, and others) (Lilley and Dupree, 2006).

Proteomics is one of the new approaches to diseases and epidemiology in fish, giving us an insight on the protein abundance and modification patterns in the organism (Rodrigues *et al.*, 2012; Peng, 2013). Different areas of proteomics use in aquaculture are represented in Figure 1.4.

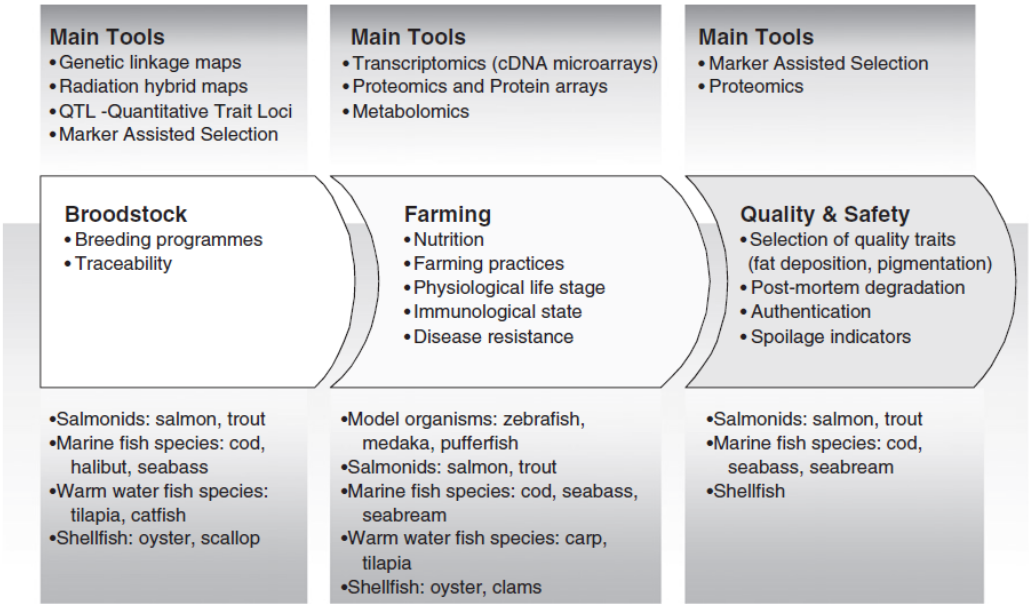

Figure 1.4 - Areas of possible use of proteomics as a tool for investigation in aquaculture (adapted from Rodrigues *et al.*, 2012)

As we can see, they are used in many different areas research in aquaculture (welfare, nutrition, health, quality, or safety) were proteomics approaches are, by its nature, easily applied to model or non-model species with an increasing success (Rodrigues *et al.*, 2012).

Proteomics is also one of the new approaches to diseases and epidemiology in fish, giving us an insight on the protein abundance and modification patterns in the organism (Rodrigues *et al.*, 2012; Peng, 2013). This can be extremely useful in the study of the functional and physiological responses of fish to the parasite *A. ocellatum*, giving us a better insight of the mechanisms involved in the process and the identification of possible biomarkers, since protein profiles appear to be specific to particular stressors (Alves *et al.*, 2010; Cox and Mann, 2011).

In the case of this study, proteomics can be extremely useful in the study of the functional and physiological responses of fish to the parasite *A. ocellatum*, giving us a better insight of the mechanisms involved in the process and the identification of possible biomarkers, since protein profiles appear to be specific to particular stressors (Alves *et al.*, 2010; Cox and Mann, 2011). Between all the proteomes, the plasma proteome is a unique biomarker source since it reflects the global expression of all cellular genomes (Isani *et al.*, 2011) and hence should be a good source of biomarkers in an *A. ocellatum* outbreak.

1.7 Objective of This Work

In this work we will analyse the proteome and relate it with osmoregulatory and stress indicators of gilthead sea bream plasma, in order to enlighten the different physiological responses when exposed to an *A. ocellatum* outbreak.

II. Material and Methods

2.1. Experimental Design

One hundred and fourteen gilthead sea breams, with a mean body weight of 87.2±17 gr, were placed in 200 L rectangular plastic tanks (Control – without external parasites, T1 – with *A. ocellatum*, T2 - with *A. ocellatum* and other monogenetic ectoparasites) in duplicate, at Aquaculture Research Centre, National Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal). Fish were kept at 22±0.2 ºC, in closed recirculation seawater systems, artificial aeration and 24h light photoperiod. Fish were contaminated with tomonts of *A. ocellatum* obtained from contaminated fish at EPPO – IPMA Aquaculture Research Station.

2.2. Sampling

Two samples of water, one at the beginning and other at the end of the experience, were obtained in order to access the number of dinoflagellates per milliliter in the tanks by the Utermöhl method for quantitative phytoplankton analysis (Karlson, 2010).

Fish contamination was accessed by microscopical observation of a wet mount of the first two branchial arches from two fish killed with a cut in the spine, after 12 and 18h after contamination.

When the contamination reached 500 parasites per branchial arc, 4 random fish from each tank were anesthetized with 2-phenoxyethanol (Sigma Aldrich), and approximately 1 ml of blood was withdrawn using syringes heparinized with 1% EDTA. The blood was centrifuged at 2500 rpm for 10 minutes and plasma was collected and kept at -80ºC for subsequent analysis. Fish

were killed with an overdose of anesthetic, measured, weighted, liver and spleen weight were annotated for condition index (CI), hepatosomatic index (HSI) and spleen somatic index (SSI). For stress and osmoregulatory parameters, blood was collected into 1ml heparinized syringes from a puncture of the caudal peduncle (25,000 units of ammonium heparin (Sigma H6279)/3 ml saline solution 0.9 % NaCl) from all the remaining fish (N=11) from Control and T1 (T2 is only used as a positive control for proteomic analysis). Plasma was separated from cells by the centrifugation of the whole blood (10 min, 2500 rpm, room temperature), snap-frozen in liquid nitrogen and stored at -80ºC until analysis of osmolarity, pH, hormonal (cortisol) and metabolic (glucose, lactate, and total protein concentrations) parameters. From each fish, the second gill arch on the dorsal side was removed, dried with an absorbent paper and 3–5 filaments were cut using fine-point scissors. Biopsy samples were placed into 100 µl of ice-cold sucrose-EDTA-imidazole (SEI) buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at -80ºC until analysis.

2.3. Stress and Osmoregulatory Indicators

2.3.1 Analytical Procedures

Plasma glucose and lactate levels were measured using commercial kits from Spinreact (Glucose-HK Ref. 1001200; Lactate Ref. 1001330) adapted to 96-well microplates. Plasma proteins were determined in diluted plasma samples 1:5 (v/v) with the QCA Total Proteins kit (Química Clínica Aplicada S.A., Barcelona, Spain). All assays were performed with a Tecan Sunrise microplate reader, using Magellan v2.5 software for Windows (Tecan Austria, Salzburg). Plasma cortisol levels were quantified with an ELISA kit (EA65, Oxford Biomedical Research, MI, USA) modified and adapted to fish, according to Herrera et al. (in press). Cortisol was extracted from 20 µl plasma in 200 µl diethyl ether. The lower limit of detection (81% binding) was 0.1 ng/ml plasma.

Osmolarity of the plasma samples were analyzed with a cryo-osmometer (OSMOMAT 030, Gonotec), and pH was determined with a pH portable device (pH Spear, Oakton®, Eutech Instruments).

2.4. Statistical Analysis

The data were statistically analysed with IBM SPSS Statistics for Windows, Version 21.0. (IBM Corp. Released 2012. Armonk, NY: IBM Corp).

2.5. Proteomics

2.5.1 Strip pH Range Selection

Prior to protein separation, quantification of protein was performed using the Bradford assay (Bio-Rad), using bovine serum albumin as standard. Two-dimensional difference gel electrophoresis was used to separate proteins according to their isoelectric point (pI) in the first dimension and their molecular weight (Mw), considered as second dimension.

Proteins from one of the treatments were used and rehydration buffer (6M urea, 2M thiourea, 4% CHAPS, 0.02 % (w/v) DTT, 0.002% bromophenol blue, 0.5% (v/v) IPG buffer pH 4-7) was added to complete 450 µl. Rehydration was performed passively for 14 hours using IPG box (GE Healthcare).

For the best pH range selection, different ranges were tested. We used a 24 cm Immobiline™ DryStrips (GE Healthcare) linear pH 3-10, a 24 cm Immobiline™ DryStrips (GE Healthcare) with linear pH 4-7 and a 7 cm Readystrip (Bio rad) pH 7-10.

The isoeletric isoelectric focusing (IEF) was made in 5 steps:
Strip pH 3-10: at 500V gradient 1 hr, at 500V step-n-hold 1 hr, at 1000V gradient 1 hr, at 8000V gradient 3 hrs, and at 8000V step-n-hold for a total of 60000 Vhr.
Strip pH 4-7: at 500V gradient 1 hr, at 500V step-n-hold 1 hr, at 1000V gradient 1 hr, at 8000V gradient 3 hrs, and at 8000V step-n-hold for a total of 60000 Vhr.
Strip pH 7-10: at 100V step-n-hold 2 hr, at 200V step-n-hold 2 hr, at 500V step-n-hold 2 hr, at 1000V step-n-hold 2 hr, at 2000V step-n-hold 2 hr, at 4000V step-n-hold 2 hr, and at 5000V step-n-hold for a total of 60000 Vhr.

Before second dimension, strips were reduced and alkylated using 6 ml of an equilibration buffer (1.5M Tris-HCl pH 8.8, 6M urea, 30% (v/v) glycerol, 0.007M SDS, a few grains of

bromophenol blue) with 1% (w/v) DTT or 2.5% (w/v) iodoacetamide respectively for 15 min each. Strips were loaded onto 12.5% Tris-HCl SDS-PAGE gels and run in an Ettan DALTsix Vertical System (for the 24cm strips) or mini-Protean cell (Bio-Rad) for the 7 cm strip, at 10 mA/gel for 1 hour followed by 60 mA/gel using a standard Tris-Glycine-SDS running buffer, until the bromophenol blue line reaches the end of the gel.
The gels were then stained with colloidal Coomassie blue, scanned and analysed.

2.5.2 Protein Labeling for DIGE

Prior to protein separation, quantification of protein was performed using the Bradford assay (Bio-Rad), using bovine serum albumin as standard. Two-dimensional difference gel electrophoresis was used to separate proteins according to their isoelectric point (pI) in the first dimension and their molecular weight (Mw), considered as second dimension, using fluorescent dyes. Samples were adjusted to pH 8.5 with 0.1 M NaOH and 50 µg of proteins were minimally labelled with 400 pmol of fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethylformamide following manufacturer's instructions (5 nmol labelling kit, GE Healthcare). Labelling was performed on ice for 30 minutes in the dark and quenched with 1 mM of lysine for 10 min. Four samples per dietary treatment were labelled with Cy3 and four with Cy5 to reduce impact of label differences, while an internal standard consisting of equal amounts of protein from all samples was labelled with Cy2.

2.5.3 Protein Separation by 2D Gel Electrophoresis

Labelled proteins from each dietary treatment plus 50 µg of internal standard were mixed together and rehydration buffer (6M urea, 2M thiourea, 4% CHAPS, 0.02 % (w/v) DTT, 0.002% bromophenol blue, 0.5% (v/v) IPG buffer pH 4-7) was added to complete 450 µl. Rehydration was performed passively for 14 hours using IPG box (GE Healthcare) on 24 cm Immobiline™ DryStrips (GE Healthcare) with linear pH 4-7, continued by isoelectric focusing (IEF) in 5 steps: at 500V gradient 1 hr, at 500V step-n-hold 1 hr, at 1000V gradient 1 hr, at 8000V gradient 3 hrs, and at 8000V step-n-hold for a total of 60000 Vhr. Before second dimension, strips were reduced and alkylated using 6 ml of an equilibration buffer (1.5M Tris-HCl pH 8.8, 6M urea, 30% (v/v) glycerol, 0.007M SDS, a few grains of bromophenol blue) with 1% (w/v) DTT or 2.5% (w/v) iodoacetamide respectively for 15 min each. Strips were loaded onto 12.5% Tris-HCl SDS-PAGE gels and run in an Ettan DALTsix Vertical System at

10 mA/gel for 1 hour followed by 60 mA/gel using a standard Tris-Glycine-SDS running buffer, until the bromophenol blue line reaches the end of the gel.

2.5.4 Gel Image Acquisition, Analysis and Statistics

Gels were scanned on a Typhoon scanner 9400 (GE Healthcare) using three laser emission filters (520BP40 for Cy2, 580BP30 for Cy3 and 670BP30 for Cy5) at 100 µm resolution. Images were analyzed with SameSpots (Totallab, United Kingdom) with filters for average normalized volume ≤ 10,000 and spot area ≤ 500. All the significantly different spots ($p<0.05$ by ANOVA and a frequency discovery rate (FDR)) resultant of protein abundance data (expressed in relation to the internal standard) that presented differences between T1 and the other treatments, and the spots with higher average normalized volume that presented differences between T1 and T2 versus control were manually excised from preparative gels stained with colloidal Coomassie blue.

2.5.5 Protein Identification by MS Analysis of Peptides and Database Search

The proteins of the previously selected spots were identified by LC-MS/MS. Proteins were broken up into peptides by tryptinization, using the following methodology: Spots of interest were washed in 200 µl of 100 mM ammonium bicarbonate for 30 minutes to 1 hour on a shaker. Wash was discarded and 200 µl of 50% acetonitrile / 100 mM ammonium bicarbonate was added for 30 minutes to 1 hour on a shaker. Wash was discarded and 50 µl acetonitrile was added to the spots and left to actuate for 10 minutes. Solvent was discarded and spots are dried on a vacuum centrifuge for at least 30 minutes. Twenty µl of trypsin was added to each spot and allowed to digest overnight at 37ºC. After digestion 20 µl of 5% FA acid is added to the gel pieces and left to incubate on a shaker for 20 minutes. Forty µl of acetonitrile was added and left again on a shaker for 20 minutes. Liquid was transferred to a new microplate and dried on a vacuum centrifuge for a couple of hours.

Then the obtained peptides were electrosprayed into the mass spectrometer using a quadrupole and subsequently an orbitrap connected to an LC ultimate 3000 nanosystem (Waters). The flow rate of the loading pump was 25 µl/min, at 37ºC, 44.7 bar. The resulting MS/MS data were used

as input in the SEQUEST software using the Actinopterygii subset of the NCBInr database. These searches were performed assuming the formation of single-charged peptides, carbamidomethylation of cysteine residues, possible oxidation of methionine residues and up to 1 missed cleavage. Mass tolerance was 10 ppm for MS data and 0.5 Da for MS/MS data.

III. Results

. Dinospore Counting and Branchial Analysis

The dinospore counts in the water from the different treatments are indicated in Figure 3.1.

