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| Complete List of Authors: | Moreira, Márcio; IPMA - Portuguese Institute for the Sea and Atmosphere, , EPPO - Aquaculture Research Station<br>Schrama, Denise; Universidade do Algarve, FCT<br>Soares, Florbela; IPMA,<br>Wulff, Tune; Danmarks Tekniske Universitet, The Novo Nordisk Foundation Center for Biosustainability<br>Pousão-Ferreira, Pedro; Instituto Portugues do Mar e da Atmosfera, EPPO<br>Rodrigues, Pedro; FCT |
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# Physiological responses of reared sea bream (*Sparus aurata* Linnaeus, 1758) to an *Amyloodinium ocellatum* outbreak

*Márcio Moreira[1], Denise Schrama[2], Florbela Soares[1], Tune Wulff[3], Pedro Pousão-Ferreira[1], Pedro Rodrigues[2*]*

[1]*IPMA - Portuguese Institute for the Sea and Atmosphere, EPPO - Aquaculture Research Station, Av. 5 de Outubro s/n, 8700-305 Olhão, PORTUGAL*

[2]*CCMAR – Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, PORTUGAL*

[3] *The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kogle Allé 6, 2970 Hørsholm, DENMARK*

** Corresponding author (e-mail: pmrodrig@ualg.pt, Adress: CCMAR – Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, PORTUGAL, Contacts: +351289800100, Ext:7855)*



## Abstract

Amyloodiniosis represents a major bottleneck for semi-intensive aquaculture production in Southern Europe, causing extremely high mortalities. *Amyloodinium ocellatum* is a parasitic dinoflagellate that can infest almost all fish, crustacean and bivalves that live within its ecological range. Fish mortalities are usually attributed to anoxia, associated with serious gill hyperplasia, inflammation, haemorrhage and necrosis in heavy infestations; or with osmoregulatory impairment and secondary microbial infections due to severe epithelial damage in mild infestation. However, physiological information about the host responses to *A. ocellatum* infestation is scarce. In this work we analysed the proteome of gilthead sea bream (*Sparus aurata*) plasma and relate it with haematological and immunological indicators, in order to enlighten the different physiological responses when exposed to an *A. ocellatum* outbreak. Using 2D-DIGE, immunological and haematological analysis and in response to the *A. ocellatum* contamination we have identified several proteins associated with acute phase response, inflammation, lipid transport, homeostasis, and osmoregulation, wound healing, neoplasia, and iron transport. Overall, this preliminary study revealed that amyloodiniosis affects some fish functional pathways as revealed by the changes in the plasma proteome of *S. aurata,* and that the innate immunological system is not activated in the presence of the parasite.

**Keywords:** Amyloodinium ocellatum, gilthead sea bream, proteomics, physiological responses

Running title: Fish physiological responses to amyloodiniosis

## 1. Introduction

Nowadays, aquaculture is the food industry with the highest annual growth rate (5,8% average growth rate between 2005-2014, 4,9% in 2014) (FAO, 2014; FAO, 2016). The World Bank (2013) and Lem, Bjorndal & Lappo (2014) predicts a growth of more than 50% until 2030, supplying over 60% of fish destined for direct human consumption. However, a higher development of aquaculture is constrained by different causes.

Fish diseases are one of the main problems in aquaculture, especially in intensive fish farming, representing severe annual costs to producers (Murray & Peeler, 2005). Parasitic diseases caused by obligate or opportunistic pathogens can have a major impact on global finfish and shellfish aquaculture, representing a key constraint to production, sustainability and economic viability of aquaculture facilities in many regions (Shinn, Pratoomyot, Bron, Paladini, Brooker & Brooker, 2015). Also global warming tends to increase the frequency of the outbreaks by increase of the ecological range of diseases and abundance of parasites, and a diminished immunocompetence in the hosts (Marcogliese, 2008; FOESA, 2013; Cristina García Diez & Remiro Perlado, 2014; FAO, 2015; Mohan, 2015).

Amongst them, amyloodiniosis is a parasitic disease that represents a major threat for semi-intensive aquaculture, especially in Southern Europe, being one of the most serious impediments for

the production of several warm water aquaculture species (Noga, Ullal. Corrales & Fernandes, 2011).

This disease is caused by the dinoflagellate *Amyloodinium ocellatum* (Brown), that provokes fast and asymptomatic outbreaks, with acute morbidity and mortality (around 100%) in brackish and marine warm water fish on different aquaculture facilities worldwide (Becker, 1977; Lawler, 1980; Paperna, Colorni, Ross & Colorni, 1981; Lauckner, 1984; Noga, 2006; Soares, Quental-Ferreira, Cunha & Pousão-Ferreira, 2011). This parasite has also the capacity for hyperparasite some parasites of fish like *Neobenedenia melleni* (MacCallum) (Monogenea: Capsalidae) in gilthead sea bream (*Sparus aurata* L.) (Colorni, 1994), and inflict moderate to intense tissue reaction in several commercially important shrimp species (Aravindan, Chaganti & Aravindan, 2007). The affected producers can have a major economic impact, as demonstrated by the reported cases of *A. ocellatum* in a milkfish (*Chanos chanos* Forsskål) hatchery in 2004 (Cruz-Lacierda, Maeno, Pineda & Matey, 2004) and a case in Nile tilapia (*Oreochromis mossambicus* W. K. H. Peters) in the Salton Sea, California, United States (Kuperman & Matey, 1999) had a total cost loss of 20,000 USD and 6-77 million USD respectively (Shinn *et al.*, 2015).

Biologically *A. ocellatum* is a dinoflagellate with a life cycle that comprises three stages: trophont (parasitic state, attached to fish gills and skin), tomont (encapsulated state, a cyst that develops after the trophont leaves the fish); and dinospores (free living state, released from the tomont) (Kuperman & Matey, 1999; Landsberg, Blakesley, Reese, Mcrae & Forstchen, 1988; Woo, 2007). Each tomont can produce up to 256 dinospores in 3 days at 25 ºC, each one capable to infect a new host and produce a trophont (Brown & Hovasse, 1946).

