UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Heavy Metal Immunotoxicology
and Skin Mucus in Fish

Inmunotoxicología producida por Metales
Pesados y Caracterización del Moco
de Piel en Peces

D. Francisco A. Guardiola Abellán

2014

UNIVERSIDAD DE
MURCIA

D. Francisco Javier Martínez López, Profesor Titular de Universidad del Área de Fisiologia Animal y **Presidente Comisión Académica programa doctorado** * Biología de peces: aspectos básicos y aplicados,

INFORMA:

Que vista la solicitud de autorización de presentación de tesis doctoral de D. Francisco Antonio Guardiola Abellán, titulada "Heavy metal immunotoxicology and skin mucus in fish", realizada bajo la inmediata dirección y supervisión de Dª Mª Ángeles Esteban Abad, D. Alberto Cuesta Peñafiel y D. José Meseguer Peñalver , y evaluado el expediente completo, la Comisión Académica del Programa de Doctorado, en sesión celebrada el día 12 de Mayo de 2014, y de conformidad con lo establecido en el artículo 21 del "Reglamento por el que se regulan las enseñanzas oficiales de doctorado de la Universidad Murcia", resolvió la autorización de presentación de la tesis doctoral.

Asimismo, le envía el informe de la Comisión de Rama de Conocimiento de Ciencias sobre la propuesta de expertos que pueden formar parte del tribunal que ha de juzgarla, junto con los preceptivos informes de idoneidad.

Murcia, a 15 de Mayo de 2014

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**Informe del Departamento para alumnos del RD 778/1998.*
**Informe de La Comisión Académica del Programa para alumnos del RD 56/2005 y RD 1393/2007.*

Dª. Mª Ángeles Esteban Abad, Profesor Titular de la Universidad de Murcia, D. Alberto Cuesta Peñafiel, Profesor Titular de la Universidad de Murcia, y D. José Meseguer Peñalver, Catedrático de Universidad de Murcia, del Área de Biología Celular, en el departamento de Biología Celular e Histología,

AUTORIZAN

La presentación de la Tesis Doctoral titulada **"Heavy metal immunotoxicology and skin mucus in fish"**, realizada por D. Francisco Antonio Guardiola Abellán bajo nuestra inmediata dirección y supervisión, en el Departamento de Biología Celular e Histología, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 20 de Mayo de 2014

Fdo.: Mª Ángeles Esteban | Fdo.: Alberto Cuesta | Fdo.: José Meseguer

UNIVERSITÀ
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ALDO MORO

dipartimento di
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To Whom It May Concern,

The PhD Thesis entitled "Heavy metal immunotoxicology and skin mucus in fish" is presented by Mr. Francisco Guardiola Abellan and focuses on the main immunophysiological effects due to the exposure of *Sparus aurata* to heavy metals and the role exerted by skin layer in terms of defence mechanisms.

Particularly, in the first section of the PhD Thesis, a detailed investigation was shown screening the histopathological consequences on fish health of the exposure to arsenic, cadmium and mercury which were appropriately selected as target metals due to the potential risk of contamination of water for aquaculture.

In the second section of the PhD Thesis, a deep research was conducted concerning the barrier of skin mucus and its interactions with pathogens which can attack fish health. At the beginning, the characterization of the physico-chemical properties of the skin was described and, then, the comparison of the skin defence in several model fishes allowed an interesting interpretation of the mechanisms involved in such defence. The two sections are well connected each other in a rational architecture of the work.

Overall, the topic of Mr. Francisco Guardiola Abellan's PhD Thesis is very appealing for its involvement in the scenario of aquaculture and, for this purpose, several advanced techniques belonging to immunology and physiology field have been exploited to elucidate the behavior of cell fish. In addition, Mr. Francisco Guardiola Abellan's chemical background is also well documented by the studies performed in his PhD Thesis, so revealing a multidisciplinary perspective of the scientific issues and the Mr. Francisco Guardiola Abellan's ability to manage with different methodologies which is crucial for the interpretation of biological phenomena. At this regard, during his PhD Thesis, Mr. Francisco Guardiola Abellan's education has included the research visiting stay abroad on behalf as Erasmus Placement Fellow at *Dipartimento di Scienze e*

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Tecnologie Biologiche Chimiche e Farmaceutiche at University of Palermo (Italy) and the participation at several national and international Conferences. Altogether, such experiences have contributed not only to highlight the topic of the PhD Thesis, but also to cultivate good skills for the presentation and discussion of the resulting data.

Notably, all the extensive research work performed during Mr. Francisco Guardiola Abellan's PhD Thesis allowed the publication of 15 articles on peer reviewed journals, 1 chapter of book and 4 manuscripts currently under review.

Such a number of publications are an outstanding performance for a PhD student who has developed his research work within five years and, moreover, it clearly indicates a high impact activity of the results produced in the international scientific community of expertise in fish biology.

Tacking into account all the above mentioned goals arising from the PhD Thesis, I strongly recommend the "European mention" for Francisco Guardiola Abellan's PhD Thesis and an invitation to go ahead in his career of motivated investigator in the Academia or in the Companies.

Bari, 21 May 2014

Dr. Adriana Trapani *Adriana Trapani*

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UNIVERSITÀ DEGLI STUDI DI MESSINA
DIPARTIMENTO DI SCIENZE BIOLOGICHE ED AMBIENTALI

Prof. Caterina Faggio

Messina, 20 May 2014

To Whom It May Concern,

The research of the doctoral thesis of Dr. **Francisco Guardiola Abellan** entitled: "***Heavy metal immunotoxicology and skin mucus in fish***" investigates and explores in an excellent way on the immunophysiolgy of teleost fish and on effects of different heavy metals on the humoral immunity. It also presents substantial elements of originality; in fact Dr. Guardiola has made a fundamental contribution to scientific research in the field of the immune system of teleost fish, facing the argue in an innovative key. The thesis is innovative in relation to the topics, referring in complete and thorough manner to the trend of international research on the subject cited above. This has allowed to develop the scientific approach from a theoretical phase to a phase application. The knowledge of the immune system of fish, in general, and of the species of interest in aquaculture, in particular, has become one of the primary objectives in research applied to aquaculture. The PhD thesis is distinguished, in fact, for the ability to combine theoretical and experimental analysis of a proposal phase and realization of the results achieved. The research findings are interesting and analyzed with a strong critical sense which allows to identify issues that may be the subject of subsequent scientific insights. Excellent narrative skills inherent in the doctoral thesis that through careful selection of content and graphical form turns out to be already in this first draft of an excellent reference for experts and technicians in the field of scientific reference.

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The results are very original and interesting. Dr. Francisco Guardiola therefore has demonstrated an excellent understanding of the issues addressed in the presentation and strong critical spirit. In addition, the considerable number of papers (over 15) within three years is an outstanding performance for a PhD student. Therefore, I strongly support to be awarded the PhD with "European Mention".

Prof. Caterina Faggio

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SUMMARY

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SUMMARY

During the present PhD Thesis, we have studied the immunotoxicological effects of waterborne exposure to arsenic, cadmium and mercury in the gilthead seabream (*Sparus aurata* L.) (first part). Furthermore, different constitutive humoral defence mechanisms of the skin mucus of gilthead seabream have been identified and compared with those present in the serum of this fish species and with those present in skin mucus of several marine fish (second part). Finally, the effects of these heavy metals in the skin mucosal immunity of gilthead seabream, which is a species with the highest rate of production in Mediterranean aquaculture, were evaluated (third part). The study has a total number of seven chapters.

The first part of this Thesis contains three chapters (1, 2 and 3).

1. Firstly, we have evaluated the effects of waterborne exposure to sub-lethal concentrations of arsenic (As), cadmium (Cd) and mercury (Hg) in the teleost fish gilthead seabream, with special emphasis in the innate immune response. In the first chapter, it was determined the As concentration in liver and muscle of exposed fish, showing As-accumulation in the liver after 30 days of exposure. Moreover, the hepatosomatic index was increased at significant extent after 10 days while returned after 30 days to control values. Histological alterations in the liver were observed including hypertrophy, vacuolization and cell-death processes. Focusing on the immunological response, the humoral immune parameters (seric IgM, complement and peroxidase activities) were not affected to a statistically significant extent. On the other hand, the cellular innate parameters of head-kidney leucocyte peroxidase, respiratory burst and phagocytic activities were significantly increased after 10 days of exposition compared to the control fish. Overall, As-exposition in the seabream affects the immune system and could interfere with fish biology, aquaculture management or human consumers.

2. Similarly, in the second chapter, organo-somatic changes, Cd accumulation in liver and muscle, liver histology and humoral and cellular immune responses were determined in gilthead seabream exposed to waterborne Cd. Results showed that exposure to Cd induced no alterations on spleen and liver organo-somatic indexes whilst produced progressive deleterious morphological alterations in liver and exocrine pancreas that correlated with the hepatic Cd-accumulation. Regarding the immunotoxicological potential, Cd-exposure produced a reduction in the serum complement activity and leucocyte respiratory burst to a significant extent after 10 and 30 days whilst the serum peroxidase activity and leucocyte phagocytosis were increased at different sampling times. On the other hand, serum IgM levels and leucocyte peroxidase activity resulted unaltered. The present results seem to indicate that seabream specimens exposed to Cd in the present conditions suffer acute toxicity and and might be also considered a potential risk for human consumers.

3. In the third chapter, we have evaluated the effects of Hg in gilthead seabream. Firstly, toxicological effects were confirmed because Hg waterborne-exposed seabream specimens showed increased liver antioxidant enzymes (superoxide dismutase, catalase and glutathione reductase) after 2 days, higher hepatosomatic index after 10 days as well as histopathological alterations in the liver and skin as well as up-regulation of the expression of genes related to xenobiotic metabolism (CYP1A1), cellular stress (HSP-70 and HSP-90) and apoptosis (CASP-3) in the skin, but not in the head-kidney. Regarding the immune system, serum complement and peroxidase activities were increased by Hg waterborne-exposure but only the first reached significance after 30 days of treatment. On the other hand, head-kidney leucocyte peroxidase, respiratory burst and phagocytic activities were increased though only leucocyte phagocytosis and peroxidase activity did to a significant extent after 10 and 30 days, respectively. In general, our data demonstrate that waterborne exposure to sublethal Hg produce acute toxicological effects and increased immune parameters in gilthead seabream.

The second part of this Thesis also contains three chapters (4, 5 and 6).

4. The aim of chapter fourth was identify and characterize different constitutive humoral defence mechanisms of the skin mucus of gilthead seabream (*Sparus aurata*). Thus, the levels of total immunoglobulin M, several enzymes and proteins (peroxidase, lysozyme, alkaline phosphatase, esterases, proteases and antiproteases), as well as the bactericidal activity against opportunist fish pathogens (*Vibrio harveyi*, *V. anguillarum* and *Photobacterium damselae*) and non-pathogenic bacteria (*Escherichia coli* and *Bacillus subtilis*) were measured in the skin mucus and compared with those found in the serum. This study demonstrates that gilthead seabream skin mucus contains lower levels of IgM, similar levels of lysozyme, alkaline phosphatase and proteases, and higher esterase, peroxidase and antiprotease activities than serum. In addition, skin mucus revealed stronger bactericidal activity against tested fish pathogen bacteria compared to the serum activity, whilst human bacteria can even grow better in the presence of mucus. These results could be useful for better understand the role of the skin mucus as a key component of the innate immune system, which are of vital importance for fish health and consequently for aquaculture management.

5. Thus, in the fifth chapter, we evaluated physico-chemical and biological parameters in the skin mucus of five species of teleosts: gilthead seabream (*Sparus aurata* L.), European sea bass (*Dicentrarchus labrax* L.), shi drum (*Umbrina cirrosa* L.), common dentex (*Dentex dentex* L.) and dusky grouper (*Epinephelus marginatus* L.). Thus, protein concentration, pH, conductivity, redox potential, osmolarity, density and viscosity were measured, as well as differential scanning calorimetry (DSC). It was observed a correlation among pH, conductivity and redox potential in the skin mucus of all fish tested. Moreover, it was generally observed a clear interrelation between density and osmolarity as well as between density and temperature. Viscosity showed an indirect shear- and temperature-dependent behaviour. Finally, microcalorimetric measurements confirmed proteins with different structures which would be more stable in *S. aurata* and *D. labrax* than in the rest of species studied. The results have been discussed with the aim of elucidating the possible relationship between physico-

chemical and biological parameters of the skin mucus with the disease susceptibility due to the differential presence and activity of antibacterial factors.

6. In the last chapter of this second part, the sixth, terminal carbohydrate composition, levels of total IgM antibodies and several immune-related enzymes (proteases, antiproteases lysozyme, peroxidase, alkaline phosphatase and esterases,) as well as the bactericidal activity (against fish pathogenic *V. harveyi, V. angillarum, P. damselae* and non-pathogenic bacteria *E. coli, B. subtilis, Shewanella putrefaciens*) were identified, measured and compared in the skin mucus of the five marine teleosts mentioned above. First, lectin binding results suggests that skin mucus glycoproteins contain, in order of abundance: N-acetylneuraminic acid, glucose, N-acetyl-glucosamine, N-acetyl-galactosamine, galactose and fucose residues. Second, results showed that while some immune activities were very similar in the studied fish (e.g. IgM and lysozyme activity) other such as protease, antiprotease, alkaline phosphatase, esterase and peroxidase activities varied depending on the fish species. Highest levels of peroxidase and protease activity were found in *U. cirrosa* while *E. marginatus* and *S. aurata* showed the highest levels of alkaline phosphatase and esterase activities, respectively. Moreover, skin mucus of *S. aurata* revealed the highest bactericidal activity against pathogenic bacteria, contrarily to what happened with non-pathogenic bacteria (*E. coli* and *B. subtilis*). Thus, study of the variations in the carbohydrate profile and immune-related components of the fish skin mucus could help to understand the fish resistance as well as the presence and distribution of pathogens and magnitude of infections, aspects that are of major importance for the aquaculture industry.

Finally, the last part of this Thesis includes one chapter, the chapter seven.

7. In this chapter, it was investigated whether the skin mucus innate immune parameters determined in the gilthead seabream are affected by the heavy metals assayed above. Terminal carbohydrate composition, levels of total IgM antibodies and several enzymes and proteins (peroxidase, lysozyme, alkaline phosphatase, esterases, ceruloplasmin, proteases and antiproteases), as well as the bactericidal activity against opportunist fish pathogens (*V. harveyi*, *V. anguillarum* and *Photobacterium damselae*)

and non-pathogenic bacteria (*Escherichia coli*, *Bacillus subtilis* and *Shewanella putrefaciens*) were determined in the skin mucus of seabream specimens exposed to waterborne arsenic, cadmium and methylmercury. Moreover, immunoblotting and HPLC analysis were performed. This study demonstrates some changes in the mucus composition and immune functions after heavy metal exposure. Overall, carbohydrate profile suffered little changes and most of the enzymatic activities were increased after exposition. Interestingly, Hg evoked the most important increments (IgM levels, fucose-binding lectin and bactericidal activity) in the skin mucus. Protein profiles obtained by SDS-PAGE and HPLC showed little variations in the seabream mucus after exposure to heavy metals. The results could be useful for better understanding the role and behaviour of the mucosal immunity in skin as a key component of the innate immune system against pollutants and some of these parameters could be useful as biomarkers in fish toxicology.

INTRODUCTION

Heavy metal immunotoxicology and skin mucus in fish

FA Guardiola 2014

INTRODUCTION. Heavy metal immunotoxicology and skin mucus in fish

0. OVERVIEW

Global demand for seafood, particularly fish products, has tripled between 1,961 and 2,001 due to population growth and increased consumption of fish *per capita*. According to the FAO (Food and Agriculture Organization of the United Nations), extractive fishing only covers 60% of the annual world fish production [1], a situation in which aquaculture is seen as the only way to satisfy the demand in the near future. In fact, aquaculture production has increased from representing 9% of the fisheries resources in 1,980 to a current 43%, actually, and it is estimated that in 2,030 more than 65% of the aquatic food will come from aquaculture [1]. Concretely, Spain is the third member state of the European Union (EU) with a higher production of fish from aquaculture as well as in production of gilthead seabream (*Sparus aurata* L.), emerging as an area of economic activity of great strategic importance. Furthermore, it is located in the 20th position in the world ranking of aquaculture producers and 9th in the world ranking of exporters of fish [2].

The success of modern aquaculture is based on the control of the reproduction, a good knowledge of the biology of the farmed fish, on technology innovation, and on the development of a specific feed. Nevertheless, there are some important challenges to develop productive, feasible, and sustainable aquaculture in present intensive systems. One of these challenges is that in large-scale production facilities where aquatic animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often results in economic losses [3]. In intensive farming, animals are subjected to stress conditions that weaken their immune system, increasing susceptibility to pathogens and thus favouring the emergence of diseases. Thus, it has long been suspected a link between environmental contamination and disease in fish populations [4]. This connection could be due to the impairment of the innate immune system [5] where the structure and cellular composition of the epidermis (one of main innate immune barriers) as well as mucosal surfaces can be affected by stressors, such as pathogens and environmental contaminants [6–8].

Many of the environmental pollutants affect to the different aquatic animals to certain degree depending on the toxic substance, concentration, duration, self-life and animal behaviour and biology [9]. Furthermore, direct exposure to environmental contaminants of toxic substances in bivalves, crustaceans, molluscs or fish led to bioaccumulation, an important issue that needs to be controlled mainly in those species destined for human consume. Therefore, farmers have to know and control the impact of the environmental contaminants in the species produced for humans [9]. In this specific field, relevant fish species for aquaculture are less used in toxicological experiments. Moreover, the impact of the environmental contaminants in the immune response of these fish, and consequently in the disease resistance, have received much less attention [9].

The gilthead seabream (*Sparus aurata* L.), the main specie studied in the present PhD thesis, is a protandrous hermaphrodite species belonging to the family *Sparidae*, which represents half of marine fish production that, along with European sea bass (*Dicentrarchus labrax* L.), form the culture of excellence in the South Atlantic and Mediterranean area of Spain. This has done that, in recent years, it has become a kind important for research [10] focusing on aspects of their culture, such as the implementation of techniques for improving their reproductive success, survival and growth [11].

Therefore, knowledge of the immune system of fish, in general, and of the species of interest in aquaculture, in particular, has become one of the primary objectives in research applied to aquaculture. Further, fish occupy a key phylogenetic position in the evolution of vertebrates representing the first animal group that has a well structured innate and adaptive immune system, so that the study of the immunology of this group of vertebrates has a dual basic scientific and phylogenetic interest. In this context, the aim of this work was to characterize the skin mucosa surfaces of different species with importance for aquaculture as well as the immunotoxicological effects of three heavy metals (arsenic, cadmium and methylmercury) on the gilthead seabream (*Sparus aurata* L.), with special interest in their effects in the skin mucosa

1. THE IMMUNE SYSTEM OF TELEOSTS

The term immunity is used to designate immune reaction against foreign agents, including microorganisms (viruses, bacteria, fungi, protozoa and multicellular parasites) and macromolecules (proteins and polysaccharides) without the consequences of such pathological reaction [12]. The immune system is composed of cells and molecules that are responsible for this immunity, while the collective and coordinated response to the components of the immune system to foreign substances constitutes the immune response. The immune response always starts with the recognition of the pathogen or foreign material and its purpose is to develop a mechanism able to clear it [12,13].

In general, the immune system of fish is very similar to higher vertebrates, but has some important differences. In all vertebrates, including fish, the immune response may be of two types: innate (also known as natural or non-specific) and adaptive (also called acquired or specific), whereas invertebrates possess only innate immune response [12]. The fish constitute the first group of vertebrates displaying cellular and humoral responses that have the characteristics of specificity and memory [14]. Teleost are the first animal group that have an innate and adaptive immune system well structured and differentiated. Regarding to the innate immune response, this includes physical barriers (epithelium and mucous membranes), cellular effectors (phagocytic cells and nonspecific cytotoxic cells) and humoral factors (complement and other acute-phase proteins), acting as first line of defense against infection until the specific response is activated, whilst the adaptive response has cellular (lymphocytes) and humoral (secreted antibodies) components which appears exclusively in vertebrates (see Fig. 1) [12]. Both responses are greatly interconnected and involve a wide variety of innate and adaptive components. The immune response always starts with the innate response followed by the activation of lymphocytes and the adaptive response.

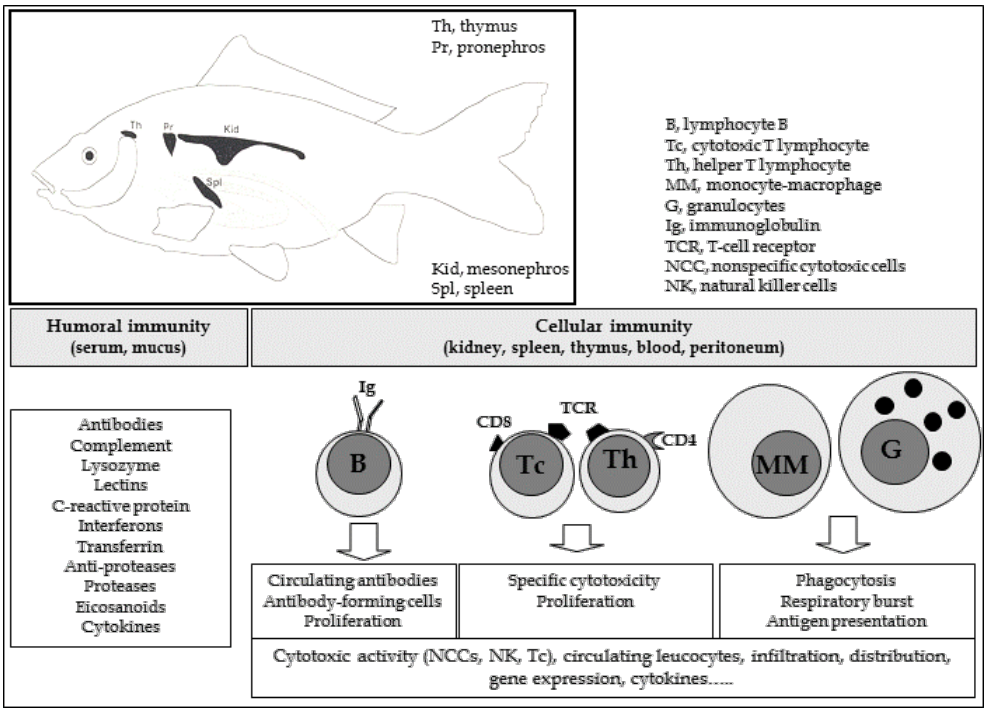

Fig. 1. Fish immune system organization and representative humoral and cellular immune responses [9].

1.1. Lymphomieloid organs

The organs of the immune system of fish are also essentially similar to those of mammals, comprising hematopoietic tissue (generating the blood cells of the myeloid line: erythrocytes, monocyte-macrophages, granulocytes and thrombocytes), lymphoid tissue (generating the blood cells of the lymphoid line: lymphocytes), primary organs (where lymphocyte maturation occurs) and secondary organs (where, mainly, mature lymphocytes come in contact with antigens) [15]. In fish, all tissues and organs show hematopoietic and lymphoid capacity, so they are called lymphomyeloid tissues and organs [16]. Nonetheless, the kidney, a primary organ, is the main hematopoietic organ, which is structured in three parts: the anterior or cephalic (head-kidney, HK), with lymphoid and hematopoietic function; posterior or caudal, with renal function; and the intermediate, which shares both functions. The second most important primary organ is the thymus, which appears near the gills and is mainly composed of T lymphocytes, being considered as the main source of mature T lymphocytes. Moreover, the spleen is the main secondary organ of teleost fish. It shows few lymphocytes that greatly increase after antigen administration, finding both T and B lymphocytes [16].

An important component of the lymphoid tissue is associated with mucus, forming the MALT (mucosa-associated lymphoid tissue). In fish, the MALT is composed by populations of dispersed cells including T and B lymphocytes, macrophages, plasma cells, granulocytes and mast cells. According to anatomical location, the MALT in teleost fish is subdivided into gut-associated lymphoid tissue (GALT), gill-associated lymphoid tissue (GIALT) and skin-associated lymphoid tissue (SALT) [17]. Since the most studied has been the GALT [18] a part of this present PhD thesis will focus on the study of the SALT, which we will review later in more detail in the section 2.

1.2. Humoral responses

These responses are mediated by a number of soluble factors with different scope and action that may be part of the innate or adaptive system. Mostly represent a set of proteins and glycoproteins with defence functions which are found in serum, mucus and eggs [19,20].

Among the humoral factors of the innate immune system, which may be in soluble form or membrane receptors, the most important is the complement system. This system consists of a complex cascade of enzymatic glycoproteins, which acts signalling the presence of potential pathogens and contributing to their degradation through chemotaxis and opsonization. The complement system is well developed in fish and includes classical, alternative and lectin pathways. These three pathways can lead to the formation of the membrane attack complex and cell lysis or increase phagocytosis by opsonization of the pathogen and the activation of the adaptive immune response through the classical pathway [21,22]. Other important humoral factors are lytic enzymes or lysins. Within this group hydrolases, such as lysozyme and chitinase, cathepsin, the lytic pathway of complement and other hemolytic and bacteriolytic enzymes are included [23]. There are two known isoforms of lysozyme (14 and 20 kDa) that show bactericidal activity by breaking the wall of Gram-positive bacteria and act as an opsonin by activating the phagocytosis or indirectly activating of polymorphonuclear leukocytes and macrophages [24].

Furthermore, other humoral factors are the lectins, proteins which are highly specific sugar-binding and are involved in recognition processes both at molecular and cellular levels. Highlight the lectin that binds mannose (MBL), which can act as an opsonin or

agglutinin [25] and pentraxins (proteins composed of five identical subunits including the C-reactive and serum amyloid P protein). The C-reactive protein (CRP) and serum amyloid protein (SAP) are mainly synthesized by hepatocytes as part of the acute-phase response to trauma or infection. Its main function is to precipitate C lipopolysaccharide (LPS) present in the bacterial walls and interact with C1q to activate the classical complement cascade [26]. All of them are characterized as main factors in the acute-phase response and important pathogen recognition proteins [27].

Besides those already mentioned, include as key elements in the innate defense the antimicrobial peptides (AMPs), which are low-molecular-weight peptides that have bactericidal properties against different pathogens [28–30] such as protease inhibitors (α2-macroglobulin and α1-antitrypsin) that block bacterial lytic enzymes [31]; bacterial growth inhibitors such as transferrin, antiproteases and ceruloplasmin, which prevents or slows the growth of bacteria [32]; and viral replication inhibitors such as interferon [33–35]. Currently, several peptides from different species have been isolated, amongst which are pleurocidins [36], piscidins [37], cathelicidins [38,39], defensin-type molecules [40,41] and others [28,42].

On the other hand, humoral factors involved in specific immune responses are immunoglobulins (Ig antibody) expressed as membrane molecules of B lymphocytes or secreted into the plasma. Until recently, it was believed that the fish had only two classes of immunoglobulins, IgM and IgD. Furthermore, it was generally accepted that IgM was the only immunoglobulin capable of responding to an antigen in both mucosal and systemic form, lacking thus the fish an immunoglobulin specialized mucosal surfaces [43]. IgD has been also identified in fish, although their functional relevance remains to be determined [44]. Nevertheless, after the analysis of multiple genomes of teleost fish, it was discovered a new immunoglobulin isotype called IgT in rainbow trout [45], IgZ in zebrafish [46,47] and IgH in pufferfish [48]. Recently, the protein structure, production and potential role in immunity of IgT was studied [49,50], showing that the IgT of rainbow trout is an immunoglobulin specialized in the immune responses of the intestinal and skin mucosa, while IgM appears to be specialized in systemic immunity.

1.3. Cellular responses

The immune system cells are classified into three main types: monocytes-macrophages, granulocytes and lymphocytes. These cells interact between them resulting in different, innate and adaptive immune responses [51,52].

Important players in the initiation of the innate immune response are the toll-like receptors (TLRs), a family of transmembrane proteins of type I. They are responsible for the recognition of pathogen-associated molecular patterns (PAMPs) and induction of the leucocyte activation pathways leading to pathogen clearance. In recent years, several studies in fish have revealed a remarkable specificity of the innate response that had not been observed before [53–55]. TLRs have been identified in several fish species, showing an organization, expression, and similar features to those found in mammals [56].

Cell-types involved in nonspecific immune response of fish are monocytes-macrophages, granulocytes, platelets and natural killer cells [15,33,51,57]. All these cell-types showed a clear functional homology to mammalian leucocytes, although there are morphological and ultrastructural differences which also exist between different species of fish, especially in the case of granulocytes [58,59]. The monocyte-macrophages are the phagocytic cells for excellence and are especially abundant in the kidney. Functionally, they are more important in innate immunity because are highly phagocytic and can secrete free radicals of oxygen (ROIs) and nitrogen (RNIs) capable of killing a wide variety of pathogens such as viruses, bacteria and parasites [51,60,61]. Furthermore, they can be the initiator of activation and regulation of the specific immune response [62–64]. The process of phagocytosis in fish has the same steps as described for mammalian leucocytes, finishing with three mechanisms responsible for the killing of phagocytized microorganisms: the lysosomal enzymes able to digest the ingested pathogens into the phagolysosome, the production of ROIs with a rapid and abrupt increase in the rate of oxygen consumption which is known as respiratory burst and is independent of mitochondrial respiration and the production of nitric oxide (NO) and other RNIs; all of them showing bactericidal activity [65,66].

Non-specific cytotoxic cells, NCC, in fish are functionally equivalent to natural killer cells (NK) of mammals, forming a heterogeneous population of cells with typical

morphological characteristics of monocytes-macrophages, granulocytes and lymphocytes [67,68] able to kill tumor cells, xenogeneic cells, virus-infected cells and parasites. Thus, while in common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*) the three leucocyte-types are playing the NCC activity against tumor cell lines [68–70] in tilapia (*Tilapia mossambica* and *Tilapia honorum*) and European sea bass (*Dicentrarchus labrax*) it is mediated by macrophages [71].

B lymphocytes are involved in the humoral response (secreting Ig), while T lymphocytes are responsible for cell-mediated responses, cytokine secretion and also act as helper cells of B lymphocytes [72]. B lymphocytes are involved in the humoral response (secreting antibodies) always in the presence of macrophages as accessory cells, which produce interleukin (IL)-1 (necessary for this response). The antibodies are responsible for antigen neutralization, precipitation and agglutination, opsonization and activation of the classical complement pathway. A crucial aspect of the immune response is specific immunological memory, thus the secondary antibody production in teleosts is often more extensive and rapid than the primary, but this immunological memory is not as developed as in mammals [15,20,52]. T lymphocytes are responsible for cell-mediated responses, cytokine secretion and also act as helper cells of B lymphocytes [72]. The recognition of antigen by T lymphocytes is produced only when are presented properly. This occurs when an antigen presenting cell (APC), such as macrophages or B lymphocytes, after antigen phagocytosis and digestion, shows or present antigen small peptide fragments in its membrane. These are associated with glycoproteins of major histocompatibility complex of class II (MHC II) forming a complex which is placed on the surface of APC, then can be recognized by T lymphocytes. In fish, although information is more limited than in the case of mammals, there is antigen presentation and restriction to MHC [51,64]. Further, lymphocytes may produce cytokines that activate macrophages after stimulation with an antigen [73], showing, therefore, a coordinated and mutual control among the innate and adaptive response.

1.4. Cytokines

There is evidence that in fish, as in mammals, exist a network of cytokines and chemokines that influence the innate and acquired immune response. But these

molecules also regulate many other important biological processes, including cell growth and activation, inflammation, tissue repair, fibrosis and morphogenesis.

Cytokines are proteins (usually glycoproteins) with a low molecular weight (usually no more than 8-25 kDa) secreted by immune cells (mainly macrophages and T lymphocytes) in response to pathogens, their products or other related signals and that act at very low concentrations on cells of the immune system in an autocrine (recognizing a receptor on the cell membrane of the producer cell) or paracrine (acting on different than the secretory cell, but without a long-haul) way [74,75]. Despite being a heterogeneous group of proteins, they are considered as a protein family from a functional point of view, since not all of them are chemically related but share common properties [76]. However, some cytokines share a high homology (about 30%), like interleukin (IL)-1β and IL-1α, or tumoral necrosis factor (TNF)-α and TNFβ. In addition, there are subfamilies with a really high structural homology (about 80%), like the interferon α (IFNα) subfamily with about 20 members. In addition, they have a very short half life and exhibit pleiotropic attributes (regulate different functions), redundant, synergism, antagonism and induction in cascade [75].

Cytokines mediate effector phases in both innate and adaptive immunity [77]. Most of the research has been focused on interleukins (ILs), tumor necrosis factors (TNFs), interferons (IFNs), transforming growth factors (TGFs) migration inhibitory factors (MIFs), the colony stimulating factors (CSFs) and chemokines. In the innate immunity, cytokines are produced mainly by mononuclear phagocytes and so are usually called monokines. Monokines are produced by mononuclear phagocytes in response to microorganisms and upon T lymphocytes antigen stimulation as part of adaptive immunity. However, most of the cytokines involved in adaptive immunity are produced by activated T lymphocytes and these molecules are referred to as lymphokines. Lymphokines present a double function, either regulating the proliferation and differentiation of different lymphocytes populations or participating in the activation and regulation of inflammatory cells (mononuclear phagocytes, neutrophils and eosinophils). Both lymphocytes and mononuclear phagocytes produce other cytokines known as colony stimulating factors (CSFs), which stimulate the proliferation and differentiation of immature leukocytes in the bone marrow. Some other cytokines known as chemokines are chemotactic for specific cell types.

Although cytokines are made up of a diverse group of proteins, they share some features such as: (i) they are produced during the effector stages of the innate and adaptive immunity, and regulate the inflammatory and immune response; (ii) their secretion is brief and auto-limited, in general, cytokines are not stored as preformed molecules, and their synthesis is initiated by a new genetic transcription; (iii) a particular cytokine may be produced by many different cellular types; (iv) a particular cytokine may act on different cell types; (v) cytokines usually produce different effects on the same target cell, simultaneously or not; (vi) different cytokines may produce similar effects; (vii) cytokines are usually involved in the synthesis and activity of other cytokines; (viii) cytokines perform their action by binding to specific and high affinity receptors present on the target cell surface; (ix) the expression of cytokine receptors is regulated by specific signals (other cytokines or even the same one); (x) for many target cells, cytokines act as proliferation factors [77].

In fish, cytokines are grouped into growth factors (TGFβ1, TGFβ2, TGFβ3, etc.) [78–80], pro-inflammatory cytokines (IL-1b, TNF-a, IL-18, IL-6, etc.) [81–85], chemokines (CC, CXC, CX_3C, CXCL-8, etc.) [86–88], immunosuppressive or anti-inflammatory cytokines (IL-10, IL-19, IL-20, IL-22, etc.) [89,90] and IFNs [91–93].

2. THE SKIN MUCOSA

In the last years, mucosal immunity has focused our attention but most of the information relies on the study of the MALT [94]. In this section, we will focus on skin-associated lymphoid tissue (SALT), one of the least studied tissues with immunological role. In general, the fish MALT constitutes a very large area for the possible microbial invasion [95] and contains defence mechanisms (both innate and adaptive) that constitute the first line of defence against a broad spectrum of pathogens present in the aquatic environment [72,94]. Functions of this system appear to be related with to the ability to trap antigens and release IgT and IgM involved in responses against several pathogens [17,50].

2.1. Skin-associated lymphoid tissue (SALT)

The mucosal surfaces of fish are important sites of microbial exposure [96]. In addition to being physical barriers, mucosal surfaces, more concretely skin mucus, are also active immunological sites armed with cellular and humoral defences (see Fig. 2). Thus, these surfaces contain B lymphocytes and immunoglobulins, which play a pivotal role in the maintenance of mucosal homeostasis (reviewed by [97]). Since these surfaces represent the interface between each animal and the external environment, they are exposed more than any other site, to a continuous offensive of microbes and stressors.