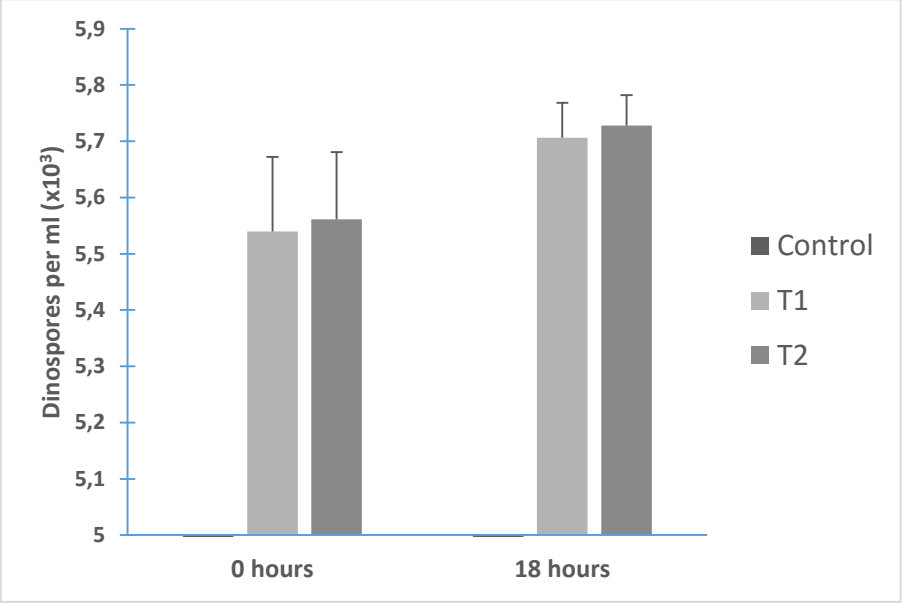

Figure 3.1 - *Amyloodinium ocellatum* dinospores concentration in the water from different treatments, T1, T2 and Control (N=11, Error bars= standard deviation).

The number of dinospores per ml in T1 and T2 tanks was very high, indicating a large potential infective population of parasites present in the tanks. No parasites were found in the control tank.

After 18 hours, the observation of the branchial arches indicated that Control tanks had no *A. ocellatum* trophonts in the gills. T1 had a total of 497.25±14.50 *A. ocellatum* trophonts per branchial arc, and T2 had 50.52±10.96 monogeneans (mainly *Dactylogirus* sp.) and 79.13± 20.71 *A. ocellatum* trophonts per branchial arc. In figure 3.2 there are some images of gilthead sea bream branchia from the Control, T1 and T2.

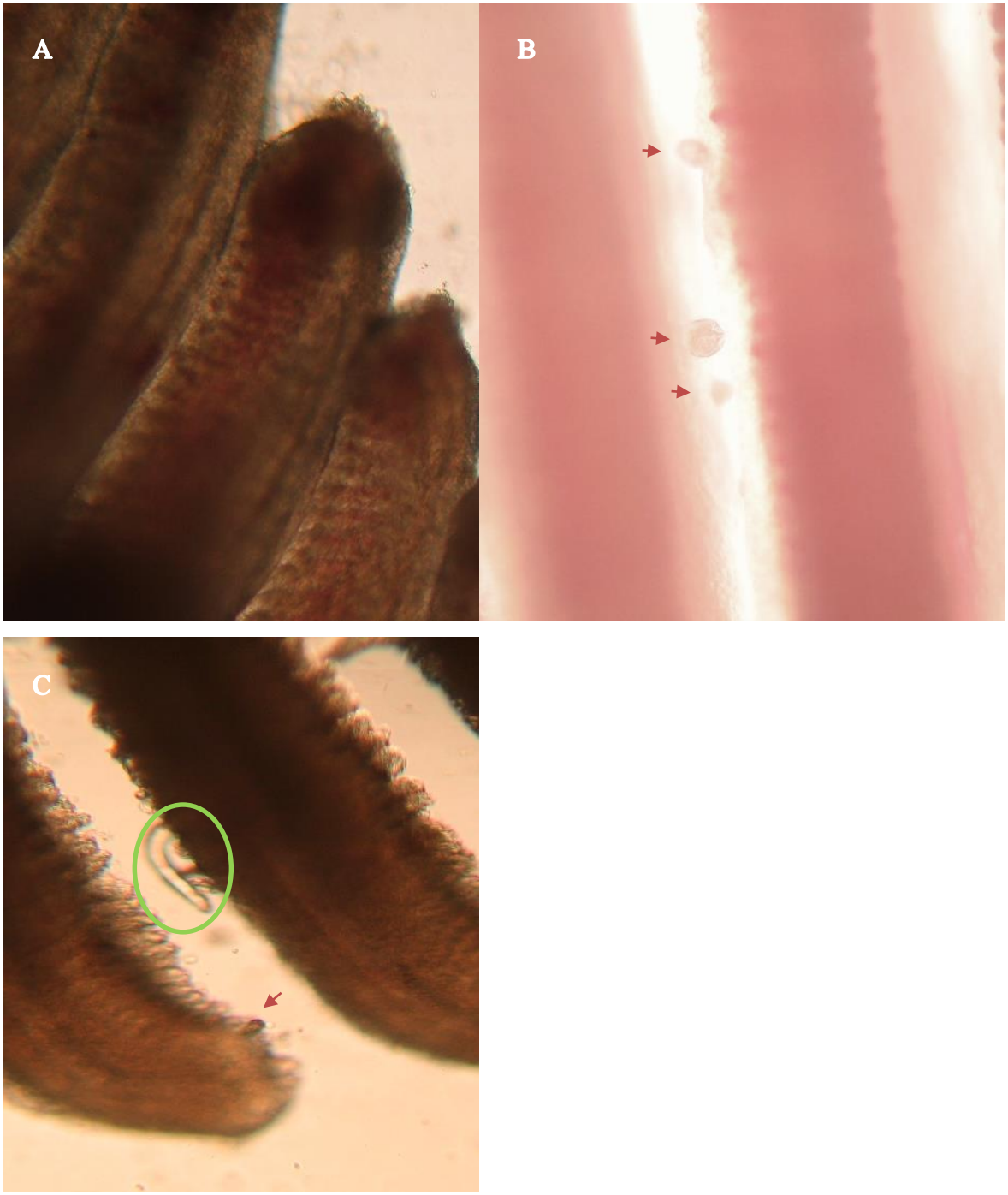

Figure 3.2 - Images of gilthead sea bream *(Sparus aurata)* branchia from the different treatments: Control (A, 40x amp.), T1 (B, 40x amp.) and T2 (C, 40x amp.) after 18h of exposition to an *Amyloodinium ocellatum* outbreak. The red arrows point out the *Amyloodinium ocellatum* trophonts and the green circle the monogenean parasite *Dactylogirus* sp.

These results assure that there was no cross-contamination to the Control tanks, and that the parasite populations for T1 and T2 are the expected for the experiment. However, the lower number of *A. ocellatum* trophonts was unexpected.

Condition Index, Hepatosomatic Index, Spleen Somatic Index

The result of IC, HSI and SSI are represented in figure 3.3.

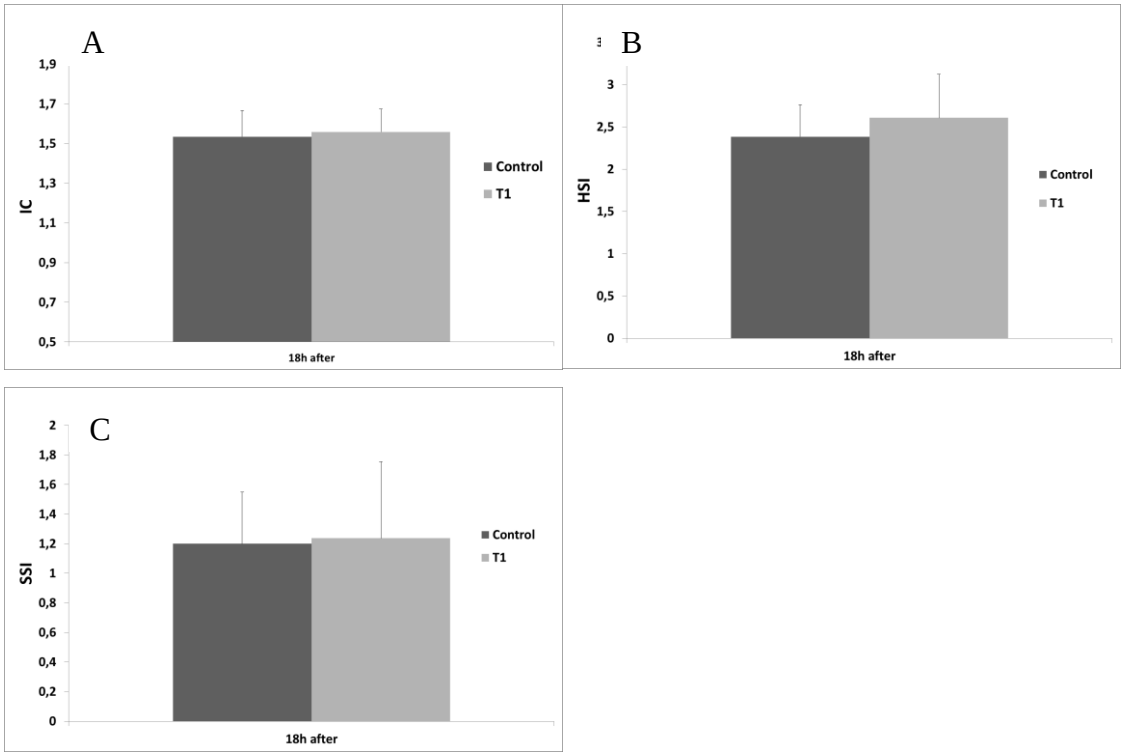

Figure 3.3 - Gilthead sea bream *(Sparus aurata)* IC (A), HSI (B) and SSI (C) values in Control and T1 after 18h of exposition to an *Amyloodinium* ocellatum outbreak (N=11, Error bars= standard deviation).

The graphics above indicate that there are no significant differences ($p<0.05$) between control and T1 at 18h in gilthead sea bream exposed to *A. ocellatum* on IC, HSI and SSI.

Stress, Metabolic and Osmotic indicators

The results of the different stress and osmoregulatory indicators analysed are indicated in Figure 3.4.

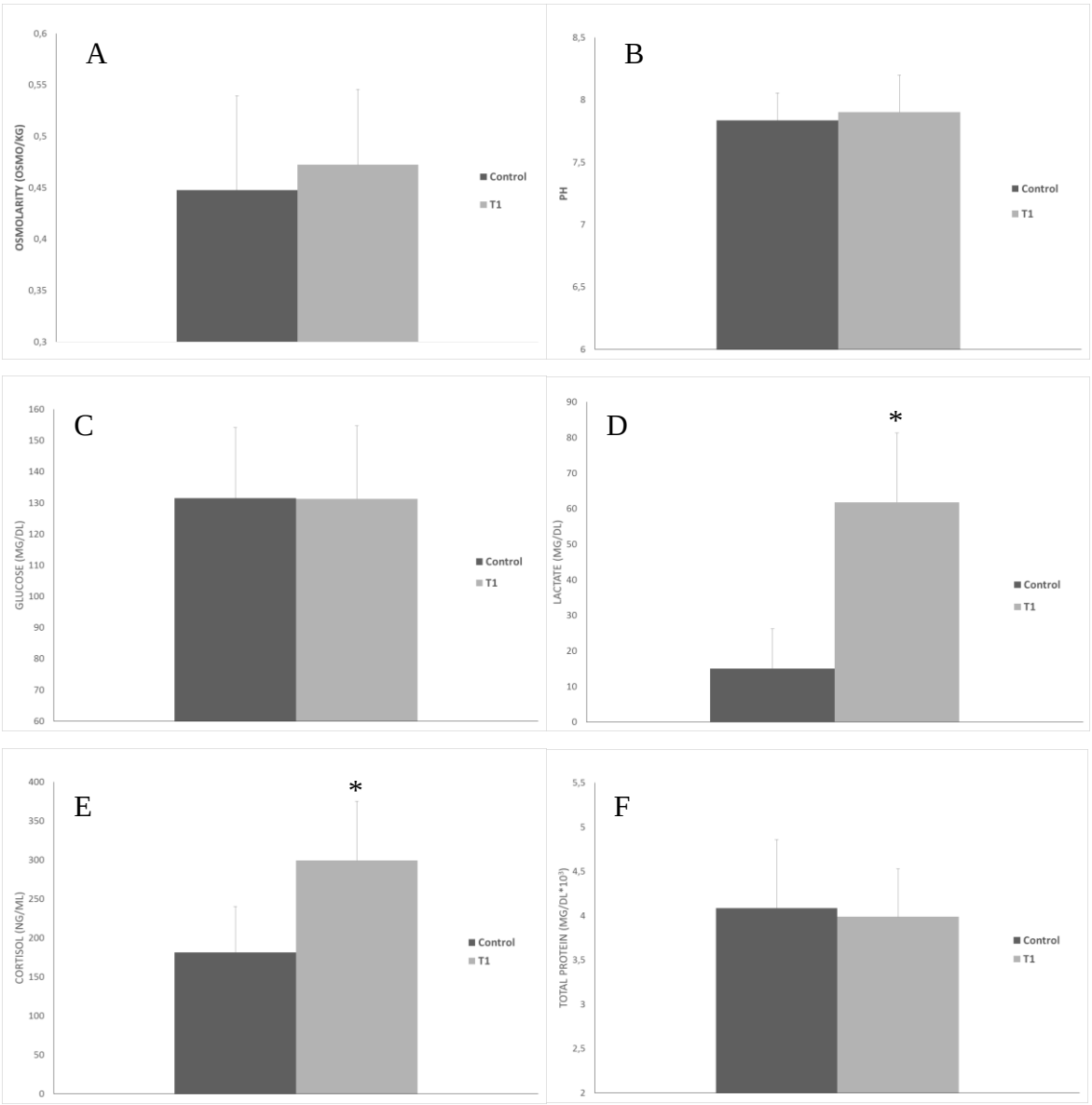

Figure 3.4 - Gilthead sea bream *(Sparus aurata)* osmolarity (A), pH (B), Glucose (C), Lactate (D), Cortisol (E) and Total protein (F) levels in Control and T1 after 18h of exposition to an *Amyloodinium ocellatum* outbreak. (N=11, Error bars= standard deviation. The asteriscus (*) marks the test where is statistical difference between treatments).

We can observe that there are significant differences ($p<0.05$) in the cortisol and lactate levels between Control and T1. T1 has higher levels of cortisol and lactate than control.

The rest of the tests (glucose, osmolarity, pH and total protein) didn´t present significant differences ($p<0.05$) between Control and T1.