The symptomatology of this disease is characterized by sudden changes in fish behaviour, with jerky movements, swimming at the water surface, decreased appetite, increased respiratory rate and gathering at the surface or in areas with higher dissolved oxygen concentrations (Soares *et al.*,

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

These reported immunological reactions and the development of resistance to *A. ocellatum* by fish previously contaminated with the parasite (Cobb, Levy & Noga, 1998) means that an immunological approach can be used to access a possible immunological response from the organism to *A. ocellatum* presence. Lysozyme makes part in an extensive battery of defence mechanisms in fish, such as bacteriolysis and opsonization of the bacterial wall, and it is present in lymphoid tissues, mucus, plasma, and other body fluids (Magnadottir, 2006). It's a membranolitic enzyme that measures the immune system activity against bacteria and an important index of innate immunity of fish (Saurabh & Sahoo, 2008). Lysozyme can provide us information about the activation of the innate immune system and about the existence of a possible secondary bacterial infections due to severe epithelial damage (Corrales *et al.*, 2010).

Taking in account the damage that *A. ocellatum* can cause in the gills, haematological analysis could be a good approach for accessing possible responses from fish to a parasite outbreak. Haematological analysis can be especially important as a direct or inferential indicator to monitor fish health, since they can provide information related to fish tolerance to stress, metabolic

disorders, reproductive dysfunctions or pathologies, like the ones caused by external agents such as parasites (Clauss, Dove & Arnold, 2008; Del Rio-Zaragoza, Hernandez-Rodriguez & Buckle-Ramirez, 2008; Buscaino, Filiciotto, Buffa, Bellante, Di Stefano, Assenza, Fazio, Caola & Mazzola, 2010; Del Rio-Zaragoza, Fajer-Avila, Almazan-Rueda & Abdo de la Parra, 2011). Because of this, these kind of analyses are being gradually introduced to determining the health status of these animals (Percin & Konyalioglu, 2008).

In recent years a large amount of large-scale approaches in biology that promise to revolutionize systems-level understanding of cellular and organism functions has been developed. These fields, referred to as ‘‘omics’’, can assess the structure and functioning of organisms with a comprehensive and unprecedented level of detail (Joyce & Palsson, 2006). Amongst these, one of the most promising as a potentially new approach to conventional methods is Proteomics (Alves, Cordeiro, Silva, Richard, de Vareilles, Marino, Di Marco, Rodrigues & Conceição, 2010). Proteomics constitutes one of the new best approaches for better understanding fish diseases and epidemiology, giving us an insight on the protein abundance and modification patterns in the organism (Rodrigues, Silva, Dias & Jessen, 2012; Peng, 2013). This information can be extremely useful in the study of fish physiological responses to the parasite *A. ocellatum*, allowing 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 & Mann, 2011). Two of the most common techniques are 2D Fluorescence Difference Gel Electrophoresis (DIGE), and shotgun techniques like iTRAQ or RP-HPLC (Martyniuk, Alvarez & Denslow, 2012).

Blood plasma has been used in proteomics studies because is considered a biological fluid of primary importance for analysis, since it is a solution with several proteins and peptides bathing cells and tissues of the whole organism, acting as a mirror/reporter of physiological or pathological conditions (Isani, Andreani, Carpenè, Di Molfetta, Eletto & Spisni, 2011) and hence should be a good source of biomarkers in an *A. ocellatum* outbreak.

Gilthead sea bream was used in this experiment due to its economic importance in Southern Europe aquaculture. This species has been used for several years as a model in marine fish physiology because of the vast knowledge accumulated, especially to several ambiental stressors (Isani *et al.*, 2011).

In this work we will analyse and compare the proteome of gilthead sea bream plasma and analyse haematological and immunological indicators, to enlighten the different physiological responses when exposed to an *A. ocellatum* outbreak.



## 2. Material and Methods

### *2.1 Fish and rearing conditions*

One hundred and fourteen gilthead sea breams, with a mean body weight of 87.2 ± 17.1 gr and a mean body length of 17.73 ± 1.14 cm were reared in duplicate 200 L rectangular plastic tanks (Control - fish without parasites, T1 – fish with *A. ocellatum*, T2 – fish with a lower amount of *A. ocellatum* and monogenic parasites like *Dactylogirus* sp. or *Lamellodiscus* sp. N=19), at Aquaculture Research Centre, National Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal). Fish were kept at 22±0.2 ºC, for 5 days for acclimation in closed recirculation seawater systems, artificial aeration and natural photoperiod. Fish on tanks from T1 and T2 were infected with *A. ocellatum* tomonts obtained from an amyloodiniosis outbreak in an EPPO – IPMA earthen pond.

*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, Caroline & Bresnan, 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, at 12 and 18h after contamination (according to preliminary tests).

At 18h, when the contamination reached 500 parasites per branchial arc, 4 random fish from each tank were selected, in order to get 8 biological replicates per treatment. They were then anesthetized with 2-phenoxyethanol (Sigma Aldrich), and approximately 1 ml of blood was withdrawn using syringes heparinized with 1% EDTA. This process was performed in less than 3 minutes. The blood was then centrifuged at 2500 rpm for 10 minutes, and plasma was collected and kept at -80ºC for subsequent proteomic analysis.

For haematological and immunological analysis, blood samples were collected from all the remaining fish (N=11) from Control, T1 and T2. Blood was taken by caudal vein puncture with a 1 ml heparinized syringe. Collection of blood samples was completed within 5 min of capturing the fish to minimize handling stress.

Infected fish were then killed with an overdose of anesthetic according with the EU Directive 2010/63/EU for animal experiments.

The first two branchial arches of all the fishes (N=15) were also withdrawn for parasite counting by microscopical observation.