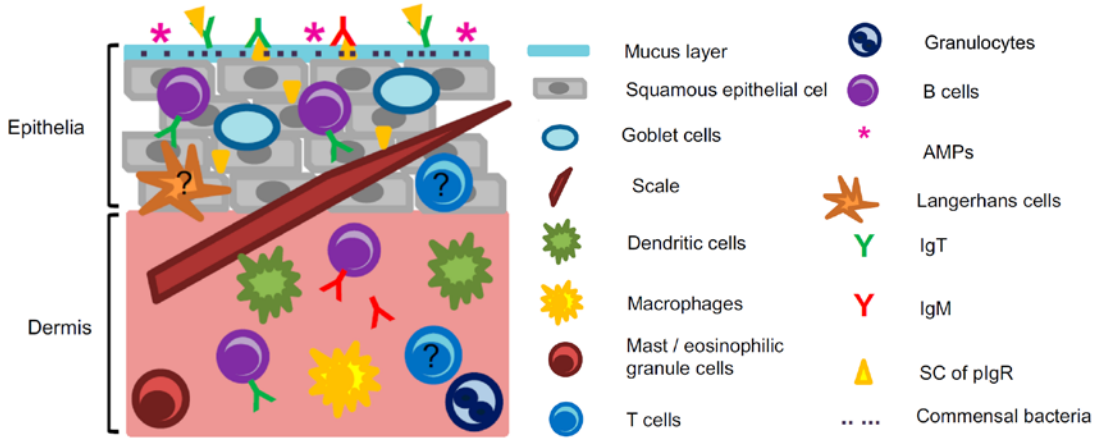

Fig. 2. Schematic representation of teleost fish skin [98]. AMPs: antimicrobial peptides; Ig: immunoglobulin; SC of pIgR: secretory component of the polymeric immunoglobulin receptor. Elements that are suspected to be present in a tissue, but have not been studied so far are marked as unknown (?) [99].

Therefore, fish skin mucus acts as a natural, physical, biochemical, dynamic, and semipermeable barrier that enables the exchange of nutrients, water, gases, odorants, hormones, and gametes [96]. The skin mucus is mainly composed of water and glycoproteins [100,101], conjugated with a large content of high-molecular-weight oligosaccharides, called mucins [102–104]. Among its functions, skin mucus is involved in fish respiration, ionic and osmotic regulation, reproduction, locomotion, defence against microbial infections, disease resistance and protection, excretion or communication [105,106]. Concomitantly, mucus plays a critical role in the defence mechanisms of the fish by also acting as a biological barrier [107–109]. Skin mucus has evolved to have robust mechanisms that can trap and immobilize pathogens before they

can contact epithelial surfaces, because it is non-permeant to most bacteria and other pathogens [103]. This occurs because in this mucus layer, particles, bacteria, or viruses are entrapped and removed from the mucosa by the water current [110]. Furthermore, the epidermal mucus is continuously replaced and the mucus layer thickness and composition prevents the pathogen adherence to the underlying tissues and provides a medium in which antibacterial mechanism may act [111–113].

The external mucous gel forms a layer of adherent mucus covering the living epithelial cells [114], and it is secreted by epidermal goblet cells [115]. As already mentioned previously, the predominant molecules present in mucus are the mucins. Mucins are high-molecular-weight glycoproteins that contain one or more protein domains with sites of extensive O-glycan attachment. Along with mucins, a complex mixture of other proteins, ions and lipids are also found in mucus, creating an ideal niche for microbial adherence and growth [98]. In fact, these mucins exert a mechanical barrier by serving as filters for pathogens and preventing pathogen adherence to the underlying tissues [113]. At this respect, mucin carbohydrates may act as microorganism receptors playing a decisive role in either pathogen expulsion or settlement and invasion [116,117]. Moreover, the skin mucus serves as a biological barrier since its continuous production of numerous substances involved in the immune response [113,118]. To date, there is a limited knowledge about the defence mechanisms of the epidermal mucus of fish, although both constitutive and inducible innate defence mechanisms are found [33]. Immune molecules in fish mucus include glycoproteins, lysozyme, immunoglobulins, complement proteins, lectins, agglutinin, calmodulin, interferon, C-reactive protein, flavoenzymes, proteolytic enzymes and antimicrobial peptides [23,105,113,119–121] which exert inhibitory or lytic activity against different type of pathogens [118,122]. This mucus composition determines its adhesiveness, viscoelasticity, transport and protective capacity [98]. Unfortunately, the complete repertoire of immune factors present in the skin mucus and their precise role on fish immunology and defence is poorly understood [123] and it is restricted to a few fish species, mainly freshwater.

2.2. Humoral responses of mucosal skin immunity

It is well-known that fish mucosal secretions carry out a wide variety of innate immune molecules including complement proteins, lysozyme, proteases, esterases,

AMPs and immunoglobulins, as the principal components of the humoral adaptive immune response [96]. The relative contribution of each one of the innate components to the total immune response appears to be variable amongst different teleost species. Interestingly, differences in the levels of innate immune molecules in the mucus may also reflect certain ecological strategies [98].

Among the most important humoral substances found are the complement system and AMPs, due to the importance of the first in bridging innate and adaptive immunity and of the latter in regulating commensals and pathogens. The presence of some complement components has been demonstrated in skin mucosal tissues of teleost fish. For example, C7 expression can be detected in skin of carp [124] and grass carp (*Ctenopharyngodon idella*) [125]. To date, few studies have tackled the role of complement in teleost skin mucus, although an important function of complement in the killing of pathogens in these surfaces is suspected. Contrarily, in the last years, identification of skin mucus AMPs has attracted great interest since this tissue seem to be the major source of AMPs (see Table 1) with approximately 70% of all AMPs expressed in this mucosal tissue, compared to 52% and 29% expressed in the gills and the gut, respectively [98]. Fish produce many different AMPs with anti-bacterial, anti-viral and anti-fungal activities [98,126]. Nevertheless, studies of teleost AMPs are still in its infancy and more studies are needed to better understand their specific role in mucosal immunity and their effects on commensals and pathogens.

Moreover, the principal components of the humoral adaptive immune response are the immunoglobulins. Vertebrates, due to the particular characteristics of mucosal surfaces, have specialized Igs in their mucosal surfaces. In teleosts, three Ig isotypes have been described for the nonce (IgM, IgD and IgT). In the case of IgM, it represents the main Ig in the plasma of teleosts and the main player in systemic immune responses. Further, IgM is also present in mucosal secretions of gut and skin [17] and is involved in responses against several pathogens. Furthermore, newly it has been demonstrated that the IgM heavy chain of fugu (*Takifugu rubripes*) acts as an N-acetyl-glucosamine (GlcNac) binding protein having a potent inhibitory effect on the growth of many kinds of bacteria [127]. IgD is known to be expressed in all immune tissues at the transcript level while its function still remains uncertain [128]. In the case of IgT, similar to mammalian IgA, is the only teleost Ig isotype with a specialized mucosal function as demonstrated in the gut of rainbow trout [43,50].

Table 1. AMPs identified in teleost skin.

AMP	Host species	References
Parasin I	Catfish	[129]
Pleurocidin	Winter flounder	[130]
Congerin	Japanese conger eel	[131]
Hipposin	Halibut	[132]
Oncorhycin III]	Rainbow trout	[133]
Piscidin	7 perciform species	[134]
Epinecidin-1	Grouper	[135]
Efap	Brown-spotted grouper	[136]
Hbb P-1	Channel catfish	[137]
omDB-2, -3, 4	Rainbow trout	[138]
AJN-10	Japanese eel	[139]
AS-hepcidin 2 and 6	Black porgy	[140]
Pelteobagrin	Yellow catfish	[141]
b-defensin 1 and 2	Carp	[142]
TP2-3	Nile tilapia	[143]
YFGAP	Yellowfin tuna	[144]
Cathelicidin-1 and 2, hepcidin, LEAP-2	Rainbow trout	[145]
Piscidin 1 and 2, b-defensin, hepcidin, cathelicidin-1	Atlantic cod	[146]

2.3. Cellular responses of skin mucosal immunity

These barriers are formed by epithelial cells, mucus-producing cells, neuroendocrine cells and an intrinsic immune system. Epithelial cells interact directly with pathogens and commensals which express pattern recognition receptors (PRRs) including lectins, nod-like receptors (NLRs) and toll-like receptors (TLRs).

Other components in cellular response are mast/eosinophilic granule cells (EGCs), which are most abundant in the gills, gut and skin and functionally show close similarity to the mast cells of mammals. A common feature in many teleost mucosal tissues is the recruitment of EGCs to sites of inflammation (reviewed by [147]). These cells from mucosal tissues have been studied with respect to their AMP content; nevertheless,

aside from their localization and AMP content, the information about the biology or function of these innate immune cells in mucosal immunity is scarce [98]. Moreover, macrophages and granulocytes are also present in skin of teleost fish [98].

In the cellular adaptive immunity response, the main cells that act are B and T lymphocytes. Amongst other functions, the main role of B lymphocytes appears to be the recognition of antigens in their native form and the production of Igs against those antigens. Moreover, T cells play an indispensable role in cell-mediated immunity and as they interact with the bacteria present in mucosal surfaces it seems they are very important in making tolerance or immunity against the commensal microbiota normally resident in the gastrointestinal tract [148]. Nevertheless, there are few functional studies of teleost T cells in mucosal surfaces. Particularly, at the transcript level in the skin, expression analyses suggest an increase of TCRα and CD4-1 genes in Atlantic salmon after salmon louse infection [149] while was observed a down-regulation of some T cell markers [149] and also tyrosine kinases after cortisol treatment [150]. Therefore is likely that T cells present in fish skin participate in skin immune responses although this hypothesis is not confirmed yet.

3. IMMUNOTOXICOLOGY IN FISH

Immunotoxicology, a specific subspecialty within the field of toxicology, has earned increasing interest in the last decade due to the increased knowledge of immunology and the importance of the immune response in maintaining the integrity of the organisms [151]. Moreover, considerable attention has been focused on the applicability and predictability of laboratory-animal-based assays for immunotoxicity in safety assessment studies [152]. Thus, field and semi-field experiments are good to have suspicions about the contaminant presence but the setup of laboratory experiments with controlled parameters and precise and pure compounds are strictly necessary to understand the impact on fish immune response and their potential mechanisms [9]. Though fish immunotoxicology is a relatively new field of study, is rapidly expanding as more and more techniques and reagents become available for use in teleost species [153–155].

Environmental contaminants are widely distributed in aquatic environments, and although many of them are prohibited or restricted they are usually very persistent in the nature [9] and capable of disrupt fish immunocompetence [156–158]. Stresses imposed on the immune system of fish by environmental pollutants may not always be overtly apparent since stressor agents may directly kill the fish or indirectly aggravate disease states by lowering resistance and allowing the invasion of environmental pathogens [159]. Furthermore, chemical exposure has the potential to interfere with critical phases of the fish immune response, by destroying, sensitizing, or otherwise altering cellular function (e.g., blocking phagocytic activity, inducing or inhibiting cell proliferation, or reducing precursor cell formation). Although the exact relationship between environmental pollution and disease in aquatic organisms is still uncertain, immunosuppression is the strongly supported hypothesis by which aquatic pollutants are thought to increase disease prevalence in exposed fish [159,160].

To better understand the effects of pollutant chemicals on fish immunocompetence, a battery of immune assays has been employed in such models as Nile tilapia (*Oreochromis niloticus*) [161], rainbow trout (*Oncorhynchus mykiss*) [162], Chinook salmon (*Oncorhynchus tshawytscha*) [163], mummichog (*Fundulus heteroclitus*) [164], turbot (*Scophthalmus maximus*) and Japanese medaka (*Oryzias latipes*) [165]. Some of the immune assays more commonly used in fish models include macrophage functions (e.g phagocytosis and ROI production), nonspecific cytotoxic cell activity, number of circulating leucocytes and lymphoid organ cellularity, complement fixation, lymphocyte proliferative responses, cytotoxic T-lymphocytes and antibodies (circulating antibody levels or antibody-forming cell numbers), immune-tissue organo-somatic idexes, delayed hypersensitivity response and host-resistance to pathogens [5,166–168].

Despite the fact that fish may be negatively impacted by these polluting chemicals, little is known regarding the effects of chemicals (either alone or as mixtures) on the fish immune response. In light of increasing social and political pressure to use non-mammalian systems for predicting human health risks and the need to develop biomarkers for assessing the biological effects of environmental stress, more studies are needed to better understand chemical-induced effects on aquatic species. Furthermore, fish represent an extremely diverse group of organisms (>25,000 species), but it would

be anticipated that at least some could serve as a nearly ideal alternate model for investigating immunotoxicity in mammalian species [151].

3.1. Immunotoxicology by heavy metals

Contamination of aquatic habitats with heavy metals from various industrial and mineral mining sources it is still a problem to solve. The present interests in mineral mining, energy development and use, and dredging will undoubtedly result in further pollution of aquatic environments by such metals as arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), copper (Cu), chromium (Cr) and zinc (Zn). Aquatic ecosystem impacts include contamination of sediments and the water column, accumulation of pollutants in biota over a wide area, and apparent increases in pollutant-related anomalies in the residing species [151]. Thus, heavy metals in these ecosystems are receiving more and more attention. Among the adverse effects, they can produce mortality, alterations in hematological parameters, metabolism and development, as well as alteration of sexual maturation or immunodeficiency [9]. Focusing on the last aspect, laboratory and field studies have demonstrated that exposure to certain metals alters both humoral and cellular responses of the innate and adaptive immune functions, as well as interfering with host resistance against infectious pathogens. Some heavy metals may transform into the persistent metallic compounds with higher toxicity, which can be bioaccumulated in the organisms and magnified in the food chain, thus threatening human health [169].

3.1.1. Arsenic

The semimetal arsenic (As) is an important environmental toxicant, which has been associated with multitude of animal and human health problems; although, its impact on fish immune system has not been extensively investigated. Nevertheless, the immunotoxicological effects of arsenic reduced the leucocyte respiratory burst, expression of some immune-relevant genes and disease resistance in zebrafish (*Danio rerio*) [170,171] in a similar fashion than in the catfish *Clarias batrachus* [172,173]. Studies in fish were either conducted *in vitro* or, even when conducted *in vivo,* used exposition levels not reported in nature (0.5-100 μM) and different effects on the immune system were demonstrated [172,174–176]. In addition, As-exposure produces a selective head-kidney macrophage death [177], down-regulates the synthesis of

macrophage-derived cytokines such as TNFα (tumour necrosis factor-alfa) and IFN-γ (interferon gamma) [178] and decreases the phagocytic activity of macrophages [176] and respiratory burst of embryos [171]. Finally, the spread and persistence of viral and bacterial pathogens is also associated to arsenic-exposed fish [171,172,176].

3.1.2. Cadmium

Cadmium (Cd) is a nonessential heavy metal causing great toxicity and represents the major aquatic pollutant in many parts of the world [179]. The effects of Cd on innate immune function represent the best studied area of Cd-induced immunotoxicity in fish [159,180–184]. Among the first observations, Robohm [185] found that Cd treatment inhibited the antibody levels in cunners (*Tautogolabrus adspersus*) and enhanced the antibody levels and chemotactic activity of peritoneal exudate cells in striped bass (*Morone saxatilis*). In rainbow trout exposed to 2 ppb of Cd (level found in some contaminated waters), the lysozyme activity was unaffected while the macrophage functions, phagocytosis and production of ROIs, were significantly impaired [183]. These authors also demonstrated that Japanese medaka (*Oryzias latipes*) leucocytes increased their production of ROIs and phagocytic functions without any change in many haematological parameters or antibody levels [186].

In vivo studies by Albergoni and Viola [187,188] assessing the effects of waterborne Cd exposure on humoral immunity demonstrated that catfish exposed for 7 days to 10, 20, or 30 μg Cd L^{-1} (as $CdCl_2$) had significantly reduced titers of total nonspecific Ig; however, levels returned to control values in fish exposed for an additional week. This response may have been due to initial toxicity followed over time by induction of protective enzymes (e.g., metallothionein). Response to a specific antigen was assessed in the aforementioned studies by immunization of Cd-exposed fish with sheep red blood cells (sRBCs). Studies demonstrated that catfish exposed to 20 μg Cd L^{-1} required a shorter amount of time than controls to reach a peak of anti-sRBC IgM levels. Moreover, fish exposed to Cd for 2 weeks prior to immunization reached peak antibody response more quickly and demonstrated a significant increase in antibody titer. Although contradictory findings have been reported [185,189], similar stimulatory effects have been observed in Cd-exposed rainbow trout (*Oncorhynchus mykiss*) following challenge with *Vibrio anguillarum* [190] and in metal-exposed striped bass (*Morone saxatilis*) challenged with *Bacillus cereus* [185]. Given that the

immunomodulatory effects of Cd in mammals as well as in fish depends on dose, mode of Cd exposure, and time of exposure in relation to immunization in both fish [187,188], contradictory results are not surprising.

Waterborne Cd exposure studies by Zelikoff et al. [183], Voccia et al. [191], and Sanchez-Daron et al. [182] examined the effects of low-dose in rainbow trout (*Oncorhynchus mykiss*) and demonstrated suppressive effects on PMA-stimulated H_2O_2 production. Moreover, in the European sea bass, while *in vivo* exposure had a similar inhibitory effect on phagocytic functions the *in vitro* treatment produced an increment [180]. In the case of juvenile common carp experimentally infected with the blood parasite, *Sanguinicola inermis* (Trematoda: *Sanguinicolidae*) there were tissue changes and while the counts of neutrophils, eosinophils and thrombocytes increased in the thymus the number of neutrophils in the pronephros was reduced due to Cd^{2+} treatment (0.1 mg L^{-1}) [192]. More recently, the Cd exposure has been related to the increase of melano-macrophage centres on several fish tissues [193]. In the hybrid tilapia (*Oreochromis niloticus* × *O. aureus*), the Cd exposure increased the lysozyme activity but greatly reduced the alternative complement activity [194]. Overall, results of the aforementioned *in vivo* and *in vitro* studies demonstrate the sensitivity of fish phagocytes to the immunomodulating effects of Cd. In addition, the findings suggest that ROI production may be the most sensitive indicator of immunotoxic effects associated with exposure to low, environmentally relevant doses of Cd. In fact, its applicability as a biomarker in fish to predict the toxicological impact of contaminated aquatic environments has been suggested [159,183,184].

Although only a limited number of studies in fish have examined the effects of Cd on specific cell-mediated immunity, strong evidence exists demonstrating altered T-lymphocyte proliferative responses [182,187,188,190,191]. Voccia et al. [191] demonstrated that waterborne exposure to Cd at either 1 or 5 ppb depressed proliferation of mitogen-stimulated anterior kidney and thymic lymphocytes. Proliferative responses of thymic lymphocytes were depressed at both concentrations, while lymphoproliferation by kidney cells was reduced only at the highest Cd concentration.

3.1.3. Mercury

Contamination of the aquatic environment by mercury (Hg) has been recognized as a potential environmental and public health problem for over 40 years [151]. It has been estimated that industrial effluents have increased the concentrations of Hg in rivers and lakes by 90 ng L^{-1} per year [195]. Furthermore, in aquatic systems, metallic, inorganic or organic Hg can be biotransformed to its most toxic form, methylmercury (MeHg), by different sulphate reducing microorganisms present in sediments [196].

Mercury, and derivatives such as methylmercury, induces organ lesions as well as neurological, haematological and immunological disorders [197]. Inorganic Hg has also been shown to damage fish liver and skin [197–199], as well as arrest gonadal growth and reduce the gonadosomatic index of catfish [200]. Immunotoxic effects following Hg exposure range from depressed hematopoiesis and enzyme activity. Investigators have observed diverse effects ranging from low-dose activation to high-dose inhibition of fish immune cell function following Hg exposure [191,201].

First evidences, in rainbow trout, described a decrease in the number of mucous-producing cells and mucus production after exposure to mercury and methylmercury, which can be associated to impaired immunity [202]. Further in the same species, head-kidney macrophages exposed for several weeks to 0.5 ppb Hg exhibited diminished phagocytosis, respiratory burst activity, and Ig levels [182]. Afterwards, serum C-reactive protein was increased in freshwater murrel (*Chana punctatus*) [203] and major carp (*Catla catla*) [204] by exposure to mercury. Nevertheless, plasmatic lysozyme of plaice was decreased after exposure to sublethal doses of mercury [205]. In contrast, blue gourami (*Trichogaster trichopterus*) showed increased kidney and plasma lysozyme activity, but at the same time reduced the production of agglutinating specific antibodies after chronic exposure to 0.045 or 0.09 mg Hg^{2+} L^{-1} [201]. Further evidences have been obtained *in vitro* where blue gourami lymphocytes incubated with mercury showed increased proliferation at low dosages, which was reversed by higher levels (>0.045 mg L^{-1}) [201]. In the marine fish *Sciaenops ocellatus*, mercury treatment (≤10 μM) produced a high-dose inhibition and a low-dose (0.1 to 1 μM) activation of leukocytes as determined by Ca-mobilization and tyrosyne phosphorilation of proteins [206]. *In vitro*, treatment with $HgCl_2$ induced apoptosis in head-kidney macrophages as well as reduced the ROIs production and the benefits of macrophage-activating factors

(MAF) in the European sea bass (*Dicentracrchus labrax*) [207]. More recently, it has been documented that largemouth bass (*Micropterus salmoides*) naturally inhabiting Hg-contaminated waters suffered immunosuppression though the effects might be not exclusive to Hg [208]. Taken together, the mentioned studies support the hypothesis that immunotoxic doses of Hg alter fish immunity, thus modifying the ability to regulate the magnitude and specificity of a competent immune response.

Finally, taking into account that very little is known about the impact of waterborne exposure to heavy metals in fish biology, immunotoxicology and concretely on skin mucus of fish, we have developed this Thesis with the objective to throw some light in the potential risk of heavy metals contamination to cultured fish biology and in turn to human consumption.

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OBJECTIVES

Heavy metal immunotoxicology and skin mucus in fish

FA Guardiola 2014

OBJECTIVES

This work has the following specific objectives:

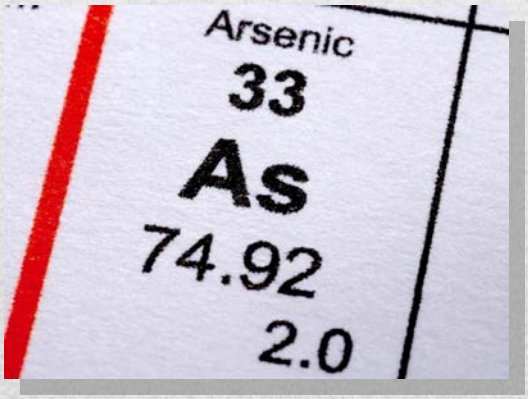
1. Evaluate some toxicological effects of waterborne exposure to arsenic, cadmium and mercury in the gilthead seabream (*Sparus aurata* L.) with especial emphasis in the immune response.

2. Identify and characterize the immunological and physico-chemical parameters of the skin mucus from several marine teleost fish and, in the case of gilthead seabream compare with the immunity present in the serum.

3. Study of the innate immune repertoire functioning at the skin mucus of gilthead seabream exposed to waterborne arsenic, cadmium and mercury.

CHAPTER 1

Immunotoxicological effects of inorganic arsenic on gilthead seabream (*Sparus aurata* L.)



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CHAPTER 1. Immunotoxicological effects of inorganic arsenic on gilthead seabream (*Sparus aurata* L.)

ABSTRACT

Arsenic (As) has been associated with multitude of animal and human health problems; however, its impact on host immune system has not been extensively investigated. In fish, there are very few works on the potential risks or problems associated to the presence of arsenic. In the present study we have evaluated the effects of exposure (30 days) to sub-lethal concentrations of arsenic (5 µM As_2O_3) in the teleost fish gilthead seabream (*Sparus aurata*), with special emphasis in the innate immune response. The arsenic concentration was determined using atomic fluorescence spectrometry (AFS) in liver and muscle of exposed fish showing As-accumulation in the liver after 30 days of exposure. The hepatosomatic index was increased at significant extent after 10 days while returned after 30 days of exposure to control values. Histological alterations in the liver were observed including hypertrophy, vacuolization and cell-death processes Focusing on the immunological response, the humoral immune parameters (seric IgM, complement and peroxidase activities) were no affected to a statistically significant extent. Regarding the cellular innate parameters, head-kidney leucocyte peroxidase, respiratory burst and phagocytic activities were significantly increased after 10 days of exposition compared to the control fish. Overall, As-exposition in the seabream affects the immune system and how this might interfere with fish biology, aquaculture management or human consumers should focus further investigations. This paper describes, for the first time, the immunotoxicological effects of arsenic exposure in the gilthead seabream, which is a species with the highest rate of production in Mediterranean aquaculture.

1. INTRODUCTION

Metals and semi-metals are naturally present in earth and enter into aquatic environments by various geochemical processes. Moreover, anthropogenic sources of metals including mining, metalworking and industrial processes also contribute to environmental, included aquatic, concentrations of metals [1]. It is also known that heavy metal contamination in aquatic systems is one of the most critical environmental issues [2]. Apart from the negative aspects that pollutants may have on the aquatic organisms their effects on those destined to human consume have additional importance. Thus, chemicals used in aquaculture, as well as the contaminants present in the surrounding waters, could negatively impact animal health and/or consumers [3]. Fish are known to be the greatest inputs of toxic trace elements (total and organic mercury, selenium, cadmium, lead, arsenic, copper, chromium, iron, manganese, molybdenum, vanadium and zinc) for humans, but limited data on the differences between farmed and wild fish are available [4]. Much attention has focused on the potential human exposure to metals, particularly mercury and arsenic, via the consumption of both farm-raised fish and wild-caught species [5–8].

The semimetal arsenic (As) is an important environmental toxicant produced as a byproduct of smelting, fossil fuel combustion, and pesticide production [9]. Arsenic is a naturally occurring element found in soil, air, and water [10–12], where it is taken up by various organisms [9,13]. In nature, arsenic exists in the inorganic and organic forms, being the inorganic form the most toxic [14,15]. Toxicological studies in mammals have documented that As-exposition causes melanosis, keratosis and skin hyperpigmentation in humans [16] and it might also act as a carcinogen [17]. At cellular level, arsenic induces oxidative stress that causes DNA damage through the production of superoxide and hydrogen peroxide [18,19], disrupts mitosis and promotes apoptosis [20,21], impairs cellular metabolism [22] and can induce genotoxicity and lipid peroxidation [23]. Moreover, arsenic is also immunotoxic exhibiting its effects on a variety of immune responses [24–26], resulting in a general immunosuppression and increased susceptibility to infection [21,27,28].

Very little is known about the arsenic toxicity in fish biology. Environmental arsenic primarily accumulates in retina, liver and kidney of exposed fish. There are reports suggesting that arsenic induces oxidative stress [29], liver inflammation [30], hyperptrophy, production of stress proteins [31] and apoptosis of fish hepatocytes [32]. Regarding the immunotoxicological effects of As, earlier studies in fish were either conducted *in vitro* or, even when conducted *in vivo*, used exposition levels not reported in nature (0.5-100 µM) [9,14,32–35] and different effects on the immune system were demonstrated [15,33,34,36]. In addition, As-exposure produces a selective head-kidney macrophage (HKM) death [37], down-regulates the synthesis of macrophage-derived cytokines such as TNFα (tumour necrosis factor-alfa) and IFN-γ (interferon gamma) [38] and decreases the phagocytic activity of macrophages [33] and respiratory burst of embryos [12]. Thus, Datta et al. [14,37] suggested that fish macrophages are the main targets for the arsenic toxicity. Finally, the spread and persistence of viral and bacterial pathogens is also associated to arsenic-exposed fish [12,33,34].

Since very little is known about the specific effects of arsenic on fish immune response gilthead seabream (*Sparus aurata* L.) specimens were exposed to a sub-lethal dose of inorganic arsenic. We aimed to evaluate its accumulation in liver and muscle as well as its immunotoxicological impact. Potential risks for fish health and consumers will be discussed.

2. MATERIAL AND METHODS

2.1. Fish care and maintenance

Thirty-six specimens (41.5 ± 28.15 g body weight and 13.65 ± 2.65 cm body-length) of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.), obtained from *Doramenor Acuicultura S.L.* (Murcia, Spain), were kept in seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia. The water was maintained at 20 ± 2°C with a flow rate of 1,500 l h^{-1} and 28‰ salinity. The photoperiod was of 12 h light: 12 h dark and fish fed with a commercial pellet diet (Skretting) at a rate of 2% body weight day^{-1}. Fish were allowed to acclimatise for 15 days before the start of the experimental trial. They were starved for 24 h prior to sampling and sacrificed by an overdose of MS222 (Sandoz, 100 mg ml^{-1} water) [39].

All experimental protocols were approved by the Bioethical Committee of the University of Murcia.

2.2. Arsenic exposure

Fish were divided into two tanks and remained unexposed (control group) or exposed to arsenic (5 μM As_2O_3; Fluka Analytical). The exact quantity of arsenic trioxide (previously dissolved in a small volume of water) was administered directly into the aquarium water. Six fish per tank and group were sampled after 2, 10 or 30 days of exposition.

2.3. Fish sampling

Fish were dissected and the whole fish, liver and spleen weighted. Fragments of liver and muscle were obtained and stored at -80 ºC for As determination while fragments of liver from processed for histology. Blood samples were obtained from the caudal vein of each specimen with a 27-gauge needle and 1 ml syringe. After clotting at 4ºC, each sample was centrifuged and the serum removed and frozen at -80ºC until use. The head-kidney (HK) was excised from each fish under sterile conditions, cut into small fragments and transferred to 8 ml of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's osmolarity to gilthead seabream plasma osmolarity of 353.33 mOs), 2% foetal calf serum (FCS, Gibco), 100 i.u. ml^{-1} penicillin (Flow) and 100 μg ml^{-1} streptomycin (Flow)] [40]. Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 μm), washed twice (400 g, 10 min), counted and adjusted to 10^7 cells ml^{-1} in sRPMI. Cell viability was determined by the trypan blue exclusion test.

2.4. Liver and muscle analysis of total arsenic

Frozen liver and muscle samples were lyophilized and 100-200 mg of the resulting powder were placed in Teflon vessels with 3 ml of water, 2 ml of concentrated H_2O_2 and 5 ml of concentrated HNO_3 acid solution. The digestion of the samples was carried out using a Milestone ETHOS Plus Microwave system operating with a standard program (85, 200, 210 and 0ºC during 2, 8, 10 and 20 min., respectively), since it has been demonstrated to be an efficient methodology for determining total As (Shah et al., 2009). Finally, 50 μl of the solutions were used to determine the arsenic concentration

using atomic fluorescence spectrometry with an automated continuous flow hydride generation (HG-AFS) spectrometer (PSA Millenium Excalibur 10055). Quality control of the analytical was used of reference materials: DOLT-2 Dogfish liver, DORM-2 Dogfish muscle. The recovery obtained with the reference materials was above 93% in all cases. Data are presented as µg As per kg dry-weight tissue.

2.5. Determination of organo-somatic indexes

Whole body, spleen and liver were weighted. The organo-somatic index (OSI) for spleen and liver was calculated with the following formula: OSI = (g tissue g body^{-1}) × 100.

2.6. Light microscopy

Liver samples were fixed with 10 % neutral buffered formalin (Panreac) for 24 h. After serial dehydration steps in alcohol, samples were embedded in paraffin (Thermo Scientific), sectioned at 5 µm, mounted and stained with haematoxylin-eosin (H-E). Slides were analysed by a light microscope (Leica 6000B) and images were acquired with a Leica DFC280 digital camera.

2.7. Immune parameters

2.7.1. Natural haemolytic complement activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [41]. Equal volumes of SRBC suspension (6%) in phenol red-free Hank's buffer (HBSS) containing Mg^{+2} and EGTA (ethylene glycol tetraacetic acid) were mixed with serially diluted serum to give final serum concentrations ranging from 10% to 0.078%. After incubation for 90 min at 22ºC, the samples were centrifuged at 400 g for 5 min at 4°C to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader (BMG labtech-Fluostar galaxy). The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 µl of distilled water or HBSS to 100 µl samples of SRBC, respectively.

The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting Y $(1\text{-}Y)^{-1}$ against the volume of serum added (ml) on a log-log

scaled graph. The volume of serum producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} obtained for each experimental fish.

2.7.2. Serum and leucocyte peroxidase activity

The peroxidase activity in serum or leucocytes was measured according to Quade and Roth [42]. Briefly, 15 µl of serum were diluted with 135 µl of HBSS without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. 50 µl of 20 mM 3,3',5,5'- tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM H_2O_2 were added. To determine the leucocyte peroxidase content, 10^6 HK leucocytes in sRPMI were lysed with 0.002% cetyltrimethylammonium bromide (Sigma) and, after centrifugation (400 g, 10 min), 150 µl of the supernatants were transferred to a fresh 96-well plate containing 25 µl of 10 mM TMB and 5 mM H_2O_2. In both cases, the colour-change reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid and the optical density was read at 450 nm in a plate reader. Standard samples without serum or leucocytes, respectively, were used as blanks.

2.7.3. Serum IgM level

Total serum IgM levels were analyzed using the enzyme-linked immunosorbent assay (ELISA) [43]. Thus, 20 µl per well of 1/100 diluted serum were placed in flat-bottomed 96-well plates in triplicate and the proteins were coated by overnight incubation at 4ºC with 200 µl of carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with PBT (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA, Sigma) in PBT, followed by three rinses with PBT. The plates were then incubated for 1 h with 100 µl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer, Sigma). After exhaustive rinsing with PBT the plates were developed using 100 µl of a 0.42 mM TMB solution, prepared daily in a 100 mM citric acid/sodium acetate buffer, pH 5.4, containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µl of 2M H_2SO_4 and the plates were read at 450 nm. Negative controls consisted of samples

without serum or without primary antibody, whose OD values were subtracted for each sample value.

2.7.4. Respiratory burst activity

The respiratory burst activity of gilthead seabream HK leucocytes was studied by a chemiluminescence method [44]. Briefly, samples of 10^6 leucocytes in sRPMI were placed in the wells of a flat-bottomed 96-well microtiter plate, to which 100 µl of HBSS containing 1 µg ml^{-1} phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma) were added. The plate was shaken and luminescence immediately read in a plate reader (BMG labtech-Fluostar galaxy) for 1 h at 2 min intervals. The kinetics of the reactions were analysed and the maximum slope of each curve was calculated. Luminescence backgrounds were calculated using reagent solutions containing luminol but not PMA.

2.7.5. Phagocytic activity

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by gilthead seabream HK leucocytes was studied by flow cytometry [45]. Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to $5x10^7$ cells ml^{-1} of sRPMI. Phagocytosis samples consisted of 125 µl of labelled-yeast cells and 100 µl of HK leucocytes in sRPMI (6.25 yeast cells:1 leucocyte). Samples were mixed, centrifuged (400 g, 5 min, 22ºC), resuspended and incubated at 22ºC for 30 min. At the end of the incubation time, samples were placed on ice to stop phagocytosis and 400 µl ice-cold PBS was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 40 µl ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HK leucocytes were included in each phagocytosis assay.

All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 3,000 cells, which were acquired at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (granularity) (SSC) and forward scatter (size) (FSC), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were made on a computerised system. The fluorescence histograms represented the relative fluorescence on a logarithmic

scale. The cytometer was set to analyse the phagocytic cells, showing highest SSC and FSC values. Phagocytic ability was defined as the percentage of cells with one or more ingested bacteria (green-FITC fluorescent cells) within the phagocytic cell population whilst the phagocytic capacity was the mean fluorescence intensity. The quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson).

2.8. Statistical analysis

The results are expressed as mean ± standard error, SE. Data were statistically analysed by one-way analysis of variance (ANOVA). Differences were considered statistically significant when $P \leq 0.05$.

3. RESULTS

3.1. Arsenic is accumulated in the liver

The concentrations of inorganic As analysed in muscle and liver are shown in Table 1. In muscle, the As concentration was always below the quantification limit (cl; <0.1 µg kg dry-weight^{-1}). Similarly, in liver, the inorganic As concentration in the unexposed fish or exposed for 2 or 10 days was undetected. However, As was detected in the liver of all the seabream specimens after 30 days of exposure with a concentration of 429.6 ± 59.8 µg kg dry-weight^{-1}.

3.2. As-exposure increases the hepatosomatic index

The organo-somatic index for gilthead seabream spleen was found to exhibit slight variations along the exposition time to arsenic but never reached a significant extent (Fig. 1). On the contrary, the hepatosomatic index was increased after 2 and 10 days of As-exposition, being statistically significant after 10 days of As-exposure compared to those obtained in control or unexposed fish (Fig. 1). However, hepatosomatic index returned to control values in the last sampling of the trial.

Table 1. Concentration of inorganic As (µg kg dry-weight^{-1}) determined in muscle and liver samples from gilthead seabream specimens unexposed or exposed to 5 µM of As_2O_3. cl: quantification limit (0.1 µg kg dry-weight^{-1}).