Proteomic Analysis

3.4.1 Selection of pH Range

The gels obtained for the different pH ranges are present in figures 3.5, 3.6 and 3.7:

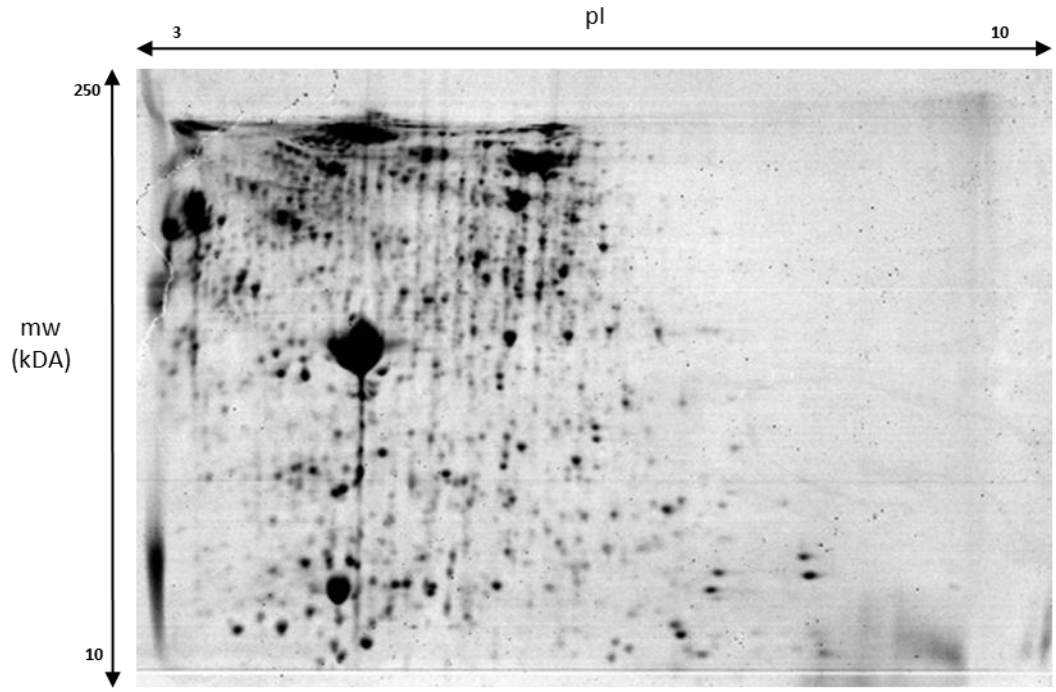

Figure 3.5 - Gel with pH 3-10 range obtained for gilthead sea bream *(Sparus aurata)* plasma

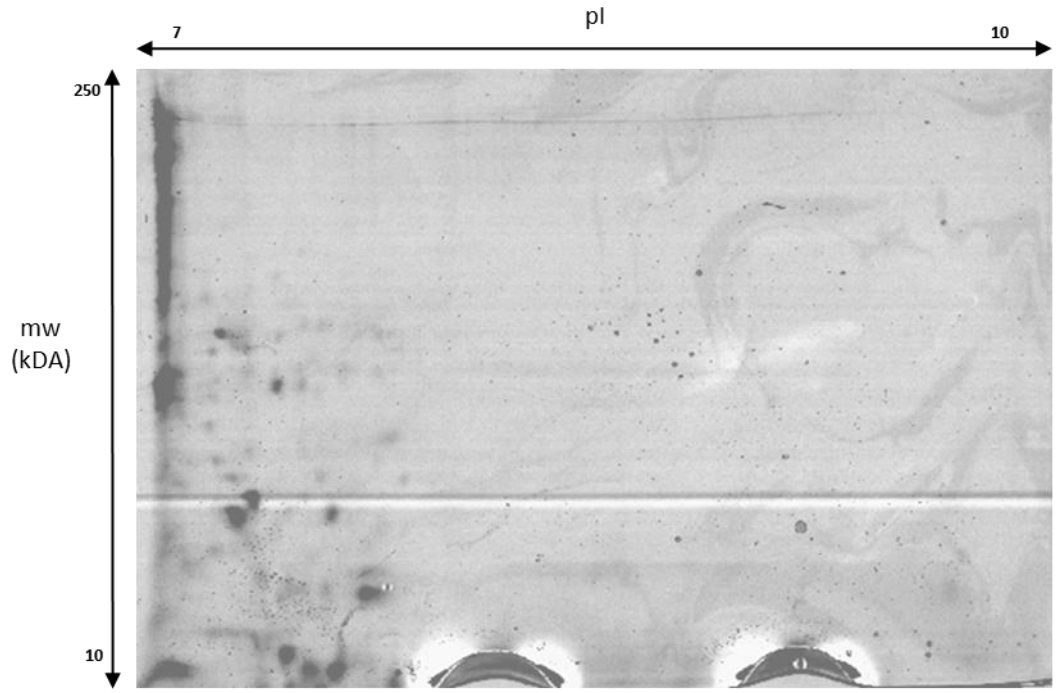

Figure 3.6 - Gel with pH 7-10 range obtained for gilthead sea bream *(Sparus aurata)* plasma

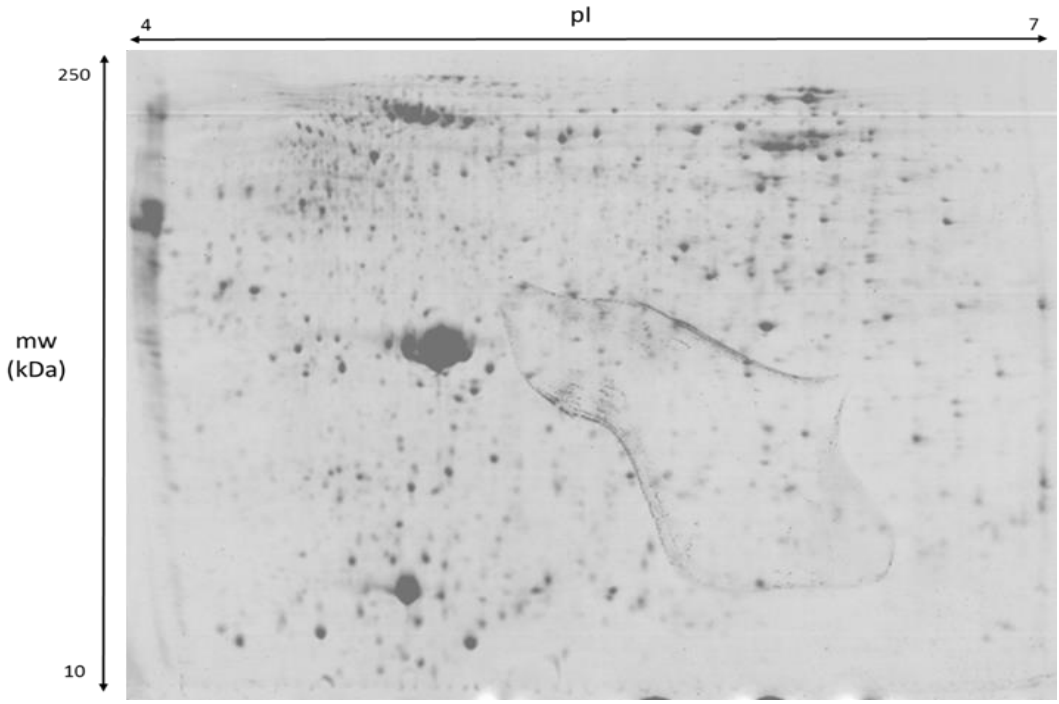

Figure 3.7 - Gel with pH 4-7 range obtained for gilthead sea bream *(Sparus aurata)* plasma

From these gels, we can observe that in the end part of the pH 3-10 gel there is almost no spots present, which is confirmed by the pH 7-10 gel that presents 40 spots (approximately).

The pH 4-7 gel is the one that presents the best spot coverage and resolution, that will allow an easy excision of the spots of interest for analysis and also a better identification of proteins

3.4.2. DIGE, Selection of Spots and Protein Identification

The MS analysis yield a total of 2078 different spots, from which 752 had differences in the spot volume between treatments. After FDR, we have obtained 409 spots. From those, and as indicated in section Material and Methods, we selected the spots indicated in the 2-DE gel presented in Figure 3.8. These spots were selected because they presented volume differences between T1 and the rest of the treatments, and could be specific reaction of the organism to *A. ocellatum*; or they presented volume differences between T1, T2 and Control, that could be generalistic indicators of organism response to parasitism or specific reaction of the organism to *A. ocellatum*; and the spot with higher volume differences between all the treatments.

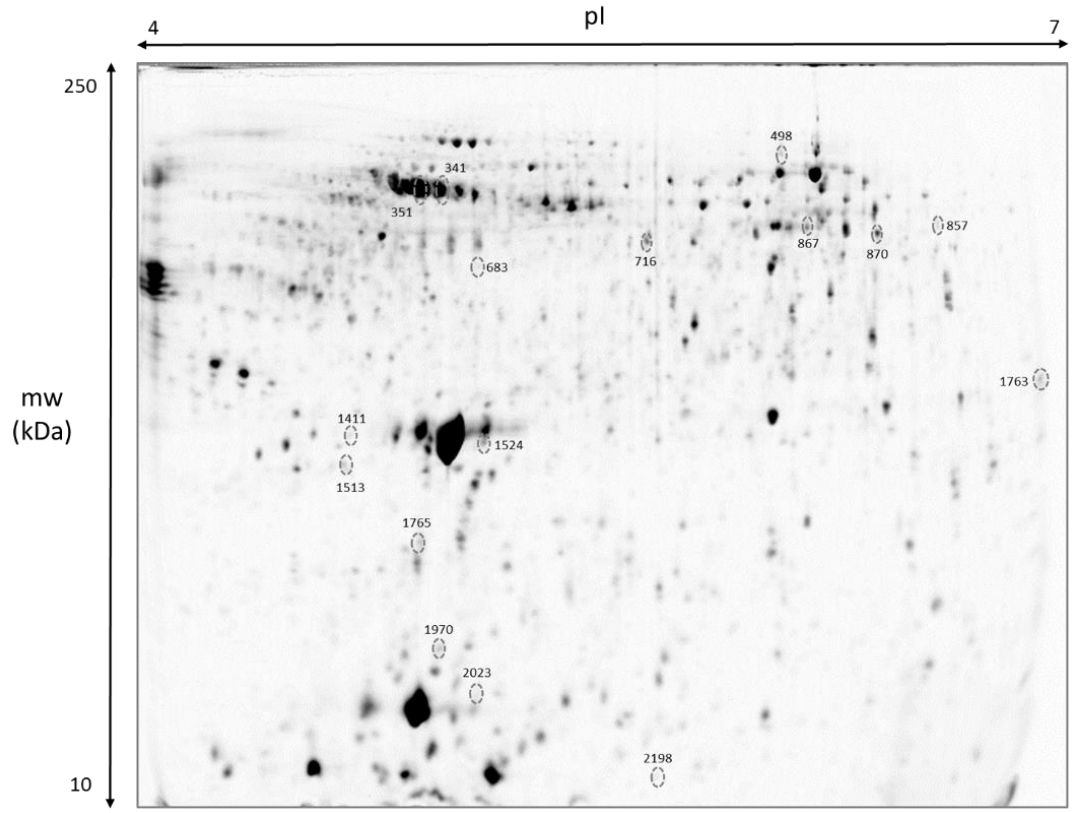

Figure 3.8 - Representative 2D PAGE of gilthead sea bream *(Sparus aurata)* plasma representing the spots selected for analysis.

The selected individual peptides that were identified by LC-MS/MS were used to perform a BLAST search restricted to the Actinopterygii class, and the results obtained are summarized in Table 3.1.

In our work, we found some proteins that can be related with several physiological functions that can be correlated with those symptoms.

We have obtained four matches for different isoforms of Apolipoprotein A-I, six matches for isoforms of Transferrin, three matches for isoforms of Warm temperature acclimation related 65 kDa protein, and matches for Uncharacterized protein OS Takifugu rubripes GN LOC101068102 PE 4 SV 1, Uncharacterized protein OS Lepisosteus oculatus GN KRT9 2 of 3 PE 3 SV 1, Estrogen-regulated protein OS Sparus aurata PE 2 SV 1 and Chromosome 7 SCAF15042 whole genome shotgun sequence Fragment OS Tetraodon nigroviridis GN GSTENG0003380800. In Table 3.2, we indicate the expression and possible functions of the peptides obtained.

Table 3.1 - Detailed list of selected protein spots identified by LC-MS/MS differentially expressed in the plasma of gilthead sea bream *(Sparus aurata)* exposed to an *Amyloodinium ocellatum* outbreak.

Nº.	Accession	Description	mW T/C (Da)	pI T/C	Score	Peptides	Sequence Coverage (%)	Anova	Fold	best peptide match: sequence
1970	Apolipoprotein tr|O42175|APOA1_SPAAU	A I OS Sparus aurata GN apoa1 PE 2 SV 1	29615/8781	5,03 / 4,7	8737,304	22	37,6923	0,000543	1,627199	SSLAPQNEQLK
1524	tr|O42175|APOA1_SPAAU	Apolipoprotein A I OS Sparus aurata GN apoa1 PE 2 SV 1	29615/22196	5,03/4,9	22277,95	51	70	0,004577	1,305117	SSLAPQNEQLK
1765	tr|O42175|APOA1_SPAAU	Apolipoprotein A I OS Sparus aurata GN apoa1 PE 2 SV 1	29615/14313	5,03/6,2	9561,762	19	27,3077	0,000486	1,915019	IQANVEETK
2023	tr|O42175|APOA1_SPAAU	Apolipoprotein A I OS Sparus aurata GN apoa1 PE 2 SV 1	29615/7713	5,03/4,8	15933,77	36	46,9231	0,000495	1,964107	SSLAPQNEQLK
1513	tr|Q4RIP0|Q4RIP0_TETNG	Chromosome 7 SCAF15042 whole genome shotgun sequence Fragment OS Tetraodon nigroviridis GN GSTENG0003380800	216349/20089	5,91/4,45	98,7483	7	1,407	9,48E-05	6,98901	EELERER
1411	tr|D0VB93|D0VB93_SPAAU	Estrogen-regulated protein OS Sparus aurata PE 2 SV 1	25589/22870	4,49/4,5	671,5168	3	14,3478	0,001297	1,841448	FGGCHDYQVR
498	tr|F2YLA1|F2YLA1_SPAAU	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	74234/66470	5,88/5,8	106,0871	6	8,9725	0,001353	1,92155	EPIAAASSSALR

716	tr|F2YLA1|F2YLA1_SPAAU	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	74234/54998	5,88/5,1	4035,147	27	16,932	0,006367	1,381493	VPAHAVVTR
857	tr|F2YLA1|F2YLA1_SPAAU	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	74234/46888	5,88/6,15	2100,655	17	15,0507	0,004126	1,413968	APIDNYQACHLVK
867	tr|F2YLA1|F2YLA1_SPAAU	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	74234/51290	5,88/5,8	3374,932	18	14,3271	0,002922	1,322411	APIDNYQACHLVK
870	tr|F2YLA1|F2YLA1_SPAAU	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	74234/47358	5,88/6	5406,854	24	30,6802	0,001358	1,345001	VVDFLQDQQTK
1763	tr|F2YLA1|F2YLA1_SPAAU	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	74234/13753	5,88/6,2	902,5396	8	5,0651	0,001184	1,317303	APAFSCVK
2198	tr|W5N831|W5N831_LEPOC	Uncharacterized protein OS Lepisosteus oculatus GN KRT9 2 of 3 PE 3 SV 1	88937/4074	4,67/5,3	80,5252	3	3,3048	0,002665	1,410271	LAADDFR
1277	tr|H2S183|H2S183_TAKRU	Uncharacterized protein OS Takifugu rubripes GN LOC101068102 PE 4 SV 1	55432/31781	7,84/4,7	1922,945	5	8,998	5,03E-05	1,83739	LDGSVDFGR
683	tr|F1C794|F1C794_PERFV	Warm temperature acclimation protein 65 2 Fragment OS Perca flavescens GN Wap65 2 PE 2 SV 1	19643/54452	4,86/4,8	295,481	1	8,1395	0,000706	1,968914	CAGIEFDAITPDEK
341	tr|C0L788|C0L788_SPAAU	Warm temperature acclimation related 65 kDa protein OS Sparus aurata PE 2 SV 1	49126/69175	5,33/4,8	1072,78	8	12,9412	0,000137	2,026232	FSEESDHVER
351	tr|C0L788|C0L788_SPAAU	Warm temperature acclimation related 65 kDa protein OS Sparus aurata PE 2 SV 1	49126/71990	5,33/4,9	4686,333	35	37,6471	0,00044	1,658337	FSEESDHVER

Table 3.2 - Profile expression and possible function of the identified peptides in gilthead seabream (*Sparus aurata*) exposed to an *Amyloodinium ocellatum* outbreak.