*2.3 Haematological analysis*

The determination of the haematocrit (HCT) value was made by the methodology described by Soares, Leitão, Moreira, Teixeira de Sousa, Almeida, Barata, Feist, Pousão-Ferreira & Ribeiro (2012). The rest of the blood (N=11) was transferred to a clean slide and was used for haematological determination. Blood cells were air dried and stained with Wright-Giemsa. The number of erythrocytes, thrombocytes, leucocytes, monocytes and granulocytes were counted microscopically. T-student statistical analysis of the data was performed in IBM SPSS Statistics for Windows, Version 21.0. (IBM Corp. Released 2012. Armonk, NY: IBM Corp).

*2.4 Immunological analysis*

Plasma lysozyme activity was assessed using a turbidimetric assay (Ellis, 1990) adapted to 96-well microplates. In summary, lyophilized *Micrococcus lysodeikticus* (Sigma) were dissolved in in 0.05 M sodium phosphate buffer at pH 6.2 (0.3 $mgml^{-1}$), and this solution was used as a substrate for the plasma lysozyme. Triplicates of each sampled fish plasma (15 µl) from Control, T1 and T2 (N=11) were added to 250 µl of the bacterial suspension, and the reduction in absorbance at 450 nm was measured at 0.5 and 4.5 min of reaction. A unit of lysozyme activity was defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per min. T-student statistical analysis of the data was performed in IBM SPSS Statistics for Windows, Version 21.0. (IBM Corp. Released 2012. Armonk, NY: IBM Corp).

*2.5 Protein labeling for 2D-DIGE*

Prior to protein separation, quantification of protein in plasma 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 labeled with 400 pmol of fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethylformamide following manufacturer's instructions (5 nmol labeling kit, GE Healthcare). Labeling was performed on ice for 30 minutes in the dark and quenched with 1 mM of lysine for 10 min. Four samples per treatment (Control, T1 and T2) 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 labeled with Cy2.

*2.6 Protein separation by 2D gel electrophoresis*

Labeled proteins from each 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 with the samples and internal standard were loaded onto 12.5% Tris-HCl SDS-PAGE gels and run in an Ettan DALTsix Vertical System at 10 $mAgel^{-1}$ for 1 hour followed by 60

mAgel$^{-1}$ using a standard Tris-Glycine-SDS running buffer, until the bromophenol blue line reaches the end of the gel.

*2.7 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 Progenesis SameSpots™ 2DE gel analysis software (Nonlinear Dynamics, United Kingdom) with filters for average normalized volume ≤ 10,000 and spot area ≤ 500. A one-way ANOVA was then applied on resulting protein abundance data (expressed in relation to the internal standard), having considered proteins with a $P < 0.05$ as being likely to be affected by the presence of *A. ocellatum*. All the significantly different spots were then subjected to a Tukey test, to rule out false positives resultant of protein abundance data. These statistical tests were made using R package (version 3.1.1) (R Core Team, 2013). From these spots, we selected the ones that presented differences between T1 and the other treatments (indicators of a specific reaction from the host to *A. ocellatum*), and the spots with higher average normalized volume that presented differences between T1 and T2 versus control (possible generalist indicators of a response from the host to ectoparasites). The selected spots were then manually excised from preparative gels post-stained with colloidal Coomassie Blue, for digestion and identification.

*2.8 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 peptide containing samples were run on a SYNAPT G2 HDMS mass spectrometer (Waters) coupled to a nanoAcquity UPLC system (Waters). Each sample was first trapped using a C18 trap column (C18 symmetry, 5 µm, 180 µm x 20 mm, Waters) and subsequently separated using a C18 nanoanalytical column (BEH130 C18 1.7 µm, 75 µm x 200 mm, Waters). During separation the flow rate of the loading pump was 0.3 $\mu lmin^{-1}$, using two mobile phases, A (deionized water with 0.1% formic acid) and B (Acetonitrile with 0.1% formic acid). During the 20 minutes gradient B was increased from 1 to 45%. Data was collected on the mass spectrometer employing the positive ion MSe acquisition method (cycle time 0.8 s). The resulting data were used as input in the ProteinLynxGlobalServer (PLGS v.2.5.3). First spectra were generated using the following parameters:

- **Chromatographic peak width**: Automatic
- **MS TOF resolution**: Automatic
- **Lock Mass for charge 1**: 556.2771$Dae^{-1}$
- **Lock Mass Window**: 0.25 Da
- **Low energy threshold**: 80 Counts
- **Elevated energy Threshold**: 20 Counts
- **Intensity threshold**: 750 Counts

Raw data files Protein identification was obtained by using the Protein Lynx Global Server (PLGS) software v2.5.3 (Water corporation) using the in-build MSe search function against the databases

described below. The search parameters were trypsin as enzyme, carboxamidomethyl on cysteine as fixed modification and oxidation of methioninen as partial modification while allowing one missed cleavage.

First searches were done against a database generated from uniprot including all reviewed proteins from the taxonomy Actinopterygii [7898]. In the database was added a list of known contaminants.

If not hit were obtained searches were done against the database all proteins from the taxonomy Actinopterygii [7898]. In the database was added a list of known contaminants.

Finally, all hits were manually inspected for verification and to avoid false positives.

## 3. Results

### *3.1 Amyloodinium ocellatum dinospore and trophont counts*

The dinospore counts in the different treatments are indicated in Figure 1.

*Suggested location for figure 1*

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. 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.

*3.2 Hematological analysis*

The obtained results for the HCT analysis are described in Table 1.

*Suggested location for Table 1*

The obtained results for the cell count results are described in the Table 2.

*Suggested location for Table 2*

The HCT and cell count values did not present any statistical differences between treatments.

*3.3 Immunological analysis*

The obtained results for the lysozyme analysis are described in Figure 2.

*Suggested location for figure 2*

The lysozyme values did not present any statistical differences between treatments. Moreover, the values obtained were at the minimum, and there was bacterial growth in several samples. This explains the dispersion of the data. This also means that lysozyme wasn´t active in almost all the samples.