Exposition (days)	Muscle		Liver	
	Unexposed	Exposed	Unexposed	Exposed
2	<cl	<cl	<cl	<cl
	<cl	<cl	<cl	<cl
	<cl	<cl	<cl	<cl
	<cl	<cl	<cl	<cl
	<cl	<cl	<cl	<cl
	<cl	<cl	<cl	<cl
10	<cl	<cl	<cl	<cl
	<cl	<cl	<cl	<cl
	<cl	<cl	<cl	<cl
	<cl	<cl	<cl	<cl
	<cl	<cl	<cl	<cl
	<cl	<cl	<cl	<cl
30	<cl	<cl	<cl	306.4
	<cl	<cl	<cl	644.1
	<cl	<cl	<cl	273.5
	<cl	<cl	<cl	550.2
	<cl	<cl	<cl	348.2
	<cl	<cl	<cl	455.2

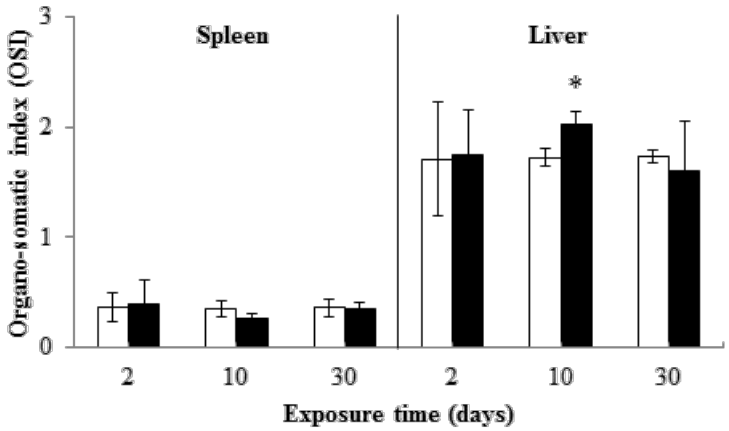

Fig. 1. Organo-somatic index (%) of spleen and liver in gilthead seabream specimens unexposed (control; white bars) or after exposure to arsenic (5 µM; black bars). Bars represent the mean ± S.E. (n=6). Asterisk denotes significant differences between unexposed and As-exposed groups ($P \leq 0.05$).

3.3. Histological alterations

Liver from arsenic-exposed gilthead seabream specimens revealed considerable histological changes increasing with the exposure time (Fig. 2). While after 2 days of exposition any evident changes were observed in the liver, after 10 days of exposure the hepatocytes showed initiation of vacuolization and cells with elongated nucleus (apoptotic/necrotic) (Fig. 2B). After 30 days of exposition, livers revealed architectural loss in the hepatocytes, high cytoplasmic vacuolization and presence of apoptotic/necrotic cells (Fig. 2C).

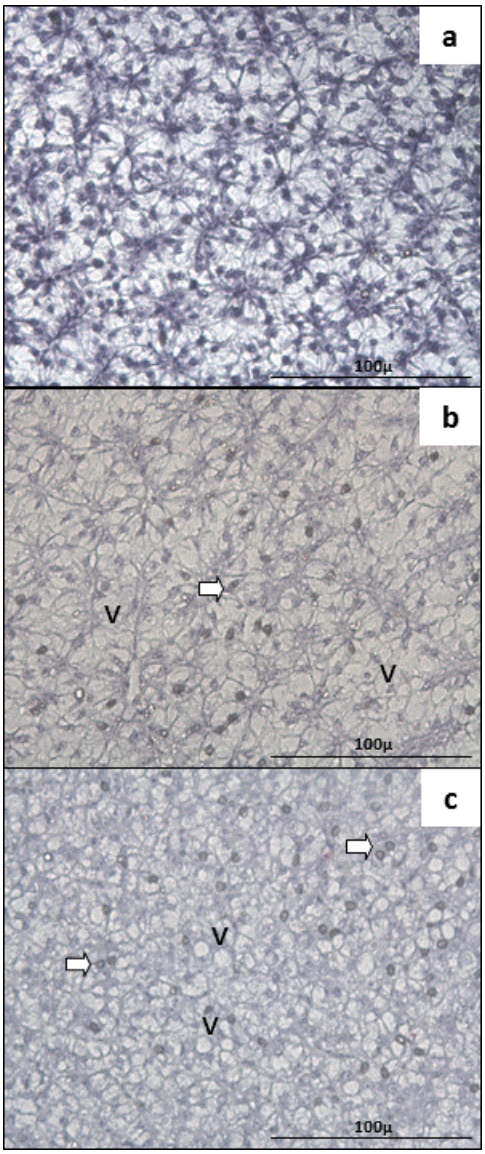

Fig. 2. Representative histology of liver gilthead seabream samples stained with hematoxylin-eosin from gilthead seabream specimens unexposed (control) (a) or exposed for 10 (b) or 30 (c) days to 5 µM arsenic. Micrographs (b, c) show vacuolated hepatocytes; V, vacuoles, arrow, hepatocyte nucleus.

3.4. Effect of As in the humoral immune parameters

The determined gilthead seabream humoral immune parameters were not affected to a significant extent by As-exposition (Table 2). Specimens maintained in presence of arsenic showed variations in the haemolytic complement activity respect to the values obtained in the serum from specimens in control group (unexposed with As), with a decrease and increase after 2 and 30 days, respectively. Seric peroxidase activity was always increased after As-exposition while IgM level was reduced in seabream specimens after 2 and 30 days.

Table 2. Humoral immune activities in the serum of gilthead seabream specimens unexposed (control) and As-exposed (5 µM). Data represent the mean ± S.E. (n=6).

Activities	Experimental groups	Days of exposition		
		2	10	30
Natural haemolytic complement activity (ACH_{50} units ml^{-1})	Unexposed	11.27 ± 3.09	11.24 ± 1.60	10.74 ± 0.23
	As- exposed	7.25 ± 2.82	11.32 ± 1.91	14.76 ± 5.50
Peroxidase activity (units ml^{-1})	Unexposed	25.25 ± 3.09	25.87 ± 6.35	26.02 ± 6.14
	As-exposed	31.88 ± 4.85	31.20 ± 3.26	32.17 ± 8.14
Immunoglobulin M (OD 450 nm)	Unexposed	0.23 ± 0.01	0.23 ± 0.02	0.24 ± 0.01
	As-exposed	0.20 ± 0.02	0.23 ± 0.03	0.19 ± 0.02

3.5. As-exposition increased the cellular innate immune parameters

Peroxidase activity (Fig. 2), respiratory burst activity (Fig. 3), and phagocytic ability (Fig. 4A) of head-kidney leucocytes isolated from fish exposed to As for 10 days was increased in a statistically significant manner respect to the activity found in leucocytes isolated from unexposed fish. Nevertheless, no statistical deviations were found in fish treated with arsenic for 2 or 30 days. The phagocytic capacity of leucocytes isolated from fish exposed to As failed to show significant variations at any experimental time (Fig. 4B).

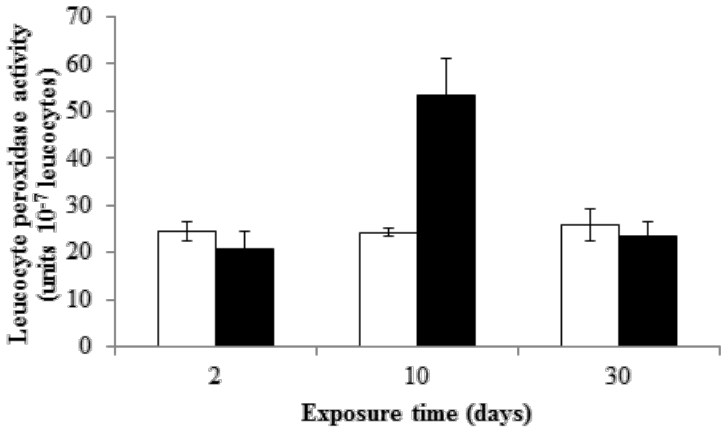

Fig. 3. Leucocyte peroxidase activity (units 10^{-7} leucocytes) in head-kidney leucocytes of gilthead seabream specimens unexposed (control; white bars) or exposed to 5 µM of arsenic (black bars). Bars represent the mean ± S.E. (n=6). Asterisk denotes significant differences between unexposed and exposed groups ($P \leq 0.05$).

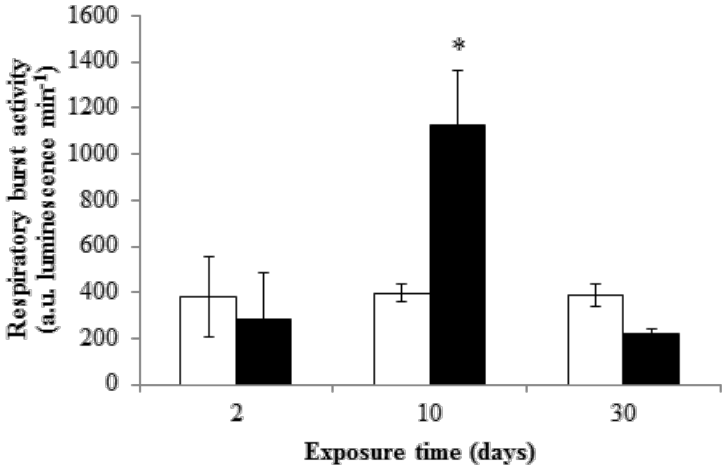

Fig. 4. Respiratory burst activity (slope min^{-1}) in head-kidney leucocytes of gilthead seabream specimens unexposed (control; white bars) or exposed to µM of arsenic (black bars). Bars represent the mean ± S.E. (n=6). Asterisk denotes significant differences between unexposed and exposed groups ($P \leq 0.05$).

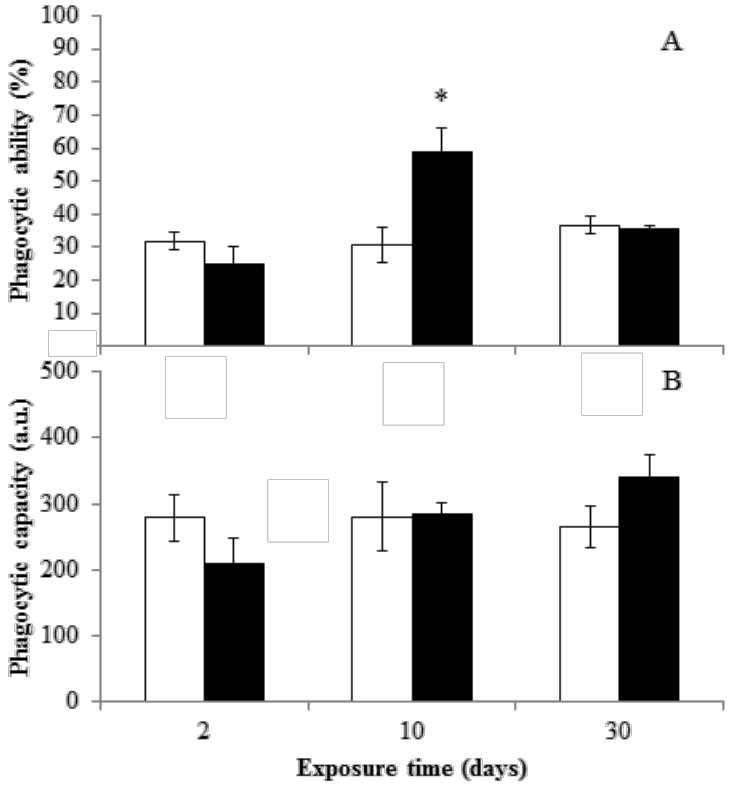

Fig. 5. Phagocytic ability (%) (A) and capacity (a.u.) (B) in head-kidney leucocytes of gilthead seabream specimens unexposed (control; white bars) or exposed to 5 µM of arsenic (black bars). Bars represent the mean ± S.E. (n=6). Asterisk denotes significant differences between unexposed and exposed groups ($P \leq 0.05$).

4. DISCUSSION AND CONCLUSIONS

The occurrence of pollutants, including heavy metals, in aquatic environments influences the health and survival of fish by several mechanisms, including depression of the immune system [14,46]. In addition, they are bioaccumulated and transferred through food chains [47] becoming a potential risk for humans as final consumers [48–50].

In non-contaminated environments, total As concentration in seawater and marine sediments are usually within the range of 1-2 µg L^{-1} and 3-15 µg g^{-1}, respectively [51]. In the present study, gilthead seabream were exposed to a high but sub-lethal concentration of arsenic (5 µM or 989 µg L^{-1}), which might be considered as an extreme

case of contamination (around 1,000-fold the seawater level) and is within the range of As tested in previous studies developed in fish (0.5-100 μM) [14,15,32,34,35,37]. Exposition of seabream specimens to this high concentration resulted in neither mortality nor visible negative toxic effects.

The accumulation pattern of contaminants in fish, and in other aquatic organisms, depends on their uptake and elimination rates [52,53]. Basically, fish assimilate As by ingestion of particulated material suspended in water, food ingestion, ion-exchange of dissolved metals across lipophilic membranes (e.g., the gills and the skin) and adsorption by tissue and membrane surfaces [54]. The only available results in fish have demonstrated that most of the interiorized As was accumulated in the intestine, stomach, liver, and gill of tilapia (*Oreochromis mossambicus*) rather than in the muscle [15]. In agreement with this previous study, our results confirm that As accumulates in the liver of seabream after 30 days and concomitantly, confirm that As do not accumulate in the muscle, a fact that it is very important since this specie is destined to human consume. Further studies are still needed to monitor the muscle accumulation of contaminants and the potential impacts for aquaculture and consumers.

Organo-somatic indexes are commonly used as biomarkers for toxicity [55] and in the case of arsenic, reductions in the liver [30], spleen [33] and head-kidney [14,33,34] indexes have been described in fish. In the present study, the organo-somatic index for seabream spleen was not significantly affected by the As-exposition. However, exposure to arsenic resulted in a time-dependent increase of the hepatosomatic index after 2 and 10 days, being statistically significant at day 10, and returned to the control values at the end of the trial. This increase in the liver size has been suggested to be due to either hyperplasia or hypertrophy of hepatocytes [56] whilst in seabream we found hypertrophy and swollen of hepatocytes, formation of vacuoles inside the cell, deposition of glycogen and appearance of cells with deformed nucleus in gilthead seabream. Moreover, previous data identifying decrease of the fish hepatosomatic index for chronic or long As-expositions were associated with increase in the apoptosis of hepatocytes and to a reduction in their protein content and/or proliferation because these cells are potential targets of this metal [32,57,58]. Besides this, our data after 30 days of exposition also describe histological alterations with increase in the hepatocytes cell-death structures and vacuolization though the hepatosomatic index was not changed.

Fish have proven to be valuable model systems for aqua-toxicological studies acting as important environmental sentinels [12], as well as for the estimation of heavy metals pollution and their potential risk as consequence of their consumption [48–50]. Gilthead seabream specimens exposed to As did not show any statistically significant variations in the humoral immune parameters analyzed in the present study. The natural haemolytic complement activity measured in serum specimens showed a little decrease after 2 days of As-exposition but an increase at 10 and 30 days with respect to fish in the control group (unexposed). Most complement components are synthesized in the liver but leucocytes also account for their production at lower scale [59]. Since the liver damage and As-accumulation partly coincides with slight increases of the seric complement and peroxidase activities this could suggest that leucocytes could now been the main source of such proteins and that they have been activated to produce them. Other hypothesis could be that hepatocytes, before being completely damaged, increase the production of important proteins involved in general homeostasis or defense under certain cell-stress situations. Following this hypothesis, exposure of cells to a variety of stress factors elicits an up-regulation of a number of cytoprotective systems [60], amongst which the heat shock response (heat shock proteins, HSPs) is one of the most studied [61]. However, these hypotheses need further confirmation. The peroxidase activity, related to the myeloperoxidae and/or eosinophil peroxidase activity, is assumed to be a good marker for leucocyte activation [45]. Polymorphonuclear cells release these peroxidases into the blood in response to infection, therefore elevated serum levels of peroxidases could identify many pathological situations [45,62,63]. This is the first work studying the seric peroxidase level in fish exposed to pollutants and the obtained results suggest that peroxidase could be included as a new bioindicator of pollutants but further works should confirm this asseveration. Regarding the serum IgM levels, we found that seabream specimens maintained in presence of arsenic showed a slight decrease at 2 and 30 days and small increase with respect to unexposed individuals, suggesting an interference of As in the number and viability of seabream B-lymphocytes. This is in agreement with previous studies showing that arsenic trioxide can induce apoptosis of lymphocytes through oxidative stress leading to lymphocytopenia in human [64,65] and reduction of Ig production in fish [26,33,34,66]. Moreover, and confirming that As exposure interferes with the humoral immune responses, the production of interleukin (IL)-4 was decreased in *Clarias batrachus* specimens subjected to a higher concentration of arsenic (42.42 µM) during longer

exposition time (150 days) [34]. Further studies are needed to understand the immunotoxicological effects and mechanisms of action of As in the humoral immune parameters of fish.

Cellular innate immune parameters revealed increased phagocytic ability, peroxidase and respiratory burst activities of seabream HK leucocytes at 10 days of As-exposition. Fish phagocytes (macrophages and/or granulocytes) engulf invading pathogens and destroy them by producing ROS (reactive oxygen species) such as superoxide anions (O^{2-}) or hydrogen peroxide (H_2O_2) that serves as the first line of defense [67]. Recently, a study suggests a potential mechanism for arsenic induced toxicity whereby arsenic disrupts mitochondria function that leads to an increase in intracellular ROS [68]. In addition, peroxidase is also produced at the end of the oxidative burst cascade increasing the line of defense. Regarding the phagocytic activity in fish, *Clarias batrachus* exposition to 42.42 µM arsenic for 21 days or to 0.5 µM for 1 or 30 days decreased it [14,33]. On the other hand, in rats, phagocytic activity was significantly increased after oral administration of As (20 ppm) for 4 weeks but decreased after 12 weeks [69]. These data are in line with our results in seabream and indicate that As-expositions for short time or low dosages could increase some immune responses. In line with this, there are also several studies documenting arsenic-induced alterations in the ROS production [70,71]. Our results also show an increase in ROS production after 10 days of seabream exposure to As corroborating previous data obtained after exposition to 0.5 µM of As both *in vitro* and *in vivo* [14]. All the same, this is in contrast to a study of Hermann and Kim [9] who observed a reduction in ROS production in zebrafish embryos following *in vivo* exposure to arsenic although perhaps, these apparent differences are due to the maturation of the immune system. *In vitro* studies demonstrated that lower As concentrations (0.5 µM) induced ROS generation in head-kidney macrophages of *C. batrachus* contributing to changes in cell functioning and apoptosis. Furthermore, maximum arsenic-induced ROS level was observed after 2 h of incubation although the levels declined rapidly and reached basal levels at 16 h [37]. In addition, arsenic induced lipid peroxidation in HK macrophages, being the major ROS producers [37]. Thereby, the ROS generated due to arsenic stress activates the extracellular signal-regulated kinase [35] and induce DNA damage and apoptosis in a variety of cells [72]. Strikingly, seabream HK leucocytes concomitantly enhanced all the assayed activities indicating a real activation of seabream phagocytes (macrophages

and acidophilic granulocytes) mainly at 10 days. In a similar way, human macrophages exposed to macrophage colony stimulating factor and As changed their morphology, phenotype and functions resulting in higher survival and increased activities [73]. Unfortunately, the mechanism underlying this leucocyte activation is unknown and the role of As in the immunity deserves further evaluation.

To conclude, the present results demonstrate that exposure of gilthead seabream to 5 μM inorganic arsenic produced a bioaccumulation in the liver after 30 days of exposition. Moreover, short expositions (10 days) produced an increment in the hepatosomatic index, histopathological alterations in the liver and increase of the phagocyte innate immune responses. Strikingly, when fish are really accumulating As and show hepatic-adverse effects the cellular immune response is not diminish. Further studies should elucidate the potential risks for fish health at longer exposition times and whether this could contribute to significant effects on the food chains by bioaccumulation.

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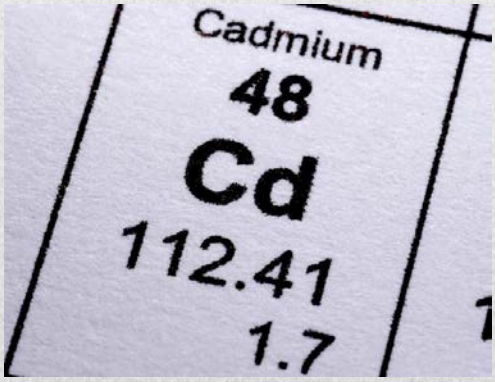
CHAPTER 2

Accumulation, histopathology and immunotoxicological effects of waterborne cadmium on gilthead seabream (*Sparus aurata*)



Guardiola FA, Cuesta A, Meseguer J, Martínez S, Martínez-Sánchez MJ, Pérez-Sirvent C, Esteban MA. Accumulation, histopathology and immunotoxicological effects of waterborne cadmium on gilthead seabream (*Sparus aurata*). Fish Shellfish Immunol 2013;1–9.

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CHAPTER 2. Accumulation, histopathology and immunotoxicological effects of waterborne cadmium on gilthead seabream (*Sparus aurata*)

ABSTRACT

Studies in fish have demonstrated that Cd-exposure produce skeletal deformities and alterations in tissue morphology, enzyme activities, stress response, ion regulation and immune response. In the present work, gilthead seabream (*Sparus aurata*) specimens were exposed to waterborne Cd (5 µM $CdCl_2$ or 1 mg L^{-1}) for 2, 10 or 30 days. Organo-somatic changes, Cd accumulation, liver histology and humoral and cellular immune responses were determined. Results showed that exposure of seabream specimens to Cd induced no alterations on spleen and liver organo-somatic indexes whilst produced progressive deleterious morphological alterations in liver and exocrine pancreas that correlated with the hepatic Cd-accumulation. Regarding the immunotoxicological potential, strikingly, Cd-exposure produced a reduction in the serum complement activity and leucocyte respiratory burst to a significant extent after 10 and 30 days whilst the serum peroxidase activity and leucocyte phagocytosis were increased at different sampling times. On the other hand, serum IgM levels and leucocyte peroxidase activity resulted unaltered. The present results seem to indicate that seabream exposed to Cd in the present conditions suffer toxicity.

1. INTRODUCTION

Marine aquaculture production is vulnerable to adverse impacts of disease and environmental conditions. Metals and metalloids (metal-like elements) are naturally present in the environment and enter aquatic systems via various geochemical processes and anthropogenic sources (including mining, metalworking and industrial processes) [1]. In general, the land–ocean inputs are the main source of the heavy metal fluxes and sea contamination [2]. It is known that the contamination chain of heavy metals almost always follows the cyclic order: industry, atmosphere, soil, water, phytoplankton, zooplankton, fish and human. Heavy metals can be accumulated by marine organisms through a variety of pathways, including respiration, adsorption and ingestion and often reach the human body by ingestion [3]. Among the metals, mercury (Hg), arsenic (As), lead (Pb) and cadmium (Cd) are classified as potentially toxic heavy metals because they are very harmful, even at low concentrations, when ingested over a long time period. Thus, effects of heavy metals on nutrition, reproductive cycle, immune status and the environmental differences influencing bioaccumulation are required [4]. At practical level, bioaccumulation and immunotoxicological negative effects are the most important for aquaculture industry since fish can get contaminated for human consumers and be more susceptible to stress and diseases.

Cadmium (Cd^{2+}) is a nonessential divalent metal ion that dissolves in water or deposits in sediment and it constitutes a contamination source for the various aquatic food chain links [5]. Even though the importance of Cd^{2+} as environmental health hazards is now widely appreciated, the specific mechanisms by which it produces its adverse effects have yet to be fully elucidated. The molecular mechanism accounting for most of the biological effects of Cd are not well-understood and the toxicity targets are largely unidentified [6]. Studies developed in mammals demonstrate that Cd accumulates and negatively affects several organs (kidney, lung, bones, placenta, brain and the central nervous system), damages to the reproductive, hepatic, haematological and immunological functions and is carcinogenic [7–9]. In fish, Cd accumulates at high concentrations in tissues [10–14] and some studies have also described alterations in their physiology including skeletal deformities, tissue morphology, enzyme activities, stress response or ion regulation [15–24]. The immunotoxicological aspects, so

important for aquaculture in certain regions, have been slightly evaluated and resulted in controversial results [25]. Whilst Cd-exposure produced a decrease in antibody levels, lysozyme and complement activity and phagocytic responses (phagocytosis and production of reactive oxygen species (ROS)) other studies have also demonstrated the opposite depending on the study, fish specie, exposure route, tissue or immune response assayed [24,26–34]. Further studies are still needed to ascertain the physiological effects, including immunotoxicology, of Cd exposure in fish and their mechanisms.

Taking into account the importance of Cd bioaccumulation in fish and the potential immunotoxic effects we performed this study in gilthead seabream (*Sparus aurata*), the major cultured fish specie in the Mediterranean area. So far, it is known that gilthead seabream exposed to Cd by water (up to 11 days) [35,36] or intraperitoneal injection (up to 6 days) [16,37–39] accumulated the heavy metal in gills, liver, blood, gut or kidney, increased liver metallothionein (MT), ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST) enzymes, hepatosomatic index and serum cortisol whilst unaffected osmoregulation functions and gene expression of heat shock protein (HSP)-70 and glutathione peroxidase (GPX) 1. Furthermore, Cd exposure down-regulated the osteocalcin gene expression and suggested its role in skeletogenesis disturbance [36]. This lack of knowledge about a long waterborne exposure to Cd and the immunotoxicological potential impact in gilthead seabream led us to carry out this work. Thus, we exposed seabream specimens to Cd at a concentration found in a highly contaminated aquatic environment (5 µM or 1 mg $CdCl_2$ L^{-1}) and evaluated the accumulation on liver and muscle, the alterations in the liver and pancreas structure and, for the first time, the impact on the innate immune responses. Additionally, potential risks for fish health and consumers will be discussed.

2. MATERIAL AND METHODS

2.1. Fish and rearing conditions

Thirty-six specimens (38.5 ± 14.15 g body weight and 13.55 ± 1.43 cm body-length) of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.), obtained from *Doramenor Acuicultura S.L.* (Murcia, Spain), were kept in seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia in recirculation systems. The water was maintained at 20 ± 2°C with a flow rate of 1,500 l h^{-1} and 28‰

salinity. The photoperiod was of 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting) at a rate of 2% body weight day^{-1}. Fish were allowed to acclimatise for 15 days before the start of the experimental trial. They were starved for 24 h prior to sampling and sacrificed by an overdose of MS222 (Sandoz, 100 mg ml^{-1} water) [40]. All experimental protocols were approved by the Bioethical Committee of the University of Murcia.

2.2. Cadmium exposure

In previous studies, the LC_{50} dose for Cd-water exposure in seabream larvae (11.6-12.8 mg body weight) was of 15.3 mg L^{-1} and the effects of 0.1 to 10 mg Cd L^{-1} have been also reported [35,36]. Fish were randomly assigned and divided into two identical tanks and remained unexposed (control group) or exposed to a sublethal dosage of cadmium chloride (1 mg $CdCl_2$ L^{-1}, 5 µM; Sigma). The exact quantity of cadmium chloride (previously dissolved in a small volume of water) was administered directly into the aquarium water. Six fish per tank and group were sampled at 2, 10 or 30 days of exposure.

2.3. Sample collection

Fish were dissected under sterile conditions and the whole fish, liver and spleen weighted. Fragments of liver and muscle were obtained and stored at -80ºC for later determination of Cd accumulation. Liver fragments were also sampled for histology. Blood samples were obtained from the caudal vein of each specimen with a 27-gauge needle and 1 ml syringe. After clotting at 4ºC, each sample was centrifuged and the serum was removed and frozen at -80ºC until use. Head-kidney (HK) was excised, cut into small fragments and transferred to 8 ml of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's osmolarity to gilthead seabream plasma osmolarity of 353.33 mOs), 2% foetal calf serum (FCS, Gibco), 100 i.u. ml^{-1} penicillin (Flow) and 100 µg ml^{-1} streptomycin (Flow)] [41]. Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 µm), washed twice (400 g, 10 min), counted and adjusted to 10^7 cells ml^{-1} in sRPMI. Cell viability was determined by the trypan blue exclusion test.

2.4. Determination of organo-somatic indexes

Whole body, spleen and liver were weighted and the organo-somatic indexes (OSI) were calculated with the following formula: OSI = (g tissue g^{-1} body) × 100.

2.5. Muscle and liver analysis of total cadmium

Frozen muscle and liver samples were lyophilized and 100-200 mg of the resulting powder were placed in Teflon vessels with 3 ml of water, 2 ml of concentrated H_2O_2 and 5 ml of concentrated HNO_3 acid solution. The digestion of the samples was carried out using a Milestone ETHOS Plus Microwave system operating with a standard program (85, 200, 210 and 0ºC during 2, 8, 10 and 20 min., respectively) (Sastre et al., 2002). Finally, 50 µl of the solutions were used to determine the cadmium concentration using Electrothermal Atomic Absorption Spectrometry (ETAAS). The accuracy of our results was also evaluated through the analysis of two reference materials (DOLT-2 Dogfish liver and DORM-2 Dogfish muscle). Data are presented as µg Cd per g dry-weight tissue.

2.6. Microscopic study

Samples of liver from all specimens (control/unexposed and cadmium exposed groups) were fixed in 10% neutral buffered formalin at room temperature for 24 h. After serial dehydration steps in alcohol, samples were embedded in paraffin. The blocks of embedded tissue were sectioned at 5 µm, and sections were routinely stained with haematoxylin-eosin (H-E) and mounted on DPX. Images were acquired with a Leica DFC280 digital camera attached to a light microscope (Leica 6000B).

2.7. Immune parameters

2.7.1. Natural haemolytic complement activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [42]. Equal volumes of SRBC suspension (6%) in phenol red-free Hank's buffer (HBSS) containing Mg^{+2} and EGTA were mixed with serially diluted serum to give final serum concentrations ranging from 10% to 0.078%. After incubation for 90 min at 22ºC, the samples were centrifuged at 400 g for

5 min at 4°C to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 µl of distilled water or HBSS to 100 µl samples of SRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting $Y\ (1\text{-}Y)^{-1}$ against the volume of serum added (ml) on a log-log scaled graph. The volume of serum producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} obtained for each experimental group.

2.7.2. Serum and leucocyte peroxidase activity

The peroxidase activity in serum or HK leucocytes was measured according to Quade and Roth [43]. Briefly, 15 µl of serum were diluted with 135 µl of HBSS without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. 50 µl of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma) and 5 mM H_2O_2 were added. To determine the leucocyte peroxidase content, 10^6 HK leucocytes in sRPMI were lysed with 0.002% cetyltrimethylammonium bromide (CTAB; Sigma) and, after centrifugation (400 × g, 10 min), 150 µl of the supernatants were transferred to a fresh 96-well plate containing 25 µl of 10 mM TMB and 5 mM H_2O_2. In both cases, the colour-change reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid and the optical density was read at 450 nm in a plate reader. Standard samples without serum or leucocytes, respectively, were used as blanks. One unit was defined as the amount producing an absorbance change of 1.

2.7.3 Serum IgM level

Total serum IgM levels were analyzed using the enzyme-linked immunosorbent assay (ELISA) [44]. Thus, 20 µl per well of 100-fold diluted serum were placed in flat-bottomed 96-well plates in triplicate and the proteins were coated by overnight incubation at 4ºC with 200 µl of carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with PBT (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA) in PBS, followed by three rinses with PBT. The plates were then incubated for 1 h with 100 µl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in

blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer). After exhaustive rinsing with PBT the plates were developed using 100 µl of a 0.42 mM TMB solution, prepared daily in a 100 mM citric acid/sodium acetate buffer, pH 5.4, containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µl of 2M H_2SO_4 and the plates were read at 450 nm. Negative controls consisted of samples without serum or without primary antibody, whose OD values were subtracted for each sample value.

2.7.4. Respiratory burst activity

The respiratory burst activity of gilthead seabream HK leucocytes was studied by a chemiluminescence method [45]. Briefly, samples of 10^6 HK leucocytes in sRPMI were placed in the wells of a flat-bottomed 96-well microtiter plate, to which 100 µl of HBSS containing 1 µg ml^{-1} phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma) was added. The plate was shaken and immediately read in a plate reader for 1 h at 2 min intervals. The kinetics of the reactions were analysed and the maximum slope of each curve was calculated. Luminescence backgrounds were calculated using reagent solutions containing luminol but not PMA.

2.7.5. Phagocytic activity

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by gilthead seabream HK leucocytes was studied by flow cytometry [46]. Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to 5×10^7 cells ml^{-1} of sRPMI. Phagocytosis samples consisted of 125 µl of labelled-yeast cells and 100 µl of HK leucocytes in sRPMI (6.25 yeast cells:1 leucocyte). Samples were mixed, centrifuged (400 g, 5 min, 22º C), resuspended and incubated at 22ºC for 30 min. At the end of the incubation time, the samples were placed on ice to stop phagocytosis and 400 µl ice-cold PBS was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 40 µl ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HK leucocytes were included in each phagocytosis assay.

All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 3,000 cells, which were acquired

at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (granularity) (SSC) and forward scatter (size) (FSC), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were made on a computerised system. The fluorescence histograms represented the relative fluorescence on a logarithmic scale. The cytometer was set to analyse the phagocytic cells, showing highest SSC and FSC values. Phagocytic ability was defined as the percentage of cells with one or more ingested yeast cells (green-FITC fluorescent cells) within the phagocytic cell population. The relative number of ingested yeasts per cell (phagocytic capacity) was assessed in arbitrary units from the mean fluorescence intensity of the phagocytic cells. The quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson).

2.8. Statistical analysis

All measurements were performed on three replicates. The results in figures are expressed as mean ± standard error (SEM). Data were statistically analysed by the t-Student test. Differences were considered statistically significant when $p \leq 0.1$ or $p \leq 0.05$.

3. RESULTS

3.1. Organo-somatic indexes are not affected by Cd-exposure

Spleen and liver organo-somatic indexes for Cd-exposed seabream specimens exhibited slight variations along the exposure time to Cd although never reached a significant extent after 2, 10 or 30 days (Fig. 1).

3.2. Cadmium is accumulated in liver and muscle

The concentrations of Cd determined in gilthead seabream muscle and liver are shown in Figure 2. The Cd concentration present in muscle samples was undetected in the case of unexposed (quantification limit, ql<0.1 µg kg^{-1}) fish. However, in Cd-exposed seabream specimens the Cd concentrations were very low but detectable in 12 out 18 sampled fish. In the case of liver, Cd was detected in all fish at higher levels than in the muscle indicating that the liver accumulates higher amounts of Cd than muscle. The Cd levels increased over the trial at all the sampling times and the concentrations in Cd-exposed were significantly much higher than in unexposed specimens. The mean

liver Cd concentration between exposed and unexposed fish was of 4.9-, 3.8- and 12.7-fold after 2, 10 and 30 days of exposure, respectively.

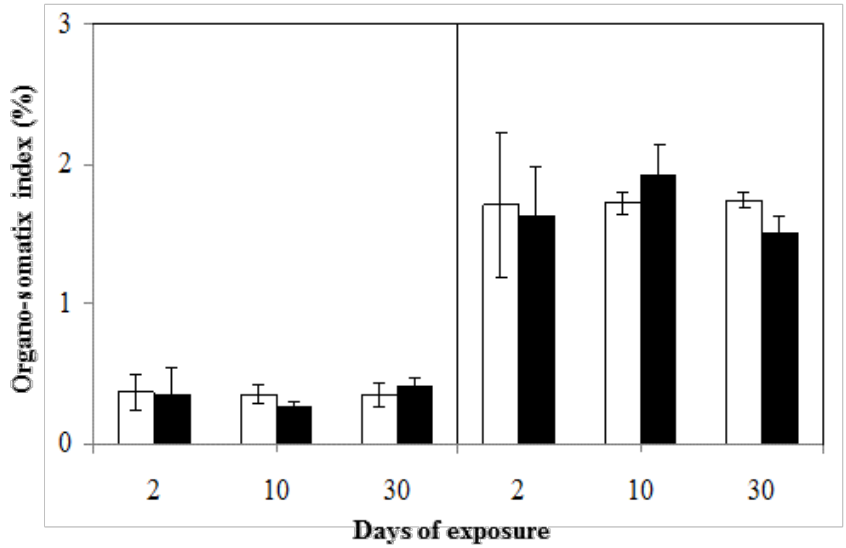

Fig. 1. Spleen and liver organo-somatic indexes (%) of gilthead seabream specimens unexposed (control; white bars) or exposed to 1 mg $CdCl_2$ L^{-1}. Bars represent the mean ± SEM (n=6).