Nº	Description	Fold	Expression	Main possible function
1970	A I OS Sparus aurata GN apoa1 PE 2 SV 1	1,627199	T1 < T2, Control	Lipid metabolism/Immune system/Osmorregulation
1524	Apolipoprotein A I OS Sparus aurata GN apoa1 PE 2 SV 1	1,305117	T1 < T2, Control	
1765	Apolipoprotein A I OS Sparus aurata GN apoa1 PE 2 SV 1	1,915019	T1 < T2, Control	
2023	Apolipoprotein A I OS Sparus aurata GN apoa1 PE 2 SV 1	1,964107	T1 < T2, Control	
683	Chromosome 7 SCAF15042 whole genome shotgun sequence Fragment OS Tetraodon nigroviridis GN GSTENG0003380800	1,968914	T1 < T2, Control	Stress response
1513	Estrogen-regulated protein OS Sparus aurata PE 2 SV 1	6,98901	T1 > T2, Control	Immune system
498	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	1,92155	T1 > T2, Control	Iron transport / homeostasis / Immune system
716	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	1,381493	T1 > T2, Control	
857	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	1,413968	T1 > T2, Control	
867	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	1,322411	T1 > T2, Control	
870	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	1,345001	T1 > T2, Control	
1763	Transferrin OS Sparus aurata GN Tf PE 2 SV 1	1,317303	T1 > T2, Control	
341	Uncharacterized protein OS Lepisosteus oculatus GN KRT9 2 of 3 PE 3 SV 1	2,026232	T1 > T2, Control	Stress response
1411	Uncharacterized protein OS Takifugu rubripes GN LOC101068102 PE 4 SV 1	1,841448	T1, T2 > Control	No ID available. Unknown function.
1277	Warm temperature acclimation protein 65 2 Fragment OS Perca flavescens GN Wap65 2 PE 2 SV 1	1,83739	T1, T2 > Control	Blood clotting
2198	Warm temperature acclimation related 65 kDa protein OS Sparus aurata PE 2 SV 1	1,410271	T1, T2 < Control	Inflammatory response
351	Warm temperature acclimation related 65 kDa protein OS Sparus aurata PE 2 SV 1	1,658337	Control > T1 > T2	Stress response

IV. Discussion

Since an outbreak of *A. ocellatum* is normally associated with mortality, mainly due to anoxia or osmoregulatory impairment and secondary microbial infections due to severe epithelial damage (Lawler, 1980; Noga, 2012), a high population of dinospores of the parasite was needed, in order to maximize the hypothesis of expected changes in the fold of proteins related with the physiological response of the host to the parasite.

The observed lower load of *A. ocellatum* in T2 was unexpected, since there is no reference in the literature to a lower load of this parasite in the presence of other parasites, like trematodes. Even so, this fact can have two explanations: intraspecific competition between parasites for space in the branchia (Mladineo *et al.*, 2010; Loot *et al.*, 2011); or an unspecific reaction from the immune system to the presence of ectoparasites (Bayne and Gerwick, 2001; Tort *et al.*, 2004, Tort, 2011), that constitutes an obstacle to the fixation of *A. ocellatum* dinospores.

As for the IC, HSI and SSI, there are no significant differences between Control and T1. Since the experiment as last only 18h, this could explain the absence of differences between treatments. In literature also doesn´t exist any information about variation in IC or HSI in an ectoparasites outbreak.

Nogueira (2014) reported histological changes in the spleen of meagre in response to an *A. ocellatum* outbreak, which could represent some change in the SSI. However, there were no significant differences ($p<0.05$) between Control and T1 for SSI in this experiment. This could be due to the duration of the experiment, or to a different physiological response of the spleen of gilthead sea bream to an *A. ocellatum* outbreak. Further histological studies will be need in order to elucidate this point.

The pH range selection of the strip to the proteomic analysis indicated that the ideal pH range for this analysis was 4-7. In the 3-10 gel, almost all the spots were in the 3-7 pH range, which was confirmed by the 7-10 gel that demonstrated that only 40 spots (approximately) were observed in this gel. So the pH 4-7 gel was the selected, because it had almost all the spots

present in the 3-10 gel, and the better resolution of the spots in the gel allowed an easier excision of the spots for posterior analysis.

In the identified proteins, we have identified several spots (in Table 3.2) as Apolipoprotein A-I (ApoAI), as being down regulated in T1 gilthead sea bream plasma. In this species, this protein is produced in the liver and intestine (Llewellyn *et al.*, 1998) and it has several physiological roles. It is involved in lipid absorption in the intestine (Concha *et al.*, 2005; Panzenbock *et al.*, 2000; Geay *et al.*, 2011) and also in the transport of cholesterol to the liver by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase (Ibarz *et al.*, 2010; Geay *et al.*, 2011). It´s also involved in innate immunity response as antimicrobial protein (Concha *et al.*, 2003; Concha *et al.*, 2004; Sarropoulou *et al.*, 2009; Rajan *et al.*, 2013) with roles in inhibition of bacterial endotoxin, antiviral activity, and inhibition of inflammatory cytokines (Ulevitch and Weinstein 1981; Johnston *et al.*, 2008), neutralization of LPS (Johnston *et al.*, 2008; Ulevitch and Weinstein, 1981) or associated with C3 complex in cod (*Gadus morhua*) (Magnadottir, 2006) and in rainbow trout (*Oncorhynchus mykiss*) (Villarroel et al. 2007). They can also have antioxidant activity (Panzenbock *et al.*, 2000) and osmoregulatory functions in teleosts (Smith *et al.*, 2005), with a possible role in the hyperosmotic regulation on fish (Chen *et al.*, 2009).
The fact that usually an immune response is correlated with an increase or no change of ApoAI (Concha *et al.*, 2003; Concha *et al.*, 2004; Villarroel *et al.*, 2007; Bo *et al.*, 2012; Wu *et al.*, 2012) levels in plasma could rule out the possible role of this protein in response to an outbreak of *A. ocellatum*. But, since Smith *et al.* (2005) correlate the expression of ApoAI as a determinant of the barrier properties of the freshwater fish gill (particularly in terms of modulating trans-cellular permeability the trans-cellular conductance in branchial epithelia) and Chen *et al.* (2009) indicate a downregulation of ApoAI when ayu (*P. altivelis*) passes from freshwater to brackish water, the observed downregulation of ApoAI could be related with the physiological impairment or epithelial damage of the branchia by *A. ocellatum*.

Six spots (in Table 3.2) were identified as Transferrin (Tf), which was upregulated in T1 on gilthead sea bream plasma. The observed differences in pI were also observed by Jurado *et al.* (2015) in gilthead sea bream mucus, and they can possibly be explained by the different levels of glycosylation, since this protein has various sites for O- and N-glycosylation in mammals (Satomi *et al.*, 2004).

Transferrin is a protein with a central role in iron metabolism by binding and transporting iron (Zanuzzo *et al.,* 2015), thus making it unavailable for catalysis of superoxide radical formation via Fenton reactions (Neves and Rodrigues, 2009). Tf has also a role in homeostasis (Crichton *et al.,* 2002; Gomme and Bertolini, 2005) and oxidative stress (Zheng and Sun, 2010) response. Tf is also associated with a role in the innate immune system response (Congleton and Wagner, 1991; Chen *et al.,* 2009; Garcia-Fernandez and Blanco, 2011) since it binds to iron, creating an environment with low levels of iron, unfit to microorganism's survival. In case of inflammation, Tf can act mostly as a negative acute phase protein (Ritchie *et al.,* 1999), and there are also some referenced cases where they can act as a positive acute phase protein (Congleton and Wagner, 1991; Ritchie *et al.,* 1999; Bayne and Gerwick, 2001).

The overexpression of Transferrin in T1 can be explained as an immune response against the parasite *A. ocellatum*, since Tf expression increases in liver in case of infection and iron overload and decreasing in iron deficiency, and in the brain, Tf expression was also increased in infection (Neves and Rodrigues, 2009). Since in response to cellular damage and/or infection, proteolytic cleavage products of Tf serve as "alarms" for the immune system, activating fish macrophages that produce nitric oxide in response (Stafford and Belosevic, 2001; Sarropoulou *et al.,* 2009). Addis *et al.* (2010) also demonstrates a upregulation in transferrin in gilthead sea bream exposed to *Moraxella* sp. that can be due to intraperitoneal inflammation, Poochai *et al.* (2014) refer an upregulation of transferrin in response to a *Streptococcus agalactiae* challenge in Nile tilapia (*Oreochromis niloticus*), and Bayne and Gerwick (2001) also refer an upregulation of Tf in rainbow trout. Contradictory results were pointed out by Garcia-Fernandez and Blanco (2011) that expected a downregulation of this protein in response to an immune challenge.

Three spots were identified as warm temperature acclimation related 65 kDa (Wap65) protein (in Table 3.2). This group of proteins can have different roles. They can be associated with the acclimation of different fish species to warmer temperatures (Choi e*t al.,* 2008; Addis *et al.,* 2010; Pierre *et al.,* 2010; Pierre *et al.,* 2011; Li and Chen, 2013), with a possible role in maintaining proteins in their correct folding (Kikuchi *et al.,* 1995), they can act as metalloendopeptidases in liver of ayu (Li *et al.,* 2011), and have a role in immune system regulation (Picard and Schulte, 2004) associated with acute immune response mechanisms (Sha *et al.,* 2008; Shi *et al.,* 2010; Sarropoulou *et al.,* 2010; Bich Hang *et al.,* 2013) and function through the complement activation pathways when microbial infection occurs (Diaz-Rosales *et*

al., 2014). Diaz-Rosales *et al.* (2014) also suggests potential anti-inflammatory properties of Wap65 in turbot (*Scophtalamus maximus*), especially as mediators of an anti-inflammatory response.

This protein presents different expression patterns, being upregulated in T1 in relation to Control and T2 in one of the spots, downregulated in T1 in relation to Control and T2 in other spot, and other that is down regulated in T1 in relation to Control and upregulated in relation to T2. This can be explained by the fact that most teleost species presents two Wap65 genes and several isoforms of this protein (Eissa and Wang, 2014), being Wap65-1 widely and constitutively expressed and slightly modulated by temperature, while Wap65-2 is more strongly regulated by an increase of temperature, bacterial and virus infection or LPS stimulation (Sarropoulou *et al.*, 2010; Sha *et al.*, 2008) and the fact that transcriptional patterns of Wap65 genes to different stimulatory treatments are variable and contradictory (Diaz-Rosales *et al.*, 2014).

Due to these facts, and even taking in account that Wap65 in gilthead sea bream exposed to *Moraxella* sp. (Addis *et al.*, 2010) is upregulated, and that Wap65 can also be upregulated in thermal stress, but not in osmotic stress in in olive flounder (*Paralichthys olivaceus*) (Choi, 2010) and black porgy (*Acanthopagrus schlegeli*) (Choi *et al.*, 2008), we cannot correlate de expression of this protein to a specific response to an *A. ocellatum* outbreak.

The uncharacterized protein OS Takifugu rubripes GN LOC101068102 PE4 SV1, in which the closest ID by BLAST (http://www.uniprot.org/blast/uniprot/2014090493DNYK5ZU1) is fibrinogen beta chain protein, that plays an important role with fibrin in blood clotting (Geay *et al.*, 2011), fibrinolysis, cellular and matrix interactions, inflammation, wound healing, neoplasia and can have an immune role in the liver of fishes (Bohne-Kjersem *et al.*, 2009; Xie *et al.*, 2009). The overexpression of this protein in treatment T1 and T2 can be related to a response to the lesions produced by the ectoparasites in gilthead sea bream gills or because of their role as an acute phase protein, as a coagulation factor, as reporter by Bayne and Gerwick (2001) and L. Tort (2004).

The uncharacterized protein OS Lepisosteus oculatus GN KRT9 2 of 3 PE 3 SV 1, in which the closest ID by BLAST (http://www.uniprot.org/blast/uniprot/B2015092498OFPN8JTE?sort=e-val&desc=false) is Keratin, it´s a cytoskeletal protein whose primary function is to protect cells from mechanical and non-mechanical injuries (Coulombe and Omary, 2002). Recent reports

have also shown that keratin from fish mucus possesses antibacterial activity owing to its pore-forming properties (Molle *et al.*, 2008). Keratin turnover is dependent on ubiquitin-proteasome pathway and it's expression levels can be altered upon cell injury (Rogel and Ridge, 2010).

The tr|D0VB93|D0VB93_SPAAU is an uncharacterized protein with no known function.

As for the osmoregulatory indicators the results showed no significant differences ($p<0.05$) between the pH and osmolarity between Control and T1. This is not in agreement with Noga (2012) and Lawler (1980), which referred the existence of osmoregulatory impairment, inflammation, necrosis and damage to the branchia of the fishes as consequences from infestations of *A. ocellatum.* It is also not in agreement with the differences observed in the expression of ApoAI and Transferrin in our experiment (two proteins that can have osmoregulatory functions).
This observation in pH and osmolarity can be explained by Bayne and Gerwick (2001), that say that when the organisms produce more proteins in response to an exterior stimulus, the organisms tend to control the osmolarity and pH of the blood by decreasing the production of others, which means that even in an *Amyloodinium* outbreak, we could not observe differences in the plasma pH and osmolarity. This can also explain the absence of differences in the total protein levels in gilthead sea bream plasma between treatments.

In the stress and metabolic indicators, we have observed significant differences ($p<0.05$) between treatments in cortisol and lactate analysis, and no difference in glucose analysis.
Plasma cortisol in fish increase after stress exposure (primary response), and is related with the activation of the acute phase protein system and the regulation of the immune system (Bayne and Gerwick, 2001). This could explain the observed differences in the expression of Tf, ApoAI, Wap65 and fibrinogen beta chain protein, which are proteins that belong to acute phase response system and immune response system (Bayne and Gerwick, 2001; Tort *et al.*, 2004; Boulton *et al.*, 2015).
High cortisol levels can also help to balance of blood potassium and sodium ions level in animal body (Kousha, 2013), which also contributes to explain the absence of differences in pH and osmolarity analysis between treatments.

Elevated levels of cortisol also induces higher concentrations of glucose and lactate (secondary response), respectively, caused by extended glycogenolysis and gluconeogenesis through the degradation of glycogen (Olsen *et al*., 2008) and change the lactate to the form of pyruvate (pyruvic acid) in the liver of fish (Philp *et al*., 2005). But in this experiment there are no significant differences in the glucose levels, but only in lactate levels.

So an increased plasma glucose concentration can indicate a mobilization of this metabolite to provide extra energy resources, enabling the fish to overcome the disturbance (Wendelaar Bonga, 2007). Soengas *et al*. (2007) also says that the increased energy requirements due to high cortisol levels are fulfilled first by glucose and subsequently by lactate.

So the absence of differences in the glucose analysis, and the differences observed in lactate levels could mean that the organism already has depleted the glucose reserves, and that is using the lactate as energy source for the organism.

So, how all this pieces fit together in a physiological response to the presence of the parasite *A. ocellatum*?

One possible interpretation is that the presence of the parasite induces a stress response by the fish (Landsberg *et al.,* 1998). This is revealed by the higher amount of cortisol in T1 in relation to Control after 18h. The cortisol induces the activation of the acute phase response proteins, which is demonstrated in this work by the higher volume of the spots related to the proteins Tf, Wap65 and fibrinogen beta chain protein.