*3.4 Proteomic analysis*

The plasma proteome analysis revealed a total of 2078 different protein spots, from which 752 had differences in the protein spot volume between treatments. This number was reduced to 397 protein spots after Tukey test. From these, we have selected the protein spots indicated in the 2D gel presented in Figure 3, following the methodology described in Material and Methods. These protein spots were selected based on their volume differences. Volume differences between T1 and the rest of the treatments might indicate a specific reaction of the organism to *A. ocellatum*. Volume differences between T1, T2 and Control might indicate a generalist response to ectoparasites. The protein spot that presented a higher volume difference between all the treatments was also selected.

*Suggested location for figure 3*

*3.5 Protein spot sequencing and identification*

The selected individual peptides that were identified by LC-MS/MS were used to perform a BLAST search restricted to the Actinopterygii class. In our work we have obtained one match for Apolipoprotein A-I, two matches for Hemopexin, 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 and Estrogen-regulated protein OS Sparus aurata PE 2 SV 1. The obtained results, spot volume profile and possible main function are summarized in in Table 3.

Suggested location for table 3

## 4. 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.

As mentioned in the results section, 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. However, the number of trophonts of *A. ocellatum* attached to the branchia in T1 was high and the load of *A. ocellatum* in T2 was low. There is no reference in the literature to a lower load of this parasite in the presence of other parasites, like trematodes. This lower load of *A. ocellatum* trophonts in T2 can have two explanations: a) intraspecific competition between parasites for space in the branchia (Vidal-Martínez, Pech, Sures, Purucker & Poulin, 2009; Mladineo, Petrić, Šegvić & Dobričić, 2010; Loot, Poulet, Brosse, Tudesque, Thomas & Blanchet, 2011); or b) an unspecific reaction from the immune system to the presence of ectoparasites (Bayne & Gerwick, 2001; Tort, Balasch & MacKenzie, 2004; Tort, 2011), that constitutes an obstacle to the fixation of *A. ocellatum* dinospores. Nevertheless, this lower count in T2 allows us to use it as a treatment condition with lower parasite load, useful to discard possible false positives, and also as an indicator of generalist responses to the parasite (volume differences between T1, T2 and Control).

In the haematological analysis, it is described by various authors that the presence of *A. ocellatum* provokes several lesions and hyperplasia of the respiratory epithelium accompanied by lamellar fusion, detachment of the epithelium, venous sinus dilatation, aneurysm formation and rupture of the lamellar epithelium, haemorrhage, necrosis and lymphocytic inflammatory reaction (Lawler,

1980; Noga, 2012). Moreover, changes in haematocrit can be interpreted as a strategy to increase oxygen carrying capacity of the blood when fish are exposed to a stressful event (Ortuño, Estebán & Meseguer, 2001). All of these could induce several changes in the cell blood composition (Guerra-Santos, Albinati, Moreira, Lima, de Azevedo, Costa, de Medeiros & Lira, 2012), as reported by Del Rio-Zaragoza *et al.* (2011), for spotted rose snapper (*Lutjanus guttatus* Steindachner) infected by dactylogyrid monogeneans.

However, there were no statistical differences between treatments in this study for HTC and blood cell count. This is in agreement with the data obtained by Guerra-Santos *et al.* (2012) for cobia (*Rachycentron canadum* L.) infected with *A. ocellatum*, but we have to take in account that one possible explanation for these results could arise from the fact that haematological data on fish are quite difficult to interpret, mainly because of variations caused by both internal and external factors (Fazio, Filiciotto, Marafioti, Di Stefano, Assenza, Placenti, Buscaino, Piccione & Mazzola, 2012). This could mask possible differences between treatments.

Regarding the immunological analysis, the lysozyme values indicate that there could be no innate immunological response to the presence of the parasite, which is in agreement with the results obtained for the contamination of gilthead seabream by other ectoparasites (Sitjà-Bobadilla, Calduch-Giner, Saera-Vila, Palenzuela, Álvarez-Pellitero & Pérez-Sánchez, 2008; Guardiola, Cuesta, Arizcun, Meseguer & Esteban, 2014; Henry, Nikoloudaki, Tsigenopoulos & Rigos, 2015). This could be due to immuno-suppression of the hosts by the parasites by different strategies as exploitation of the immune system (Buchmann, 2000), evasion mechanisms based on antigen variation or mimicry, modification of the host's immune system, resistance to host's cellular or humoral factors (Rodriguez-Tovar, Wright, Wadowska, Speare & Markham, 2002), or the development of a thick parasite tegument containing inhibitors of the complement cascade (Buchmann & Lindenstrøm, 2002).

The absence of significant differences in the values between treatments can also indicate the absence of a possible secondary bacterial infection (Guardiola *et al.*, 2014).

In the proteomic analysis, the selected protein spots were identified as Apolipoprotein A-I (1 match), Hemopexin (2 matches), Uncharacterized protein OS Takifugu rubripes GN LOC101068102 PE 4 SV 1 (1 match), Uncharacterized protein OS Lepisosteus oculatus GN KRT9 2 of 3 PE 3 SV 1 (1 match) and Estrogen-regulated protein OS Sparus aurata PE 2 SV 1 (1 match). Apart from Estrogen-regulated protein OS *Sparus aurata* PE 2 SV 1, that has no known function, all other proteins are related to different metabolic functions, as lipid transport, osmoregulation and antioxidant activity, iron transport, immune system and cell injury, fibrinolysis, cellular and matrix interactions, inflammation, wound healing and neoplasia.

In lipid transport, we have identified several spots as Apolipoprotein A-I (ApoAI), as being down regulated in T1 gilthead sea bream plasma. This protein is produced in the liver and intestine (Llewellyn, Ramsurn, Wigham, Sweeney & Power, 1998), and it is involved in lipid absorption in the intestine (Panzenbock, Kritharides, Raftery, Rye & Stocker, 2000; Concha, Lopez, Villanueva, Baez & Amthauer, 2005; Geay, Ferraresso, Zambonino-Infante, Bargelloni, Quentel, Vandeputte, Kaushik, Cahu & Mazurais, 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, Costa, Harrison & Power, 2010; Geay *et al.*, 2011). This could be related with the lipid metabolism in our study, as a reflex of lipid recruitment to metabolic processes (Geay *et al.*, 2011). However, further studies would be needed in order to enlighten the role of this protein in the lipid metabolism of gilthead seabream exposed to *A. ocellatum*.