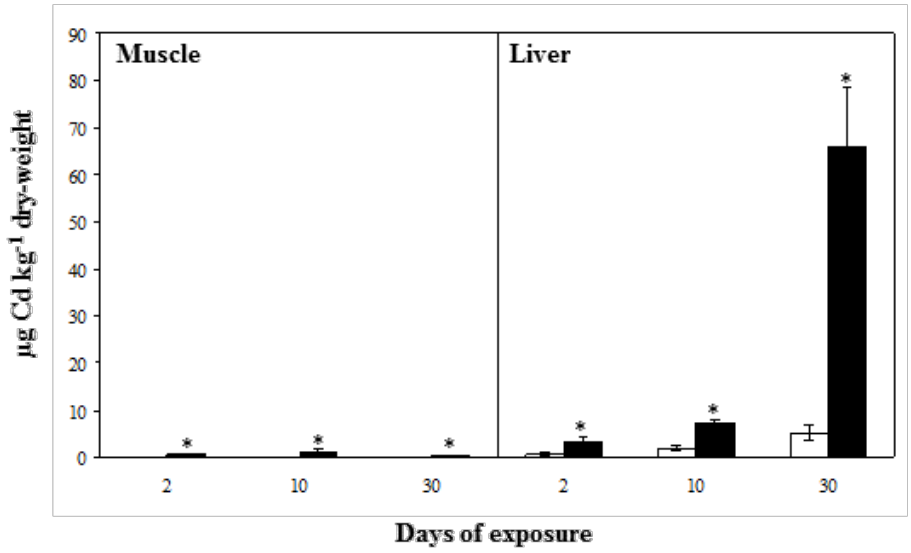

Fig. 2. Concentration of cadmiun (µg kg^{-1} dry-weight) determined in muscle and liver samples of unexposed (control) or exposed to 1 mg $CdCl_2$ L^{-1} gilthead seabream specimens. Bars represent the mean ± SEM (n=6). For each tissue, asterisk denotes significant differences ($p \leq 0.05$) with the control. ql: quantification limit (0.1 µg kg^{-1} dry-weight).

3.3. Hepatic histology is altered by Cd exposure

The liver is made of hepatocytes surrounded by sinusoids and between two neighbouring sinusoids; the hepatocytes are arranged as cords, generally two cells in thickness. Hepatocytes show a characteristic very finely vacuolated, eosinophilic cytoplasm and a very patent and round nucleus (Fig. 3A). Liver from seabream specimens exposed to Cd (Fig. 3B-D) suffered several and progressive deleterious changes that increased with the exposure time. More concretely, after 10 days (Fig. 3C) hepatocytes started to show vacuolated cytoplasm and the nuclei was displaced to the cell periphery. Finally, in liver from specimens exposed to Cd for 30 days (Fig. 3D) the typical cellular organization is lost and it was not possible to differentiate neither the cords of hepatocytes nor the sinusoids in the parenchyma. Furthermore, all the hepatocytes showed quite large vacuoles and characteristic peripheral nuclei. It is also remarkable that in liver from exposed fish, a high presence of blood cells can be usually observed in the lumen of the vessels, some of them being adhered to the endothelial cells.

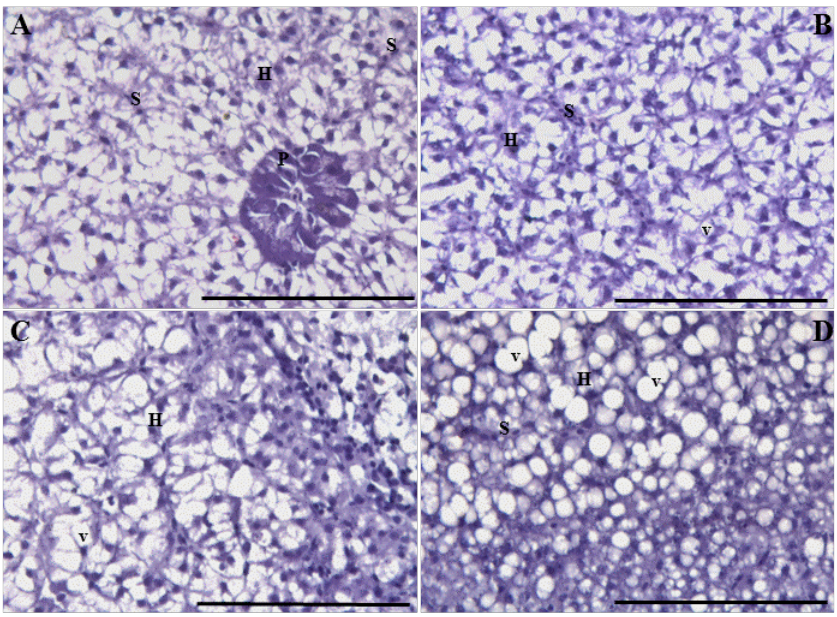

Fig. 3. Representative micrographs of liver sections from gilthead seabream specimens unexposed (control, A) or exposed to 1 mg $CdCl_2$ L^{-1} for 2 (B), 10 (C) or 30 (D) days and stained with haematoxylin-eosin. Bars: 100 µm. H, hepatocyte; S, sinusoid; P, exocrine pancreas; v, vacuole.

The pancreatic exocrine tissue appears into the liver around the portal vein, is separated from the hepatocyte cords by a thin septa of connective tissue and it is organized in acini (Fig. 3A and 4A). The exocrine pancreatic cells are characterized by the presence of secretion granules, usually located at the apical portion of the cell, and a round and basal nucleus. The exocrine pancreatic tissue of gilthead seabream was also progressively affected by Cd exposure (Fig. 4) in its structure, organization and abundance of granules (Fig. 4B-D). After 2 days of exposure the acini wall was greatly reduced and therefore, the lumen was increased (Fig. 4B). The pancreatic organization was more drastically affected at days 10 and 30 (Figs. 4C and D) and, blood cells, mainly acidophilic granulocytes, appear surrounding the pancreatic tissue.

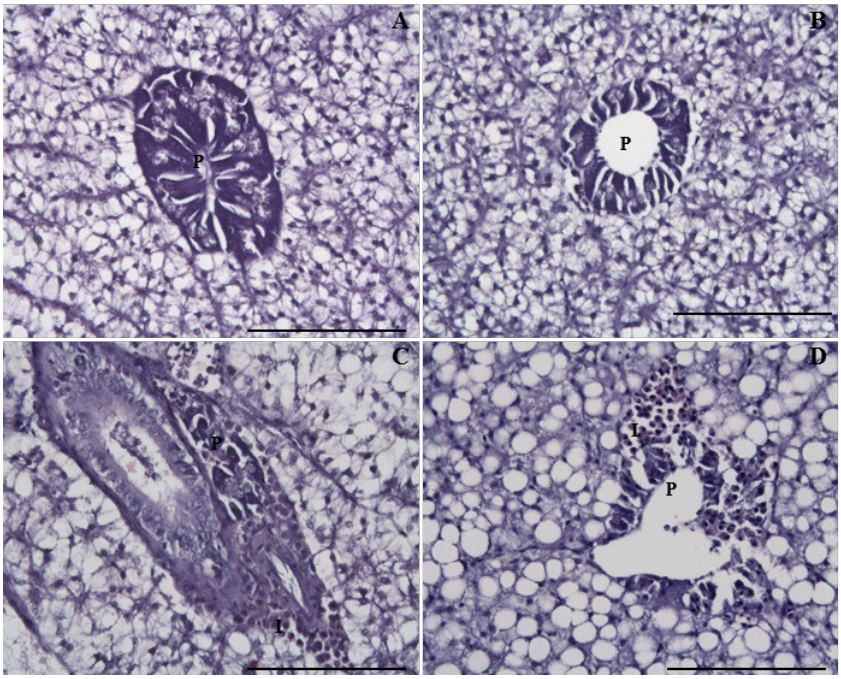

Fig. 4. Representative micrographs from the exocrine pancreas of gilthead seabream specimens unexposed (control, A) or exposed to 1 mg $CdCl_2$ L^{-1} for 2 (B), 10 (C) or 30 (D) days and stained with haematoxylin-eosin. Bars: 100 µm. P: pancreas; I, blood infiltration.

3.4. Immunotoxicological effects of Cd

Gilthead seabream humoral immunity was differently affected by Cd exposure. The haemolytic complement activity of Cd-exposed specimens was always similarly decreased but only after 10 and 30 days reached a statistically significant level ($p \leq 0.05$) (Fig. 5). By contrast, the peroxidase activity in serum was always increased upon Cd-

exposure but only after 2 and 10 days did to a significant extent (Fig. 6). Finally, the serum total IgM levels increased after 10 (p=0.09) and 30 days of exposure to Cd but filed to reach significance (Table 1).

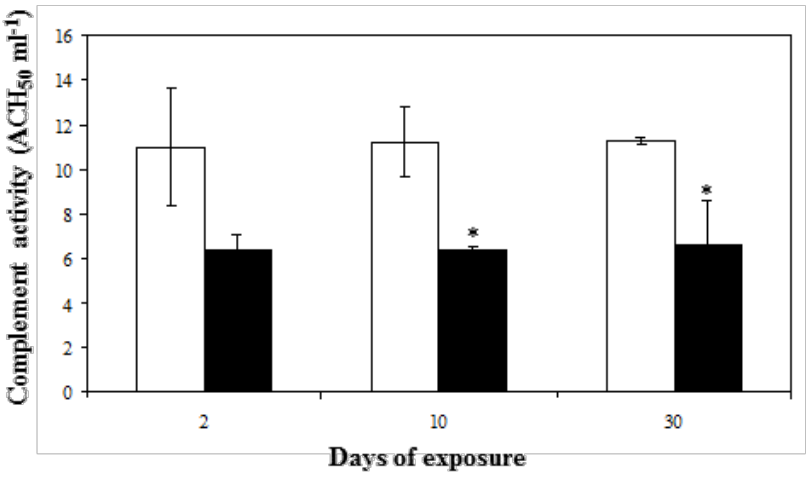

Fig. 5. Complement activity in the serum of gilthead seabream specimens unexposed (control; white bars) or exposed to 1 mg $CdCl_2$ L^{-1} (black bars). Bars represent the mean ± SEM (n=6). Asterisk denotes significant differences ($p \leq 0.05$) with the control.

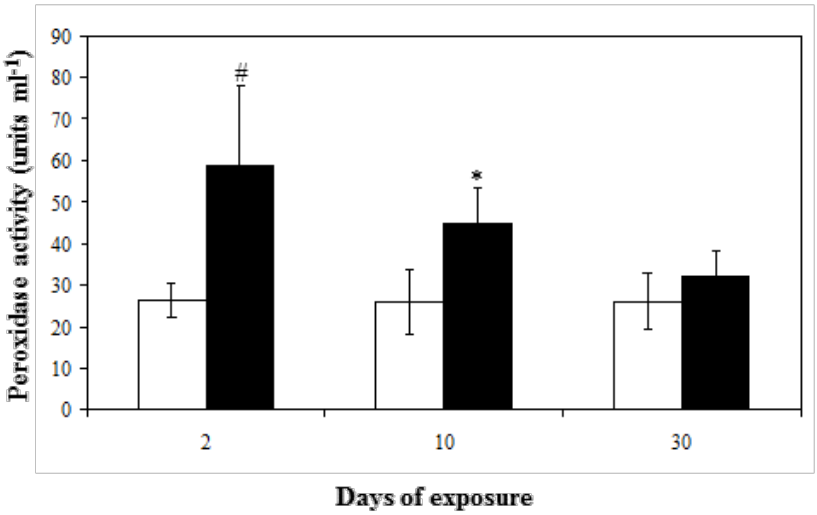

Fig. 6. Peroxidase activity in the serum of gilthead seabream specimens unexposed (control; white bars) or exposed to 1 mg $CdCl_2$ L^{-1} (black bars). Bars represent the mean ± S.E. (n=6). Symbols # and * denote significant differences ($p \leq 0.1$ and $p \leq 0.05$, respectively) with the control.

Seabream innate cellular immune parameters, determined as phagocyte-cell responses, were also differently affected by waterborne Cd. Firstly, leucocyte peroxidase activity was unaffected by the Cd exposure (Table 1). On the other hand, the respiratory burst of head-kidney leucocytes was significantly reduced after 10 ($p=0.077$) and 30 ($p=0.014$) days of exposure compared to the activity in unexposed or control fish (Fig. 7). By contrast, the percentage of phagocytic cells was unaffected (Fig. 8) whilst the phagocytic capacity was significantly increased after 30 days of Cd-exposure.

Table 1. Immune parameters of gilthead seabream specimens unexposed (control) or exposed to 1 mg $CdCl_2$ L^{-1}. Data represent the mean ± SEM (n=6).

Activities	Experimental groups	Days of exposure		
		2	10	30
Immunoglobulin M	Unexposed	0.230 ± 0.01	0.249 ± 0.02	0.248 ± 0.01
(OD 450 nm)	Cd-exposed	0.221 ± 0.02	0.298 ± 0.02	0.293 ± 0.03
Leucocyte peroxidase	Unexposed	24.5 ± 2.6	24.3 ± 1.1	24.2 ± 3.6
(units 10^{-7} cells)	Cd-exposed	25.8 ± 2.7	28.1 ± 3.9	21.3 ± 2.1

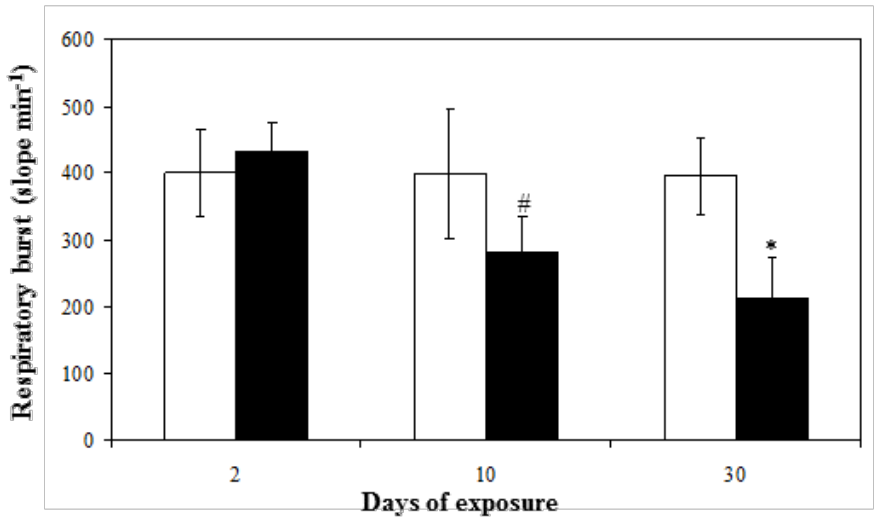

Fig. 7. Respiratory burst of head-kidney leucocytes of gilthead seabream specimens unexposed (control; white bars) or exposed to 1 mg $CdCl_2$ L^{-1} (black bars). Bars represent the mean ± SEM (n=6). Symbols # and * denote significant differences ($p\leq0.1$ and $p\leq0.05$, respectively) with the control.

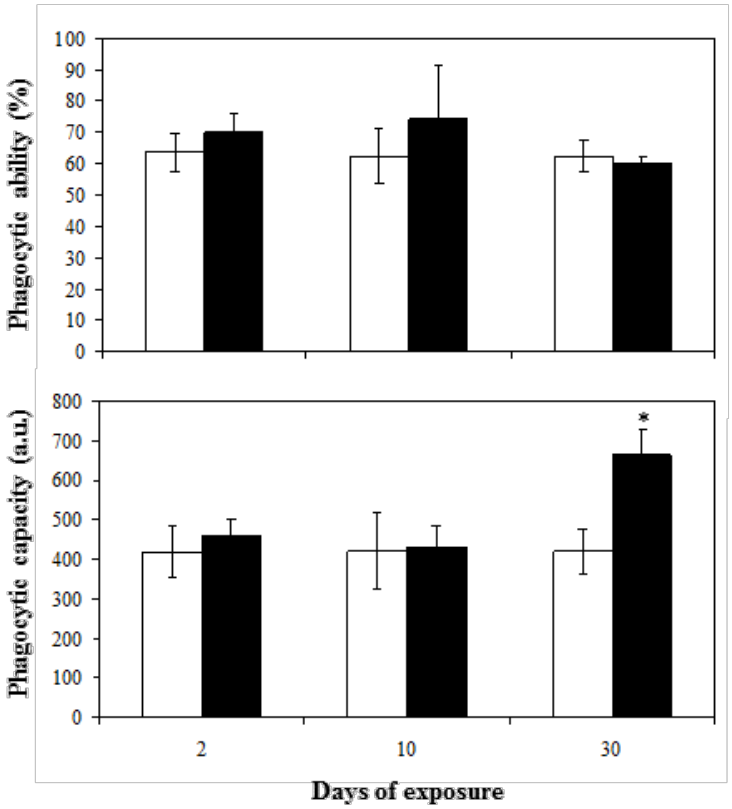

Fig. 8. Phagocytic ability (%) and capacity (arbitrary units; a.u.) of gilthead seabream specimens unexposed (control; white bars) or exposed to 1 mg $CdCl_2$ L^{-1} (black bars). Bars represent the mean ± SEM (n=6). Symbol * denotes significant differences ($p \leq 0.05$) with the control.

4. DISCUSSION AND CONCLUSIONS

Heavy metals are serious pollutants because they are stable compounds not readily removed by oxidation, precipitation or any other natural processes and they are discharged into the marine environment from different natural and anthropogenic sources (e.g. industrial and domestic swage, natural runoff and petroleum activities) and can harm both marine species and ecosystems, due to their persistence, bioaccumulation and biomagnification into the food chains [3,8,47]. Among the heavy metals, Cd may lead to contamination of soils and waters [9], reach shellfish and finfish [48–50] and could entry into the human food chain. All these considerations constitute the main reason of choosing Cd as a heavy metal for the present research while gilthead seabream was chosen as a fish model because its importance in marine aquaculture and commercial interest.

Most of the published information on organisms as bioindicators of pollution have concentrated on invertebrates, mainly molluscs and crustaceans, and less in fish [4]. It has been established that Cd occurs in the aquatic organisms and marine environment only in trace concentrations [51] and in the ocean seawater, the concentration of Cd ranged from approximately 0.01 to 42 μg L^{-1} [52]. In previous works, seabream specimens were exposed by intraperitoneal injection (200 μg to 2.5 mg Cd kg^{-1} biomass; up to 6 days) [16,37–39] or waterborne (from 0.1 to 20 mg L^{-1}; up to 11 days) [35,36]. In seabream larvae, the LC_{50} for Cd was of 15.3 mg L^{-1} [36]. Therefore, we have exposed seabream with a sublethal dose of waterborne Cd and evaluated a longer exposure time, up to 30 days. Furthermore, and for comparisons, we have chosen cadmium chloride, as in all the assays in seabream, with the knowledge that it is less toxic than cadmium nitrate and cadmium for blood cells when studied *in vitro* [53].

It is generally accepted that heavy metal uptake mainly occurs from water, food and sediment [51]. Moreover, a study carry out on three benthic fish species has demonstrated that Cd concentrations in fish organs follow a similar trend with the concentrations measured in the sediment and the water [4]. While the influence of fish age with Hg concentrations has been unambiguously shown for several fish species [54], it is still difficult to distinguish a clear tendency for Cd [54,55]. In general, Cd is highly accumulated in the gut usually followed by the gills, kidney, liver and muscle and the waterborne exposure gives higher accumulation levels than food intake [56]. Our present results are in accordance to previous data indicating that Cd concentration in seabream muscle was very low to undetectable. Moreover, these levels in muscle decrease over the time suggesting that the Cd directly entries by the skin and not from the inside by the blood route. Previous data reported Cd concentrations in cultured seabream flesh of 1.36 μg kg wet-$weight^{-1}$ and 370 μg kg^{-1} dry-weight [57,58]. In fact, these fish could still be considered adequate for human consume taken into account the maximum permitted levels of Cd into fish (0.5 μg Cd g^{-1} dry-weight; European Commission Regulation Nº 1881/2006) and the tolerable weekly intake (7 μg kg^{-1} body-weight; Food and Agriculture Organization (FAO)/World Health Organization Committee). By contrast, liver represents an important storage of metals in animals and there is a great relationship between the liver and environment concentrations of cadmium [4,56,59,60]. Thus, the liver is more often recommended as the environmental indicator tissue of pollution than other fish organs [4]. In the present study, the

accumulation of Cd in seabream livers greatly increased, up to 12-fold, with the experimental time demonstrating that Cd is bioaccumulated and can be transmitted to the next step in the food chain. It is also known that this accumulation might be affected by the diet, age, salinity, size, season, pH, temperature, etc. In previous works on seabream, liver accumulation also occurred by Cd injection (up to 160-fold) or water exposure (4 to 5-fold) [35]. Further studies are still needed to ascertain the Cd accumulation and distribution in fish tissues with special emphasis to those destined for human consume.

The present results also show that spleen and liver organo-somatic indexes of seabream specimens did not suffer any alteration as a consequence of the tested Cd concentration after 30 days of exposure. However, microscopic study of the liver demonstrated important and progressive alterations without any hepatosomatic index change as previously shown [61]. Histopathological changes in liver from Cd-exposed seabream, as well as in other fish, included loss of the typical cellular organization, alterations in hepatic cells (increased vacuolation, atrophy, necrosis, peripheral displacement of nuclei, decrease in the size of the nuclei and nucleoli, indistinguishable cell membranes), increase in connective tissue, immune-cells infiltration, formation of macrophage granulomas or congestion in blood vessels [61–64]. Furthermore, and for the first time, we also found alterations in the pancreatic exocrine tissue including loss of the typical organization, decrease of acini walls, increase in the acini lumen, and decrease of exocrine granules as well as infiltration of blood cells, mainly acidophilic granulocytes. Although Cd may injure hepatocytes directly, there are compelling reasons to believe that hepatocellular injury is *in vivo* produced as the result of ischemia caused by damage to endothelial cells. Studies over the past 20 years have shown that Cd, at relatively low, sublethal concentrations, can target vascular endothelial cells at a variety of molecular levels, including cell adhesion molecules, metal ion transporters and protein kinase signalling pathways [65]. Perhaps, additional alterations to those observed in liver also occurred in seabream kidney or other intern organs and tissues as a consequence of the Cd exposure, and the present results show some morphological evidences to suspect an influence of Cd on blood and/or endothelial cells after a careful examination of the liver sinusoids. Further focus on this aspect should be undertaken in fish.

At physiological level, some studies have evaluated the Cd impact on the immune response of fish and this is the first one in seabream, one of the most economically important cultured finfish of seawaters. Our results look contradictory but follow the general data found in the literature. At humoral level, we found no variation on the seric total IgM levels whilst the levels of specific antibodies after Cd-exposure have been found to be significantly reduced in cunners (*Tautogolabrus adspersus*) and common carp (*Cyprinus carpio*), increased or decreased in rainbow trout (*Oncorhynchus mykiss*) or unaltered in medaka (*Oryzias latipes*) [29,31,32,34,66]. Regarding the alternative complement activity, seabream specimens showed a very similar significant decrease along the exposure time which coincided with those found in the hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) [24]. This could be related to the histopathology of liver since most of the complement components are synthesized in the liver but also, at low levels, in leucocytes [67]. Therefore, is tempting to speculate that alterations in the liver are concomitant with decreased serum complement activity as in this study. However, this is not always happening. The serum peroxidase activity was, by contrast, increased after short exposure times (2 and 10 days). The peroxidase activity, related to the myeloperoxidase and/or eosinophil peroxidase activity, is released by polymorphonuclear cells into the blood in response to infections; therefore elevated serum levels of peroxidases could identify many pathological situations [46,68]. In a similar fashion, As-exposure also produced increased serum peroxidase activity in seabream [69] and suggests that peroxidase could be included as a new bioindicator of pollutants but further works should confirm this. Similar dual behavior in fish exposed to Cd has been observed showing some humoral immune activities increased together with others decreased. This needs further investigation to understand the reasons behind these interesting findings and at which extent the liver or the leucocytes account for some of the humoral activities.

At cellular level, we also found a dual immunotoxicological pattern for Cd as with other pollutants [25]. The seabream respiratory burst was impaired, the phagocytosis increased and the peroxidase activity unaffected in head-kidney leucocytes from Cd-exposed specimens. In addition, this is confirmed by the literature. For example, phagocytosis and ROS production was impaired in rainbow trout, dab (*Limanda limanda*) and European sea bass (*Dicentrarchus labrax*) [26,28,29,33,70] but enhanced in medaka [34] after *in vivo* exposure to sublethal doses of cadmium. Moreover, *in vitro*

studies also revealed that leucocyte treatment with Cd produced increased or decreased phagocyte-related functions [26,53]. Moreover, lymphocyte viability and functions seem to be more affected than phagocyte cells by Cd exposure [53]. Some studies have shown variable effects in the number of leucocytes after Cd-exposure [27,30,32,71]. In one study it has been shown a decrease in the number of neutrophils in the pronephros and, at the same time, increases in the thymus of parasitized and Cd-exposed fish indicating that they are mobilized from the kidney to the thymus [30]. Other authors have demonstrated an elevation of circulating leucocytes after Cd-exposure [27] which could also explain the increase in serum peroxidase observed in the seabream. In our study, the leucocyte mobilization has been also detected by liver infiltration of seabream acidophilic granulocytes, the functional analogous to the neutrophils. It is known that fish kidney accumulates and is affected by Cd exposure [31,35,38,39,63]. However, little attention has been paid to the haematological process in this tissue, the main for fish. Recently, common carp exposed to Cd showed increased both apoptosis and proliferation in the kidney haematopoietic tissue but the proliferation/apoptosis ratio was always higher than 1 indicating that the tissue is actively repaired and not destroyed by the Cd [72]. However, the question about why fish head-kidney leucocytes show activated and decreased phagocyte functions at the same time remains unsolved and deserves further analysis.

To conclude, our results corroborated that gilthead seabream exposed to waterborne cadmium greatly accumulates the metal in the liver. The spleen and liver organo-somatic indexes are unchanged though deleterious histopathological alterations were observed in the liver and pancreatic tissues increasing with the exposure time. Moreover, it produced bivalent effects on the immune response. Whilst the serum complement and HK leucocyte respiratory burst were significantly decreased the serum peroxidase and leucocyte phagocytic activities were increased by Cd exposure. The present results seem to indicate that seabream specimens exposed to Cd in the present conditions suffer acute toxicity and are not considered a risk for human consume.

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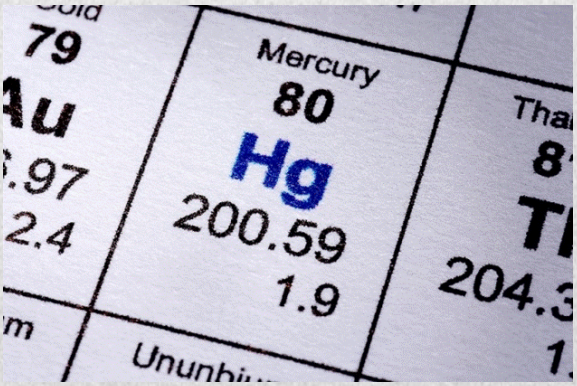
CHAPTER 3

Waterborne methylmercury produces structural damage and changes antioxidant and immune status in the gilthead seabream (*Sparus aurata* L.)



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CHAPTER 3. Waterborne methylmercury produces structural damage and changes antioxidant and immune status in the gilthead seabream (*Sparus aurata* L.)

ABSTRACT

In the aquatic systems, the organisms are continuously exposed to several chemicals. Among them, mercury is an environmental contaminant that causes acute and chronic damage to multiple organs. In fish, practically all organic mercury is in the form of methylmercury (MeHg), which has been associated with animal and human health problems. In the present study we have evaluated the effects of waterborne-exposure to sub-lethal concentrations of MeHg (10 μg L^{-1}) on the teleost fish gilthead seabream (*Sparus aurata*). Firstly, toxicological effects were confirmed because MeHg waterborne-exposed seabream specimens showed increased liver antioxidant enzymes (superoxide dismutase, catalase and glutathion reductase) after 2 days, higher hepatosomatic index after 10 days, histopathological alterations in the liver and skin as well as up-regulation of the expression of genes related to xenobiotic metabolism (CYP1A1), cellular stress (HSP-70 and HSP-90) and apoptosis (CASP-3) in the skin, but not in the head-kidney. Regarding the immune system, serum complement and peroxidase activities were increased by MeHg waterborne-exposure but only the first reached significance after 30 days of treatment. On the other hand, head-kidney leucocyte peroxidase, respiratory burst and phagocytic activities were increased though only leucocyte phagocytosis and peroxidase activity did to a significant extent after 10 and 30 days, respectively. According to our knowledge, this paper describes for the first time, the effects of waterborne MeHg exposure in the gilthead seabream immunity.

1. INTRODUCTION

Methylmercury (MeHg), the most common and toxic form of organic mercury, is an environmental contaminant produced from metallic, inorganic or organic mercury by different sulphate reducing microorganisms present in sediments [1]. Mercury is released into the environment by anthropogenic and natural activities, transformed to MeHg by anaerobic bacteria and rapidly taken up by living organisms and biomagnified through the marine food chain, including shellfish and fish [2], reaching concentrations of 10,000-100,000 times greater in fish than in the water itself [3]. Eventually, MeHg can represent a hazard to higher trophic levels and, in fact, human beings are mainly exposed to mercury by dietary fish consumption [4,5]. Therefore, much care is needed when trading wild and cultured aquatic animals for human consume.

In mammals, numerous experimental and epidemiological studies have demonstrated that exposure to MeHg is associated with neurotoxic effects as the brain is the main target organ [6–8]. Concretely, MeHg interferes with the structural design of the developing brain [9] and the temporal sequencing of cell adhesion molecules that guide neuronal migration and connections [10]. Furthermore, MeHg has been also related with altered immune system [11], kidney [12], cardiovascular system [13], gene expression profile [14], oxidative stress [15] and lipid peroxidation [7]. Similarly, in fish, practically all organic mercury is in the form of MeHg [16]. This is, as other chemical substances, absorbed across the skin or the gills enters the blood stream and reach internal organs [16] where it can be accumulated in multiple tissues and organs (e.g., muscle) [17,18]. Liver and kidney are primarily involved in its biotransformation or elimination, respectively [19]. Several studies have pointed out how MeHg dietary intake or injection affect to the fish biology. MeHg altered several functions in the fish brain including cell structural degeneration, calcium homeostasis, oxidative system, metabolic markers or visual deficits [20–23]. The toxicological effects of MeHg are also important in the reproductive organs. Thus, exposure to MeHg revealed toxic effects in gonads [24], decreased reproduction ratio [25], suppressed reproductive behaviour following maturation [26], increased apoptosis of ovarian follicular cells [27] and steroidogenic gonadal cells [27] as well as altered sperm motility and reproductive success [28]. In addition, other effects of MeHg include alterations in the mitochondrial

energy metabolism in skeletal muscle [29], inhibition of the thioredoxin system in the liver [22], delayed mortality syndrome [30], decreased larval swimming speed [31], impaired survival of larvae after maternal exposure to MeHg [32], delayed growth [33], hyperplasia in the gill epithelium as well as alterations in the gene expression profiles of several organs (including liver, gonad and muscle) [24,29,34,35]. These effects demonstrate that fish suffer important alterations after MeHg exposure.

Unfortunately, to our days few studies have explored the effects of mercury on the immune system responses of vertebrates including fish (see [36]). Thus, among the immunotoxicological effects, it has been observed that exposures to low concentrations of Hg produce immune activation while exposures to high concentrations produced the opposite. For example, *in vitro* exposure to 0.045 mg L^{-1} $HgCl_2$ (165 nM) induced lymphocyte mitosis in blue gourami (*Trichogaster trichopter*) whilst higher concentrations inhibited it [37]. However, in the case of European sea bass (*Dicentrarchus labrax*) head-kidney macrophages exposed to 2-20 µM the macrophage activation factor (MAF)-induced respiratory burst and phagocytic activities were abrogated [38]. Recently, it was documented that largemouth bass (*Micropterus salmoides*) naturally inhabiting Hg-contaminated waters suffered immunosuppression though the effects might be not exclusive to Hg [39].

More specifically, waterborne exposure to 0.05 ppb $HgCl_2$ produced inhibition of phagocytosis, respiratory burst, lymphocyte mitogenesis and immunoglobulin M (IgM) production but increased lysozyme activity in the rainbow trout (*Oncorhynchus mykiss*) [40] whilst failed to change them in the Japanese medaka (*Oryzias latipes*) [41]. Furthermore, exposition to MeHg decreased the specific antibody titers in blue gourami (waterborne, 9 ppb) [42], increased blood leucocyte and neutrophil counts in the tigerfish (*Hoplias malabaricus*) (intraperitoneal injection, 0.075 µg g^{-1}) [43], and altered the expression of immune-related genes in the Atlantic cod (*Gadus morhua*) (dietary, 2 mg kg^{-1}) [44]. Due to the importance of the immune system in the disease resistance and the pointed negative effects of MeHg in fish this relation deserves further characterization.

Taken into account that very little is known about the impact of MeHg in fish biology and concretely on fish immunity, the objective of the present study was to

investigate the effects of the sublethal waterborne-exposure to MeHg (10 μg L^{-1}) for 2, 10 or 30 days in the teleost gilthead seabream (*Sparus aurata*), with special emphasis to the innate immune activity. Furthermore, other aspects such as histopathology, liver antioxidant enzymes and detoxification metabolism were also assessed and discussed. The information will throw some light in the potential risk of MeHg contamination to cultured fish biology and in turn to human consumption.

2. MATERIAL AND METHODS

2.1. Animals

Thirty-six (121 ± 30 g weight and 20 ± 1.5 cm length) specimens of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.), obtained from the *Instituto Español de Oceanografía* (Mazarrón, Spain), were kept in re-circulating seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia. The water temperature was maintained at 20 ± 2°C with a flow rate of 900 l h^{-1} and 28‰ salinity. The photoperiod was of 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting, Spain) at a rate of 2% body weight day^{-1}. Fish were allowed to acclimatise for 15 days before the start of the experimental trial. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.2. Experimental design

Fish were randomly assigned and divided into two identical tanks, one was maintained unexposed (control group) and the other was exposed to 10 μg L^{-1} (40 nM) of methylmercury (II) chloride (CH_3HgCl, Sigma). Six fish per tank and group were sampled after 2, 10 or 30 days of exposure.

2.3. Sample collection

Specimens were weighted, the blood samples were collected from the caudal vein with an insulin syringe and the skin, head-kidney (HK), liver and spleen dissected under sterile conditions. The liver and spleen were weighted. The blood samples were left to

clot at 4°C for 4 h and later the serum was collected after centrifugation (10,000 g, 5 min, 4°C) and stored at -80 °C until use. Fragments of liver and skin were processed for light microscopy analysis. Liver fragments were also processed for evaluating antioxidant enzymes. Skin and HK fragments were stored in TRIzol Reagent (Invitrogen) at -80ºC for gene expression analysis. Other HK samples were cut into small fragments and transferred to 8 ml of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's osmolarity to seabream plasma osmolarity of 353.33 mOs), 2% foetal calf serum (FCS, Gibco), 100 i.u. ml^{-1} penicillin (Flow) and 100 µg ml^{-1} streptomycin (Flow)] for leucocyte isolation [45]. Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 µm), washed twice (400 g 10 min), counted (Z2 Coulter Particle Counter) and adjusted to 10^7 cells ml^{-1} in sRPMI. Cell viability was higher than 98%, as determined by the trypan blue exclusion test. All the cellular immune functions were performed only in viable cells.

2.4. Determination of organo-somatic indexes and condition factor

Whole body, liver and spleen were weighted and the organo-somatic index (OSI) for liver and spleen was calculated with the following formula: OSI = (g tissue g $body^{-1}$) × 100. Condition factor (K) was calculated according to the following formula: K = (g body cm $length^{-3}$) × 100.

2.5. Antioxidant enzyme assays

Superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) enzymatic activities were measured in the liver of the specimens. Liver samples were homogenized mechanically (Pellet pestles, Sigma) in potassium phosphate buffer pH 7.0 and centrifuged (10,000 g, 10 min) obtaining the supernatants which were used to determine the enzymatic activities. The SOD activity was determined by the inhibition of the reduction of cytochrome C at 550 nm [46]. The superoxide radical anion was generated *in situ* by the xanthine oxidase reaction, and detected for spectrophotometer by monitoring the formation of the reduced form of cytochrome C at 550 nm (Thermo Scientific model Evolution 300 dual beam spectrophotometer). Catalase activity was measured by monitoring the consumption of H_2O_2 at 240 nm [47]. This method is based on the principle that the absorbance will decrease due to the decomposition of

hydrogen peroxide by catalase at 240 nm (Thermo Scientific model Evolution 300 dual beam spectrophotometer). The amount of H_2O_2 converted into H_2O and O_2 in 1 min under standard conditions is accepted as the enzyme reaction rate. Glutation reductase was measured by the method modified by Carlberg and Mannervik [48]. The reaction was initiated by the addition of 0.1 mM NADPH to the mixture of enzyme in 50 mM potassium phosphate buffer pH 7.0 containing 2 mM EDTA and 0.5 mM GSSG. The change in absorbance was monitored at 340 nm for 3 min by a spectrophotometer (Thermo Scientific model Evolution 300 dual beam spectrophotometer). One unit of GR activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NADPH per minute (ε340 nm for NADPH 6.22 mM^{-1} cm^{-1}).