Metabolically, cortisol also induces the metabolization of glucose and subsequently lactate to cope with the higher energy requirements of the organism that is subjected to stress (Soengas *et al*., 2007; Olsen *et al*., 2008). After 18h (sampling), the lactate levels are higher in T1 and there is no significant differences in the glucose levels between treatments. This can indicate a depletion of the glucose levels in the organism, that explain the higher levels of lactate, a sub product of the glycolysis metabolic pathway (Soengas *et al*., 2007).

We also know that parasites can induce an innate immune response in fishes (Tort, 2011) and that secondary microbial infections due to severe epithelial damage (Lawler, 1980; Noga, 2012), could also activate an immune response from the organism. This could explain the difference in the volume of the spots related with the Tf, Wap65, fibrinogen beta chain and possibly keratin proteins.

The severe epithelial damage caused by the parasite *A. ocellatum* can also induce anoxia and osmoregulatory impairment in the organism (Lawler, 1980; Noga, 2012). This could explain

the volume difference observed in the spots related with proteins with possible functions homeostasis and osmoregulatory functions (ApoAI, Tf), inflammation (Wap65, fibrinogen beta chain), oxidative stress (Tf), and wound healing and neoplasia (fibrinogen beta chain and keratin).

But with possible osmoregulatory impairment and all the production of metabolites and proteins, there should be changes in the pH and osmolarity in fish plasma. But cortisol has also a function in the regulation of the homeostasis in plasma (Kousha, 2013), that could explain the absence of pH and osmolarity between treatments observed at the time of sampling.

However, this is only a possible interpretation of the obtained results. Further studies will be needed to elucidate not only how these proteins are modulate along an *A. ocellatum* outbreak, but also to discover more proteins involved in the physiological response of gilthead sea bream to this parasite.

4.1. General Conclusions

Overall, this preliminary study revealed that amyloodiniosis can upregulate or downregulate proteins with known functions on the immune response and acute phase response, and physiological responses related with inflammation, lipid transport, homeostasis, osmoregulation, wound healing, inflammation, neoplasia, oxidative stress, and iron transport in gilthead sea bream exposed to *A. ocellatum*. Some of this data were also confirmed by the osmotic and stress parameter analysis.

Further interdisciplinary studies will be needed to elucidate the role of these proteins in an amyloodiniosis outbreak.

This was the first time that proteomics was used for the assessment of the physiological changes in the plasma of fishes exposed to *A. ocellatum*. The results obtained demonstrate that proteomics can be an excellent tool for a wider analysis of the physiological responses to *A. ocellatum* outbreaks.

The results obtained in this work, even if preliminary, can be the origin of future works. They could not only to improve the knowledge about physiological response of fishes to this parasite,

but also, based on the obtained knowledge, to develop products to improve the physiological resistance of the fishes to *A. ocellatum*

4.2. Future perspectives

This work was only an initial study in order to verify the viability of using proteomic techniques in the analysis of the physiological responses of gilthead sea bream to an *A. ocellatum* outbreak.

This study opens different work areas in the analysis of the organism response to an *A. ocellatum* outbreak.

Further studies will be needed not only to analyze the proteomic profile of the blood plasma at different hours of contamination, but also to see the proteomic response in different organs to an *A. ocellatum* outbreak.

Based on the fact that we know that *A. ocellatum* act on different physiological areas, we can develop and test products or improve feeding for the critical time of infestation of this parasite, in order to improve the physiological resistance of the fishes. This will allow that fish could resist more time to *A. ocellatum* outbreaks, and give more time to the producers to react and treat the affected fishes.

V. Bibliography

Aardt, W.J., J. Booysen. 2004. 'Water Hardness and the Effects of Cd on Oxygen Consumption, Plasma Chlorides and Bioaccumulation in *Tilapia sparrmanii'*, *Water SA*, 30: 57–64.

Abowei, J.F.N., O.F. Briyai, S.E. Bassey. 2011. 'A Review of Some Basic Parasite Diseases in Culture Fisheries Flagellids, Dinoflagellides and Ichthyophthriasis, Ichtyobodiasis, Coccidiosis Trichodiniasis, Heminthiasis, Hirudinea Infestation, Crustacean Parasite and Ciliates', *British Journal of Pharmacology and Toxicology*, 2(5): 213-226.

Abreu, P.C., R.B. Robaldo, L.A. Sampaio, A. Bianchini, C. Odebrecht. 2005. 'Recurrent Amyloodiniosis on Broodstock of the Brazilian Flounder *Paralichthys orbignyunus*: Dinospore Monitoring and Prophylactic Measures'. *Journal of the World Aquaculture Society*, 36(1): 42–50.

Addis, M., R. Cappuccinelli, V. Tedde, D. Pagnozzi, I. Viale, M. Meloni, F. Salati, T. Roggio, S. Uzzau. 2010a. 'Influence of *Moraxella* sp. colonization on the kidney proteome of farmed gilthead sea breams (*Sparus aurata*, L.)', *Proteome Science*, 8: 50.

Addis, M.F., R. Cappuccinelli, V. Tedde, D. Pagnozzi, M.C. Porcu, E. Bonaglini, T. Roggio, S. Uzzau. 2010b. 'Proteomic analysis of muscle tissue from gilthead sea bream (*Sparus aurata*, L.) farmed in offshore floating cages', *Aquaculture*, 309: 245-52.

Aiello, P., A. D'Alba. 1986. '*Amyloodinium ocellatum* infestation in yellowtail, *Seriola dumerili*, intensively reared in Sicily, Italy', *Bulletin of the European Association of Fish Pathologists*, 6: 110–111.

Alvarez-Pellitero, P., A. Sitja-Bobadilla, A. Franco-Sierra. 1993. 'Protozoan parasites of wild and cultured sea bass, *Dicentrarchus labrax* (L.), from the Mediterranean area'. *Aquaculture and Fisheries Management*, 24: 101–108.

Alves, R.N., O. Cordeiro, T.S. Silva, N. Richard, M. de Vareilles, G. Marino, P. Di Marco, P.M. Rodrigues, L.E.C. Conceição. 2010. 'Metabolic molecular indicators of chronic stress in gilthead seabream (*Sparus aurata*) using comparative proteomics', *Aquaculture*, 299 (1–4): 57-66.

Aravindan, N.; K. Chaganti; S. Aravindan. 2007. 'Protozoan parasites in commercially important shrimp species from northeast coast of Andhra Pradesh, India', *Journal of Experimental Zoology*, 10 (1): 9-20.

Askarian, F., A. Kousha. 2009. 'The influence of photoperiod in farming beluga sturgeon (*Huso huso*): evaluation by growth and health parameters in serum', *Journal of Fisheries and Aquatic Science*, 4: 41-49.

Balcázar, José Luis, Ignacio de Blas, Imanol Ruiz-Zarzuela, David Cunningham, Daniel Vendrell, and José Luis Múzquiz. 2006. 'The role of probiotics in aquaculture', *Veterinary Microbiology*, 114: 173-86.

Bank, The World. 2013. "Fish to 2030 – Prospects for Fisheries and Aquaculture." In, 1-77. The World Bank.

Bayne, C.J., L. Gerwick. 2001. 'The acute phase response and innate immunity of fish', *Developmental & Comparative Immunology*, 25: 725-43.

Barbaro, A., A. Francescon. 1985. 'Parassitosi da *Amyloodinium ocellatum* (Dinophyceae) su larve di *Sparus aurata* allevate in un impianto di riproduzione artificiale'. *Oebalia*, 11: 745–752.

Baticados, M.C.L., G.F. Quinitio. 1984. 'Occurrence and pathology of an Amyloodinium-like protozoan parasite on gills of grey mullet, *Mugil cephalus* '. *Helgolander Meeresunters*, 37: 595-601.

Becker, C.D..1977. 'Flagellate parasites of fishes'. In: Krier, J.P. (ed.) Parasitic Protozoa. Academic Press, New York, pp. 357–416.

Beranova-Giorgianni, S.. 2003. 'Proteome analysis by two-dimensional gel electrophoresis and mass spectrometry: strengths and limitations', *TrAC Trends in Analytical Chemistry*, 5: 273–281.

Bich Hang, B.T., S. Milla, V. Gillardin, N.T. Phuong, P. Kestemont. 2013. 'In vivo effects of *Escherichia coli* lipopolysaccharide on regulation of immune response and protein expression in striped catfish (*Pangasianodon hypophthalmus*)', *Fish and Shellfish Immunology*, 34: 339-47.

Bo, J., J.P. Giesy, R. Ye, K.-J. Wang, J.-S. Lee, W.T.A. Doris. 2012. 'Identification of differentially expressed genes and quantitative expression of complement genes in the liver of marine medaka *Oryzias melastigma* challenged with *Vibrio parahaemolyticus*',

Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 7: 191-200.

Bohne-Kjersem, A., A. Skadsheim, A. Goksøyr, B.E. Grøsvik. 2009. 'Candidate biomarker discovery in plasma of juvenile cod (*Gadus morhua*) exposed to crude North Sea oil, alkyl phenols and polycyclic aromatic hydrocarbons (PAHs)', *Marine Environmental Research*, 68: 268-77.

Bolasina, S.N.. 2011. 'Stress response of juvenile flounder (*Paralichthys orbignyanus*, Valenciennes 1839) to acute and chronic stressors', *Aquaculture*, 313: 140-143.

Boulton, K., E. Couto, A.J. Grimmer, R.L. Earley, A.V.M. Canario, A.J. Wilson, C.A.Walling. 2015. 'How integrated are behavioral and endocrine stress response traits? A repeated measures approach to testing the stress-coping style model.', *Ecology and Evolution*, 5(3): 618–633.

Brown, E. M., R. Hovasse. 1946. '*Amyloodinium ocellatum* Brown, a peridinian parasite on marine fishes: A complementary study', *Proc. Zool. Soc. Lond*. 116: 33-46.

Brunt, J., R. Hansen, D.J. Jamieson, B. Austin 2008. 'Proteomic analysis of rainbow trout (*Oncorhynchus mykiss*, Walbaum) serum after administration of probiotics in diets', *Veterinary Immunology and Immunopathology*, 121 (3–4): 199-205.

Buckland-Nicks, J., T. Reimchen. 1995. 'A novel association between an endemic stickleback and a parasitic dinoflagellate. 3. Details of the life cycle', *Archiv für Protistenkunde*, 145: 165–175.

Cannon, W.B.. 1935. 'Stresses and strains of hemostasis', *The American Journal of the Medical Sciences*, 189: 1-14.

Carragher, J.F., J.P. Sumfter, T.G. Pottinger, A.D. Pickering. 1989. 'The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson', *General and Comparative Endocrinology*, 76: 310-321.

Cecchini, S., M. Saroglia, G. Terova, F. Albanesi. 2001. 'Detection of antibody response against *Amyloodinium ocellatum* (Brown, 1931) in serum of naturally infected European sea bass by an enzyme-linked immunoabsorbent assay (ELISA) ', *Bulletin of the European Association of Fish Pathologists*, 21: 104-108.

Chen, J., Y. H. Shi, H. Q. Hu, H. Niu, and M. Y. Li. 2009a. 'Apolipoprotein A-I, a hyperosmotic adaptation-related protein in ayu (Plecoglossus altivelis)', *Comp Biochem Physiol B Biochem Mol Biol*, 152: 196-201.

Chen, J., H. Y. Yang, Y. H. Shi, and M. Y. Li. 2009b. 'An interaction between leukocyte cell-derived chemotaxin 2 and transferrin of ayu, Plecoglossus altivelis', *Fish Shellfish Immunol*, 26: 536-42.

Choi, C.Y., K.W. An, Y.K. Choi, P.G. Jo, B.H. Min. 2008. 'Expression of warm temperature acclimation-related protein 65-kDa (Wap65) mRNA, and physiological changes with increasing water temperature in black porgy, *Acanthopagrus schlegeli*', *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 309A: 206-14.

Choi, C.Y.. 2010. 'Environmental stress-related gene expression and blood physiological responses in olive flounder (*Paralichthys olivaceu*s) exposed to osmotic and thermal stress', *Animal Cells and Systems*, 14: 17-23.

Chonchuenchob, P., S. Sumpawapol, A. Mearoh. 1987. 'Diseases of cage-cultured sea bass (*Lates calcarifer*) in southestern Thailand', *Australian Centre for International Agricultural Research Proceedings Series*, 20: 194-197.

Chong Y.C., T.M. Chao. 1986. 'Common Diseases of Marine Foodfish', *Singapore Primary Production Department*: n/a-n/a.

Cobb, C.S., M.G. Levy, E.J. Noga. 1998a. 'Development of immunity by the tomato clownfish Amphiprion frenatus to the dinoflagellate parasite *Amyloodinium ocellatum*', *Journal of Aquatic Animal Health*, 10: 259–263.

Cobb, C.S., M.G. Levy, E.J. Noga. 1998b. 'Acquired immunity to amyloodiniosis is associated with an antibody response', *Diseases of Aquatic Organisms*, 34: 125–133.

Colorni, A. 1989. 'Pathology of marine warmwater finfish in Israel: problems and research', *Advances in Tropical Aquaculture*, AQUACOP, IFREMER, Actes de Colloque 9: 133-142.

Colorni, A. 1994. 'Hyperparasitism of *Amyloodinium ocellatum* (Dinoflagellida: Oodinidae) on *Neobenedenia melleni* (Monogenea: Capsalida) '. *Diseases of Aquatic Organisms*, 19: 157-159.

Colorni, A., A. Ullal, G. Heinisch, and E. J. Noga. 2008. 'Activity of the antimicrobial polypeptide piscidin 2 against fish ectoparasites', *Journal of Fish Diseases*, 31: 423-32.

Concha, M.I., S. Molina, C. Oyarzún, J. Villanueva, R. Amthauer. 2003. 'Local expression of apolipoprotein A-I gene and a possible role for HDL in primary defence in the carp skin', *Fish and Shellfish Immunology*, 14: 259-73.

Concha, M.I., V.J. Smith, K. Castro, A. Bastias, A. Romero, and R.J. Amthauer. 2004. 'Apolipoproteins A-I and A-II are potentially important effectors of innate immunity in the teleost fish *Cyprinus carpio*', *European Journal of Biochemistry*, 271: 2984-90.

Concha, M. I., R. Lopez, J. Villanueva, N. Baez, and R. Amthauer. 2005. 'Undetectable apolipoprotein A-I gene expression suggests an unusual mechanism of dietary lipid mobilisation in the intestine of *Cyprinus carpio*', *The Journal of Experimental Biology*, 208: 1393-9.

Congleton, J.L., E.J. Wagner. 1991. 'Acute-phase hypoferremic response to lipopolysaccharide in rainbow trout (*Oncorhynchus mykiss*)', *Comparative Biochemistry and Physiology Part A: Physiology*, 98: 195-200.

Corrales, J., I. Mulero, V. Mulero, E.J. Noga. 2010. 'Detection of antimicrobial peptides related to piscidin 4 in importante aquacultured fish', *Developmental and Comparative Immunology*, 34: 331–343.

Coulombe, P.A., M.B. Omary. 2002. ''Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments', *Current Opinion in Cell Biology*, 14: 110-22.

Cox, J., M. Mann. 2011. 'Quantitative, high-resolution proteomics for data-driven systems biology', *Annual Review of Biochemistry*, 80: 273–99.