The protein ApoAI can also have antioxidant activity (Panzenbock *et al*., 2000) and osmoregulatory functions in teleosts (Smith, Wood, Cash, Diao & Part, 2005), with a possible role in the hyperosmotic regulation on fish (Chen, Shi, Hu, Niu & Li, 2009). 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 (*Plecoglossus altivelis* Temminck & Schlegel) passes from freshwater to brackish water, the observed downregulation of ApoAI can be related with the physiological impairment or epithelial damage of the branchia by *A. ocellatum*.

ApoAI could be involved in innate immunity response as antimicrobial protein (Concha, Molina, Oyarzún, Villanueva & Amthauer, 2003; Concha, Smith, Castro, Bastias, Romero & Amthauer, 2004; Sarropoulou, Sepulcre, Poisa-Beiro, Mulero, Meseguer, Figueras, Novoa, Terzoglou, Reinhardt, Magoulas & Kotoulas, 2009; Rajan, Lokesh, Kiron & Brinchmann, 2013) and can also have a role in the immunological response to certain ectoparasites in roho labeo (*Labeo rohita* F. Hamilton), like *Argulus siamensis* (C. B. Wilson) (Mohapatra, Karan, Kar, Garg, Dixit & Sahoo, 2016). This protein can also participate in inhibition of bacterial endotoxin, antiviral activity, anti-inflammatory, anti-apoptotic role, inhibition of inflammatory cytokines, neutralization of LPS (Ulevitch, Johnston & Weinstein, 1981; Johnston, Brown, Gauthier, Reece, Kator & Van Veld, 2008) or associated with C3 complex in cod (*Gadus morhua* L.) (Magnadottir, 2006) and in rainbow trout (*Oncorhynchus mykiss* Walbaum) (Villarroel, Bastías, Casado, Amthauer & Concha, 2007). 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, Giesy, Ye, Wang, Lee & Doris, 2012; Wu, Chen, Lin, Tzeng & Chang, 2012) levels in plasma reinforces the possibility of an absence of response of the innate immune system to *A. ocellatum*, and also the possible absence of secondary bacterial infections.

Hemopexin is a protein with an important role in iron transport, being the plasma protein with the highest binding affinity to heme among known proteins. It is mainly expressed in liver, and is the major vehicle for the transportation of heme in the plasma, since it binds and transports heme to the liver for breakdown, thus preventing heme-mediated oxidative stress and heme-bound iron loss

(Gracey, Troll & Somero, 2001; Tolosano & Altruda, 2002). Changes in the metabolism and sequestration of iron could be linked to hypoxia-induced erythropoiesis and increased demand for iron for hemoglobin synthesis (Gracey *et al.*, 2001) or for the removal of free iron from the plasma, which is a potential source of iron for pathogens (Paoli, Anderson, Baker, Morgan, Smith & Baker, 1999). The evolution of such a high affinity is a functional/structural response to the necessity of sequestering free heme within the blood stream with great efficiency (Paoli *et al.*, 1999). The higher volume observed of this protein in T1 and T2 could indicate a response to anoxia caused by ectoparasites (Buchmann & Lindenstrøm, 2002), that leads to an increased haemoglobin synthesis (Gracey *et al.*, 2001) in order to cope with a possible increased in the production of erythrocytes.

There are several identified proteins in this work that are related with cell injury, fibrinolysis, cellular and matrix interactions, inflammation, wound healing and neoplasia mechanisms, mainly in acute phase response to inflammation and injury. Hemopexin is an acute phase reactant, and its synthesis can be induced after inflammation (Hirayama, Kobiyama, Kinoshita & Watabe, 2004). 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 fish (Bohne-Kjersem, Skadsheim, Goksøyr & Grøsvi, 2009; Xie, Zhang, Lin, Wang, Zou & Wang, 2009). The overexpression of this protein in treatment T1 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 & Gerwick (2001) and Tort *et al.* (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 1, is a cytoskeletal protein whose primary function is to protect cells

from mechanical and non-mechanical injuries (Coulombe & Omary, 2002). Other report has also shown that keratin from fish mucus possesses antibacterial activity owing to its pore-forming properties (Molle, Campagna, Bessin, Ebran, Saint & Molle, 2008). Keratin turnover is dependent on ubiquitin-proteasome pathway and its expression levels can be altered upon cell injury (Rogel, Jaitovich & Ridge, 2010). The overexpression of this protein in T1 and T2 could be a response to cell injury by ectoparasites in this study.

## 5. Conclusion

All the identified proteins spots and the results obtained in the hematological analysis are part of a large physiological response of the gilthead sea bream to an *A. occelatum* outbreak.

One possible interpretation of the results is presented in figure 4.

*Suggested location for figure 4*

The presence of the parasite could induce a stress response by the fish (Landsberg *et al.*, 1988). This can induce the activation of the acute phase response proteins, which is demonstrated in this work by the variation in volume of the spots related to the proteins hemopexin, fibrinogen beta chain protein and possibly keratin proteins.

We also know that parasites could possibly induce an innate immune response in fish (Tort, 2011). However, the results obtained in the hematological analysis are puzzling, since the presence of hemorrhages in the branchia (Lawler, 1980) could lead to differences in the HTC values, and the immune response to parasites (Tort, 2011) could also provoke some differences in WBC values. The absence of WBC and lysozyme levels could indicate that *A. ocellatum* can have an evasion

system for the innate immune systems host and the inexistence of a bacterial secondary infection (Guardiola *et al.*, 2014), which could explain the ApoAI variation.

The increase of hemopexin expression could indicate a higher quantity of free heme groups in the plasma, that can be delivered to the liver for iron recycling and reuse in the production of new erythrocytes (Concha *et al.*, 2003). In this way the absence of differences in RBC in the HTC and differential cell count can be explained by erythrocyte synthesis in hematopoietic organs of fish.