2.6. Light microscopy

For light microscopy analysis, liver and skin samples were fixed with 10 % neutral buffered formalin (Panreac) at room temperature for 24 h, embedded in paraffin (Thermo Scientific) and sectioned at 5 μm (Microm). Sections were dewaxed, rehydrated and stained with haematoxylin-eosin (HE). Slides were analysed by a light microscope (Leica 6000B) and images were acquired with a Leica DFC280 digital camera.

2.7. Immune parameters

2.7.1. Natural haemolytic complement activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [49]. Equal volumes of SRBC suspension (6%) in phenol red-free Hank's buffer (HBSS) containing Mg^{+2} and EGTA (ethylene glycol tetraacetic acid) were mixed with serially diluted serum to give final serum concentrations ranging from 10% to 0.078%. After incubation for 90 min at 22ºC, the samples were centrifuged at 400 g for 5 min at 4°C to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader (BMG Labtech-Fluostar galaxy). The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 μl of distilled water or HBSS to 100 μl samples of SRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by

plotting Y $(1\text{-}Y)^{-1}$ against the volume of serum added (ml) on a log-log scaled graph. The volume of serum producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} obtained for each experimental fish.

2.7.2. Serum and leucocyte peroxidase activity

The peroxidase activity in serum or leucocytes was measured according to Quade and Roth [50]. Briefly, 15 µl of serum were diluted with 135 µl of HBSS without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. 50 µl of 20 mM 3,3',5,5'- tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM H_2O_2 were added. To determine the leucocyte peroxidase content, 10^6 HK leucocytes in sRPMI were lysed with 0.002% cetyltrimethylammonium bromide (Sigma) and, after centrifugation (400 g, 10 min), 150 µl of the supernatants were transferred to a fresh 96-well plate containing 25 µl of 10 mM TMB and 5 mM H_2O_2. In both cases, the colour-change reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid and the optical density was read at 450 nm in a plate reader. Standard samples without serum or leucocytes, respectively, were used as blanks.

2.7.3. Serum IgM level

Total serum IgM levels were analyzed using the enzyme-linked immunosorbent assay (ELISA) [51]. Thus, 20 µl per well of 1/100 diluted serum were placed in flat-bottomed 96-well plates in triplicate and the proteins were coated by overnight incubation at 4ºC with 200 µl of carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with PBT (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA, Sigma) in PBT, followed by three rinses with PBT. The plates were then incubated for 1 h with 100 µl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer, Sigma). After exhaustive rinsing with PBT the plates were developed using 100 µl of a 0.42 mM TMB solution, prepared daily in a 100 mM citric acid/sodium acetate buffer, pH 5.4, containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µl of 2M H_2SO_4 and the plates were read at 450 nm. Negative controls consisted of samples

without serum or without primary antibody, whose OD values were subtracted for each sample value.

2.7.4. Respiratory burst activity

The respiratory burst activity of gilthead seabream HK leucocytes was studied by a chemiluminescence method [52]. Briefly, samples of 10^6 leucocytes in sRPMI were placed in the wells of a flat-bottomed 96-well microtiter plate, to which 100 µl of HBSS containing 1 µg ml^{-1} phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma) were added. The plate was shaken and luminescence immediately read in a plate reader (BMG labtech-Fluostar galaxy) for 1 h at 2 min intervals. The kinetics of the reactions were analysed and the maximum slope of each curve was calculated. Luminescence backgrounds were calculated using reagent solutions containing luminol but not PMA.

2.7.5. Phagocytic activity

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by gilthead seabream HK leucocytes was studied by flow cytometry [53]. Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to $5x10^7$ cells ml^{-1} of sRPMI. Phagocytosis samples consisted of 125 µl of labelled-yeast cells and 100 µl of HK leucocytes in sRPMI (6.25 yeast cells:1 leucocyte). Samples were mixed, centrifuged (400 g, 5 min, 22ºC), resuspended and incubated at 22ºC for 30 min. At the end of the incubation time, samples were placed on ice to stop phagocytosis and 400 µl ice-cold PBS was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 40 µl ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HK leucocytes were included in each phagocytosis assay.

All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 3,000 cells, which were acquired at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (granularity) (SSC) and forward scatter (size) (FSC), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were made on a computerised system. The fluorescence histograms represented the relative fluorescence on a logarithmic

scale. The cytometer was set to analyse the phagocytic cells, showing highest SSC and FSC values. Phagocytic ability was defined as the percentage of cells with one or more ingested bacteria (green-FITC fluorescent cells) within the phagocytic cell population whilst the phagocytic capacity was the mean fluorescence intensity. The quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson).

2.8. Gene expression analysis (Real-time PCR)

After 2, 10 and 30 days of MeHg exposition, total RNA was extracted from 0.5 g of seabream head-kidney and skin using TRIzol Reagent [54]. It was then quantified and the purity was assessed by spectrophotometry; the 260:280 ratios were 1.8-2.0. The RNA was then treated with DNase I (Promega) to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SuperScript III reverse transcriptase (Invitrogen) with an oligo-dT_{18} primer. The expression of selected genes (Table 1) was analysed by real-time PCR, which was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 µl of 2 x SYBR Green supermix, 5 µl of primers (0.6 µM each) and 5 µl of cDNA template) were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min at 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1α (EF1α) RNA content in each sample. The primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples.

2.9. Statistical analysis

All measurements were performed on three replicates. The results in figures are expressed as mean ± standard error (SEM). Data were statistically analysed by the t-Student test using SPSS 19 to determine differences between unexposed and methylmercury-exposed groups. Differences were considered statistically significant when $p \leq 0.05$.

Table 1. Primers used for real-time PCR

Gene name	Gene abbreviation	GenBank number	Primer sequences (5′→3′)
Elongation factor 1α	Ef1α	AF184170	CTGTCAAGGAAATCCGTCGT TGACCTGAGCGTTGAAGTTG
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	AF011223	GCATCAACGACCGCTTCAACGC CCTACAACCTTCTCATCCGACATCTGG
Heat-Shock Protein-70	HSP-70	EU805481	AATGTTCTGCGCATCATCAA GCCTCCACCAAGATCAAAGA
Heat-Shock Protein-90	HSP-90	DQ524994	GGAGCTGAACAAGACCAAGC AGGTGATCCTCCCAGTCGTT
Metallothienein-A	MTA	X97276	ACAAACTGCTCCTGCACCTC CAGCTAGTGTCGCACGTCTT
Caspase-3	CASP-3	EU722334	CTGATCTGGATGGAGGCATT AGTAGTAGCCTGGGGCTGTG

3. RESULTS

3.1. Methylmercury increased the hepato-somatic index

Hepato-somatic index of seabream specimens exposed to waterborne MeHg showed a significant increment after 10 days compared with the values obtained in unexposed fish (Fig. 1A). On the contrary, spleen-somatic index (Fig. 1A) and condition factor (K; Fig. 1B) resulted unaffected to a significant level by seabream exposure to MeHg.

3.2. Short exposure to MeHg increased the antioxidant enzyme activities

All the antioxidant enzymes studied in the liver (SOD, CAT and GR) showed increased activity after 2 days of MeHg exposition though only the SOD and CAT enzymes reached a statistically significant level compared with the values obtained in control fish (unexposed) (Fig. 2). Afterwards, very faint effects were observed throughout the trial.

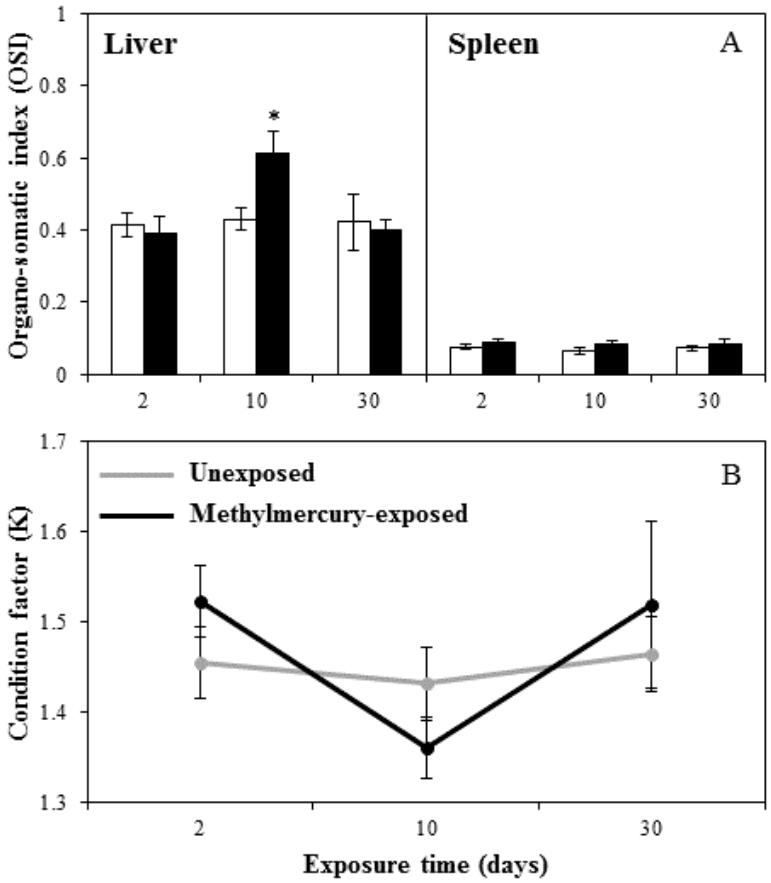

Fig. 1. (A) Spleen and liver organo-somatic index (OSI, %) of gilthead seabream specimens unexposed (control; white bars) or exposed to methylmercury (10 μg L^{-1}; black bars). (B) Condition factor (K) of gilthead seabream specimens unexposed (control; grey line) or exposed to methylmercury (10 μg L^{-1}; black line). Bars or lines represent the mean ± SE (n=6). Asterisk denotes significant differences according to a t-Student test between unexposed and methylmercury-exposed groups ($p \leq 0.05$).

3.3. MeHg produced histopathological alterations in the skin and liver

Waterborne methylmercury altered the morphology of both skin and liver being the changes observable since 2 days of exposition time although much more evident when increasing the exposure time.

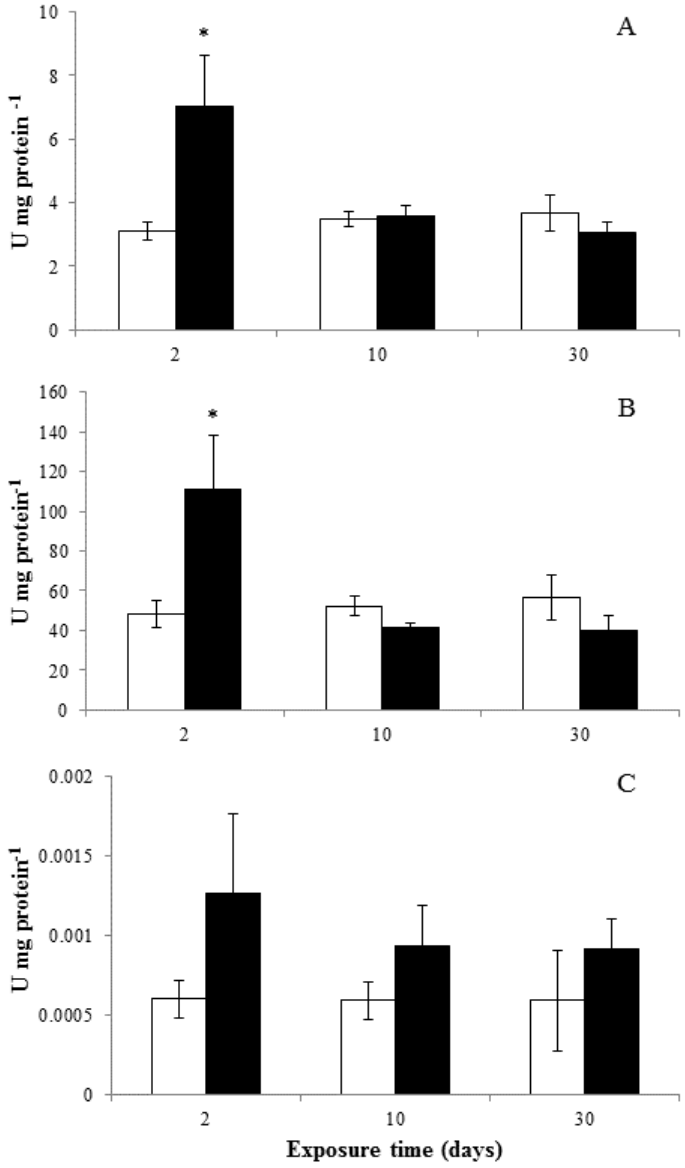

Fig. 2. (A) Superoxide dismutase (SOD), (B) catalase (CAT) and (C) glutathione reductase (GR) activity (U mg protein^{-1}) in liver of gilthead seabream specimens unexposed (control; white bars) or exposed to methylmercury (10 µg L^{-1}; black bars). Bars represent the mean ± SEM (n=6). Asterisks denote significant differences according to a t-Student test between unexposed and methylmercury-exposed groups ($p \leq 0.05$).

Gilthead seabream skin had the typical organisation of teleost skin and was composed of three well defined layers: the epidermis, dermis and hypodermis which overlaid a fat layer that varied in thickness. The non-keratinized epidermis is a scamous stratified epithelium with goblet cells while the dermis and the hypodermis are connective tissue (Fig. 3).

The epidermis (5–10 cells thick) consists entirely of live cells, of which the majority is squamous cells and the minority is mucous cells. The hypodermis consists of loosely organized collagen fibres and rich supply of vessels (Fig. 3A). The epidermis from MeHg-exposed seabream specimens was thicker and showed a more disorganized cell arrangement (more evident in the basal part of the epithelium) than the epidermis of control (unexposed) fish (Fig. 3B). In the epithelium, it is observed cell degeneration characterized by swollen epidermal cells (intracellular edema) with pyknotic (condensed) nuclei.

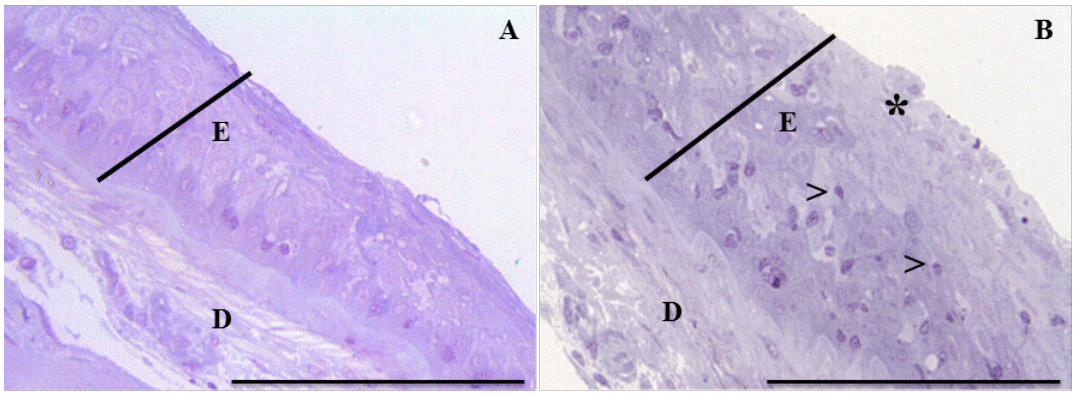

Fig. 3. Micrographs of skin sections from gilthead seabream unexposed (control) (A) and exposed for 30 days to methylmercury (10 µg L^{-1}) (B) stained with hematoxylin-eosin. E, epidermis, D, dermis. arrow picnotic nuclei. Bar = 100 µm.

Gilthead seabream hepatocytes are located between the sinusoids (which usually have circulating cells in the lumen, mainly erythrocytes) forming cord-like structures known as hepatic cell cords. Hepatocytes have a roundish polygonal cell body containing a clear spherical nucleus with usually one nucleolus (Fig. 4A). The main observed alterations in the liver were the loss of the parenchyma organization, increased hepatocyte vacuolation, displacement of the nuclei of the hepatocytes to the cell periphery and congestion of blood vesels (Fig. 4B-D). After exposition of seabream to MeHg, some progressive deleterious alterations were observed in the liver by light microscopy and consisted of progressive altered hepatocyte distribution (Fig. 4B-D). Liver from specimens exposed for 2 days showed vacuolized hepatocytes, while hypertrophied hepatocytes were also observed after 10 and 30 days of MeHg exposition (Figs. 4C and 4D).

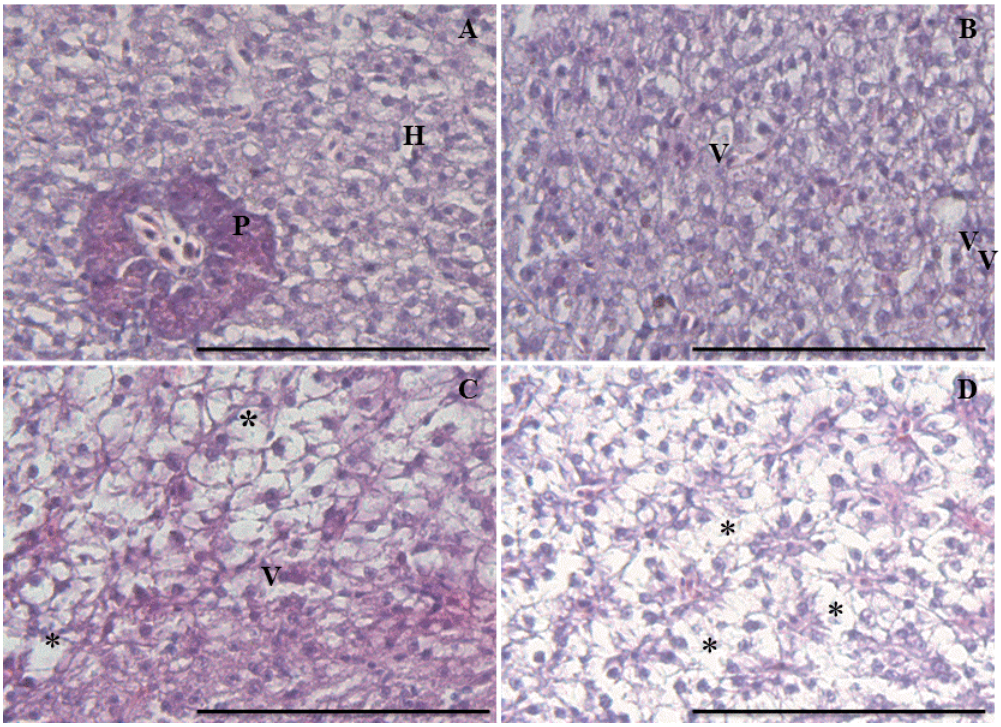

Fig. 4. Micrographs of liver sections from gilthead seabream unexposed (A) and exposed to methylmercury (10 µg L^{-1}) for 2 (B), 10 (C), and 30 (D) days stained with hematoxylin-eosin. H, hepatocyte; S sinusoid; *, focal necrosis; arrow, hypertrophic hepatocytes; Fd, fatty degeneration; V, vacuolization. Bar = 100 µm.

3.4. Waterborne methylmercury induced the immune response

Gilthead seabream humoral immune parameters were differently affected by MeHg exposure (Table 2). The haemolytic complement activity of specimens exposed to MeHg was always more elevated than the values obtained from control (unexposed fish). However, the observed increments were only statistically significant after 30 days of exposure. Seric peroxidase activity was always increased in specimens exposed to MeHg but the increments were not statistically significant for any tested time compared to the values from control fish. Finally, the seric total IgM level was increased and reduced in seabream specimens after 2 and 10 and 30 days of exposition to MeHg, respectively, respect to the levels obtained in the serum from unexposed fish (control group), although the detected variations were no statistically significant.

Table 2. Humoral immune parameters in the serum of gilthead seabream specimens unexposed (control) or after exposure to methylmercury (10 µg L-1). Data represent the mean ± SEM (n=6). Asterisk denotes significant differences according to a t-Student test between unexposed and methylmercury-exposed groups (P≤0.05).

Activities	Experimental groups	Days of exposition		
		2	10	30
Natural haemolytic complement activity (ACH_{50} units ml^{-1})	Control	6.09 ± 1.26	5.88 ± 0.07	6.18 ± 0.49
	10 µg L^{-1}	8.59 ± 1.79	9.11 ± 2.34	17.06 ± 1.98*
Peroxidase activity (units ml^{-1})	Control	16.81 ± 5.38	17.41 ± 1.18	17.92 ± 1.09
	10 µg L^{-1}	16.78 ± 5.98	21.62 ± 1.66	21.50 ± 2.25
Immunoglobulin M (OD 450 nm)	Control	0.142 ± 0.012	0.154 ± 0.018	0.145 ± 0.011
	10 µg L^{-1}	0.161 ± 0.011	0.163 ± 0.017	0.113 ± 0.016

On the other hand, innate cellular immune parameters of gilthead seabream specimens were generally increased after MeHg exposure. Firstly, leucocyte peroxidase activity showed an increase at all sampling times, being statistically significant at 30 days of exposition respect to the activity found in leucocytes isolated from unexposed (control) fish (Fig. 5). Similarly, the respiratory burst of head-kidney leucocytes showed an increase throughout of trial but any statistically significant difference was observed (Fig. 6). In the same way, the percentage of phagocytic cells (Fig. 7A) and the phagocytic capacity (Fig. 7B) of leukocytes isolated from HK were increased after 10 days of MeHg exposure. After 2 and 30 days of exposition time, both phagocytic parameters resulted unaffected to a significant level as a consequence to the exposition to MeHg.

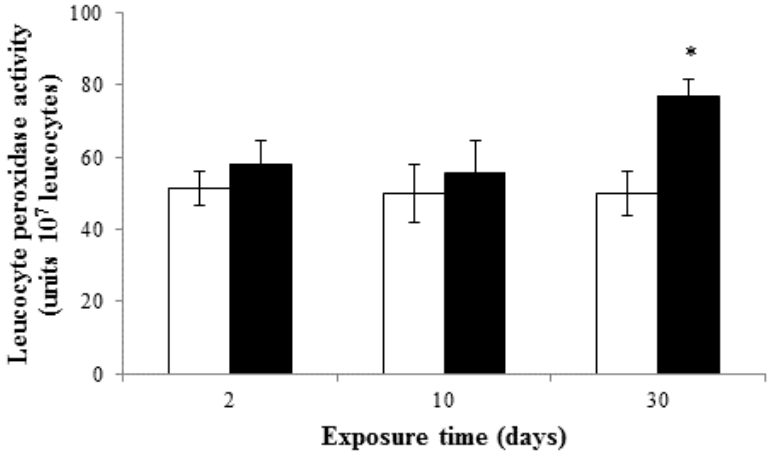

Fig. 5. Peroxidase activity (units 10^{-7} leucocytes) of head-kidney leucocytes from gilthead seabream specimens unexposed (control; white bars) or exposed to methylmercury (10 μg L^{-1}; black bars). Bars represent the mean ± SEM (n=6). Asterisk denotes significant differences according to a t-Student test between unexposed and methylmercury-exposed groups ($p \leq 0.05$).

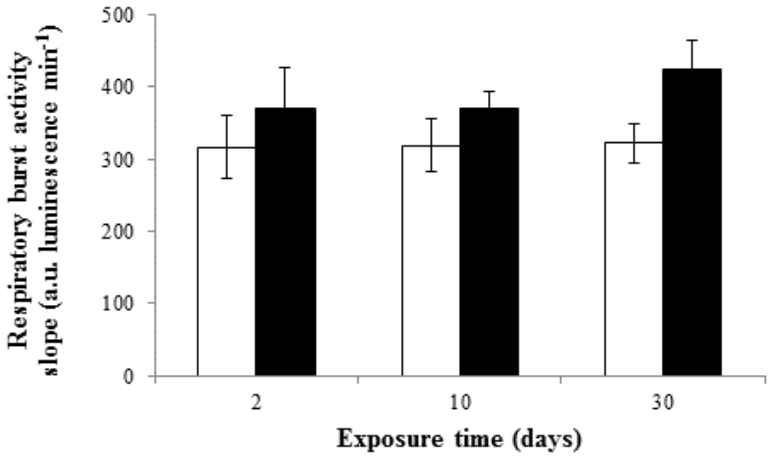

Fig. 6. Respiratory burst activity (slope min^{-1}) of head-kidney leucocytes from gilthead seabream specimens unexposed (control; white bars) or exposed to methylmercury (10 μg L^{-1}; black bars). Bars represent the mean ± SEM (n=6).

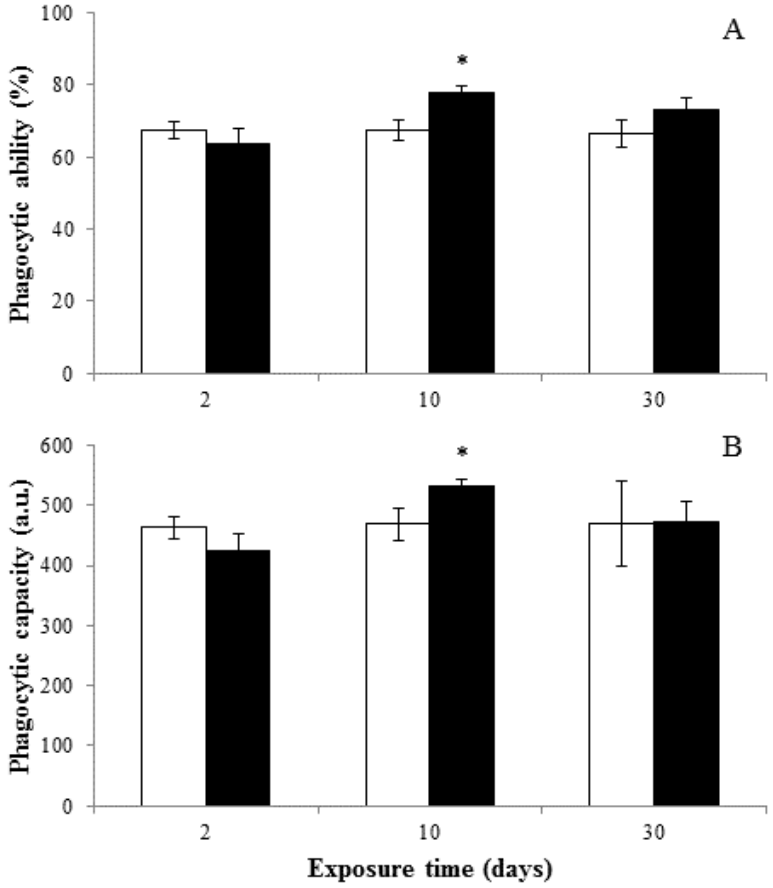

Fig. 7. (A) Phagocytic ability (%) and (B) capacity (a.u.) of head-kidney leucocytes from gilthead seabream specimens unexposed (control; white bars) or exposed to methylmercury (10 μg L^{-1}; black bars). Bars represent the mean ± SEM (n=6). Asterisks denote significant differences according to a t-Student test between unexposed and methylmercury-exposed groups ($p \leq 0.05$).

3.5. Methylmercury greatly altered the gene expression in the skin but not in the head-kidney

The expression of genes related to xenobiotic metabolism, CYP1A1 (cytochrome P450), cellular stress, HSP-70 (heat-shock protein 70) and HSP-90 (heat-shock protein 70), uptake, transport and regulation of metals, MTA (metallothionein-A) and apoptosis cell-death, CASP-3 (caspase-3) in the HK of seabream specimens exposed to MeHg did not suffer any statistically significant variations compared to the control group (unexposed) (Fig. 8A). By contrast, in the skin there was an up-regulation statistically significant of CYP1A1 after 2 and 30 days, HSP-90 gene after 10 days and HSP-70 and

CASP-3 genes after 30 days of exposure compared with the expression obtained from genes of unexposed fish (Fig. 8B).

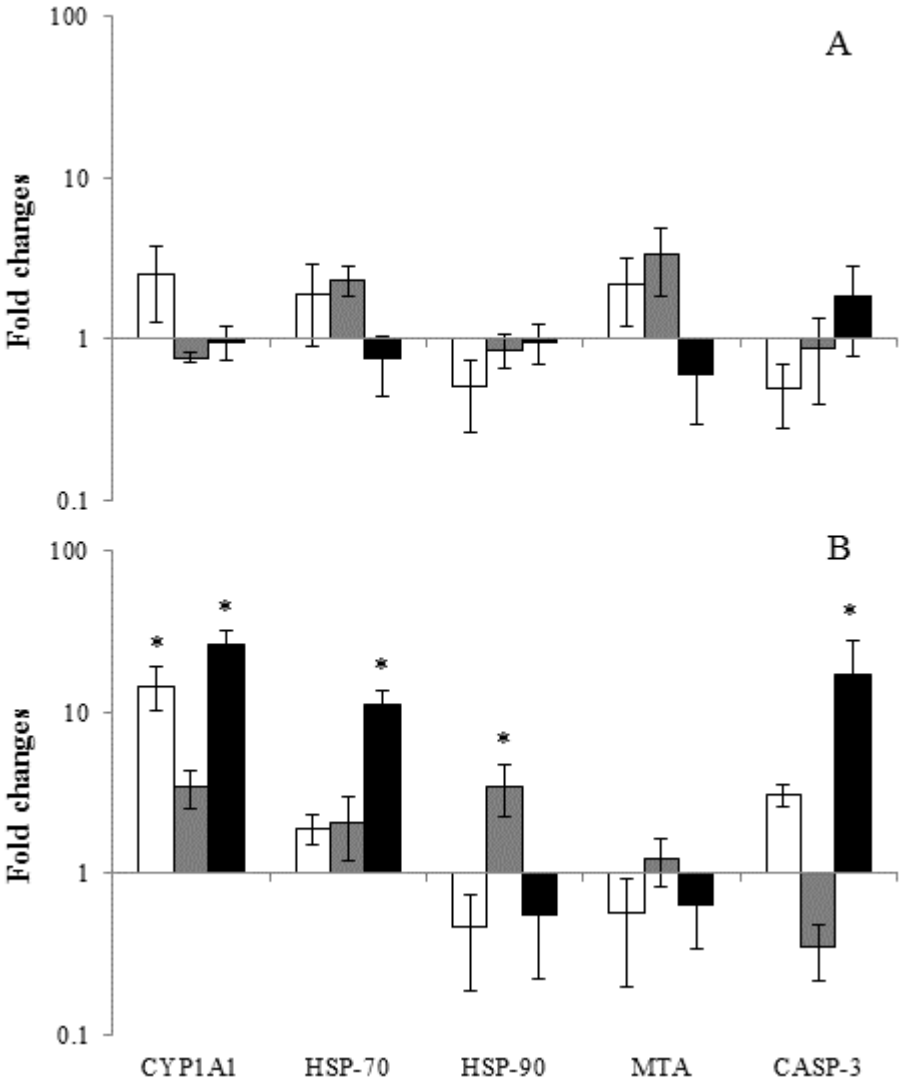

Fig. 8. Relative gene expression, determined by real-time PCR, in head-kidney (A) and skin (B) of gilthead seabream specimens after 2 (white bars), 10 (grey bars) and 30 (black bars) days of exposure to methylmercury (10 μg L^{-1}). Bars represent the mean ± SEM (n=6) fold increase relative to control. Asterisks denote significant differences according to a t-Student test between unexposed and methylmercury-exposed groups ($p \leq 0.05$).

4. DISCUSSION AND CONCLUSIONS

Actually, the fish populations in the aquatic environment are inevitably exposed to some pollutants. Although most hazardous substances are present at concentrations away below the lethal level, they may still cause serious damage to the life processes of these animals [16]. Mercury is highly neurotoxic and represents a serious risk because easily accumulates along the aquatic food chain with a biomagnification ratio in the order of 10,000–100,000 [3,55]. Mercury concentrations in the Mediterranen area are quite variable but reported levels include 0.05-2.87 µg/L in waters, < 40 µg kg^{-1} d.w. in sediments and around 70-100 µg kg^{-1} in seabream muscle fillets [56–58]. Levels of <0.43 pM MeHg have been reported in Mediterranean water [59]. Moreover, Hg represents a serious hazard for humans as the main input of Hg comes from Hg-contaminated fish [4,5]. From a toxicological standpoint, it is important to distinguish between inorganic and organic forms (as MeHg), because of differences in absorption, bodily distribution, and toxic effects [16]. Fish tissues are very sensitive indicators of aquatic pollution because they have a high Hg bioaccumulation capacity for both inorganic and organic forms [60]. In contrast to inorganic Hg, MeHg easily crosses biological membranes and is able to accumulate in the skeletal muscle where the detected concentrations could be similar to those usually observed in other organs such as liver, kidneys or brain [19,34,61,62]. Therefore, Hg represents one of the most serious aquatic pollutants for fish which can be transferred to humans.

In the present study, gilthead seabream specimens, the most important cultured fish specie in the Mediterranean area, were exposed to a sublethal concentration of waterborne MeHg (10 µg L^{-1}, equivalent to 40 nM or 10 ppb) in order to better understand the effects of this organometallic compound on fish innate immunology (e.g. complement, phagocytosis, respiratory burst and peroxidase activities). Concomitantly, other aspects related to toxicology (histopathology, liver antioxidant enzymes or detoxification metabolism) were also analysed to confirm toxicity. In several studies carried out in fish, acute toxicity effects of MeHg were present at very diverse concentrations in waterborne conditions but always the toxicity was higher than when using $HgCl_2$ form. Thus, rainbow trout specimens were exposed from 4 to 34 µg MeHg

L^{-1} resulting in fish death after >100 and 2 days, respectively [63]. Other studies used MeHg concentrations of 5-15 µg L^{-1} in zebrafish (*Danio rerio*) embryos [30] or 9 ppb in blue gourami [42]. Thus, MeHg level used herein is similar to those previously used and much higher than the reported in the Mediterranean waters of <0.43 pM [59].

Fish growth and organ-somatic indexes are valuable as simple and useful biomarkers of toxicity in fish [64]. Thus, seabream specimens exposed to waterborne MeHg showed increased hepato-somatic index after 10 days whilst the spleen-somatic index remained unaltered. These results agree with our findings in gilthead seabream exposed to waterborne As or Cd [65,66]. Concomitantly, the condition factor was unaffected by waterborne MeHg as also described after dietary intake in Atlantic salmon (*Salmo salar*) [23], Sacramento blackfish (*Orthodon microlepidotus)* [33] or trahira (*Hoplias malabaricus*) [55]. All these data together suggest changes in other tissues apart form the brain. Thus, it has been observed that MeHg can also induce a number of lesions and injuries in different fish organs such as liver, kidney and gills [43]. In our work, we have found histopathological changes in the liver of seabream exposed to waterborne MeHg that drastically increased with the prolongation of the exposure time. Similarly, De Oliveira Ribeiro et al. [67] documented important changes in the liver of arctic charr (*Salvelinus alpinus*) after MeHg dietary exposure while changes were not produced when it was administered by waterborne. Other studies using the dietary intake of MeHg have found some histological changes in the target tissue, the gut [55]. However, to our knowledge, no study using the waterborne exposure has looked at the skin, the first tissue contacting with the Hg compounds. We have found thickness of the epidermis of seabream specimens exposed to MeHg and a more disorganized cell arrangement (more evident in the basal part of the epithelium) than the epidermis of control (unexposed) fish. The morphological alterations observed in skin from MeHg exposed specimens correlated to epidermal degeneration characterized by swollen epidermal cells (intracellular edema) with pyknotic (condensed) nuclei. These alterations, not only affect the skin, an important organ for homeostasis maintenance, but also fish immune status because the skin is one of the main first line of defence and a very important portal of entry for many microorganisms. Furthermore, the fish skin has an important role in the protection against invasive pathogens. Further studies are needed to establish the real implications of this important organ in fish toxicology.