Crichton, R.R., S. Wilmet, R. Legssyer, R.J. Ward. 2002. 'Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells', *Journal of Inorganic Biochemistry*, 91: 9-18.

Cristina García Diez, C.; Remiro Perlado, J. P. 2014. *Impactos del Cambio Climático sobre la Acuicultura en España* (Oficina Española de Cambio Climático, Ministerio de Agricultura, Alimentación y Medio Ambiente. : Madrid, Spain).

Cruz-Lacierda, E. R., Y. Maeno, A.J.T. Pineda, V.E. Matey. 2004. 'Mass mortality of hatchery-reared milkfish (*Chanos chanos*) and mangrove red snapper (*Lutjanus argentimaculatus*) caused by *Amyloodinium ocellatum* (Dinoflagellida) ', *Aquaculture*, 236: 85–94.

Cui, S.-J., L.-L. Xu, T. Zhang, M. Xu, J. Yao, C.-Y. Fang, Z. Feng, P.-Y. Yang, W. Hu, F. Liu. 2013. 'Proteomic characterization of larval and adult developmental stages in *Echinococcus granulosus* reveals novel insight into host–parasite interactions', *Journal of Proteomics*, 84: 158-175.

Diaz-Rosales, P., P. Pereiro, A. Figueras, B. Novoa, S. Dios. 2014. 'The warm temperature acclimation protein (Wap65) has an important role in the inflammatory response of turbot (*Scophthalmus maximus*)', *Fish Shellfish Immunology*, 41: 80-92.

Drebes, G.. 1984. 'Life cycle and host specificity of marine parasitic dinophytes', *Helgolander Meeresuntersuchungen*, 37: 603–622.

Dowd, W.W.. 2012. 'Challenges for Biological Interpretation of Environmental Proteomics Data in Non-model Organisms', *Integrative and Comparative Biology*, 52 (5): 705-720.

Eyckmans, M., D. Benoot, G.A.A. Van Raemdonck, G. Zegels, X.W.M. Van Ostade, E. Witters, R. Blust, G. De Boeck. 2012. 'Comparative proteomics of copper exposure and toxicity in rainbow trout, common carp and gibel carp', *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, 7 (2): 220-232.

Einarsdóttir, I.E., K.J. Nilssen. 1996. 'Stress response of Atlantic salmon (*Salmo salar* L.) elicited by water level reduction in rearing tanks'. *Fish Physiology and Biochemistry,* 15: 395-400.

Einarsdóttir, I.E., K.J. Nilssen, M. Iversen . 2000. 'Effects of rearing stress on Atlantic salmon (*Salmo salar* L.) antibody response to a non-pathogenic antigen', *Aquaculture Research*, 31: 923-930.

Eissa, N., H.-P. Wang. 2014. 'Transcriptional stress responses to environmental and husbandry stressors in aquaculture species', *Reviews in Aquaculture*: n/a-n/a.

Ewart, K.V., S.C.M. Tsoi. 2004. 'Innate Immune Recognition of Pathogens in Teleost Fish'. In Molecular Aspects of Fish and Marine Biology – Vol. 3: Current Trends in the Study of Bacterial and Viral Fish and Shrimp Diseases, Edited by Leung Ka Yin, World Scientific Publishing Co. Pte. Ltd., pp. 82-116.

Fajer-Ávila, E.J., I. Abdo-de la Parra, G., Aguilar-Zarate, R. Contreras-Arce, J. Zaldivar-Ramirez, M. Betancourt-Lozano. 2003. 'Toxicity of formalin to bullseye puffer fish (*Sphoeroides annulatus* Jenyns) and its effectiveness to control ectoparasites', *Aquaculture*, 223:41–50.

FAO. 2013. 'Food Outlook - Biannual Report on Global Food Markets - June 2013', Rome.

FAO. 2014a. 'Economic analysis of supply and demand for food up to 2030 – Special focus on fish and fishery products. ', Rome: FAO.

FAO. 2014b. 'Food Outlook. Biannual Report on Global Food Markets - October 2014', Rome.

FAO. 2014c. 'The State of World Fisheries and Aquaculture 2014 (SOFIA) ', Rome.

FAO. 2015a. 'Coping with climate change – the roles of genetic resources for food and agriculture', Rome.

FAO. 2015b. ' Food Outlook - Biannual Report on Global Food Markets - May 2015. ',Rome.

FOESA. 2013. 'Cambio climático y acuicultura. ' Madrid: FOESA.

Fensome, R.A., F.J.R. Taylor, G. Norris, W.A.S. Sarjeant, D.I. Wharton, G.L. Williams. 1993. 'A classification of living and fossil dinoflagellates', *Micropaleontological Species Publication*, 7: 351.

Fensome, R.A., J.F. Saldarriaga, F.J.R. Taylor. 1999. 'Dinoflagellate phylogeny revisited: reconciling morphological and molecular based phylogenies', *Grana*, 38: 66 –80.

Fernandes de Castilho, F., T.G. Pottinger, G.L. Volpato. 2008. 'Chronic social stress in rainbow trout: Does it promote physiological habituation?', *General and Comparative Endocrinology*, 155: 141-147.

Fielder, D. S., W. Bardsley. 1999. 'A preliminary study on the effects of salinity on growth and survival of mulloway *Argyrosomus japonicus* larvae and juveniles', *Journal of the World Aquaculture Society*, 30: 380 – 387.

Francis-Floyd, R., M. R. Floyd. 2011. '*Amyloodinium ocellatum*, an Important Parasite of Cultured Marine Fish', *Southern Region Aquaculture Center*, Publication No. 4705.

Fu, S.J., Z.D. Cao, J.L. Peng. 2007. 'The Effect of Acute Stress on Post-Stress Oxygen Consumption Rate in Southern Catfish, *Silurus meridionalis* Chen. ', *International Journal of Zoological Research*, 3: 101-106.

Gallet de Saint-Aurin, D.. 1987. 'Diseases of the sea bass *Dicentrarchus labrax* in intensive rearing programs in Martinique, French West Indies', *Proceedings of the Gulf and Caribbean Fisheries Institute*, 38: 144-163.

Gallet de Saint-Aurin, D., J.C. Raymond, V. Vianas. 1989. 'Marine finfish pathology: specific problems and research in the French West Indies', *Advances in Tropical Aquaculture*, AQUACOP, IFREMER, Actes de Colloque 9: 143-160.

Garcia-Fernandez, C., J.A. Sanchez, G. Blanco. 2011. 'Characterization of the gilthead seabream (*Sparus aurata* L.) transferrin gene: genomic structure, constitutive expression and SNP variation', *Fish Shellfish Immunology*, 31: 548-56.

Gaspar, A.G.. 1987. 'Algunas enfermedades de Pompanos cultivados experimentalmente en Venezuela'. *Revista Latinoamericana de Acuicultura*, 3: 27-44.

Geay, F., S. Ferraresso, J. Zambonino-Infante, L. Bargelloni, C. Quentel, M. Vandeputte, S. Kaushik, C. Cahu, D. Mazurais. 2011. 'Effects of the total replacement of fish-based diet with plant-based diet on the hepatic transcriptome of two European sea bass (*Dicentrarchus labrax*) half-sibfamilies showing different growth rates with the plant-based diet', *BMC Genomics*, 12: 522.

Gomme, P.T., K.B. McCann, J. Bertolini. 2005. 'Transferrin: structure, function and potential therapeutic actions', *Drug Discovery Today*, 10: 267-73.

Harikrishnan, R., C. Balasundaram, M.-S. Heo. 2011. 'Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish', *Aquaculture*, 317: 1–15.

Herbert, B.R., J.L. Harry, N.H. Packer, A.A. Gooley, S.K. Pederson, K.L. Williams. 2001. 'What place for polyacrylamide in proteomics?', *Trends in Biotechnology*, 19(10): (S3–9).

Hogstrand, C., S. Balesaria, C.N. Glover. 2002. 'Application of genomics and proteomics for study of the integrated response to zinc exposure in a non-model fish species, the rainbow trout', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 133 (4): 523-535.

Ibarz, A., R. Costa, A.P. Harrison, D.M. Power. 2010. 'Dietary keto-acid feed-back on pituitary activity in gilthead sea bream: Effects of oral doses of AKG. A proteomic approach', *General and Comparative Endocrinology*, 169: 284-92.

Isani, G., G. Andreani, E. Carpenè, S. Di Molfetta, D. Eletto, E. Spisni. 2011. 'Effects of waterborne Cu exposure in gilthead sea bream (*Sparus aurata*): A proteomic approach', *Fish and Shellfish Immunology*, 31: 1051-58.

Iwama, G. K.. 1998. 'Stress in Fish'. *Annals of the New York Academy of Sciences*, 851: 304–310.

Jenkins, R.E., S.R. Pennington. 2001. 'Arrays for protein expression profiling: towards a viable alternative to two-dimensional gel electrophoresis? ', *Proteomics*, 1: 13–29.

Johnson, S.K.. 1988. 'Control of diseases in red drum. ' In :Arnold, C.R., Holt, G.J., Thomas, P. (eds.). Red Drum Aquaculture: Proceedings of a Symposium on the Culture of Red Drum and Other Warm Water Fishes, Contributions in Marine Science, Supplement to Volume 30: 113–130.

Johnston, L.D., G. Brown, D. Gauthier, K. Reece, H. Kator, P. Van Veld. 2008. 'Apolipoprotein A-I from striped bass (*Morone saxatilis*) demonstrates antibacterial activity in vitro', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 151: 167-75.

Joyce, A.R., B.O. Palsson. 2006. 'The model organism as a system: integrating 'omics' data sets'. *Nature Reviews Molecular Cell Biology*, 7:198–210

Jurado, J., C.A. Fuentes-Almagro, F.A. Guardiola, A. Cuesta, Mª Á. Esteban, M.-J. Prieto-Álamo. 2015. 'Proteomic profile of the skin mucus of farmed gilthead seabream (*Sparus aurata*)', *Journal of Proteomics*, 120: 21-34.

Kabata, Z.. 1985. 'Parasites and Diseases of Fish Cultured in the Tropics'. Taylor & Francis, London, 318 p.

Karlson, Bengt; Cusack, Caroline; Bresnan, Eileen. 2010. "Microscopic and molecular methods for quantitative phytoplankton analysis." In *IOC. Manuals and guides*, 113 UNESCO.

Ki, J.-S.. 2012. 'Hypervariable regions (V1–V9) of the dinoflagellate 18S rRNA using a large dataset for marker considerations', *Journal of Applied Phycology*, 24 (5): 1035-1043.

Kikuchi, K., M. Yamashita, S. Watabe, K. Aida. 1995. 'The Warm Temperature Acclimation-related 65-kDa Protein, Wap65, in Goldfish and Its Gene Expression', *Journal of Biological Chemistry*, 270: 17087-92.

Kingsford, E.. 1975. 'Treatment of Exotic Marine Fish Diseases'. Palmetto Publishing, St Petersburg, Florida, 90 pp..

Kizhakudan, J. K., P.E Praveena, B.A.V. Maran. 2014. 'Investigation on the mortality of juveniles in captive stock of the Indian halibut *Psettodes erumei*'. *Indian Journal of Geo-Marine Sciences* (In press).

Klyashtorin, L.B., B.P. Smirnov, 1981, 'Possibilities for Increase of Metabolism Activity in Fishes Affected by Stress and during Their Growth', *Biologicheskie Nauki*, 5: 58–61.

Kousha, A.. 2013. 'Chronic and acute stress of Atlantic salmon (*Salmo salar* L.): effect on cortisol, blood parameters and gut morphology', Master's Degree Thesis in International

Fisheries Management, Norwegian College of Fishery Sciences, University of Tromsø, 46p..

Kubilay, A., G. Ulukoy. 2002. 'The effects of acute stress on rainbow trout (*Oncorhynchus mykiss*) ', *Turkish Journal of Zoology*, 26: 249-254.

Kumar, P.R., A.K. Abdul Nazar, R. Jayakumar, G. Tamilmani, M. Sakthivel, C. Kalidas, V. Balamurugan, S. Sirajudeen, R. Thiagu, G. Gopakumar. 2015. '*Amyloodinium ocellatum* infestation in the broodstock of silver pompano *Trachinotus blochii* (Lacepede, 1801) and its therapeutic control', *Indian Journal of Fisheries*, 62 (1): 131-134.

Kuperman, B.L., V.E. Matey. 1999. 'Massive infestation by *Amyloodinium ocellatum* (Dinoflagellida) of fish in a highly saline lake, Salton Sea, California, USA', *Diseases of Aquatic Organisms*, 39: 65–73.

Kvasnička, F.. 2003. 'Proteomics: general strategies and application to nutritionally relevant proteins', *Journal of Chromatography B*, 787: 77–89.

Landsberg, J.H., K.A. Steidinger, B.A. Blakesley, R.L. Zondervan. 1994. 'Scanning electron microscope study of dinospores of *Amyloodinium* cf. *ocellatum*, a pathogenic dinoflagellate of marine fish and comments on its relationship to the Peridinales', *Diseases of Aquatic Organisms*, 20: 23 – 32.

Landsberg, J.H., B.A. Blakesley, R.O. Reese, G. Mcrae, P.R. Forstchen. 1998. 'Parasites of fish as indicators of environmental stress'. *Environmental Monitoring and Assessment*, 51: 211-232.

Lauckner, G.. 1984. 'Diseases caused by protophytans (algae) '. In: Kinne, O. (ed.) Diseases of Marine Animals, Vol. IV, Part 1, Biologische Anstalt Helgoland, Hamburg, Germany: 169–180.

Lawler, A. R.. 1977. 'Dinoflagellate (*Amyloodinium*) infestation of pompano'. In: C. J. Sindermann (ed), Disease Diagnosis and Control in North American Marine Aquaculture. Developments in Aquaculture and Fisheries Science, 6. Elsevier Scientific Publishing Company, Amsterdam, Oxford, and New York. 329 p.

Lawler, A.R.. 1980. 'Studies on *Amyloodinium ocellatum* (Dinoflagellata) in Mississippi Sound: natural and experimental hosts', *Gulf Research Reports*, 6: 403–413.

Levy, M.G., M.F. Poore, A. Colorni, E.J. Noga, M.W. Vandersea, R.W. Litaker. 2007. 'A highly specific PCR assay for detecting the fish ectoparasite *Amyloodinium ocellatum*', *Diseases of Aquatic Organisms*, 73: 219-226.

Li, C.H., J. Chen, Y.H. Shi, X.J. Lu. 2011. 'Use of suppressive subtractive hybridization to identify differentially expressed genes in ayu (*Plecoglossus altivelis*) associated with *Listonella anguillarum* infection', *Fish and Shellfish Immunology*, 31: 500-06.

Li, C.-H., J. Chen. 2013. 'Molecular cloning, characterization and expression analysis of a novel wap65-1 gene from *Plecoglossus altivelis*', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 165: 144-52.

Lilley, K.S., P. Dupree. 2006. 'Methods of quantitative proteomics and their application to plant organelle characterization'. *Journal of Experimental Botany*, 57: 1493–1499.

Llewellyn, L., V.P. Ramsurn, T. Wigham, G.E. Sweeney, D.M. Power. 1998. 'Cloning, characterisation and expression of the apolipoprotein A-I gene1 in the sea bream (*Sparus aurata*)', *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression*, 1442: 399-404.

Lom. J., A.R. Lawler. 1973. 'An ultrastructural study on the mode of attachment in dinoflagellates invading gills of Cyprinodontidae', *Protistologica*, 9: 293-309.