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 differential expression observed in the proteins with possible homeostasis and osmoregulatory functions (ApoAI), inflammation (fibrinogen beta chain, hemopexin), antioxidant activity (ApoAI), and wound healing and neoplasia (fibrinogen beta chain and keratin).



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

Overall, this preliminary study revealed that amyloodiniosis affects the expression of proteins with known functions on the acute phase response mechanism, and physiological responses related with inflammation, lipid transport, homeostasis, osmoregulation, wound healing, inflammation, neoplasia, antioxidant activity, and iron transport in gilthead sea bream exposed to *A. ocellatum*. Some of this data were also confirmed by the hematological and immunological 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 outcome of this work, even if preliminary, can be the origin of future works. They could not only improve the knowledge about the physiological response of fish to this parasite, but also help to develop new products to improve the physiological resistance of fish to *A. ocellatum*.

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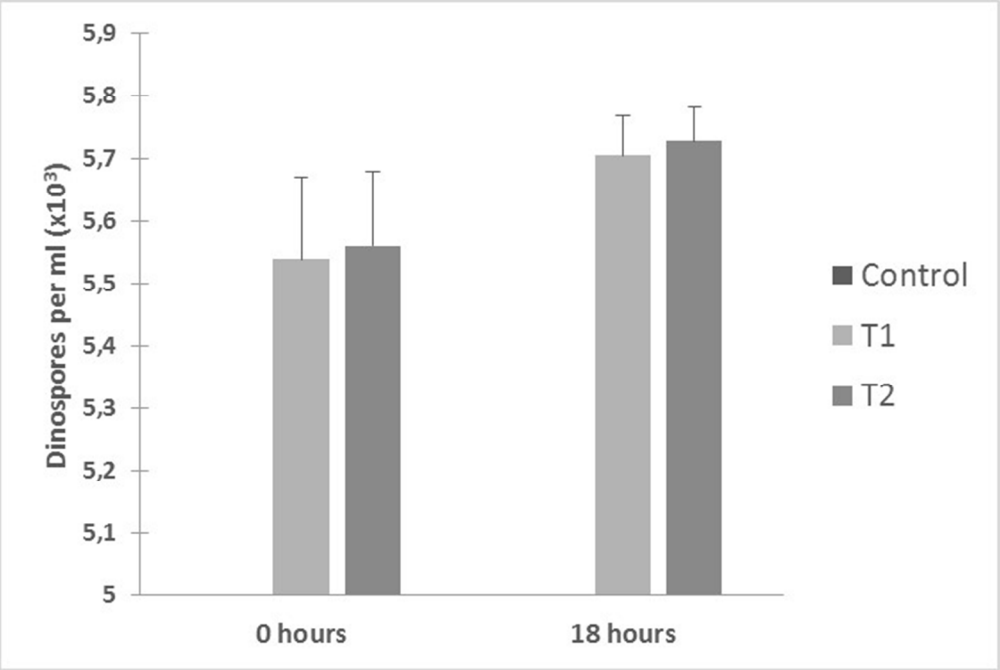


Figure 1 – Amyloodinium ocellatum dinospores concentration in the water from different treatments, T1, T2 and Control (N=11, Error bars= standard deviation).

151x101mm (118 x 118 DPI)

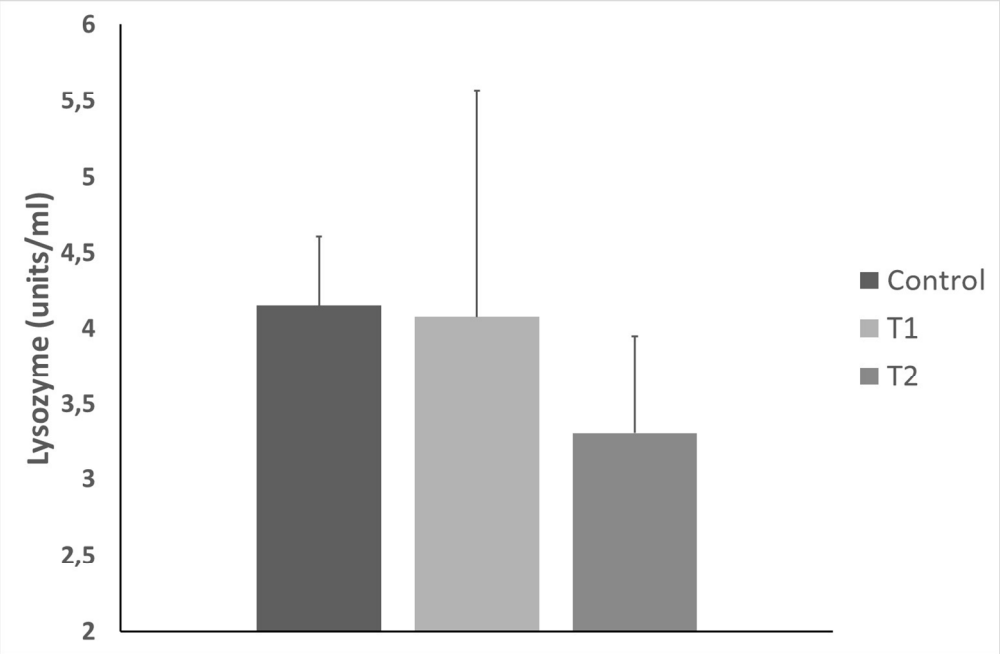


Figure 2 – Lysozyme values obtained from gilthead sea bream (Sparus aurata) plasma (N=11, Error bars= standard deviation, α=0.05).