Another important determination to evaluate toxicity is the gene expression profile. In this sense, very little is known in the case of Hg toxicity in fish. Interestingly, we found that MeHg unaltered the expression of genes related to the toxic metabolism (CYP1A1 or MTA), cell stress (HSP-70 and HSP-90) or cell death (CASP-3) in the head-kidney whilst most of them were up-regulated in the skin, correlating with the histopathological changes observed in this tissue. Strikingly, cytochrome-P450 (CYP1A1) and associated enzymes are used as biomarker of xenobiotics and are also altered upon heavy metals exposure in fish [68,69]. In gilthead seabream, CYP1A1 enzyme has been detected in epidermal skin cells (Sarasquete et al., 1999), however, this is the first study in which an up-regulation of the CYP1A1 gene expression after MeHg waterborne exposure has been reported in the skin. On the contrary, the expression of the MTA gene, which is supposed to be a very good biomarker of heavy metal toxicity, was unchanged in this study. Interestingly, and although MT, at either gene or protein levels, has been found to be affected in seabream after heavy metal exposure [70,71] or in other fish after Hg exposure [72] our data support the hypothesis that metallothioneins protect cells from inorganic Hg but not for MeHg [73], and therefore we could consider that the induction of MTA gene as a biomarker of MeHg exposure may not be good enough. Our results demonstrated higher induction of this gene in head kidney than in the skin after 2 and 10 days of MeHg exposure. The expression of cellular stress (HSP-70, HSP-90) and cell death (CASP-3) genes was up-regulated in the skin after MeHg exposure indicating that this tissue is being damaged as evidenced by the histopathological study. Whether the lack of protective MTA induction is responsible for the increased cellular stress and death in the skin deserves further analysis. In this sense, González et al. [34] revealed no direct relationship between Hg bioaccumulation levels and genetic responses in the liver, skeletal muscle and brain of zebrafish. Similarly, a microarray realized by Liu et al. [74] in MeHg-diet exposed juvenile zebrafish and rainbow trout failed to provide sufficient evidences for good teleost biomarkers for MeHg exposure, suggesting that other biomarkers will be required. Our results may suggest that CYP1A1 in the skin could be a good biomarker for MeHg-exposure, although further studies should confirm this assumption.

Regarding the oxidative stress response, SOD, CAT, GR, GPX (glutathione peroxidase), GST (glutathione S-transferase) and thioredoxins are major antioxidant enzymes involved in the protection against the deleterious effects of free radicals [75].

There are evidences showing that MeHg toxicity produces oxidative stress by both increasing the production of reactive oxygen species (ROS) [76–78] and reducing the cellular antioxidant defenses [79,80]. Therefore, it is known that inorganic Hg induced oxidative stress in fish, causing alterations in the activity of antioxidant enzymes in several tissues [15,20,22,23,55,72]. In general, the antioxidant defense mechanisms acting in liver are stronger in comparison with other tissues such as kidney and brain. Therefore, in our study, the antioxidant enzymes SOD, CAT and GR in seabream liver were evaluated. SOD and CAT activities were significantly increased after 2 days of exposure to MeHg but returned to levels of unexposed fish afterwards. However, liver GR activity was always increased though did not reached significant levels. This suggests that ROS are generated after short exposure times which can be removed by increased SOD and CAT enzymes. However, they cannot eliminate the ROS for longer times since there is no activation of SOD and CAT enzymes and then the liver show histopathological changes and an increase in the hepato-somatic index is also observed. Oxidative stress elicited by MeHg is also related to its interaction with glutathione and thioredoxin antioxidant systems, which can also acts as a possible target for MeHg neurotoxicity [22,81,82]. Nonetheless, similarly to our study, in the liver of zebra-seabreams exposed to waterborne MeHg (2 μg L^{-1}), the activity of GR increased after 28 days whilst the GPx activity was inhibited during all trial [82]. In trahira fish (*Hoplias malabaricus*) feed with MeHg there was a decrease in the liver of glutathione concentration, CAT, GST and GPx but increased SOD activity suggesting an increase in ROS and tissue damage [55]. Interestingly, Atlantic salmon SOD and GSH-Px increased in the liver and kidney, whereas decreased in the brain, after dietary MeHg for 4 months [23]. They suggested that this indicates an adaptive response of the redox-defence system in liver and kidney, as opposed to a general break-down of the redox defence system in the brain. In summary, GR increase was an important indication of liver defence mechanisms though some enzymatic systems such as SOD and CAT continued being partially impaired by MeHg after 10 days of exposure. Furthermore, reduction in SOD and CAT due to MeHg exposure could contribute to increase their toxicity, since these molecules also participate in other processes apart from redox regulation [55]. Nevertheless, the observed differences could respond to many factors such as the fish species, tissue, MeHg route, dosage and exposure times.

To date, there are very few studies about the effect of MeHg on fish immune system. We found that MeHg increased the humoral parameters evaluated herein. In other studies Hg-exposed fish showed increased lysozyme activity in rainbow trout [40] but unaffected in Japanese medaka [41]. Most complement components are synthesized in the liver but leucocytes also take part in their production at lower scale [83]. Since the liver damage partly coincides with slight increases of the serum complement and peroxidase activities this could suggest that hepatocytes, before being completely damaged, increase the production of important proteins involved in general homeostasis or defence under certain cell-stress situations. Other hypothesis could be that leucocytes could now been the main source of such proteins and that they have been activated to produce them. This pattern was also observed in seabream exposed to waterborne As [65] but opposite in Cd or deltamethrin [66,84]. The peroxidase activity, related to the myeloperoxidase and/or eosinophil peroxidase activity, could be assumed to be a good marker for leucocyte activation [53]. As MeHg did in the present study, seabream serum peroxidase was also increased by Cd and deltamethrin [66,84] but not by As [65]. Although all this data point to peroxidase activity as a good toxicity biomarker, further studies including more fish species will be needed to clearly confirm this issue. In the case of IgM, produced by B lymphocytes, it was unaffected in seabream specimens whilst inhibited by in rainbow trout (mercury chloride) and blue gurami (MeHg) [40,42], This could be due to the decrease in circulating B-lymphocytes or its IgM-production, both of which have been observed after Hg-exposure [40,42,43].

Regarding the cellular immune response, head-kidney, the main hematopoietic tissue in fish, is also a target tissue for Hg accumulation [19] suggesting that leucocyte functions might be negatively affected. Strikingly, MeHg exposure induced the phagocytosis and peroxidase activity of head-kidney leucocytes at different times as also happened in seabream exposed to other heavy metal such as Cd or As [65,66]. These data indicate that the head-kidney is not significantly altered as suggested by the lack of up-regulation of cellular stress (HSP-70 and -90) and death (CASP-3) in this organ. However, while some authors have documented that naturally or dietary Hg-exposed fish showed immunosuppression, determined as reduced numbers of head-kidney macrophages and inflammatory cells and altered expression of genes related to the inflammatory response [5,39] others have shown that waterborne Hg accumulates in the kidney and greatly alters the morphology of renal tubules [55,85] but not the

hematopoietic tissue. This could be happening in the seabream HK since leucocyte activities were increased and the gene expression was not significantly altered. Overall, data indicate that sublethal and short exposure to MeHg induced changes in genes related to immune functions.

To conclude, the present results demonstrate that gilthead seabream exposed to waterborne MeHg (10 μg L^{-1}) produced histopathological alterations in liver and skin as well as increments in the hepatosomatic index, antioxidant enzymes and innate immune parameters. Moreover, the expression of genes related to toxic metabolism, cellular stress and apoptosis were up-regulated in skin of gilthead seabream exposed to waterborne methylmercury but not in head-kidney. Overall, our data demonstrate that waterborne exposure to sublethal MeHg produce acute toxicological effects and increased immune parameters in gilthead seabream.

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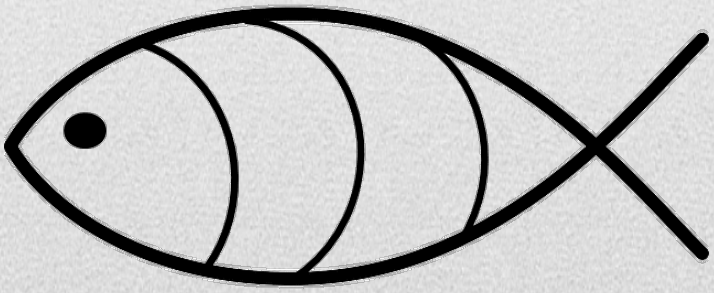
CHAPTER 4

Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*Sparus aurata*)



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CHAPTER 4. Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*Sparus aurata*)

ABSTRACT

Mucosal surfaces of fish, including skin, gill and gut, contain numerous immune substances poorly studied that act as the first line of defence against a broad spectrum of pathogens. This study aimed to identify and characterize for the first time different constitutive humoral defence mechanisms of the skin mucus of gilthead seabream (*Sparus aurata*). To do this, the levels of total immunoglobulin M, several enzymes and proteins (peroxidase, lysozyme, alkaline phosphatase, esterases, proteases and antiproteases), as well as the bactericidal activity against opportunist fish pathogens (*Vibrio harveyi, V. angillarum, Photobacterium damselae*) and non-pathogenic bacteria (*Escherichia coli, Bacillus subtilis*) were measured in the skin mucus and compared with those found in the serum. This study demonstrates that gilthead seabream skin mucus contains lower levels of IgM, similar levels of lysozyme, alkaline phosphatase and proteases, and higher esterase, peroxidase and antiprotease activities than serum. In addition, skin mucus revealed stronger bactericidal activity against tested fish pathogen bacteria compared to the serum activity, while human bacteria can even grow more in the presence of mucus. The results could be useful for better understanding the role of the skin mucus as a key component of the innate immune system with potential application for the aquaculture.

1. INTRODUCTION

The innate immune system of fish is divided into physical barriers, cellular and humoral components although there are some important differences when comparing with other vertebrates [1]. Mucosal immunity has focused our attention in the last years but most of the information relies on the study of the MALT (mucosa-associated lymphoid tissue), and specially the GALT (gut-associated lymphoid tissue) [2]. The fish MALT (present in skin, gill and gastrointestinal tract) constitutes a very large area for the possible microbial invasion [3] and contains defence mechanisms (both innate and adaptive) that constitute the first line of defence against a broad spectrum of pathogens present in the aquatic environment [2,4–8]. Since most of the infectious agents affects or initiates the process of infection in the mucous surfaces, the mucosal immune response plays a crucial role in the course of the infection [9] and different studies have begun to examine their cellular and molecular composition [10–13].

Fish skin serves as the first line of defense against a wide variety of chemical, physical and biological stressors. Secretion of mucus is among the most prominent characteristics of fish skin and this mucus plays a critical role in the animal defence acting as a natural, semipermeable, dynamic, physical, chemical, and biological barrier [14,15]. The external mucous gel forms a layer of adherent mucus covering the living epithelial cells [16], and it is secreted by epidermal goblet cells [17]. The main components of skin mucus are water and glycoproteins [18] that contain high molecular-weight oligosaccharides, called mucins [19–22]. In fact, these mucins exert a mechanical barrier by serving as filters for pathogens and preventing pathogen adherence to the underlying tissues [23]. Moreover, the skin mucus serves as a biological barrier since its continuous production of numerous substances involved in the immune response [23,24]. Till present, there is a limited knowledge about the defence mechanisms of the epidermal mucus of fishes, although both constitutive and inducible innate defence mechanisms are found [25]. Immune molecules in fish mucus include lysozyme, immunoglobulins, complement, lectins, agglutinin, calmodulin, interferon, C-reactive protein, proteolytic enzymes, antimicrobial peptides, or vitellogenin [23,26–29]. Unfortunately, the complete repertoire of immune factors present in the skin mucus and their precise role on fish immunology and defence is

poorly understood [30] and it is restricted to a few fish species, mainly freshwater. Moreover, mucus composition and immune responses varies with the fish species and with changes in the environment and its physiology. Taking into account these previous considerations, the aim of the present study was to investigate the constitutive humoral immune repertoire functioning at the skin mucus as a key component of the innate immune system of gilthead seabream (*Sparus aurata* L.), which is a species with the largest production in Mediterranean aquaculture. These activities in skin mucus will be compared with those present in seabream serum. For the first time, this study evaluate the peroxidase activity in skin mucus of a fish, revealing significant difference in this activity between skin mucus and serum samples of gilthead seabream, which may suggest that this enzyme has an important role in the mucosal defense. Knowledge of the constitutive and induced immune factors will aid to understand the mucosal immune responses in fish.

2. MATERIAL AND METHODS

2.1. Fish care and maintenance

Thirty adult specimens (125 ± 25 g body weight) of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.) obtained from the *Instituto Español de Oceanografía* (Mazarrón, Spain), were kept in seawater aquaria (250 L) in the Marine Fish Facilities at the University of Murcia. The water was maintained at 20 ± 2°C with a flow rate of 900 l h^{-1} and 28‰ salinity. The photoperiod was of 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting) at a rate of 2% body weight day^{-1}. Fish were allowed to acclimatise for 15 days before sampling. All experimental protocols were approved by the Bioethical Committee of the University of Murcia.

2.2. Skin mucus and serum collection

Fish were anesthetized prior to sampling with 100 mg l^{-1} MS222 (Sandoz). Skin mucus samples were collected from naïve specimens using the method of Palaksha et al. [10] with some modifications. Briefly, skin mucus were collected by gentle scraping the dorso-lateral surface of naïve seabream specimens using a cell scraper with enough care to avoid contamination with blood and urino-genital and intestinal excretions. In order

to get sufficient mucus, equal samples of mucus were pooled (3 pools of 10 fish) and homogenized with 1 volumes of Tris-buffered saline (TBS, 50 mM Tris–HCl, pH 8.0, 150 mM NaCl). The homogenate was vigorously shaken and centrifuged at 1,500 rpm for 10 min at 4ºC being the supernatant lyophilized (Christ) following freezing at -80ºC. Lyophilized skin mucus powder was dissolved in Milli-Q water, being the undissolved mucus portion isolated by centrifugation (1,500 rpm, 10 min, 4ºC). Protein concentration in each sample was determined by the dye binding method of Bradford [31], using bovine serum albumin (BSA, Sigma) as the standard, and adjusted to 500 µg protein ml^{-1} of skin mucus with Milli-Q water. Samples were aliquoted and stored at -20ºC until use.

Blood samples were obtained from the caudal vein of each specimen with a 27-gauge needle and 1 ml syringe. After clotting at 4ºC during 4 h, each sample was centrifuged and the serum removed and frozen at -80ºC until use. In the same way, the protein concentration present in each sample was determined and adjusted being the samples aliquoted and stored at -20ºC as previously indicated for mucus samples.

2.3. Total immunoglobulin M levels

Total IgM levels were analyzed using the enzyme-linked immunosorbent assay (ELISA) [32]. Thus, 10 µg $well^{-1}$ of skin mucus or serum proteins were placed in flat-bottomed 96-well plates in triplicate and coated by overnight incubation at 4ºC with 100 µl of 50 mM carbonate-bicarbonate buffer, pH 9.6. After three rinses with 100 µl per well of PBS-T (0.1 M phosphate buffer (PBS) and 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% BSA in PBS-T, followed by three new rinses with PBS-T. The plates were then incubated for 1 h with 100 µl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer, Sigma). After exhaustive rinsing with PBS-T the plates were developed using 100 µl of a 0.42 mM solution of 3,3',5,5'- tetramethylbenzidine hydrochloride (TMB, Sigma), prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µl of 2M H_2SO_4 and the plates were read at 450 nm in a plate reader (FLUOstar Omega, BMG Labtech). Negative controls consisted of samples without skin mucus or serum or

without primary antibody, whose optical density (OD) values were subtracted for each sample value.

2.4. Evaluation of enzyme activities

2.4.1. Lysozyme activity

Lysozyme activity was measured according to the turbidimetric method described by Parry et al. [33] with some modifications. One hundred µl of skin mucus or serum diluted 1/2 with 10 mM PBS, pH 6.2, were placed in flat-bottomed 96-well plates in triplicate. To each well, 100 µl of freeze-dried *Micrococcus lysodeikticus* in the above buffer (0.3 mg ml^{-1}, Sigma) was added as lysozyme substrate. The reduction in absorbance at 450 nm was measured after 0 and 15 min at 22ºC in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. The units of lysozyme present in skin mucus and serum were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) and the results were expressed as U mg^{-1} mucus or serum proteins.

2.4.2. Alkaline phosphatase activity

Alkaline phosphatase activity was measured by incubating an equal volume of skin mucus or serum with 4 mM p-nitrophenyl liquid phosphate (Sigma) in 100 mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$ (pH 7.8, 30ºC) as described by Ross et al. [34]. The OD was continuously measured at 1-min intervals over 3 h at 405 nm in a plate reader. The initial rate of the reaction was used to calculate the activity. One unit of activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol product in 1 min.

2.4.3. Esterase activity

Esterase activity was determined according to the method of Ross et al. [34]. An equal volume of skin mucus or serum was incubated with 0.4 mM p-nitrophenyl myristate substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30ºC). The OD and activity was determined as above.

2.4.4. Peroxidase activity

The peroxidase activity in skin mucus or serum was measured according to Quade and Roth [35]. Briefly, 30 µl of skin mucus or serum were diluted with 120 µl of Hank's buffer (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. As substrates, 50 µl of 20 mM TMB and 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid and the OD was read at 450 nm in a plate reader. Standard samples without skin mucus or serum, respectively, were used as blanks. One unit was defined as the amount producing an absorbance change of 1 and the activity expressed as U mg^{-1} mucus or serum proteins

2.4.5. Protease activity

Protease activity was quantified using the azocasein hydrolysis assay according to the method of Ross et al. [34]. Briefly, equal volume of skin mucus or serum was incubated with 100 mM ammonium bicarbonate buffer containing 0.7% azocasein (Sigma) for 19 h at 30ºC. The reaction was stopped by adding 4.6% trichloro acetic acid (TCA) and the mixture centrifuged (10,000 rpm, 10 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 µl $well^{-1}$ of 0.5 N NaOH, and the OD read at 450 nm using a plate reader. Skin mucus or serum were replaced by trypsin (5 mg ml^{-1}, Sigma), as positive control (100% of protease activity), or by buffer, as negative controls (0% activity).

2.4.6. Antiprotease activity

Total antiprotease activity was determined by the ability of skin mucus and serum to inhibit trypsin activity [36]. Briefly, 10 µl of each serum and skin mucus samples previously adjusted to 0.5 and 2 mg protein ml^{-1} were incubated (10 min, 22ºC) with the same volume of standard trypsin solution (5 mg ml^{-1}). After adding 100 µl of 100 mM ammonium bicarbonate buffer and 125 µl of 0.7% azocasein, samples were incubated (2 h, 30ºC) and, following the addition of 250 µl of 4.6% TCA, a new incubation (30 min, 30ºC) was done. The mixture was then centrifuged (10,000 rpm, 10 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 µl $well^{-1}$ of 0.5 N NaOH, and the OD read at 450 nm using a plate reader. For a positive control, buffer replaced both skin mucus or serum and trypsin, and for a negative control, buffer

replaced the skin mucus or serum. The percentage of inhibition of trypsin activity by each sample was calculated.

2.5. Bactericidal activity

Three opportunist marine pathogenic (*Vibrio harveyi, V. angillarum and Photobacterium damselae* subsp. *piscicida*) and two non-pathogenic bacteria (*Escherichia coli and Bacillus subtilis*) were used. Bacteria were grown in agar plates at 25ºC in the adequate media: tryptic soy (TSB, Sigma) for *V. harveyi, V. angillarum and P. damselae*, Luria (LB, Sigma) for *E. coli* and nutrient broth (NB) (Conda) for *B. subtilis*. Then, fresh single colonies of 1-2 mm were diluted in 5 ml of appropriate liquid culture medium and cultured for 16 h at 25°C on an orbital incubator at 200-250 rpm.

The skin mucus and serum antimicrobial activity was determined by evaluating their effects on the bacterial growth curves using the method of Sunyer and Tort [37] with some modifications. Aliquots of 100 µl of each one of the bacterial dilutions (1/10) were placed in flat-bottomed 96-well plates and cultured with equal volumes of gilthead seabream skin mucus or serum. The OD of the samples was measured at 620 nm at 30 min intervals during 24 h at 25ºC. Samples without bacteria were used as blanks (negative control). Samples without mucus or serum were used as positive controls (100% growth or 0% bactericidal activity).

2.6. Statistical analysis

Data are expressed as mean ± standard error (SE). Data were statistically analysed by one-way analysis of variance (ANOVA) to determine differences between groups. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal-Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPSS 19 and differences were considered statistically significant at a 95% of confidence level when the calculated F value for 9 degrees of freedom was not exceed the theoretical value (F = 3.17).

3. RESULTS

3.1. Skin mucus shows higher enzyme activities than serum

Peroxidase, protease, lysozyme, esterase and alkaline phosphatase activities as well as total IgM were detected in the skin mucus at 500 µg protein ml^{-1} (Fig. 1 and Table 1). However, the antiprotease activity was undetected and for this we used a higher protein concentration (2 mg protein ml^{-1}) being then detectable. When compared with the activities found in the serum, skin mucus showed very similar protease, alkaline phosphatase and lysozyme activities whilst mucus peroxidase, antiprotease and esterase activities were significantly higher (Fig. 1 and Table 1). By contrast, mucus showed significantly lower IgM levels than the serum (0.140 ± 0.015, 0.216 ± 0.018, OD 450nm, respectively).

Table 1. Enzymatic activities of peroxidase, lysozyme, alkaline phosphatase and esterase activities (U mg^{-1} protein) found in skin mucus and serum samples of gilthead seabream. Data represent the mean ± S.E. (n=30). Asterisks denote significant differences between serum and mucus samples at a 95% of confidence level when the calculated F value was not exceed the theoretical value.

		Samples	
		Mucus	**Serum**
Enzyme activities (U mg^{-1} protein)	**Peroxidase**	4.28 ± 0.89*	2.08 ± 0.79
	Lysozyme	30.47 ± 1.51	31.49 ± 1.88
	Phosphatase alkaline	32.56 ± 2.70	30.81 ± 2.10
	Esterase	104.62 ± 3.80*	54.45 ± 2.80

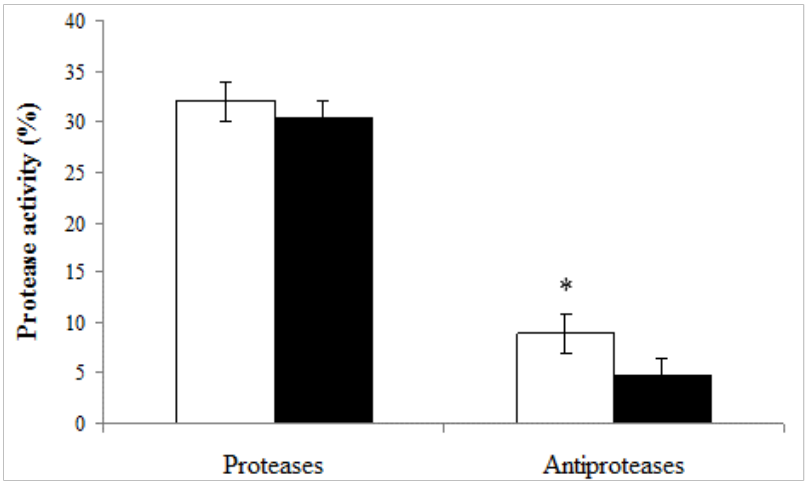

Fig. 1. Protease and antiprotease activities (%) found in serum (black bars) and skin mucus (white bars) samples of gilthead seabream. Bars represent the mean ± S.E. Asterisk denotes significant differences between serum and mucus samples at a 95% of confidence level when the calculated F value was not exceed the theoretical value.

3.2. Bactericidal activity is an important function in the skin mucus

Bactericidal activity of skin mucus and serum was found against both pathogenic and non-pathogenic bacteria (Fig. 2). Furthermore, mucus showed significantly higher capacity to kill *V. harveyi*, *V. anguillarum* or *P. damselae* bacteria, ranging 60-80%, whilst the serum only reached 42% as the greatest (Fig. 2B). Regarding the non-pathogenic *E. coli* and *B. subtilis* bacteria, neither mucus nor serum showed significant bactericidal activity (Fig. 2C). Strikingly, *B. subtilis* were able to overgrow when incubated with skin mucus indicating that it can use the mucus as a nutrient source.

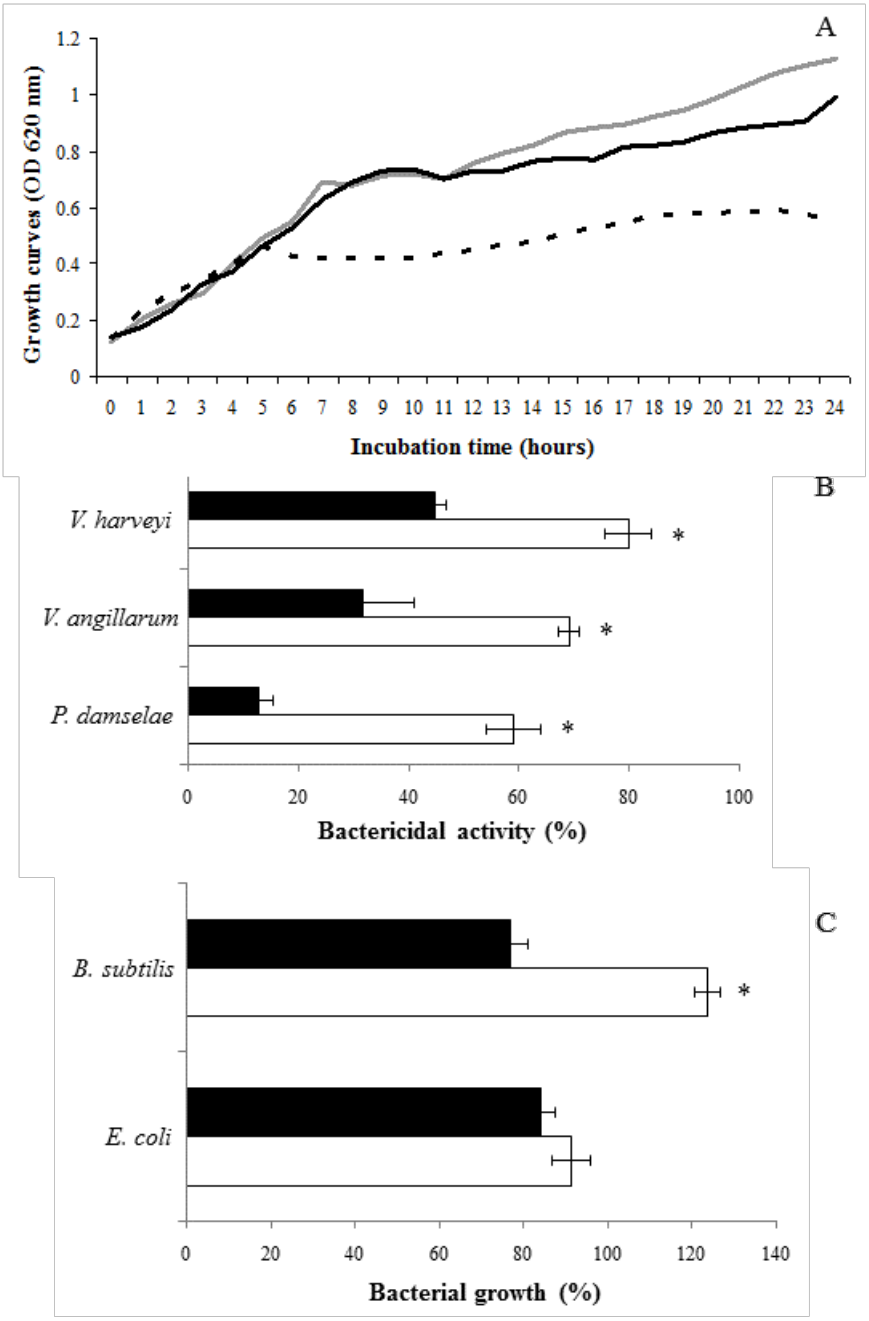

Fig. 2. Bactericidal activity in serum and skin mucus from gilthead seabream. A. Representative growth curves of *Photobacterium damselae* in culture medium (grey line) or preincubated with seabream serum (black line) or skin mucus (dash line). B. Bactericidal activity of serum (black bars) and skin mucus (white bars) samples against pathogenic (B) or non-pathogenic (C) bacteria for seabream. Bars represent the mean ± S.E. Asterisks denote significant differences between serum and mucus samples at a 95% of confidence level when the calculated F value was not exceed the theoretical value.

4. DISCUSSION AND CONCLUSIONS

From the immunological point of view, very few efforts have been made in order to study the mucosal surfaces since they are those in contact with the environment and the first line of defence. Fish are in constant interaction with a wide range of non-pathogenic and pathogenic microorganisms present in the aquatic environment and therefore have developed defence mechanisms to guarantee their survival [14]. As a clue component of innate immunity, the skin layer is the first line of defense against microorganisms, plays a frontier role in protecting fish against infections and the skin mucus contains many kinds of biologically active (including defensive) molecules [38–42]. In this sense, several studies have demonstrated that the presence and activity of immune factors in the skin mucus depends on the environmental conditions and the fish species [23,40,43–45]. We carried out this study taking in consideration the importance of the skin mucus as the first line of defense and the lack of knowledge in the case of the gilthead seabream.

All the studied molecules in the present work were constitutively present in both skin mucus and serum of gilthead seabream: immunoglobulin M, peroxidase, lysozyme, alkaline phosphatase, esterase, protease, antiprotease and antimicrobial activities. Interestingly some of them are present in the skin mucus in greater levels than in the serum indicating the immune relevance of the skin mucus. Regarding the seabream IgM, its presence in the skin mucus was significantly lower than in the serum, which correlates to results obtained in other fish species [13,46–50]. Perhaps, the results confirm the different origin of IgM present in mucus and serum [51,52] although no clear differences were detected between skin mucus and serum Ig of carp [26] and olive flounder [10] and authors suggest that it may be due to evolutionary pressure from common abundant pathogens in their surroundings. In the rainbow trout skin, about 61% of B cells were IgT^+ and 39% IgM^+ and the IgM concentration in the serum was around 1,000-fold that found in the mucus [13]. Furthermore, IgT concentration was the only one increasing in the skin mucus after bacterial or parasite infections. Thus, data from other species suggest that other Igs different of IgM could be also present in seabream skin mucus, which deserves further characterization.

Different enzymes with a putative role in the immune functions have been identified in several fish species and included in our study. Among them, alkaline phosphatase in mucus has been shown to act as an antibacterial agent, due to its hydrolytic activity, and to increase in fish during skin regeneration, because of its protective role in the initial stages of wound healing [53–56], stress [55,56] and parasitic infection [34]. For these reasons, the presence of this enzyme in epidermal fish mucus has been suggested as a potential stress indicator [34]. Moreover, seabream mucus showed very similar alkaline phosphatase activity than serum. In the case of rainbow trout, coho salmon and Atlantic salmon this enzyme was undetected in the skin mucus unless the fish were transferred from freshwater to marine water [40]. Further studies should include comparisons between different mucus-sources and serum. Another studied enzyme was the esterase being the values obtained for seabream twice in skin mucus samples than in the serum. Though there are no source-comparative studies recent data confirmed that both enzymes (alkaline phosphatase and esterase) in skin mucus of fishes increase after immunostimulant administration [57,58]. All these data seem to suggest that both enzymes could play an important role in gilthead seabream skin. Nonetheless, further studies are needed to elucidate the precise function of phosphatases and esterases because they are not well understood in relation to mucosal immunity [23]. Protesases, on the other hand, in the skin mucus may play a protective role against pathogens by both direct, cleaving their proteins [14], and indirect, by hampering their colonization and invasion, mechanisms [59]. Furthermore, proteases also activate and enhance the production of other innate immune components present in fish mucus such as complement, immunoglobulins or antibacterial peptides [60–62]. Fish epidermal mucus has been found to have different protease types: serine proteases such as trypsin [60], cysteine proteases, (e.g. cathepsin B and L) [63,64], aspartic proteases (such as cathepsin D) [65,66] and metalloproteases [40]. In our study, the protease activity was also slightly higher in mucus than in serum samples. Studies describing changes in mucus proteases following immunostimulation [57,58], stress [63,64,67,68] and infection [34,69] point to their importance in mucosal immunity. Moreover, Loganathan et al. [70] have suggested that the relative importance of protease enzymes is higher in the fish mucus than the other enzymes, for example, lysozyme, alkaline phosphatase or esterases. Interestingly, the production of antiproteases should be then low in order to maintain these high levels of mucus proteases active. This has been only studied in seabream where mucus antiproteases were undetected unless higher protein (2 mg ml^{-1})

concentrations used. Very few data are available but some antiprotease compounds have been identified and characterized in the Atlantic salmon [71] and *Takifugu pardalis* [72]. Regarding the last enzyme studied herein, the peroxidase activity in seabream skin mucus was significantly higher than in the serum. Since peroxidases are important microbicidal agents that effectively eliminate H_2O_2 and maintain the redox balance of immune system, it is tempting to consider that peroxidase in mucus will be essential for mucosal immunity and skin defense. Further characterization of the fish mucus enzymes and their precise role in the mucosal immunity is still awaiting.

One of the most practical immune functions is the direct ability to kill bacteria and this is also important in the skin mucus. This bactericidal activity can be carried out by many different compounds but unfortunately most of them are unknown. Among them one of the most characterized is the lysozyme, which level and activity in the skin mucus depends on the environmental conditions and the fish species [23,29,40]. Furthermore, the variation in mucus lysozyme activity could be related to several factors such as season [73], species and genetic variations, sex, maturity, diet, handling stress [74,75], and even with thickness of the epidermis and the mucus cell number [14]. The lysozyme activity present in skin mucus and serum of gilthead seabream showed similar values. It has been reported significantly higher lysozyme activity in skin mucus of fish reared in freshwater than the species reared in seawater [40] and the opposite [14]. Therefore, the lysozyme activity in skin mucus showed no significant correlation with other immune substances, which suggest that the lysozyme is constitutively secreted in the skin mucus of these fish species [29]. But independently of the effector molecules and the mechanisms involved in the bacterial killing, the measurement of the bactericidal activity is a very realistic approximation. In this sense, numerous reports have studied the antimicrobial activity of skin mucus extracts in a several fish species against a broad range of microbial pathogens and fungi [76–83], however, they seem to vary among fish species and can be specific towards certain bacteria [84]. Besides that, the elimination of skin mucus and subsequent challenge with *Listonella anguillarum* resulted in increased mortality in ayu and turbot [85,86] and increased susceptibility to bacterial infection in carp [87]. The three bacteria selected in the present study have great economic impact due to the losses caused in gilthead seabream farms. The genus *Vibrio*, distributed in marine and brackish environments, can cause vibriosis in fish and shellfish and there are several species causing the most economically important diseases

in marine larviculture and aquaculture [88,89], including *V. harveyi* and *V. anguillarum*. Moreover, pasteurellosis or pseudotuberculosis, is a bacterial septicaemia caused by the halophilic bacterium *Photobacterium damselae subsp. piscicida* (formerly *Pasteurella piscicida*) in a wide variety of marine fish [90]. In this paper, bactericidal activity in the skin mucus was significantly higher than in the serum and the most susceptible bacteria was *V. harveyi* followed by *V. anguillarum* and *P. damselae*. These data suggest that these pathogens are greatly refracted by intact mucus and may not enter by the naïve skin. Furthermore, we also evaluated this activity against non-pathogens for fish such as *Escherichia coli* and *Bacillus subtilis*. Surprisingly, *E. coli* viability was unaffected by serum or mucus whilst *B. subtilis* incubated with skin mucus resulted in even better growth to a significant extent. However, those factors involved in these effects are not evaluated. It has also been demonstrated that the acidic mucus extracts have potential antimicrobial activity, indicating that basic antimicrobial peptides or acidic soluble proteins are responsible for the defensive purposes against the invading pathogens [91,92]. New studies on seabream mucus extracts could be developed in order to identify the antimicrobial peptides in fish skin mucus and their role in the mucosal immunity.

In conclusion, the current study reveals that gilthead seabream skin mucus contained very similar protease, alkaline phosphatase and lysozyme activities than serum but lower IgM levels. By contrast, seabream skin mucus showed higher peroxidase, antiprotease, esterase and bactericidal activities than serum. The results could be useful for better understand the role of the skin mucus as a key component of the innate immune system, which could be beneficial in the aquaculture for fish health. This preliminary study contributes to know the normal values of important immune activities present in gilthead seabream mucus and it will help to compare to those values present in fish under different environmental conditions, infection, and disease or stress situation.