Lom, J., I. Dykova. 1992. 'Protozoan parasites of fishes'. *Developments in Aquaculture and Fisheries Science*, 26: 315.

Loot, G., N. Poulet, S. Brosse, L. Tudesque, F. Thomas, S. Blanchet. 2011. 'Determinants of life-history traits in a fish ectoparasite: a hierarchical analysis'. *Parasitology*, 138: 848-857. doi:10.1017/S003118201100014X.

Magnadottir, B. 2006. 'Innate immunity of fish (overview)', *Fish Shellfish Immunology*, 20: 137-51.

Marcogliese, D. J. 2008. 'The impact of climate change on the parasites and infectious diseases of aquatic animals', *Rev Sci Tech*, 27: 467-84.

Martemyanov, V.I.. 2009. 'The Regularities of Changes in the Sodium Ion Levels in Fish Erythrocytes during Adaptation to a Temperature', *The Biological Bulletin*, 36 (4): 412-416.

Martemyanov, V.I.. 2013. 'Patterns of changes in sodium content in plasma and erythrocytes of freshwater fish at stress.', *Journal of Ichthyology*, 53 (3): 220-224.

Martemyanov, V.I.. 2015. ' Stress reaction in freshwater fish in response to extreme impacts and during the reproduction period.', J*ournal of Coastal Life Medicine*, 3(3): 169-177.

Martin-Perez, M., J. Fernandez-Borras, A. Ibarz, A. Millan-Cubillo, O. Felip, E. de Oliveira, and J. Blasco. 2012. 'New insights into fish swimming: a proteomic and isotopic approach in gilthead sea bream', *J Proteome Res*, 11: 3533-47.

Martinez-Porchas, M., L.R. Martinez-Cordova, R. Ramos-Enriquez. 2009. 'Cortisol and glucose: reliable indicators of fish stress', *Pan-American Journal of Aquatic Sciences*, 4: 158-178.

Martyniuk, C. J., S. Alvarez, and N. D. Denslow. 2012. 'DIGE and iTRAQ as biomarker discovery tools in aquatic toxicology', *Ecotoxicology and Environmental Safety*, 76: 3-10.

Masson, I., R.B. Blaylock, J.M. Lotz. 2011. 'Susceptibility and Tolerance of Spotted Seatrout, *Cynoscion nebulosus*, and Red Snapper, *Lutjanus campechanus*, to Experimental Infections with *Amyloodinium ocellatum*', *Journal of Parasitology*, 97 (4): 577-585.

Mazeaud, M.M., F. Mazeaud, E.M. Donaldson,. 1977. 'Primary and Secondary Effect of Stress in Fish: Some New Data with a General Review', *Transactions of the American Fisheries Society*, 106: 201–212.

Mazeaud, M.M., F. Mazeaud. 1981. 'Adrenergic Responses to Stress in Fish', In Stress and Fish, Pickering, A.D.L. (Ed), Academic Press Academic: 50–75.

McCarty, R., E.L. Sabban, R. Kvetnansky. 1996. 'Stress: Molecular genetic and neurobiological advances', Harward Academic, Amesterdam, Vol. 1-2, 1002pp..

McCormick, S.D., J.M. Shrimpton, J.B. Carey, M.F. O'Dea, K.E. Sloan, S. Moriyama, B.T.H. Bjornsson. 1998. 'Repeated acute stress reduces growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol', *Aquaculture*, 168: 221-235.

Mladineo I., M. Petrić, T. Šegvić, N. Dobričić. 2010. 'Scarcity of parasite assemblages in the Adriatic-reared European sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*)', *Veterinary Parasitology*, 174 (1–2): 131-138.

Mohan, Chadag. 2015. "Climate Change and Aquatic Animal Disease.".

Molle, V., S. Campagna, Y. Bessin, N. Ebran, N. Saint, G. Molle. 2008. 'First evidence of the pore-forming properties of a keratin from skin mucus of rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*)', *Biochemistry Journal*, 411: 33-40.

Montgomery-Brock, D., V.T. Sato, J.A. Brock, C.S. Tamaru. 2001. 'The application of hydrogen peroxide as a treatment for the ectoparasite *Amyloodinium ocellatum* (Brown, 1931) on the Pacific threadfin *Polydactylus sexfilis*', *Journal of the World Aquaculture Society*, 32: 250–254.

Monti, M., M. Cozzolino, F. Cozzolino, G. Vitiello, R. Tedesco, A. Flagiello, P. Pucci. 2009. 'Puzzle of protein complexes *in vivo*: a present and future challenge for functional proteomics', *Expert Review of Proteomics*, 6: 159–69.

Mulvey, C., B. Thur, M. Crawford, J. Godovac-Zimmermann. 2010. 'How many proteins are missed in quantitative proteomics based on MS/MS sequencing methods?', *Proteomics Insights*, 3: 61.

Murray, S., M.F. Jørgensen, S.Y.W. Ho, D.J. Patterson, L.S. Jermiin. 2005a. 'Improving the Analysis of Dinoflagellate Phylogeny based on rDNA', *Protist*, 156: 269—286.

Murray, A.G., and E.J. Peeler. 2005b. 'A framework for understanding the potential for emerging diseases in aquaculture', *Preventive Veterinary Medicine*, 67: 223-35.

Neves, J.V., J.M. Wilson, P.N.S. Rodrigues. 2009. 'Transferrin and ferritin response to bacterial infection: The role of the liver and brain in fish', *Developmental & Comparative Immunology*, 33: 848-57.

Noga, E.J., C.E. Bower. 1987. 'Propagation of the marine dinoflagellate *Amyloodinium ocellatum* under germ-free conditions', *Journal of Parasitology*, 73: 924–928.

Noga, E.J., S.A. Smith, J.H. Landsberg. 1991. 'Amyloodiniosis in cultured hybrid striped bass (*Morone saxatilis* × *M. chrysops*) in North Carolina', *Journal of Aquatic Animal Health*, 3: 294–297.

Noga, E.J.. 1996. 'Fish Disease: Diagnosis and Treatment'. Iowa State University Press, Ames, Iowa, 376 pp..

Noga, E.J., U. Silphaduang, N.G. Park, J.K. Seo, J. Stephenson, S. Kozlowicz. 2009. 'Piscidin 4, a novel member of the piscidin family of antimicrobial peptides', *Comparative Biochemistry and Physiology, Part B*, 152: 299–305.

Noga, E.J., A.J. Ullal, J. Corrale, J.M.O. Fernandes. 2011. 'Application of antimicrobial polypeptide host defenses to aquaculture: Exploitation of downregulation and upregulation responses', C*omparative Biochemistry and Physiology, Part D*, 6: 44–54;

Nogueira, A.F.V.. 2014. 'Impacto fisiológico do parasita dinoflagelado *Amyloodinium ocellatum* em corvina-legítima (*Argyrosomus regius*) produzida em aquacultura',

Master Thesis in Marine Biology, Faculdade de Ciências e Tecnologia da Universidade do Algarve, 49 p..

Nolan, D.T., R.L.J.M. Op't Veld, P.H.M. Balm, S.E. Wendelaar-Bonga. 1999. 'Ambient salinity modulates the response of the tilapia, *Oreochromis mossambicus*, to net confinement', *Aquaculture*, 177: 297-309.

Oestmann, D.J., D.H. Lewis. 1996. 'Effects of 3,N-methylglucamine lasalocid on *Amyloodinium ocellatum*', *Diseases of Aquatic Organisms*, 24: 179–184.

Olsen, R.E., K. Sundell, E. Ringø, R. Myklebust, G.I. Hemre, T. Hansen, Ø. Karlsen. 2008. 'Acute stress response in fed and food deprived Atlantic cod *Gadus morhua* L.', *Aquaculture*, 280: 232-241.

Ostrowski, A.C., A. Molnar. 1998. 'Pacific Threadfin *Polydactylus sexfilis* (Moi) Hatchery Manual', Publication Nº. 132, Center for Tropical and Subtropical Aquaculture, Waimanalo, Hawaii.

Owens, L.. 2003. 'Barramundi'. In: Lucas, J.S., Southgate, P.C. (eds.). Aquaculture: Farming Aquatic Animals and Plants. Blackwell Publishing, Oxford, UK: 364–381.

Panzenbock, U., L. Kritharides, M. Raftery, K.A. Rye, R. Stocker. 2000. 'Oxidation of methionine residues to methionine sulfoxides does not decrease potential antiatherogenic properties of apolipoprotein A-I', *Journal of Biological Chemistry*, 275: 19536-44.

Paperna, I., D. Zwerner. 1976. 'Parasites and diseases of striped bass, *Morone saxatilis* (Walbaum), from the lower Chesapeake Bay', *Journal of Fish Biology*, 9: 267–287.

Paperna, I., F. Baudin Laurencin. 1979. 'Parasitic infections of sea bass, *Dicentrarchus labrax* and gilthead sea bream, *Sparus aurata*, in mariculture facilities in France', *Aquaculture*, 16: 173–175.

Paperna, I., A. Colorni, B. Ross, B. Colorni. 1981. 'Diseases of marine fish cultured in Eilat mariculture project based at the Gulf of Aqaba, Red Sea', *European Mariculture Society Special Publication*, 6: 81–91.

Paperna, I.. 1984. 'Reproduction cycle and tolerance to temperature and salinity of *Amyloodinium ocellatum* (Brown, 1931) (Dinoflagellida)', *Annales de Parasitologie Humaine et Comparee*, 59: 7-30.

Patton, W.F.. 2002. 'Detection technologies in proteome analysis', *Journal of Chromatography B - Analytical Technologies in the Biomedical and Life Sciences*, 771: 3–31.

Peng, X. X.. 2013. 'Proteomics and its applications to aquaculture in China: infection, immunity, and interaction of aquaculture hosts with pathogens', *Developmental & Comparative Immunology*, 39: 63-71.

Pereira, J. C., I. Abrantes, I. Martins, J. Barata, P. Frias, I. Pereira. 2011. 'Ecological and morphological features of *Amyloodinium ocellatum* occurrences in cultivated gilthead seabream *Sparus aurata* L.: A case study', *Aquaculture*, 310 (3-4): 289-297.

Perry, S.F., N.J. Bernier. 1999. 'The Acute Humoral Adrenergic Stress Response in Fish: Facts and Fiction', *Aquaculture*, 177: 285–295.

Person-Le Ruyet, J., K. Pichavant, C. Vacher, N. Le Bayon, A. Severe, G. Boeuf. 2002. 'Effects of O2 supersaturation on metabolism and growth in juvenile turbot (*Scophthalmus maximus* L.)', *Aquaculture*, 205: 373-383.

Philp, A., A.L. Macdonald, P.W. Watt. 2005. 'Lactate - a signal coordinating cell and systemic function', *The Journal of Experimental Biology*, 208: 4561-4575.

Picard, D.J., P.M. Schulte. 2004. 'Variation in gene expression in response to stress in two populations of *Fundulus heteroclitus*', *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 137: 205-16.

Pierre, S., S. Coupe, N. Prevot-d'Alvise, S. Gaillard, S. Richard, E. Gouze, J. Aubert, and J. P. Grillasca. 2010. 'Cloning of Wap65 in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) and expression in sea bass tissues', *Comparative Biochemistry and Physiology Part B: Biochemical Molecular Biology*, 155: 396-402.

Pierre, S., K. Tarnowska, L. Hachfi, S. Coupe, R. Simide, S. Couvray, C. Garnier, M. Grimaldi, S. Richard, S. Gaillard, N. Prevot-D'Alvise, and J. P. Grillasca. 2011. 'Effects of water temperature increase and heavy metals contamination on WAP65 gene expression in sea bass (*Dicentrarchus labrax*) liver', *Cellular & Molecular Biology (Noisy-le-grand)*, 57 Suppl: OL1614-22.

Plumb, J.A.. 1997a. 'Infections diseases of tilapia' In: Costa-Pierce, B.A., Rakocy, J.E. (eds.). Tilapia Aquaculture in the Americas, World Aquaculture Society, Baton Rouge, LA: 212–228.

Plumb, J.A.. 1997b. 'Infectious diseases of striped bass'. In: Harrell, R.M. (ed.) Striped Bass and Other Morone Culture. Elsevier Science, Amsterdam: 271–314.

Poochai, W., K. Choowongkomon, P. Srisapoome, S. Unajak, N. Areechon. 2014. 'Characterization and expression analysis of the transferrin gene in Nile tilapia

(*Oreochromis niloticus*) and its upregulation in response to *Streptococcus agalactiae* infection', *Fish Physiology and Biochemistry*, 40: 1473-85.

Pottinger, T.G., E. Mosuwe. 1994. 'The corticosteroidogenic response of brown and rainbow trout alevins and fry to environmental stress during a "critical period".', *General and Comparative Endocrinology*, 95: 350-362.

Rajan, B., J. Lokesh, V. Kiron, M. Brinchmann. 2013. 'Differentially expressed proteins in the skin mucus of Atlantic cod (*Gadus morhua*) upon natural infection with *Vibrio anguillarum*', *BMC Veterinary Research*, 9: 103.

Reed, P.; R. Francis-Floyd. 1994. 'Amyloodinium infections of marine fish', Florida: University of Florida. Florida Cooperative Extension Service. Fact Sheet VM-90.

Rensel, J.E., J.N.C. Whyte. 2003. 'Finfish mariculture and harmful algal blooms'. In: Hallegraeff, G.M., Anderson, D.M. and Cembella, A.D. (eds.) Manual on Harmful Marine Microalgae. UNESCO Publishing, Paris: 693–722.

Reyes-Becerril, M., D. Tovar-Ramírez, F. Ascencio-Valle, R. Civera-Cerecedo, V. Gracia-López, , V. Barbosa-Solomieu. 2008. 'Effects of dietary live yeast *Debaryomyces hansenii* on the immune and antioxidante system in juvenile leopard grouper *Mycteroperca rosacea* exposed to stress', *Aquaculture*, 280: 39–44.

Rigos, G., P. Christophilogiannis, M. Giahnishi, A. Andriopoulou, M. Koutsodimoy, I. Nengas, M. Alexis. 1998. '*Amyloodinium ocellatum* infestation on sharpsnout sea bream, *Puntazzo puntazzo* Cetti', *Bulletin of the European Association of Fish Pathologists*, 18: 98–200.

Rigos, G., G.M. Troisi. 2005. 'Antibacterial agents in Mediterranean finfish farming: a synopsis of drug pharmacokinetics in important euryhaline fish species and possible environmental implications', *Reviews in Fish Biology and Fisheries*, 15: 53–73.

Rimmer, M.A.. 2003. 'Barramundi'. In: Lucas, J.S., Southgate, P.C. (eds.). Aquaculture: Farming Aquatic Animals and Plants. Blackwell Publishing, Oxford, UK: 364–381.

Ritchie, R.F., G.E. Palomaki, L.M. Neveux, O. Navolotskaia, T.B. Ledue, W.Y. Craig. 1999. 'Reference distributions for the negative acute-phase serum proteins, albumin, transferrin and transthyretin: A practical, simple and clinically relevant approach in a large cohort', *Journal of Clinical Laboratory Analysis*, 13: 273-79.

Rodrigues, P.M., T.S. Silva, J. Dias, F. Jessen. 2012. 'PROTEOMICS in aquaculture: Applications and trends', *Journal of Proteomics*, 75 (14): 4325-4345.