258x169mm (150 x 150 DPI)

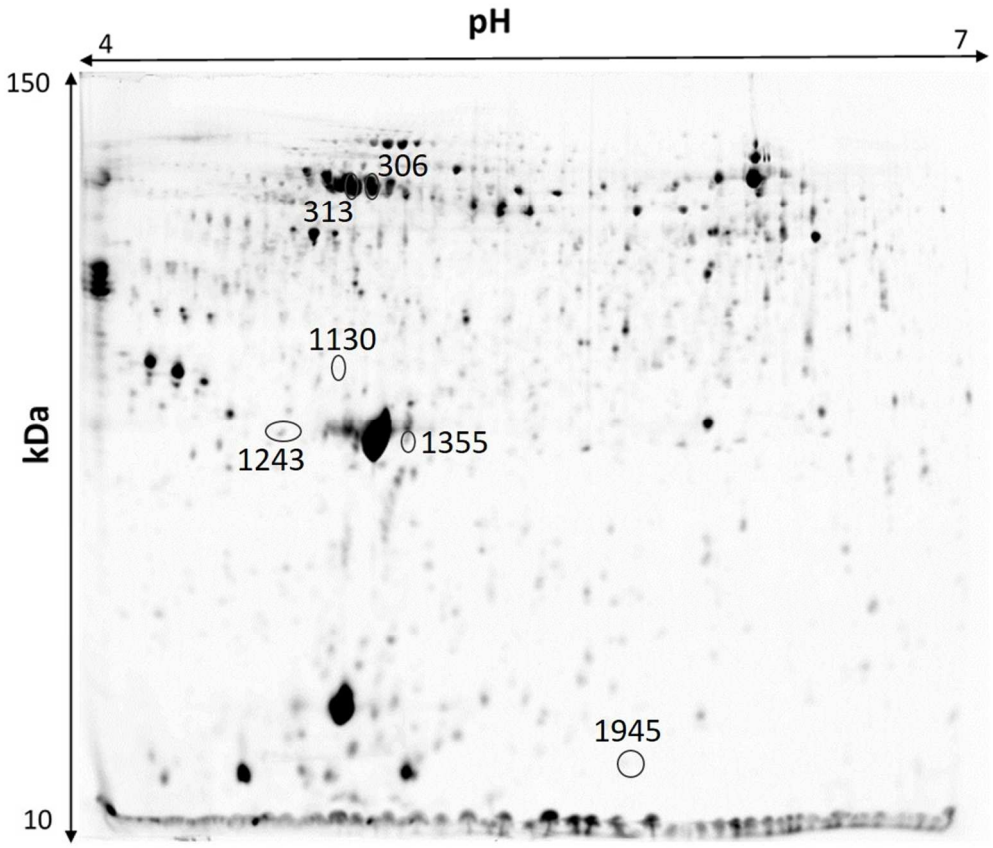


Figure 3 - Representative 2D PAGE of gilthead sea bream (Sparus aurata) plasma. The pointed circles with numbers represent the spots selected for protein analysis.

187x160mm (150 x 150 DPI)

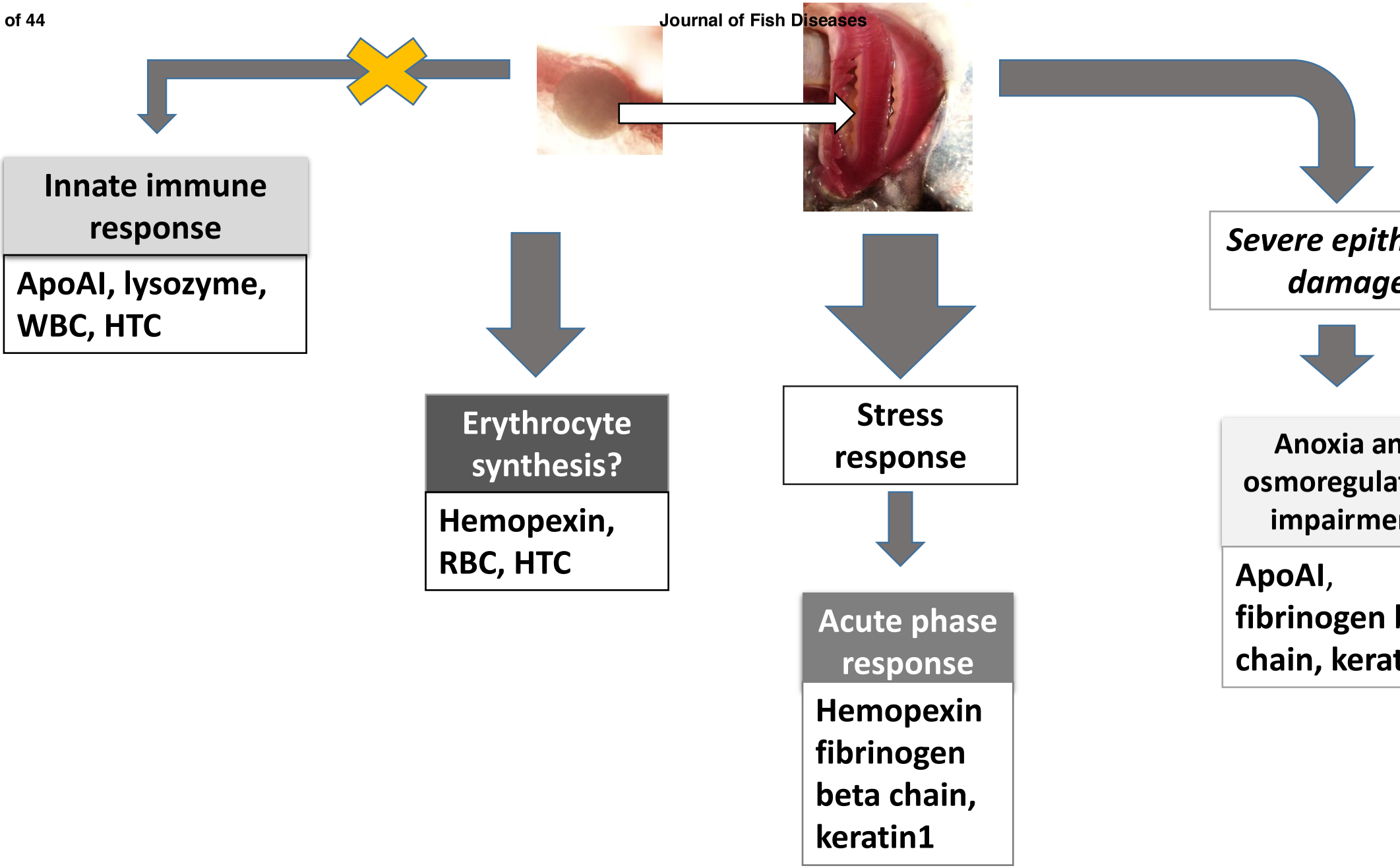
Innate immune response
ApoAI, lysozyme, WBC, HTC
Erythrocyte synthesis?
Hemopexin, RBC, HTC
Stress response
Acute phase response
Hemopexin fibrinogen beta chain, keratin1
Severe epith
damage
Anoxia an
osmoregula
impairme
ApoAI,
fibrinogen
chain, kerat