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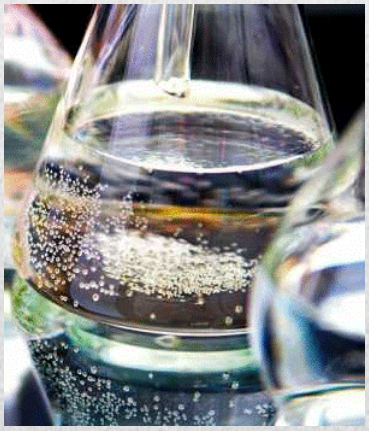
CHAPTER 5

Physico-chemical characterization of skin mucus from different species of marine teleost fish



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CHAPTER 5. Physico-chemical characterization of skin mucus from different species of marine teleost fish

ABSTRACT

Recently, the mucus-associated lymphoid tissues, and concretely the skin, have gained great interest among immunologists, and these tissues could be even more important in aquatic organisms. In fish, skin mucus has been scarcely characterized but it acts as a natural, physical, biochemical, dynamic, and semipermeable barrier that should enable the exchange of nutrients, water, gases, odorants, hormones, and gametes. In order to throw some light into its functions we have evaluated physico-chemical parameters in the skin mucus of different species of teleost such as gilthead seabream (*Sparus aurata* L.), European sea bass (*Dicentrarchus labrax* L.), shi drum (*Umbrina cirrosa* L.), common dentex (*Dentex dentex* L.) and dusky grouper (*Epinephelus marginatus* L.). Thus, protein concentration, pH, conductivity, redox potential, osmolarity, density and viscosity were measured, as well as differential scanning calorimetry (DSC). It has been generally observed a clear interrelation between density and osmolarity as well as between density and temperature. Viscosity showed an indirect shear- and temperature-dependent behaviour. Finally, microcalorimetric measurements confirmed substances with different structures which would be more stable in *S. aurata* and *D. labrax* than in the rest of fish studied. The results have been discussed with the aim of elucidating the possible relationship between physico-chemical and biological parameters parameters of the skin mucus of five different marine species of teleosts, all of them with commercial interest in the aquaculture of the Mediterranean area, with the disease susceptibility due to the differential presence and activity of antibacterial factors.

1. INTRODUCTION

Mucus is a complex viscous adherent secretion that covers the surface of most epithelia and represents an interface between the environment and the interior milieu [1], and this has probably a more vital importance in aquatic animals. In vertebrates, mucus is a viscous colloid gel that forms a layer of adherent mucus covering the epithelial cells (living cells) [2] and is secreted by various epidermal or epithelial mucus cells such as goblet cells found in mucous glands [1,3]. This mucus forms a great and vital layer in the case of skin and gut. In the fish skin, mucus plays a critical role in the animal defence acting as a natural, semipermeable, dynamic, physical, chemical, and biological barrier [4,5] which allows the exchange of nutrients, water, gases, odorants, hormones, and gametes [6]. The range of roles for fish mucus is very large and includes respiration, ionic and osmotic regulation, reproduction, locomotion, defence against microbial infections, disease resistance and protection, excretion or communication [1,7].

Mucus is primarily composed of water (approx. 95%) but also contains salts, lipids such as fatty acids, phospholipids, cholesterol and mucus glycoproteins, with molecular weights ranging from 0.5 to 20 MDa, called mucins [8,9]. In fact, these mucins exert a mechanical barrier by serving as filters for pathogens and preventing pathogen adherence to the underlying tissues [10]. These macromolecules are heavily glycosylated filamentous proteins that can form gel or non-gel structures. Moreover, they are strongly adhesive, play a major role in the defence of the mucosae [11,12], form a matrix in which a diverse range of antimicrobial molecules can be found [13], and are thought to be responsible for providing viscoelastic and rheological properties to mucosal layers [14]. The carbohydrate side chains constitute up to 80% of the total mucin mass [11,14] and give an elongated and rigid structure to the molecule, which contribute to these properties [15]. Mucin genes typically possess repetitive region/s which are rich in threonine, serine and, to a lesser extent, proline; these are the sites where glycoslyation takes place [16]. Due to their high glycosylation, many functions of the mucins depend on their carbohydrate chains, which offer wide possibilities of interactions with their environment in addition to participating to the mechanical properties of the mucus [11]. The primary functions of mucins are protection and

lubrication of epithelial surfaces [11,17], moreover mucins appear to be also involved in more complex biological processes such as epithelial cell renewal and differentiation, signalling and adhesion [15]. Thus, the main component that is responsible for the skin mucus viscous and elastic gel-like properties are the mucins.

Skin mucus has mainly attracted the focus by its properties in immunity. Apart from the physical barrier, the presence and abundance of numerous substances with immune functions has pointed to this mucus important immununological properties. In fish, the mucus contains molecules involved in the immune response such as cytokines [18], antimicrobial peptides [19,20], lysozyme [20,21], lipoprotein [22], complement [23], lectins [24–26], proteases [21,27] and antibodies [21,28–30]. Some components only have a defensive purpose [11] whereas others may also act by modifying the organization and properties of the gel [14].

Taking into account that mucus is proposed to have so many roles on the fish surface it is very surprising that the scientific literature only encloses few measurements of the physical and chemical properties, which are essential for proper biological functions. In the last decade, great strides have been made in identifying and characterizing the major oligomeric fish mucins [31,32]. Nevertheless, there have been no studies to date that relate the ensemble of their physical or chemical properties to a specific set of rheological parameters for a mucus gel, never mind its optimization to a specific function [14]. Thus, the aim of the present study was to investigate the physico-chemical parameters that possess the skin mucus of different species of marine teleosts to gain a better understanding of the biology and function of this essential barrier.

2. MATERIAL AND METHODS

2.1. Fish care and maintenance

Thirty adult specimens of each one of the following species were sampled in June 2013 from the *Instituto Español de Oceanografía* (IEO, Mazarrón, Spain) installations: gilthead seabream (*Sparus aurata* L.*)* (125 ± 25 g body weight), European sea bass (*Dicentrarchus labrax* L.*)* (100 ± 18 g body weight), shi drum (*Umbrina cirrosa* L.*)*

(565.5 ± 51 g body weight), common dentex (*Dentex dentex* L.*)* (1,600 ± 210 g body weight) and dusky grouper (*Epinephelus marginatus* L.*)* (803 ± 106 g body weight). The fish were kept in 2 m^3 tanks with a flow-through circuit, suitable aeration and filtration system and natural photoperiod. The water temperature ranged from 14.6 to 17.8°C. The environmental parameters, mortality and food intake were recorded daily.

2.2. Skin mucus collection

Fish were anesthetized prior to sampling with 100 mg l^{-1} MS222 (Sandoz). Skin mucus samples were collected according to the method of Guardiola et al. [21]. Briefly, skin mucus was collected by gentle scraping the dorso-lateral surface of naïve five specimens using a cell scraper with enough care to avoid contamination with blood and/or urino-genital and intestinal excretions. In order to get sufficient mucus to all the assays, equal samples of mucus were pooled (1 pools of 30 fish each) and stored at -80ºC.

2.3. Physico-chemical parameters

Protein concentration in skin mucus samples was determined by the Bradford method [33]. The pH measurements were done by a pH & ION-Meter GLP 22+ (Crison). Conductivity measurements were carried out at 25ºC using a Crison Conductimeter microCM 2200 and compared with the reference solution (ÉTALON CONDUCTIVITÉ 97 10 of 1413 µS cm^{-1}; Crison). Redox potential values were obtained from potentiometric measurements carried out with an electrode system consisted of a Crison platinum electrode 52-67 and an Orion Ag/AgCl double-junction reference electrode (Orion 90-02) connected to a homemade high-impedance data acquisition 16-channel box connected to a personal computer by USB (Universal Serial Bus). Redox standard solutions of 124 and 250 mV, (Fluka) were used as reference.

Osmolarity was measured using a VAPRO vapor pressure osmometer (model 5520). Density measurements were made in a densimeter (MDA5000M Anton Paar) at 12, 17 and 22 ± 0.1ºC. In this instrument the sample is introduced into a U-shaped borosilicate glass tube that is excited to vibrate at a characteristic frequency. Density is determined by the frequency changes due to the presence of the sample. All measurements were carried out in triplicate.

2.4. Mucus viscosity

For analysis, samples were thawed at room temperature in ice, briefly vortexed and centrifuged at 7,000 g for 5 min (Minispin, Eppendorf). The supernatant was collected, measured for its viscosity and the remaining mucus was stored at 20ºC for later analysis. Viscosity measurements were made in two different instruments. Kinematic viscosity was determined in a rheometer (Anton Parr MCR 102) with a plate-plate measurement system (plate model PP50, Anton Paar; plate-plate distance 0.8mm) using 1.6 ml of sample aliquots set at 12, 17 and 22 ± 0.1ºC because this range of temperature is the typical sea temperature variation between winter and summer. Therefore, to obtain a characteristic profile, viscosity was measured over a range of shear rates (11.5, 23, 46 and 115 s^{-1}). Viscosity measures were made in a constant shear rate during 200s, recording the viscosity value each second. During analysis, the 20 firsts points recorded were removed in order to eliminate artefacts at the beginning of the measure. In all cases viscosity did not show any systematic deviation from an average value. Results presented in this work are averages and standard deviations from these measurements. To obtain stable recording, mucus samples were allowed to equilibrate for 5 min after each measurement.

Dynamic viscosity was obtained in a microviscometer (Lovis 2000ME, Anton Paar) which was used to obtain the 400+ shear rates results. Samples were also treated as explained above but using 1 ml for measurement at 12, 17 and 22 ± 0.1ºC. This instrument is based on the Rolling Ball Principle. According to it, a ball rolls through a closed capillary tube, which is filled with the sample and is inclined at a defined angle. Changing the angle, one can submit the sample to different shear rates, in these experiments all slightly above 400 s^{-1}. Three inductive sensors in the tube determine the ball's rolling time between defined marks. The sample viscosity is directly proportional to the rolling time. We have not included standard deviations in our results because reproducibility of the measurement was within precision of the equipment (0.01 cm^3 g^{-1}).

2.5. Differential scanning calorimetry (DSC)

Before loading in the calorimeter cell, each of the mucus samples tested were subjected to centrifugation at 16,000 g for 5 min (Labofuge 400 R, Heraeus) to remove the particles in suspension. Measurement is carried out using a Microcal VP Differential Scanning Calorimeter (VP-DSC, Microcal, Northampton, MA, USA), being the samples subjected to 3 swept in the temperature range of 10 and 100°C and compared to reference buffer (seawater). Sample and reference cells (0.5 ml approximately each) were filled with sample. After 10 min equilibration time, the heat change was recorded at 25ºC for 120 min with a filtering period of 10 seconds, and a High Feedback Gain mode. Data were recorded and analyzed using Microcal Origin 5.0 software.

3. RESULTS

3.1. Marine fish show differential physico-chemical parameters in the skin mucus

Protein concentration, pH, conductivity, redox potential, osmolarity (Table 1) and density (Table 2) parameters were measured in the skin mucus from all marine fish. Protein concentration was similar in all fish species, although it was highest in *D. labrax* and lowest in *S. aurata*. The pH measure was very similar among species except to *D. labrax*, which was the lowest. Conductivity parameter was highest in *S. aurata* skin mucus and lowest in the case of *U. cirrosa*. Redox potential was similar in *S. aurata*, *U. cirrosa* and *D. dentex* and lowest in *D. labrax*. Osmolarity in the skin mucus was highest in the *S. aurata* and lowest in *D. labrax*. As expected, density measurements follow a similar pattern between the different temperatures tested, although a correlated decrease was observed with increasing temperature in skin mucus in all fish species resulting the highest in *S. aurata* and lowest in *D. labrax*.

Table 1. Physico-chemical and biological parameters of skin mucus of *S. aurata*, *D. labrax*, *U. cirrosa*, *D. dentex* and *E. marginatus* specimens. Data represent the value of a pool of 30 fish ± SEM of the technical replicates.

Species	Parameters measured				
	Protein (mg ml$^{-1)}$	pH	Conductivity (mS cm^{-1})	Redox potential (mV)	Osmolality (mmol kg^{-1})
Sparus aurata	0.88 ± 0.01	7.2	11.7 ± 0.6	191.9 ± 1.1	1,104 ± 4.51
Dicentrarchus labrax	1.45 ± 0.02	6.7	9.2 ± 0.5	156.5 ± 1.3	595 ± 1.76
Umbrina cirrosa	0.92 ± 0.01	7.2	7.6 ± 0.7	193.2 ± 0.9	1,010 ± 13.5
Dentex dentex	1.12 ± 0.01	7.1	9.4 ± 0.5	190.2 ± 1.2	934 ± 2.08
Epinephelus marginatus	0.90 ± 0.01	7.2	9.7 ± 0.4	178.2 ± 0.8	765 ± 2.51

Table 2. Density measurements (g ml^{-1}) of skin mucus of *S. aurata*, *D. labrax*, *U. cirrosa*, *D. dentex* and *E. marginatus* specimens. Data represent the value of a pool of 30 fish ± SEM of the technical replicates.

Species	Density measurements (g ml^{-1})		
	12ºC	17ºC	22ºC
Sparus aurata	1.028 ± 0.0017	1.027 ± 0.0016	1.026 ± 0.0016
Dicentrarchus labrax	1.014 ± 0.0017	1.013 ± 0.0016	1.012 ± 0.0016
Umbrina cirrosa	1.025 ± 0.0017	1.024 ± 0.0016	1.023 ± 0.0016
Dentex dentex	1.018 ± 0.0017	1.017 ± 0.0016	1.016 ± 0.0016
Epinephelus marginatus	1.024 ± 0.0017	1.023 ± 0.0016	1.021 ± 0.0016

Mucus viscosity for all fish species showed a non-Newtonian behaviour, concretely a shear-thinning effect. Values in Table 3 also show the usual decrease of viscosity with temperature. Generally, mucus viscosity of skin mucus of tested fish showed two different groups. One with similar and highest viscosity for *D. labrax*, *U. cirrosa* and *D. dentex* skin mucus and the other with almost identical and lowest viscosity for *S. aurata*

and *E. marginatus*, dependent of shear rate and temperature (Fig. 1). The greatest differences in kinematics viscosity were found from the lowest shear rate (11.5 s^{-1}) and temperature (12ºC), whilst slight differences were found in the highest shear rate (115 s^{-1}) and temperature tested (22ºC).

Table 3. Mucus viscosity (cP) in relation to shear rate (s^{-1}) in skin mucus of *S. aurata*, *D. labrax*, *U. cirrosa*, *D. dentex* and *E. marginatus* specimens at 12, 17 and 22 ± 0.1ºC. Measures of dynamic viscosity (400+ shear rates results) not have standard deviation because the reproducibility of the measurement is within the range of the machine (0.01). Data represent the value of a pool of 30 fish ± SEM of the technical replicates.

Species	T (ºC)	Shear rates (s^{-1})				
		11.5	**23**	**46**	**115**	**400+**
Sparus aurata	12	2.40 ± 0.17	2.21 ± 0.04	1.94 ± 0.06	1.45 ± 0.07	1.42
	17	1.49 ± 0.09	1.50 ± 0.04	1.48 ± 0.02	1.44 ± 0.06	1.25
	22	1.33 ± 0.08	1.32 ± 0.04	1.28 ± 0.02	1.25 ± 0.06	1.17
Dicentrarchus labrax	12	3.20 ± 0.20	2.90 ± 0.10	2.55 ± 0.04	2.10 ± 0.07	1.46
	17	2.60 ± 0.19	2.36 ± 0.08	2.10 ± 0.04	1.82 ± 0.06	1.29
	22	2.48 ± 0.13	2.02 ± 0.06	1.41 ± 0.03	1.29 ± 0.06	1.14
Umbrina cirrosa	12	2.92 ± 0.12	2.68 ± 0.05	2.49 ± 0.03	2.32 ± 0.06	1.63
	17	2.45 ± 0.11	2.30 ± 0.05	2.17 ± 0.02	2.06 ± 0.06	1.42
	22	2.12 ± 0.11	2.02 ± 0.05	1.92 ± 0.02	1.84 ± 0.06	1.26
Dentex dentex	12	2.76 ± 0.14	2.66 ± 0.04	2.45 ± 0.02	2.15 ± 0.06	1.56
	17	2.28 ± 0.09	2.19 ± 0.04	2.05 ± 0.02	1.88 ± 0.07	1.37
	22	1.96 ± 0.08	1.91 ± 0.04	1.82 ± 0.02	1.57 ± 0.06	1.27
Epinephelus marginatus	12	1.90 ± 0.60	1.80 ± 0.20	1.70 ± 0.10	1.70 ± 0.06	1.43
	17	1.60 ± 0.40	1.50 ± 0.16	1.51 ± 0.06	1.48 ± 0.06	1.27
	22	1.40 ± 0.20	1.30 ± 0.10	1.32 ± 0.04	1.30 ± 0.06	1.13

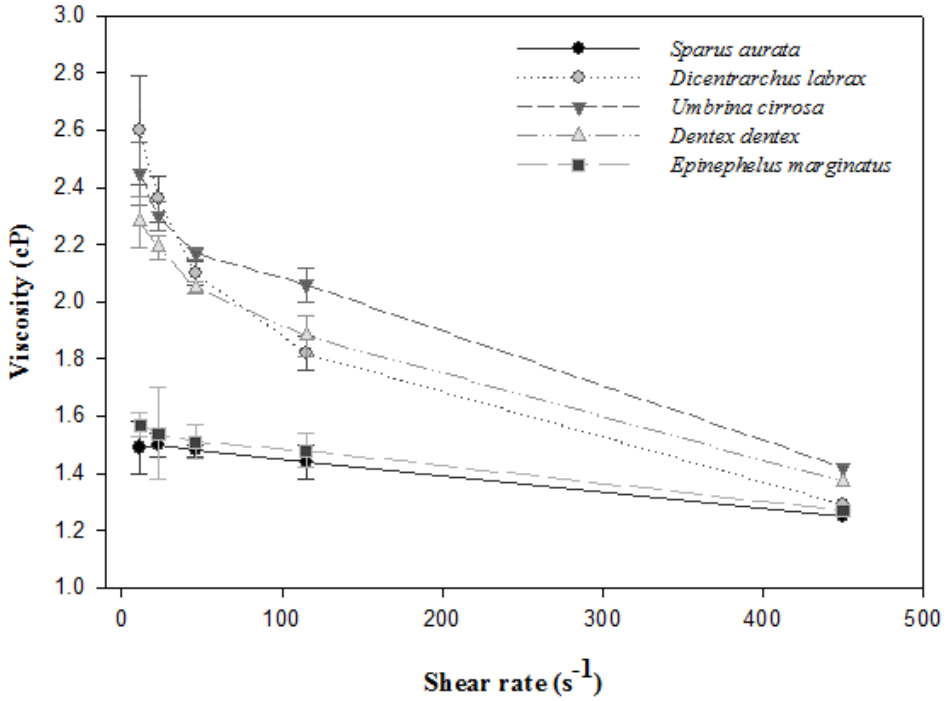

Fig. 1. Viscosity (cP) in relation to shear rate (s^{-1}) in skin mucus of *S. aurata*, *D. labrax*, *U. cirrosa*, *D. dentex* and *E. marginatus* specimens at 17 ± 0.1ºC. Lines Data represent the value of a pool of 30 fish ± SEM of the technical replicates.

3.2. Microcalorimetry measurements

All samples were subjected to three sweeps between 10 and 100ºC. Two profiles were observed in the skin mucus among fish species being *D. labrax* different to the others (Fig. 3). Moreover, differences between the three sweeps of temperature were only observed for *S. aurata* and *D. labrax* (Fig. 2). When comparing the measures between the different species it was observed higher heat release as heat capacity (Cp, cal $ºC^{-1}$) in the case of *U. cirrosa*, *S. aurata* and *D. dentex*, while it was lower in *D. labrax* and *E. marginatus* (Fig. 3A). However, when the curve of the differences between the sweep 1 and 3 was observed, it was detected higher heat release in skin mucus of *S. aurata* and *D. labrax*, contrary to what happened to the other species (Fig. 3B).

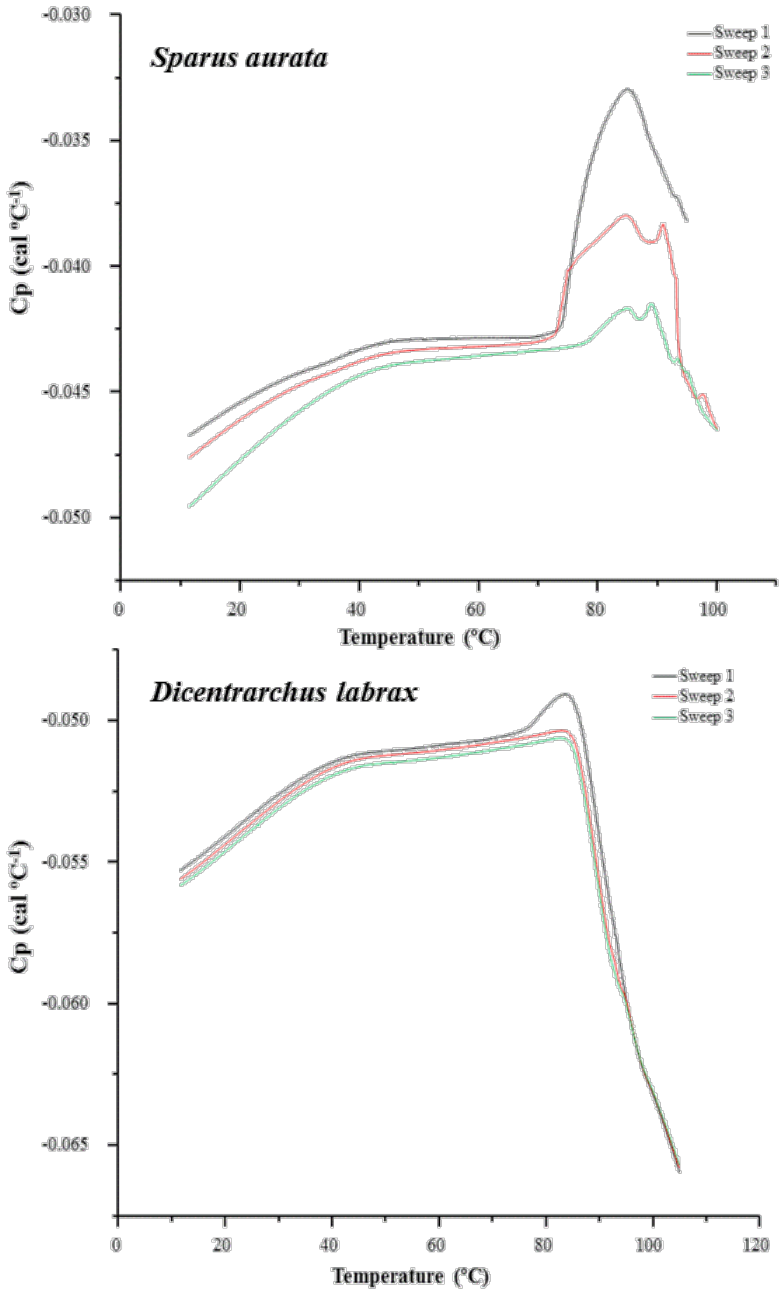

Fig. 2. Differential scanning calorimetry thermograms (Cp, cal $^{o}C^{-1}$) in skin mucus of *S. aurata* and *D. labrax* subjected to 3 swept in the temperature range of 10 and 100°C

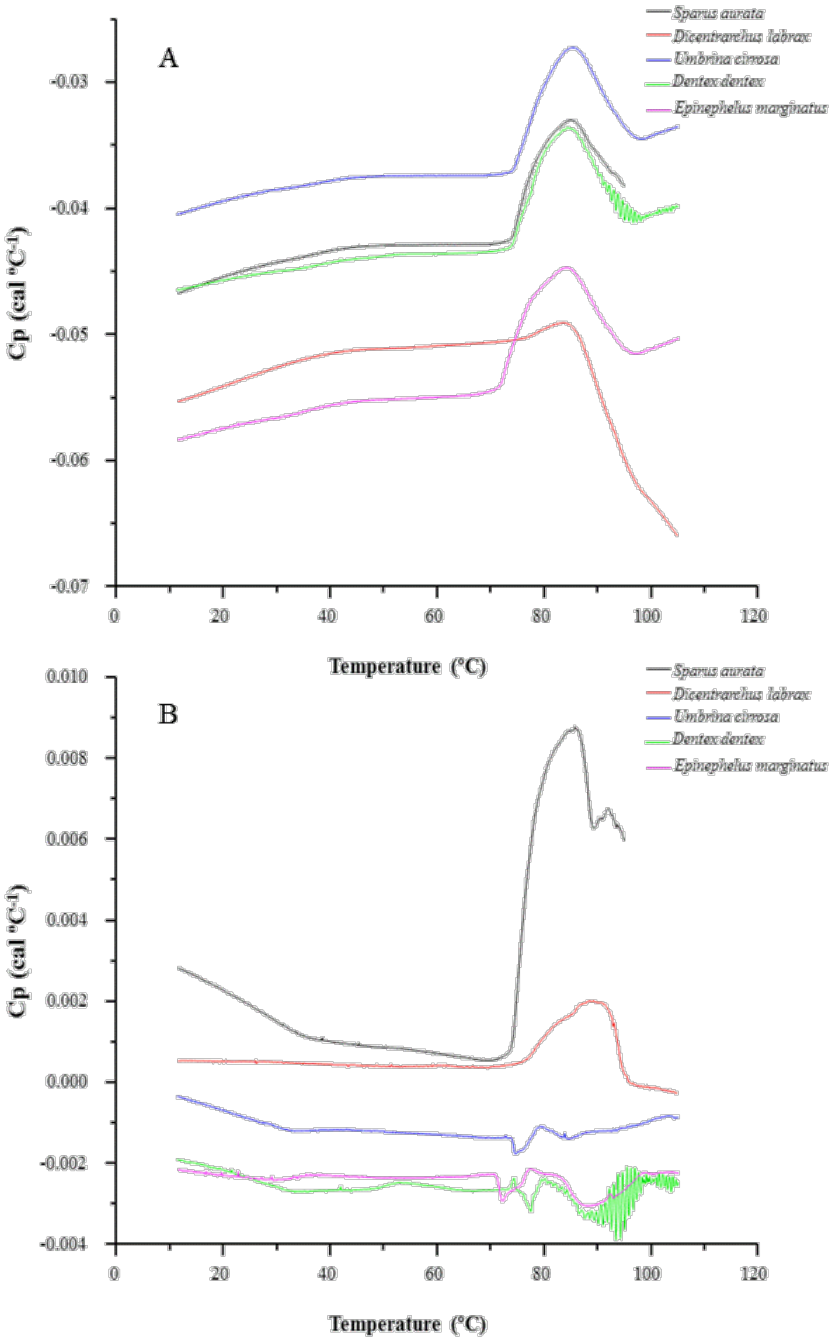

Fig. 3. Differential scanning calorimetry thermograms (Cp, cal $^{o}C^{-1}$) in skin mucus of *S. aurata*, *D. labrax*, *U. cirrosa*, *D. dentex* and *E. marginatus* specimens corresponding to the first sweep (A) and difference between first and third sweep (B) in the temperature range of 10 and 100°C.

4. DISCUSSION AND CONCLUSIONS

Mucus helps to protect epithelial surfaces in many different ways. It is a lubricant that protects against mechanical damage while enabling rapid removal of various types of aggression (such as pathogens or irritants) from the mucosal surfaces [2]. The skin surfaces, an important component of the innate immunity, is the first line of defence against microorganisms because it forms a physico-chemical barrier containing a diverse range of innate and adaptive immune factors that protects fish against infections [10,34]. The major components of the mucosal barrier are constitutively expressed, as continuous mucus production and secretion are required to replace the mucus that is degraded by bacteria [13]. The mucosal barrier itself is not a static barrier as its constituents and the released products are modulated by the microenvironment and by neural, endocrine and immune factors [13].

Some of the components present in fish mucus have been studied in several fish species such as common carp (*Cyprinus carpio*) [35], channel catfish (*Ictalurus punctatus*) [36], Japanesse eel (*Anguilla japonica*) [37,38], hagfish (*Eptatretus stoutii*) [39,40] and rainbow trout (*Oncorhynchus mykiss*) [41,42], as well as the marine species of fish present in this study [43]. Nevertheless, most of the studies focused on the disease protection and the immune properties from the biological point of view. Thus, the information about its physico-chemical parameters such as their rheological characteristics correlated with their biological properties, which could have applications for industrial purposes. Strikingly, to our knowledge, only few studies that evaluated these parameters in the skin mucus of teleosts fish exist [44,45]. Consequently, studies of these properties of skin mucus in more teleost fish species and a deeper characterization should benefit the understanding of the biology and function of mucosal barrier and their possible relationship with pathogens and disease. Thus, the aim of the present study was to investigate and compare the physico-chemical parameters that possess the skin mucus of five different marine species of teleosts, all of them with commercial interest in the aquaculture of the Mediterranean area.

Differences in mucin glycoproteins are revealed in protein (simple and complex), carbohydrate, lipid and mineral content [46,47]. The protein concentration in mucus

varies depending on many parameters such as the fish species, habitat and disease or exposition to pathogens [44,48,49]. In our study, protein concentration was similar in all fish species tested, although was highest in *D. labrax* and lowest in *S. aurata*. However these protein concentrations were higher than in salmonids [44]. These small variations can be attributed to the difference in species since all of them are in the same aquaria conditions (water composition, volume and circulation, temperature, salinity, photoperiod, etc).

The pH, conductivity, redox potential and temperature are clearly interrelated, by the amount of ions which typically have an aqueous solution. The redox potential is a measure of the activity of the electrons, which is related to the pH and oxygen content, whilst the pH is a measure of proton activity. In the case of conductivity, this is a measure of the ability of a material to allow electrical current to pass freely, which depends on the atomic and molecular structure of the material. Our results show a positive correlation between the pH, redox potential and conductivity values except for some cases such as in *U. cirrosa*, which presented low values in conductivity and higher in pH and redox potential were higher. Similarly, *D. labrax* showed a decrease in the redox potential regarding their pH and conductivity values. Practical implications of these parameters are the demonstration that bacterial attachment to fish surfaces was pH-dependent [50,51]. Balebona et al. [51] observed a similar adhesion pattern in all the strains tested at alkaline pH, being the highest adhesion in the pH range of 8.2-8.5; however, at lightly acid or neutral pH values, a differential pattern related to bacterial adhesion to mucus was obtained for the different strains tested. Therefore, further studies are needed in characterization of skin mucus parameters in order to understand their precise role in pathogen adhesion and invasion and the relation with physico-chemical parameters.

Osmolarity is a measure of the number of solute particles dissolved in a solution. Thus, osmolarity parameter in the skin mucus followed a very similar pattern to the parameters measured previously (pH and redox potential) being higher in *S. aurata*, *U. cirrosa* and *D. dentex* and lower in *D. labrax* and *E. marginatus*. These differences may indicate great ion gradients in skin mucus. Ion gradients between the surrounding water and mucus would offer a reduced ion gradient to the plasma, thereby reducing the cost of ion transport [52]. Contrarily, Roberts and Powell [44] observed slight differences of

osmolarity between three seawater fish species, which may indicate small ion gradients. Considering that the seawater osmolarity measured by us was 1,106 ± 3.2 mmol kg^{-1}, we could consider that skin mucus of *S. aurata*, *U. cirrosa* and *D. dentex* is iso-osmotic, while the skin mucus of *D. labrax* and *E. marginatus* could be categorized as hypo-osmotic to the surrounding water. Thus, in the case of iso-osmotic mucus the energy destined to ionic transport seem irrelevant, contrary to what happens in hyper-osmotic mucus [44].

Density is the relationship between the mass of a solution and the volume it occupies. Therefore, unlike the osmolarity depends on the total concentration of particles irrespective of their mass, the density depends on the number of solute particles and its mass. Thus, molecules with a high molecular weight such as mucins affects more the density that the osmolarity. In our study, density measurements followed a similar pattern between the different temperatures tested, where it was found a decrease with increasing temperature in skin mucus from all fish. Moreover, it was generally observed a positive correlation between density and osmolarity. However, no studies have related these parameters with biological functions.

On the other hand, mucus viscosity for all five species generally demonstrated non-Newtonian behaviour, where mucus exhibited a greater viscosity at low shear rates than at high shear rates. Interstingly, it has been demonstrated a non-Newtonian behaviour in the skin mucus of seawater-reared Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*), rainbow trout and plaice (*Pleuronectes platessa* L.) while that was not clearly observed when Atlantic salmon and rainbow trout were reared in freshwater [44,53]. The shear rates used in this study were the same as utilized by Roberts and Powell [44]: 11.5, 23, 46, and 115 s^{-1}, which represent velocities of 0.45, 0.91, 1.81 and 4.52 m s^{-1}. These swimming speeds lie between 0.5 (resting speed) and 2.6 m s^{-1} and have been used in laboratory swimming trials for several fish such as Atlantic salmon, brown trout, gilthead seabream and European sea bass [54–57]. Moreover, shear rates around 450 s^{-1} (corresponding to 17 m s^{-1}) were also used trying to simulate a faster swimming of fish. When fish increases their swim speed, the mucin aggregates in skin mucus become broken and elongated, and line up with the streamlines, creating a slippage plane [44]. In this way, flow resistance is reduced and viscosity becomes water-like. After, when shear stops, the viscosity of mucus recovers, restoring much of its original viscosity

within seconds [58]. This behaviour of skin mucus helps fish locomotion by reducing fluid friction and enhancing movement through water [47,59]. In our study, viscosity of skin mucus from *S. aurata*, *D. labrax*, *U. cirrosa*, *D. dentex* and *E. marginatus* showed shear-dependent behaviour where viscosity decreased with an increase in shear rate and temperature. Similarly, Chiou and Wang [45] observed the same behaviour in viscosity properties in a epithelial cell line of *Anguilla japonica*. Viscosity differences between temperature and species in our study were observed more clearly for shear rates lower than 46 s^{-1}. Analysing the species, it was observed that the viscosity was higher in *D. labrax*, *U. cirrosa* and *D. dentex* skin mucus and lower in the case of. *S. aurata* and *E. marginatus*, dependent of shear rate and temperature. Thereby, the viscosity increase may be related to the amount of protein present in the mucus, and the specimens with higher viscosity correspond to those having a higher protein concentration and vice versa. Moreover, the viscosity could be related to the osmolarity. For example, Roberts and Powell [44] observed a positive correlation between viscosity and osmolarity, with lower viscosity in fish reared in freshwater than in seawater. However, we found this correlation only for *U. cirrosa*, *D. dentex* and *E. marginatus* but this not happened for *S. aurata* and *D. labrax*. Similarly, Antonova et al. [60] found clinical synthetic lung surfactants to increase the viscosity in response to increased salinity. Another important role of this viscosity might be related to the easiness of pathogens to penetrate and cross the mucus barrier but this has never been evaluated. Therefore, further studies in viscosity of skin mucus should be performed to better understand the behavior and their properties of this surface.

Differential scanning calorimetry measures the heat capacity of states and the excess heat associated with transitions that can be induced by temperature change [61]. In our study, microcalorimetric measurements failed to show differences among the three sweeps of temperature except for *S. aurata* and *D. labrax*, where it was observed one or two substances that endure the temperature ranges established. Comparing the measures between the different species was observed higher heat release as Cp (cal $^{o}C^{-1}$) in the case of *U. cirrosa*, *S. aurata* and *D. dentex*, while it was lower in *D. labrax* and *E. marginatus*. Analyzing the differences between sweeps 1 and 3 of each specie, it was detected higher heat release in skin mucus of *S. aurata* and *D. labrax*, contrary to what happened in the rest of species. Thereby, we could say that the mucus samples have a different composition and quantity of substances in each sample, as well as diverse

populations of substances, however, we could confirm that the structures found in *S. aurata* and *D. labrax* would be more stable than in the rest of fish studied. Moreover, we could suggest that the energy released by these samples could be due to denaturation processes of different populations of proteins in the samples. Nevertheless, more studies would be needed to confirm this hypothesis.

In conclusion, we have determined and compared physico-chemical and biological parameters of the skin mucus from five marine teleost species. Thus, it has been observed a correlation among pH, conductivity and redox potential in skin mucus of all the fish tested. Moreover, it has been generally observed a clear interrelation between density and osmolarity, as well as, between density and temperature. In the case of mucus viscosity, is has been demonstrated non-Newtonian behaviour in all samples, where mucus exhibited a greater viscosity at low shear rates than at high shear rates. However, the correlation between viscosity and osmolarity was only observed for *U. cirrosa*, *D. dentex* and *E. marginatus* while that not happened for *S. aurata* and *D. labrax*. Implications of these skin mucus parameters with disease susceptibility deserve much deeper analysis. Consequently, further characterization and relationship of these parameters would be necessary to understand the biology and function of this essential barrier as is skin mucus of fish.

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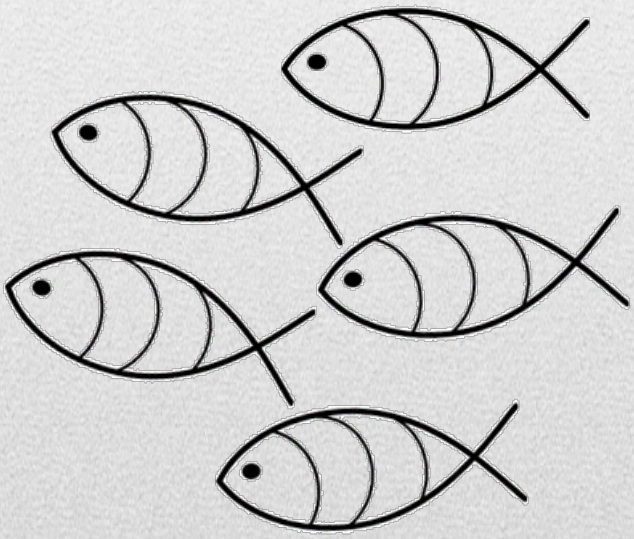
CHAPTER 6

Comparative analysis of the humoral immunity of skin mucus from several marine teleost fish



Guardiola FA, Cuesta A, Abellán E, Meseguer J, Esteban MA. Comparative analysis of the humoral immunity of skin mucus from several marine teleost fish. Fish Shellfish Immunol 2014 (submitted).