Roberts-Thomson, A., A. Barnes, D.S. Fielder, R.J.G. Lester, R.D. Adlard. 2006. 'Aerosol dispersal of the fish pathogen, *Amyloodinium ocellatum*', *Aquaculture*, 257: 18– 123.

Rogel, M.R., A. Jaitovich, K.M. Ridge. 2010. 'The Role of the Ubiquitin Proteasome Pathway in Keratin Intermediate Filament Protein Degradation', *Proceedings of the American Thoracic Society*, 7: 71-76.

Saldarriaga, J. F., F.J.R. ‘‘Max’’ Taylor, T. Cavalier-Smith, S. Menden-Deuer, P.J. Keeling. 2004. 'Molecular data and the evolutionary history of dinoflagellates', *European Journal of Protistology*, 40: 85–111.

Santos, G.A., J.W. Schrama, R.E.P. Mamauag, J.H.W.M. Rombout, J.A.J. Verreth. 2010. 'Chronic stress impairs performance, energy metabolism and welfare indicators in European seabass (*Dicentrarchus labrax*): The combined effects of fish crowding and water quality deterioration', *Aquaculture*, 299: 73-80.

Sarropoulou, E., P. Sepulcre, L. Poisa-Beiro, V. Mulero, J. Meseguer, A. Figueras, B. Novoa, V. Terzoglou, R. Reinhardt, A. Magoulas, G. Kotoulas. 2009. 'Profiling of infection specific mRNA transcripts of the European seabass *Dicentrarchus labrax*', *BMC Genomics*, 10: 157.

Sarropoulou, E., J.M. Fernandes, K. Mitter, A. Magoulas, V. Mulero, M.P. Sepulcre, A. Figueras, B. Novoa, G. Kotoulas. 2010. 'Evolution of a multifunctional gene: The warm temperature acclimation protein Wap65 in the European seabass *Dicentrarchus labrax*', *Molecular Phylogenetics and Evolution*, 55: 640-9.

Satomi, Y. , Y. Shimonishi, T. Hase, T. Takao. 2004. 'Site-specific carbohydrate profiling of human transferrin by nano-flow liquid chromatography/electrospray ionization mass spectrometry', *Rapid Communications in Mass Spectrometry*, 18: 2983-88.

Selye, H.. 1950. 'Stress and the general adaptation syndrome', *British Medical Journal*, 1: 1383-1392.

Severino, R.B.A.. 2008. 'Contributo para o conhecimento do ciclo de vida de *Amyloodinium ocellatum* e seu controlo em piscicultura', *Master Thesis in Applied Ecology*, Faculdade de Ciências da Universidade do Porto, 52p..

Sha, Z., P. Xu, T. Takano, H. Liu, J. Terhune, Z. Liu. 2008. 'The warm temperature acclimation protein Wap65 as an immune response gene: its duplicates are differentially regulated by temperature and bacterial infections', *Molecular Immunology*, 45: 1458-69.

Shi, Y., J. Chen, C. Li, M. Li. 2010. 'Molecular cloning of liver Wap65 cDNA in ayu (*Plecoglossus altivelis*) and mRNA expression changes following *Listonella anguillarum* infection', *Molecular Biology Reports*, 37: 1523-29.

Shinn, A.P., J. Pratoomyot, J.E. Bron, G. Paladini, E.E. Brooker, A.J. Brooker. 2015. 'Economic costs of protistan and metazoan parasites to global mariculture', *Parasitology*, 142: 196-270.

Shoemaker, C.A., D.H. Xu, J.J. Evans, P.H. Klesius. 2006. 'Parasites and diseases'. In: Lim, C. and Webster, C. (eds.) Tilapia: Biology, Culture, and Nutrition. Haworth Press, Binghamton, New York: 561–582.

Silva, T.S., N. Richard, J.P. Dias, P.M. Rodrigues. 2014. 'Data Visualization and Feature Selection Methods in Gel-based Proteomics', *Current Protein & Peptide Science*, 15 (1): 4-22.

Smith, S.A., M.G. Levy, E.J. Noga. 1992. 'Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of antibody to the parasitic dinoflagellate *Amyloodinium ocellatum* in *Oreochromis aureus*', *Veterinary Parasitology*, 42: 145–155.

Smith, Stephen A., Michael G. Levy, and Edward J. Noga. 1994. 'Detection of Anti-Amyloodinium ocellatum Antibody from Cultured Hybrid Striped Bass (Morone saxatilis × M. chrysops) during an Epizootic of Amyloodiniosis', *Journal of Aquatic Animal Health*, 6: 79-81.

Smith, R. W., C. M. Wood, P. Cash, L. Diao, P. Part. 2005. 'Apolipoprotein AI could be a significant determinant of epithelial integrity in rainbow trout gill cell cultures: a study in functional proteomics', *Biochimica et Biophysica Acta*, 1749: 81-93.

Soares, F., H. Quental Ferreira, E. Cunha, P. Pousão-Ferreira 2011. 'Occurrence of *Amyloodinium ocellatum* in aquaculture fish production: a serious problem in semi-intensive earthen ponds', *Aquaculture Europe*, 36 (4): 13-16.

Soares, F., H. Quental Ferreira, M. Moreira, E. Cunha, L. Ribeiro, P. Pousão-Ferreira. 2012. 'First report of *Amyloodinium ocellatum* in farmed meagre (*Argyrosomus regius*)', *European Association of Fish Pathologist Bulletin*, 32(1): 30-33.

Soengas, J.L., S. Sangiao-Alvarellos, R. Laiz-Carrión, J.M. Mancera. 2007. 'Energy metabolism and osmotic acclimation in teleost fish'. In Fish Osmoregulation (ed. B.

Baldisserotto, J. M. Mancera Romero and B. G. Kapoor), Enfield, NH: Science Publisher: 277-307.

Souza, N.A.. 2015. 'Resposta metabólica da ostra *Crassostrea gigas* na presença do parasita *Amyloodinium ocellatum*', *Master Thesis in Aquaculture and Fisheries - Aquaculture*, Faculdade de Ciências e Tecnologia da Universidade do Algarve, 45p..

Stafford, J.L., N.F. Neumann, M. Belosevic. 2001. 'Products of proteolytic cleavage of transferrin induce nitric oxide response of goldfish macrophages', *Developmental & Comparative Immunology*, 25: 101-15.

Steidinger, K.A., J.M. Burkholder, H.B. Glasgow Jr., C.W. Hobbs, J.K. Garrett, E.W. Truby, E.J. Noga, S.A. Smith. 1996. '*Pfiesteria piscicida* gen. et sp. nov. (Pfiesteriaceae fam.nov.), a new toxic dinoflagellate with a complex life cycle and behaviour', *Journal of Phycology*, 32: 157–164.

Stewart, J.E., L.J. Marks, E. Pfeiffer, B.M. Zwicker. 1988. 'Microbial utilization of the neurotoxin domoic acid: blue mussels (*Mytilus edulis*) and soft shell clams (*Mya arenaria*) as sources of the microorganisms', *Canadian Journal of Microbiology*, 44: 456–64.

Stickney, R.R. (2005). 'Aquaculture: an introductory text', 1st edition, CABI publishing, pp. 152.

Taylor, F.J.R.. 1987. 'General group characteristics and special features of interest: short history of dinoflagellate study'. In: Taylor, F.J.R. (ed.) The Biology of Dinoflagellates. Blackwell, Oxford, UK: 1–23.

The Oceanic Institute (1996). 'Final Report: Development of Mahimahi (*Coryphaena hippurus*) Aquaculture Technology 1990–1994', Section II National Oceanic and Atmospheric Administration, La Jolla, CA: 1–106.

Thomas, S., S.F. Perry. 1994. 'Influence of Initial Respiratory Status on the Short and Long Term Activity of the Trout Red Blood Cell Beta Adrenergic Na^+/H^+ Exchanger', *Journal of Comparative Physiology B. Biochemical, Systems, and Environmental Physiology*, 164: 383–389.

Tomanek, L.. 2011. 'Environmental proteomics: changes in the proteome of marine organisms in response to environmental stress, pollutants, infection, symbiosis, and development',. *Annual Review of Marine Science*, 3: 373–99.

Tonge, R., J. Shaw, B. Middleton, R. Rowlinson, S. Rayner, J. Young, F. Pognan, E. Hawkins, I. Currie, M. Davison. 2001. 'Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology'. *Proteomics*, 1: 377–396.

Tonguthai, K., S. Chinabut. 1997. 'Diseases of tilapia'. In Egna, H.S., Boyd, C.E. (eds.). Dynamics of Pond Aquaculture, CRC Press, Boca Raton, FL: 263–287.

Tort, L., J.C. Balasch, S. MacKenzie. 2004. 'Fish health challenge after stress - indicators of immunocompetence', *Contributions to Science*, 2: 443-54.

Tort, L. 2011. 'Stress and immune modulation in fish', *Developmental & Comparative Immunology*, 35 (12): 1366-1375.

Tseng, W.-Y., S.K. Ho. 1988. 'Grouper Culture—A Practical Manual', Chien Chieng Publisher, Kaohsiung, Taiwan. 200p.

Ulevitch, R.J., A.R. Johnston, D.B. Weinstein. 1981. 'New function for high density lipoproteins. Isolation and characterization of a bacterial lipopolysaccharide-high density lipoprotein complex formed in rabbit plasma', *Journal of Clinical Investigation*, 67: 827-37.

Ullal, A.J., R.W. Litaker, E.J. Noga. 2008. 'Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (*Ictalurus punctatus*, Rafinesque), *Developmental and Comparative Immunology*', 32: 1301–1312.

Van Weerd, J.H., J. Komen. 1998. 'The effects of chronic stress on growth in fish: a critical appraisal', *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 120: 107-112.

Vijayan, M.M., N. Aluru, J.F. Leatherland. 2009. 'Stress response and the role of cortisol'. In: J.F. Leatherland, P.T.K. Woo (eds), Fish Diseases and Disorders - Non-infectious Disorders, Vol. 2,. CABI Press, New York: 182-201.

Villarroel, F., A. Bastías, A. Casado, R. Amthauer, M.I. Concha. 2007. 'Apolipoprotein A-I, an antimicrobial protein in *Oncorhynchus mykiss*: Evaluation of its expression in primary defence barriers and plasma levels in sick and healthy fish', *Fish and Shellfish Immunology*, 23: 197-209.

Wedemeyer, G.A., B.A. Barton, D.J. McLeay. 1990. 'Stress and acclimation', In: C.B. Shreck, P.B. Moyle (eds), Methods for fish biology. American Fisheries Society, U.S.A.: 451-477.

Weirich, C.R.. 1997. 'Striped bass and other morone culture, Developments in aquaculture and fisheries science, Chapter 7; Transportation and stress mitigation', Elsevier Science, Vol. 30: 185-216.

Wendelaar Bonga, S.E.. 1997. 'The stress response in fish'. Physiological Reviews, 77: 591–625.

Wetmore, B.A., B.A. Merrick. 2004. 'Invited Review: Toxicoproteomics: Proteomics Applied to Toxicology and Pathology', *Toxicologic Pathology*, 32: 619-642.

Woo, P.T.K., D.W. Bruno, L.H.S. Lim. 2002. 'Diseases and Disorders of Finfish in Cage Culture'. CAB International, Wallingford, UK. 281p.

Woo, P.T.K.. 2007. 'Protective immunity in fish against protozoan diseases', *Parassitologia*, 49: 185-191.

Wu, M.S., C.W. Chen, C H. Lin, C.S. Tzeng, C.Y. Chang. 2012. 'Differential expression profiling of orange-spotted grouper larvae, *Epinephelus coioides* (Hamilton), that survived a betanodavirus outbreak', *Journal of Fish Diseases*, 35: 215-25.

Xie, F.J., Z.P. Zhang, P. Lin, S.H. Wang, Z.H. Zou, Y.L. Wang. 2009. 'Identification of immune responsible fibrinogen beta chain in the liver of large yellow croaker using a modified annealing control primer system', *Fish and Shellfish Immunology*, 27: 202-9.

Yambot, A.V., Y.L. Song. 2006. 'Immunization of grouper, *Epinephelus coioides*, confers protection against a protozoan parasite, *Cryptocaryon irritans*', *Aquaculture*, 260: 1–9.

Zanuzzo, F.S., E.C. Urbinati, M.L. Rise, J.R. Hall, G.W. Nash, A.K. Gamperl. 2015. '*Aeromonas salmonicida* induced immune gene expression in Aloe vera fed steelhead trout, *Oncorhynchus mykiss* (Walbaum)', *Aquaculture*, 435: 1-9.

Zheng, W.-J., Y.-H. Hu, L. Sun. 2010. 'Identification and analysis of a *Scophthalmus maximus* ferritin that is regulated at transcription level by oxidative stress and bacterial infection', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 156: 222-28.

VI. Annexes

Figure 6.1 – Poster presented in COST Action FA1002 Final Showcase Meeting on Farm Animal Proteomics, 17-18 November 2014, Milan (Italy).

Proteomic analysis of gilthead sea bream plasma with amyloodiniosis

Márcio Moreira[1], Denise Schrama[2], Florbela Soares[1], Pedro Pousão-Ferreira[1] and Pedro Rodrigues[2]*
[1]Aquaculture Research Centre, National Institute for the Sea and Atmosphere (IPMA), Av. 5 de Outubro s/n, 8700-305 Olhão, Portugal; marciomoreira.27@gmail.com
[2]CCMAR – Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Introduction

Diseases are one of the most important constraints in aquaculture, especially in intensive fish farming, limiting their success. The open design of many of the aquaculture systems also allows the transmission of infectious pathogens where they find ideal condition to cause a disease outbreak (Balcázar, 2006; Mladineo, 2006).

In Southern Europe, amyloodiniosis represents a major bottleneck for semi-intensive aquaculture, and is one of the most serious impediments to warm water aquaculture, with over 100 species known to be susceptible (Noga, 1996).

This disease is caused by one of the most common and important parasitic dinoflagellate in fish, *Amyloodinium ocellatum*. This parasite can affect almost all fish that live within its ecological range, causing serious morbidity and mortality in brackish and marine warm water fish in different aquaculture facilities worldwide, and is often considered the most consequential pathogen of marine fish (Paperna *et al.*, 1981). It is a 'quiet' disease since the outbreaks occur extremely rapid, and usually by the time of its detection, contaminated fish no longer respond to treatment, resulting in 100% mortality in a few days (Soares *et al.*, 2011).

The symptomatology of this disease is characterized by changes in fish behaviour, with jerky movements, swimming at the water surface and decreased appetite (Soares *et al.*, 2011). These may include increased respiratory rate and gathering at the surface or in areas with higher dissolved oxygen concentrations. Information regarding the physiological responses from the host to the parasite infestation are scarce.

Proteomics is one of the new approaches to understand fish diseases, which can elucidate the functional responses of organisms to environmental and biological challenges, thus facilitating the identification of disease biomarkers (Alves *et al.*, 2010; Cox and Mann, 2011).

In this work we will analyse the proteomic profile of gilthead sea bream (*Sparus aurata*, Linnaeus, 1758) plasma, one of the most important cultured fishes in Southern Europe (Soares *et al.*, 2011).

Figure 6.2 - Chapter of the book Farm Animal Proteomics 2014, pp. 188-193, DOI: 10.3920/978-90-8686-810-0.

Figure 6.3 - Seminar presented in Centro IFAPA Agua del Pino (Crta. El Rompido-Punta Umbría Km 3.8, Cartaya, Huelva, Spain).