Table 1 – HTC results for gilthead sea bream (*Sparus aurata*) blood (N=11).

| | Control | Treatment 1 | Treatment 2 |
|---|---|---|---|
| *Red blood cells (mm)* | **18,8**±0,23 | **18,6**±0,27 | **22,6**±0,44 |
| *Buffy coat (mm)* | 0,43±0,41 | 0,14±0,28 | 0±0 |

Table 2 – Blood cell counts for gilthead sea bream (*Sparus aurata*) blood (N=11).

| | Control | Treatment 1 | Treatment 2 |
|---|---|---|---|
| *RBC (%)* | 97,87±1,72 | 98,34±1,05 | 98,49±0,64 |
| *WBC (%)* | 0,99±0,45 | 0,92±0,26 | 1,52±0,27 |
| *Leucocytes (%)* | 0,61±0,92 | 0,26±0,33 | 0,54±0,40 |
| *Monocytes (%)* | 0,38±0,54 | 0,31±0,28 | 0,33±0,35 |
| *Neutrophyles (%)* | 0,20±0,49 | 0,14±0,18 | 0,17±0,21 |
| *Eosinophyles (%)* | 0,05±0,13 | 0,15±0,17 | 0,12±0,23 |
| *Basophyles (%)* | 0,07±0,18 | 0,06±0,35 | 0,25±0,32 |
| *Trombocytes (%)* | 0,81±1,22 | 0,74±0,84 | 0,11±0,21 |

Table 3 - 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. Asteriscus (*) in Tukey test p-value column correspond to significant statistical differences between treatments (p<0,05)

***Lipid transport/osmoregulation and immune system***

| Nº. | Accession | Description | mW T/C (Da) | pI T/C | Score | Peptides | Sequence Coverage (%) | Anova | Tukey test p-value | Fold | best peptide match: sequence | Expression |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1355 | 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 | treatment1-ctrl: 0.0025735*<br>treatment2-ctrl: 0.0155404*<br>treatment2-treatment1: 0.4634973 | 1,305 | SSLAPQNEQLK | T1, T2< Control |

***Iron transport***

| Nº. | Accession | Description | mW T/C (Da) | pI T/C | Score | Peptides | Sequence Coverage (%) | Anova | Tukey test p-value | Fold | best peptide match: sequence | Expression |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 306 | tr\|C0L788\|C0L788_SPAAU | Hemopexin | 49126/69175 | 5,33/4,8 | 1072,78 | 8 | 12,94 | 0,000137 | treatment1-ctrl: 0.0270346*<br>treatment2-ctrl: 0.0000276*<br>treatment2-treatment1: 0.0008700* | 2,026 | FSEESDHVER | T1 > T2 > Control |
| 313 | tr\|C0L788\|C0L788_SPAAU | Hemopexin | 49126/71990 | 5,33/4,9 | 4686,33 | 35 | 37,65 | 0,00044 | treatment1-ctrl: 0.0121602*<br>treatment2-ctrl: 0.0002265*<br>treatment2-treatment1: 0.0350955* | 1,658 | FSEESDHVER | |

***Cell injury, fibrinolysis, cellular and matrix interactions, inflammation, wound healing, neoplasia***

| Nº. | Accession | Description | mW T/C (Da) | pI T/C | Score | Peptides | Sequence Coverage (%) | Anova | Tukey test p-value | Fold | best peptide match: sequence | Expression |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1945 | tr\|W5N831\|W5N831_LEPOC | Uncharacterized protein OS Lepisosteus oculatus GN KRT92 of 3 PE 3 SV 1 | 88937/4074 | 4,67/5,3 | 80,53 | 3 | 3,31 | 0,002665 | treatment1-ctrl: 0.0159337*<br>treatment2-ctrl: 0.0014227*<br>treatment2-treatment1: 0.2602469 | 1,410 | LAADDFR | T1, T2 > Control |
| 1130 | tr\|H2S183\|H2S183_TAKRU | Uncharacterized protein OS Takifugu rubripes GN LOC101068102 PE 4 SV 1 | 55432/31781 | 7,84/4,7 | 1922,94 | 5 | 8,99 | 5,03E-05 | treatment1-ctrl: 0.0018758*<br>treatment2-ctrl: 0.0000442*<br>treatment2-treatment1: 0.0234588* | 1,837 | LDGSVDFGR | T1 > T2> Control |

*Unknown function*

| Nº. | Accession | Description | mW T/C (Da) | pI T/C | Score | Peptides | Sequence Coverage (%) | Anova | Tukey test p-value | Fold | best peptide match: sequence | Expression |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1243 | tr\|D0VB93\|D0VB93_SPAAU | Estrogen-regulated protein OS Sparus aurata PE 2 SV 1 | 25589/22870 | 4,49/4,5 | 671,52 | 3 | 14,35 | 0,001297 | treatment1-ctrl: 0.0049463* treatment2-ctrl: 0.0076334* treatment2-treatment1: 0.9525509 | 1,841 | FGGCHDYQVR | T1, T2 > Control |