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CHAPTER 6. Comparative analysis of the humoral immunity of skin mucus from several marine teleost fish

ABSTRACT

Fish skin mucus contains several immune substances that provide the first line of defence against a broad spectrum of pathogens although they are poorly studied to date. Terminal carbohydrate composition and levels of total IgM antibodies, several immune-related enzymes (lysozyme, peroxidase, alkaline phosphatase, esterases, proteases and antiproteases) as well as the bactericidal activity (against fish pathogenic *Vibrio harveyi*, *Vibrio angillarum*, *Photobacterium damselae* and non-pathogenic bacteria *Escherichia coli*, *Bacillus subtilis*, *Shewanella putrefaciens*) were identified and measured in the skin mucus of five marine teleosts: gilthead seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), shi drum (*Umbrina cirrosa*), common dentex (*Dentex dentex*) and dusky grouper (*Epinephelus marginatus*). First, lectin binding results suggests that skin mucus contain, in order of abundance, N-acetylneuraminic acid, glucose, N-acetyl-glucosamine, N-acetyl-galactosamine, galactose and fucose residues. Second, results showed that while some immune activities were very similar in the studied fish (e.g. IgM and lysozyme activity) other such as protease, antiprotease, alkaline phosphatase, esterase and peroxidase activities varied depending on the fish species. High levels of peroxidase and protease activity were found in *U. cirrosa* respect to the values obtained in the other species while *E. marginatus* and *S. aurata* showed the highest levels of alkaline phosphatase and esterase activities, respectively. Moreover, skin mucus of *S. aurata* revealed higher bactericidal activity against pathogenic bacteria, contrarily, to what happened with non-pathogenic bacteria (*E. coli*, *B. subtilis*). Thus, study of the variations in the carbohydrate profile and immune-related components of the fish skin mucus could help to understand the fish resistance as well as the presence and distribution of pathogens and magnitude of infections, aspects that are of major importance for the aquaculture industry.

1. INTRODUCTION

MALT (mucosa-associated lymphoid tissue) constitutes a very large area for the possible invasion of pathogens and contains defence mechanisms (both innate and adaptive) that constitute the first line of defence against a broad spectrum of pathogens present in the environment. In the case of fish, MALT is present in skin, gill and gastrointestinal tract but its composition and functional characterization has received little research interest till recent years [1]. As part of this MALT, fish skin plays a critical role in the defence mechanisms acting as the first biological barrier [2–5]. The external constituent of this barrier is a mucous gel that forms a layer of adherent mucus covering the epithelial cells (living cells) [6] and is secreted by various epidermal or epithelial mucus cells such as goblet cells [7,8]. This mucus acts as a natural, physical, biochemical, dynamic, and semipermeable barrier that allows the exchange of nutrients, water, gases, odorants, hormones, and gametes [9]. The skin mucus is mainly composed of water and glycoproteins [10,11], containing a large content of high–molecular-weight oligosaccharides, and called mucins [12–16]. Among its functions, skin mucus is involved in fish respiration, osmoregulation, reproduction, locomotion, defence against microbial infections, disease resistance and protection, excretion or communication [7,17]. Perhaps, one of the most interesting and known functions has been its relation with the immune response and disease resistance but deeper characterization is awaiting.

The immunological or protective function of epidermal mucus is the result of its mechanical and biochemical properties. Epidermal mucus is continuously replaced and the its thickness and composition prevents the pathogen adherence to the underlying tissues and provides a medium in which antibacterial mechanisms may act [18–20]. At this respect, mucin carbohydrates may act as microorganism receptors playing a decisive role in either pathogen expulsion or settlement and invasion [21,22]. Secondly, fish epidermal mucus serves as a repository of numerous innate immune components of such as glycoproteins, lysozyme, complement proteins, lectins, C-reactive protein, flavoenzymes, proteolytic enzymes and antimicrobial peptides as well as immunoglobulins (IgM and IgT) [7,20,23–25] which exert inhibitory or lytic activity against different type of pathogens [4,26]. Among them, the most characterized ones are

lysozyme and proteases. First, lysozyme is likely the most powerful bacteriolytic protein since it has the ability to cleave the bacterial peptidoglycan. Its bacteriolytic activity in fish epidermal mucus and other tissues contributes to host defence against bacterial infections [2,27–30]. Moreover, lysozyme activity in the mucus greatly varied among the fish species studied and could reflect the differential fish resistance to bacterial pathogens or the bacterial abundance/diversity in the fish environments [2,20,31]. Second, fish mucus also contains a variety of proteases which have a significant role in the innate immune mechanisms by hampering pathogen invasion and viability [11,20]. Added to this, they also activate and enhance the production of various immunological components such as complement, immunoglobulins and antimicrobial peptides [32–35]. Lastly, other innate immune-related molecules present in fish skin mucus such as esterases, phosphatases or peroxidases have received less attention. Few works have shown great variability of these immune parameters in different fish species [2,20,25, 31]. Thus, we have already demonstrated the presence of IgM, lysozyme, protease, peroxidase, esterase, alkaline phosphatase, antiprotease and bactericidal activities in gilthead seabream [25]. However, available data seem to indicate that there is no relationship between skin mucus immunity and fresh/marine fish or water cleanness. This needs further investigation at deeper level but also with the study of more fish species.

Taking in consideration the importance of the skin mucus in fish immunity and the poor characterization of the immune molecules present in it we carried out this work. Thus, we aimed to identify, measure and compare the terminal carbohydrate profile and some of the main innate immune parameters (lysozyme, protease, antiprotease, alkaline phosphatase, esterase, peroxidase and bactericidal activities) in the skin mucus of 5 marine fish species: gilthead seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), shi drum (*Umbrina cirrosa*), common dentex (*Dentex dentex*) and dusky grouper (*Epinephelus marginatus*). This information will help to understand the mucosal immunity in marine fish and the importance it may have in several aquaculture-relevant marine species.

2. MATERIAL AND METHODS

2.1. Animals

Thirty adult specimens of each one of the following species were sampled in June from the *Instituto Español de Oceanografía* (IEO, Mazarrón, Spain) installations: gilthead seabream (*Sparus aurata)* (125 ± 25 g body weight), European sea bass (*Dicentrarchus labrax)* (100 ± 18 g body weight), shi drum (*Umbrina cirrosa)* (565.5 ± 51 g body weight), common dentex (*Dentex dentex)* (1,600 ± 210 g body weight) and dusky grouper (*Epinephelus marginatus)* (803 ± 106 g body weight). The fish were kept in 2 m^3 tanks with a flow-through circuit, suitable aeration and filtration system and natural photoperiod. The water temperature ranged from 14.6 to 17.8°C. The environmental parameters, mortality and food intake were recorded daily.

2.2. Skin mucus collection

Fish were anesthetized prior to sampling with 100 mg l^{-1} MS222 (Sandoz). Skin mucus samples were collected according to the method of Palaksha et al. [30] with some modifications. Briefly, skin mucus was collected by gentle scraping the dorso-lateral surface of naïve five specimens using a cell scraper with enough care to avoid contamination with blood and/or urino-genital and intestinal excretions. In order to get sufficient mucus to all the assays, equal samples of mucus were pooled (3 pools of 10 fish each) and homogenized with 1 volume of Tris-buffered saline (TBS, 50 mM Tris–HCl, 150 mM NaCl, pH 8.0). The homogenates were vigorously shaken and centrifuged (500 g, 10 min, 4ºC) being the supernatant lyophilized following freezing at -80ºC. Lyophilized skin mucus powder was dissolved in Milli-Q water, being the undissolved mucus portion isolated by centrifugation (500 g, 10 min, 4ºC). Protein concentration in each sample was determined by the Bradford method (1976) and skin mucus samples were adjusted to 500 µg protein ml^{-1}. Samples were then aliquoted and stored at -20ºC until use.

2.3. Determination of the terminal glycosylation pattern

Glycosylation pattern in the skin mucus was determined by lectin ELISA as described previously [36]. Thus, 10 µg well^{-1} of skin mucus samples were placed in flat-bottomed 96-well plates in triplicate and coated overnight at 4ºC with the use of 50 mM carbonate-bicarbonate buffer, pH 9.6. Samples were rinsed 3 times with PBS-T (20 mM phosphate buffer (PBS) and 0.05% Tween 20, pH 7.3), blocked for 2 h at room temperature with blocking buffer (3% BSA in PBS-T) and rinsed again. Samples were then incubated for 1 h with 20 µg per well of biotinylated lectins (Table I), washed and incubated with streptavidin horseradish-peroxidase (1:1000; Life Technologies) for 1 h. After exhaustive rinsing with PBS-T the samples were developed using 100 µl of a 0.42 mM solution of 3,3',5,5'- tetramethylbenzidine hydrochloride (TMB, Sigma), prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min, stopped by the addition of 50 µl of 2M H_2SO_4 and the plates read at 450 nm in a plate reader (FLUOstar Omega, BMG Labtech). Negative controls consisted of samples without skin mucus or without lectins, whose optical density (OD) values were subtracted for each sample value. Data are presented as the OD at 450 nm for each fish specie and lectin used.

Table 1. Lectins used in ELISA, their acronym, and sugar binding.

Acronym	Lectin source	Sugar binding specificity
BSL I	*Bandeiraea simplicifolia*	α-D-galactose, N-acetyl-α-D-galactosamine
PNA	*Arachis hypogaea*	β-D-galactose
UEA I	*Ulex europeaus*	α-L-Fucose
Con A	*Canavalia ensiformis*	α-D-mannose, α-D-glucose
WFA	*Wisteria floribunda*	N-acetyl-D-galactosamine
WGA	*Triticum vulgaris*	N-acetyl-β-D-glucosamine, N-acetylneuraminic acid
LEA	*Lycopersicon esculentum*	N-acetyl-β-D-glucosamine

2.4. Total immunoglobulin M levels

Total IgM levels were analyzed for gilthead seabream and European sea bass using the enzyme-linked immunosorbent assay (ELISA) [37]. Thus, mucus proteins were coated to wells, washed and blocked as in section 2.3. The plates were then incubated for 1 h with 100 µl per well of mouse anti-gilthead seabream or anti-European sea bass IgM monoclonal antibody (1/100 in blocking buffer; Aquatic Diagnostics Ltd.), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1,000 in blocking buffer; Sigma). Washing, development and reading was carried out as above. Negative controls consisted of samples without skin mucus or without primary antibody, whose optical density (OD) values were subtracted for each sample value.

2.5. Enzymatic activities

2.5.1. Lysozyme activity

Lysozyme activity was measured according to the turbidimetric method described by Parry et al. [38] with some modifications. One hundred µl of skin mucus diluted 1/2 with 10 mM PBS, pH 6.2, were placed in flat-bottomed 96-well plates in triplicate. To each well, 100 µl of freeze-dried *Micrococcus lysodeikticus* (0.3 mg ml^{-1}, Sigma) was added as lysozyme substrate. The reduction in absorbance at 450 nm was measured after 0 and 15 min at 22ºC in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. The units of lysozyme present in skin mucus were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) and the results expressed as U mg^{-1} mucus proteins.

2.5.2. Peroxidase activity

The peroxidase activity in skin mucus samples was measured according to Quade and Roth [39]. Briefly, 30 µl of skin mucus were diluted with 120 µl of Hank's buffer (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. As substrates, 50 µl of 20 mM TMB and 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid and the OD was read at 450 nm in a plate reader. Standard samples without skin mucus samples were used as blanks. One unit was defined as the amount producing an absorbance change of 1 and the activity expressed as U mg^{-1} mucus proteins.

2.5.3. Alkaline phosphatase activity

Alkaline phosphatase activity was measured by incubating an equal volume of skin mucus samples with 4 mM p-nitrophenyl liquid phosphate (Sigma) in 100 mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$ (pH 7.8, 30ºC) as described by Ross et al. [40]. The OD was continuously measured at 1-min intervals over 3 h at 405 nm in a plate reader. The initial rate of the reaction was used to calculate the activity. One unit of activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol product in 1 min and the activity expressed as U mg^{-1} mucus proteins.

2.5.4. Esterase activity

Esterase activity was determined according to the method of Ross et al. [40]. An equal volume of skin mucus samples was incubated with 0.4 mM p-nitrophenyl myristate substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30ºC). The OD and activity was determined as above.

2.5.5. Protease activity

Protease activity was quantified using the azocasein hydrolysis assay according to the method of Ross et al. [40]. Briefly, equal volume of skin mucus was incubated with 100 mM ammonium bicarbonate buffer containing 0.7% azocasein (Sigma) for 19 h at 30ºC. The reaction was stopped by adding 4.6% trichloroacetic acid (TCA) and the mixture centrifuged (10,000 g, 10 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 µl $well^{-1}$ of 0.5 N NaOH, and the OD read at 450 nm using a plate reader. Skin mucus were replaced by trypsin solution (5 mg ml^{-1}, Sigma), as positive control (100% of protease activity), or by buffer, as negative controls (0% activity).

2.5.6. Antiprotease activity

Total antiprotease activity was determined by the ability of skin mucus to inhibit trypsin activity [41]. Antiprotease activity in skin mucus was very low and for this assay we used samples adjusted to 2 mg ml^{-1} of mucus protein instead of 0.5 mg ml^{-1} [25]. Briefly, 10 µl of skin mucus samples were incubated (10 min, 22ºC) with the same volume of a trypsin solution (5 mg ml^{-1}). After adding 100 µl of 100 mM ammonium

bicarbonate buffer and 125 µl of 0.7% azocasein, samples were incubated (2 h, 30ºC) and, following the addition of 250 µl of 4.6% TCA, a new incubation (30 min, 30ºC) was done. The mixture was then centrifuged (10,000 rpm, 10 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 µl $well^{-1}$ of 0.5 N NaOH, and the OD read at 450 nm using a plate reader. For a positive control, buffer replaced skin mucus (100% protease and 0% antiprotease activity), and for a negative control, buffer replaced the trypsin (0% protease and 100% antiprotease activity). The percentage of inhibition of trypsin activity by each sample was calculated.

2.6. Bactericidal activity

Three marine pathogenic bacteria (*Vibrio harveyi*, *V. angillarum and Photobacterium damselae* subsp. *piscicida*) and three non-pathogenic bacteria (*Escherichia coli*, *Bacillus subtilis* and *Shewanella putrefaciens*) were used to determine the bactericidal activity present in skin mucus samples. Bacteria were grown in agar plates at 25ºC in the adequate media: tryptic soy (TSB, Sigma) for *V. harveyi*, *V. angillarum*, *P. damselae* and *S. putrefaciens*, Luria (LB, Sigma) for *E. coli* and nutrient broth (NB) (Conda) for *B. subtilis*. Then, fresh single colonies of 1-2 mm were diluted in 5 ml of appropriate liquid culture medium and cultured for 16 h at 25°C at 200-250 rpm.

The skin mucus antimicrobial activity was determined by evaluating their effects on the bacterial growth curves using the method of Sunyer and Tort [42] with some modifications. Aliquots of 100 µl of each one of the bacterial dilutions (1/10) were placed in flat-bottomed 96-well plates and cultured with equal volumes of skin mucus samples. The OD of the samples was measured at 620 nm at 30 min intervals during 24 h at 25ºC. Samples without bacteria were used as blanks (negative control). Samples without mucus were used as positive controls (100% growth or 0% bactericidal activity).

2.7. Statistical analysis

The results are expressed as mean ± standard error (SE). Data were statistically analysed by one-way analysis of variance (ANOVA) to determine differences between groups. Normality of the data was previously assessed using a Shapiro-Wilk test and

homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal-Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPSS 19 and differences were considered statistically significant at a 95% of confidence level when the calculated F value for 9 degrees of freedom was not exceed the theoretical value (F = 3.17).

3. RESULTS

3.1. Glycosilation of skin mucus proteins

All the tested terminal sugar residues were present in the evaluated skin mucus samples with significant differences within the lectins and fish species (Fig. 1). The lectin binding to skin mucus was WGA>Con A>WFA>BSL I>PNA>UEA I>LEA as evidenced by the OD readings. This binding pattern suggests that terminal carbohydrates abundance in skin mucus is N-acetylneuraminic acid, glucose, N-acetyl-glucosamine, N-acetyl-galactosamine, galactose and fucose residues in decreasing order of presence. When the fish species were compared, common dentex showed the lowest carbohydrate levels, except for PNA binding, whilst the other species varied with the sugar studied.

3.2. IgM type natural antibody levels

The IgM levels present in the skin mucus samples of *S. aurata* and *D. labrax* were of 0.14 ± 0.004 and 0.13 ± 0.003, expressed as OD at 450 nm, for seabream and sea bass, respectively and showed no statistically significant differences. No commercial antibodies for the other fish species are available.

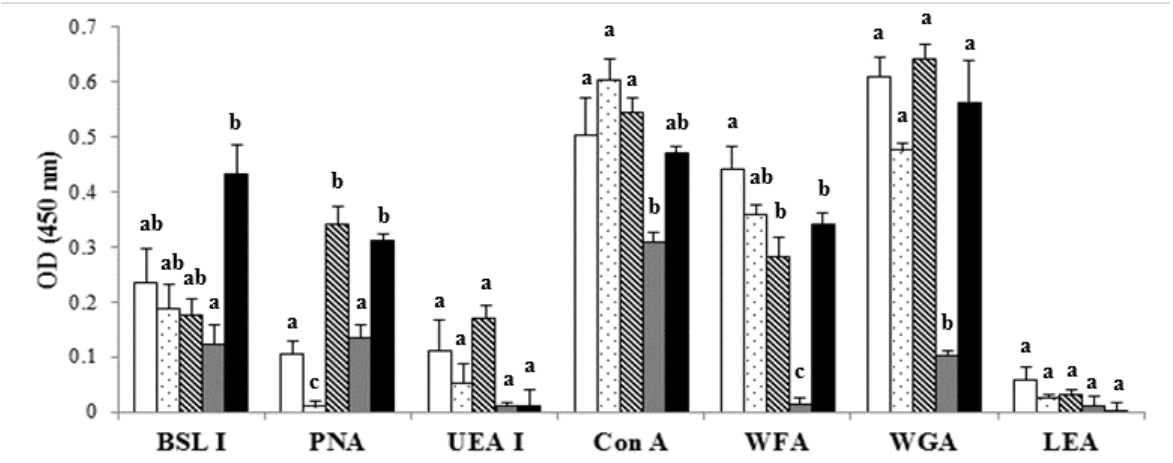

Fig. 1. Lectin binding (OD 450 nm) to carbohydrates present in skin mucus from *S. aurata* (white bars), *D. labrax* (dotted bars), *U. cirrosa* (dashed bars), *D. dentex* (grey bars) and *E. marginatus* (black bars) specimens. Bars represent the mean ± S.E. Different letters denote significant differences between fish species (P≤0.05). See Table 1 for lectin specificity.

3.3. Enzyme activities in skin mucus

Protease, antiprotease, lysozyme, peroxidase, alkaline phosphatase and esterase activities were found in the skin mucus from all the marine fish evaluated with important differences depending on the specie (Figs. 2 and 3). Lysozyme activity was very similar in all fish species (Fig. 3A). Overall, protease (Fig. 2A) and peroxidase (Fig. 3B) activities in the skin mucus followed a very similar pattern being highest in *U. cirrosa* and *D. dentex* and lowest in *D. labrax* and *E. marginatus*. Similarly, antiprotease activity was highest in *U. cirrosa* and *S. aurata* skin mucus and lowest in the case of *D. labrax* and *E. marginatus* (Fig. 2B). Skin mucus alkaline phosphatase showed the highest activity in *E. marginatus* (Fig. 3C) whilst the esterase activity (Fig. 3D) did in *S. aurata*. Interestingly, in both cases, *U. cirrosa* showed the lowest alkaline phosphatase and esterase activities.

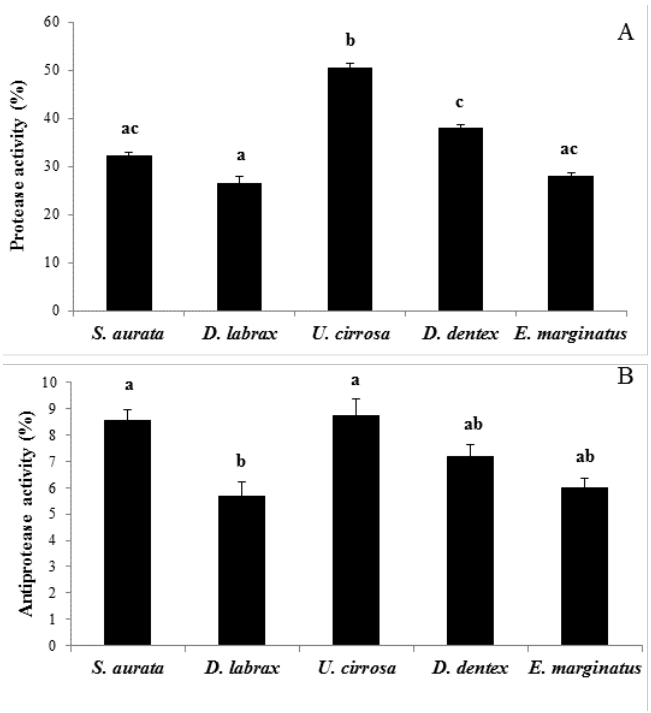

Fig. 2. Protease (A) and antiprotease (B) activity, expressed as percentage (%), in skin mucus of selected fish species. Bars represent the mean ± S.E. Different letters denote significant differences between fish species (P≤0.05).

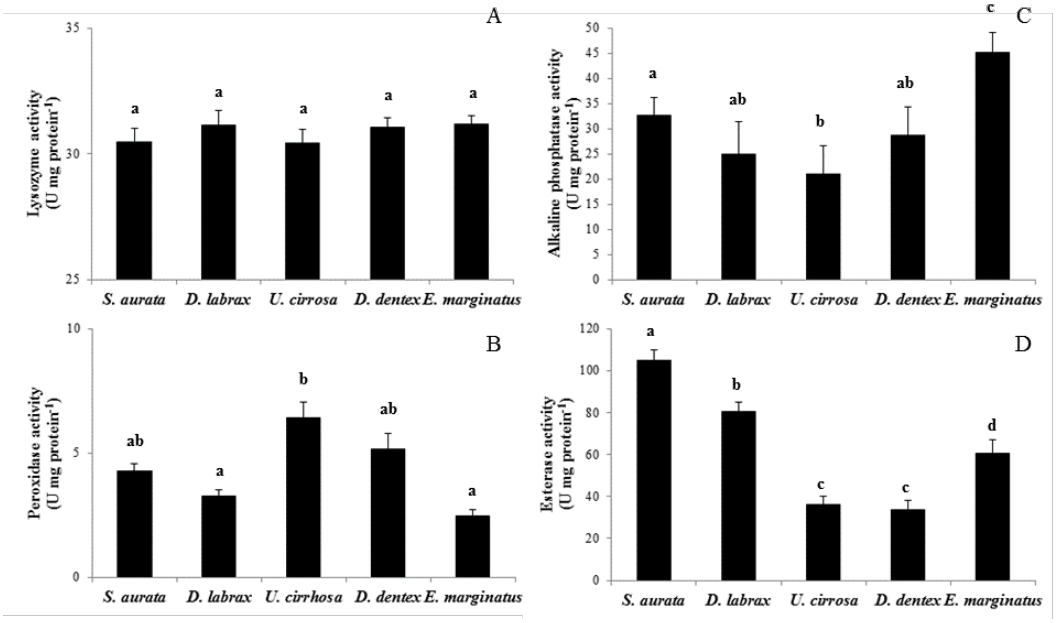

Fig. 3. Lysozyme (A), peroxidase (B), alkaline phosphatase (C) and esterase (D) activities, expressed as U mg^{-1} protein, in skin mucus of selected fish species. Bars represent the mean ± S.E. Different letters denote significant differences between fish species (P≤0.05).

3.4. Bactericidal activity

Bactericidal activity of skin mucus from *S. aurata*, *D. labrax*, *U. cirrosa*, *D. dentex* and *E. marginatus* against both pathogenic and non-pathogenic bacteria was determined (Fig. 4). Focusing on the pathogenic bacteria (Fig. 4A), the bactericidal activity followed a very similar pattern for *S. aurata*, *D. labrax*, *U. cirrosa* and *E. marginatus* where skin mucus from seabream showed the highest activity. Strikingly, in the case of *D. dentex* skin mucus, the bacteriolytic was very low against *V. harveyi* but very high against *P. damselae*. In the case of the non-pathogenic *E. coli* and *B. subtilis* the pattern was also quite similar being the lowest bactericidal activity found in the skin mucus of seabream and highest in the case of *E. marginatus*. Moreover, *B. subtilis* incubated with seabream skin mucus was able to even increase its growth instead of being killed. By contrast, in shi drum skin mucus, the bactericidal activity was very high against *E. coli* but very low against *B. subtilis*. For the probiotic *S. putrefaciens*, isolated from seabream skin [43], the bactericidal activity followed the same pattern that in the case of *V. harveyi* but with lower absolute values.

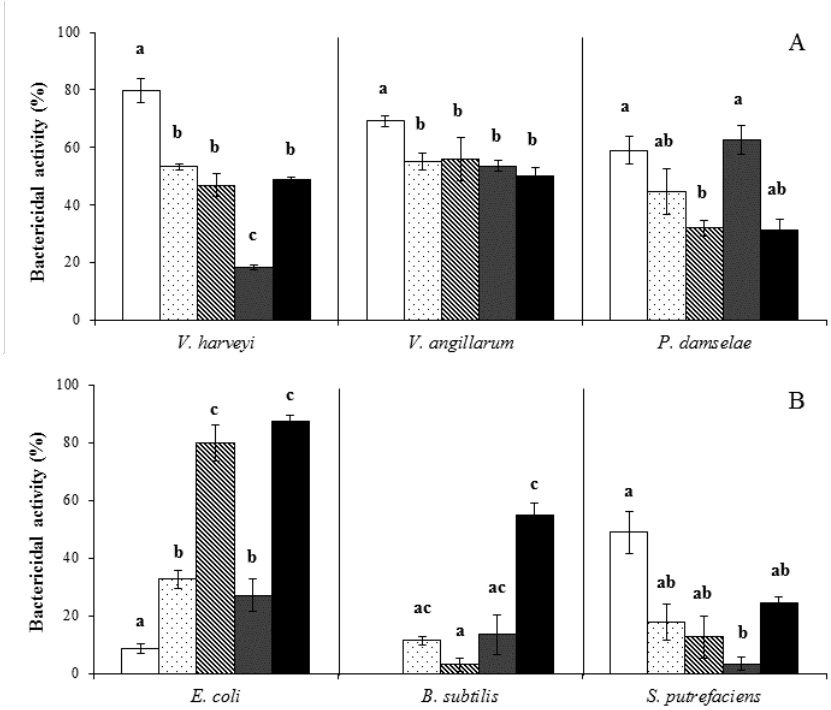

Fig. 4. Bactericidal activity (%) in skin mucus from *S. aurata* (white bars), *D. labrax* (dotted bars), *U. cirrosa* (dashed bars), *D. dentex* (grey bars) and *E. marginatus* (black bars) specimens. Bars represent the mean ± S.E. Different letters denote significant differences between fish species (P≤0.05).

4. DISCUSSION AND CONCLUSIONS

In aquatic environments, fish are in constant interaction with a wide range of pathogenic and non-pathogenic microorganisms and therefore have developed defence mechanisms to reach their survival [2]. The skin layer, an important component of the innate immunity, is the first line of defence against microorganisms because it forms a physico-chemical barrier containing a diverse range of innate and adaptive immune factors that protects fish against infections [11,20]. Strikingly, our data in seabream revealed that the skin mucus innate immune parameters (lysozyme, alkaline phosphatase, esterase, proteases, anti-proteases, peroxidase and bactericidal activities) were always higher than in the serum, indicating the great importance of the mucosal immunity as the first line of defence [25]. Moreover, the skin mucus composition and functional status is related with the environmental conditions and the interactions with commensal, symbiotic or pathogenic microbiota, and its dysfunction could involve a greater susceptibility to pathogens. Some studies have clearly demonstrated this last aspect because the elimination of skin mucus and subsequent challenge with bacterial infection resulted in increased mortality [44–46]. Therefore, is tempting to speculate that the pathogen susceptibility in the 5 fish species studied herein is different since they showed variable carbohydrate composition and immune functions in the skin mucus even considering that all fish shared the same environment (same marine installations and at the same time). Therefore, environmental factors might not be affecting to the differences found. Thus, studies of skin mucus immunology in more teleost fish species and a deeper characterization should benefit the understanding of the fish mucosal immunity and their relation with pathogens and disease.

Adhesion phenomena of pathogenic organisms to specific receptors on mucosal surfaces are extensively recognized as an important first step in the initiation of infectious diseases [47]. Many of these microorganisms use sugar-binding proteins as lectins to recognize and bind to host terminal carbohydrates [48]. The carbohydrate residues tested in this study are present in mammals and also have been observed in mucosal surfaces of fish (skin, digestive tract and gills) [21,22,49–52]. In our study, lectin binding levels to skin mucus was WGA>Con A>WFA>BSL I>PNA>UEA

I>LEA suggesting that terminal carbohydrates abundance in skin mucus is N-acetylneuraminic acid, glucose, N-acetyl-glucosamine, N-acetyl-galactosamine, galactose and finally fucose as the less abundant. This is the first comparative report about the terminal carbohydrates in fish skin mucus. N-acetylneuraminic acid provides negative charge to the mucin molecules and reduces bacterial binding [53] and it has been shown to be reduced in common carp skin mucus after bacterial infection [6]. In gilthead seabream, Con A and WGA lectin binding was high in skin mucus as it also occurs in the digestive tract [21]. Moreover, they found that all the tested terminal residues produced in the epithelial cells of the digestive tract of seabream decreased after infection with the intestinal parasite *Enteromyxum leei* [21]. Strikingly, the same group has reported that seabream intestine mucus shows high levels of galactosamine followed by fucose, neuraminic acid and lastly mannose+glucose residues and that these glycosylation is not changed upon *E. leei* infections [22] in sharp contrast to what has been reported in the intestinal cells [21]. Something similar happed in the common carp in which the lectin binding pattern in the skin cells and skin mucus was not the same and failed to follow the same profile after infection [6]. Thus, this fact deserves further characterization in order to understand the precise role of mucus carbohydrates and its role in pathogen adhesion and invasion. Furthermore, if these sugars are related to infection how the differential presence in the studied fish species is related to disease resistance or not might be worth of future investigations.

Both adaptive and innate immune factors are present in the fish skin mucus. Regarding the specific components, our data showed the presence of natural IgM in European sea bass skin mucus as it occurs in gilthead seabream [25], channel catfish (*Ictalurus punctatus*) [54], sheepshead (*Archosargus probatocephalus*) [55], common carp (*Cyprinus carpio*) [56,57], olive flounder (*Paralichthys olivaceus*) [30], rainbow trout (*Oncorhynchus mykiss*) [58] and Atlantic salmon (*Salmo salar*) [59,60]. In addition, IgT has been identified in trout skin mucus and seems to have a major role in controlling bacterial and parasite infections [61]. Further characterization of the mucus Ig repertoire, regulation and functions are needed in fish.

Enzymes in the epidermal mucus may play an important role in the fish immune functions and lysozyme, peroxidase, alkaline phosphatase, esterase, antiprotease and proteases have been identified in several fish species. Moreover, these enzymatic

activities have been compared among fish species or characterized after fish exposure to pathogens, stress or environmental factors such as temperature or salinity [2,3,20,30,31,40,62–64]. For example, our data of lysozyme activity, the most studied in fish and important against bacteria, has the same levels in all the fish though they seem to have different susceptibility to bacterial outbreaks. Another important factor is the presence of proteases which may play a protective role against pathogens by: i) directly degrading pathogens [2], ii) hampering their colonization and invasion due to modifications in the consistency of mucus surfaces and/or increasing the sloughing of these mucus layers [65], and iii) activating and enhancing the production of other innate immune components present in fish mucus such as complement, immunoglobulins or antibacterial peptides [66–68]. The role of proteases and antiproteases has been related with the defence against bacterial or parasite infections. Thus, our data show that shi drum and common dentex showed very high protease and antiprotease activities, which have been shown to be more prone to suffer diseases produced by parasites than by bacteria [69,70]. However, whether these high levels, with the same of lysozyme, are responsible to low bacterial susceptibility needs confirmation in such fish species. The other studied enzymes, alkaline phosphatase and esterase, are also present in skin mucus but their role in mucosal immunity is not well understood [20]. Nevertheless, alkaline phosphatase and/or esterases are present in fish mucus and their activity are modified with the season [71] as well as after physical or chemical stress, skin regeneration, immunostimulation and bacterial and parasitic infections [40,72–77] suggesting an important role in immunity. Our data showed a similar pattern of both enzymes in seabream, sea bass, shi drum and common dentex whilst the dusky grouper levels of phosphatase and esterase were high or low, respectively. Finally, the peroxidase activity, which acts as an important microbicidal agent that form a very toxic peroxidase-H_2O_2-halide complex has just been evaluated for the first time in seabream [25]. In our comparative study, we found similar levels for seabream, sea bass and dusky grouper and higher for shi drum and common dentex. Overall, our data show that each fish species has one or more enzymatic activities high but never all the activities are high or low at the same time. This could indicate that the immune response is always alert and the fish resistance is not limited to only one factor. Nonetheless, further studies should be performed to deepen in the knowledge of the fish mucus enzymes and their precise role in the mucosal immunity.

Evaluation of the direct lytic activity against pathogens is the most practical determination awaited for farmers whilst researchers also try to identify and characterize the molecules involved in this activity. Thus, determination of the bactericidal activity of the skin mucus might be more important than single enzymatic activities. First, some studies have revealed that the skin mucus of several fish species has a strong anti-bacterial and anti-fungal activity against a broad range of microbial pathogens and fungi [4,78–83]. Our data also confirm this and evidence that the skin mucus from the five marine fish species showed bactericidal activity against pathogenic and non-pathogenic bacteria with substantial differences among the fish species and bacterial strains. The antimicrobial activity of fish skin mucus has been observed in acidic-, organic- and aqueous-extracted mucus fractions. Though data are very variable they seem to indicate that acidic-extracted mucus contained the greatest bactericidal activity [4,84,85]. However, they state that in the aquaeous extracts the predominant bactericidal activity might reside in the lysozyme and proteases but our data do not support this hypothesis since there is no correlation between such parameters in our study. Thus, in the light of the data about fish skin mucus, the implication of other antimicrobial compounds, or the sum of many factors together, is playing part in the bactericidal activity. In this sense, several antimicrobial peptides have been identified in skin mucus that exerted bactericidal activity [4,86]. Further studies on skin mucus extracts could be developed in order to identify the antimicrobial peptides in these fish and their precise role in the mucosal immunity.

In conclusion, we have determined and compared the carbohydrate pattern and immune parameters of the skin mucus from gilthead seabream, European sea bass, shi drum, commond dentex and dusky grouper, all of them cultured or with great potential to be cultured in the Mediterranean area. Terminal carbohydrate abundance in skin mucus shows, from high to low presence, N-acetylneuraminic acid, glucose, N-acetyl-glucosamine, N-acetyl-galactosamine, galactose and fucose residues which greatly differed among the fish species. Relative to the immune parameters, though IgM level and lysozyme activity were equal other enzymatic activities and the bactericidal activity were different. Interestingly, all the fish species showed one or more activities at high levels indicating that fish are always alert and the immune response is not based on single components. Mucus from all the species also exerted bactericidal activity but this is difficult to correlate with the individual enzymatic activities. Nonetheless, the results

could be useful for better understand the role of these substances in the skin mucus as a key component of the mucosal innate immune system. Further investigations are needed to characterizate the fish mucosal immunity and the importance they have as the first line of defence.

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CHAPTER 7

Evaluation of waterborne exposition of heavy metals in skin mucus innate defence in gilthead seabream (*Sparus aurata*)

Guardiola FA, Dioguardi M, Parisi MG, Trapani MR, Meseguer J, Cuesta A, Cammarata M, Esteban MA. Evaluation of waterborne exposition of heavy metals in skin mucus innate defence in gilthead seabream (*Sparus aurata*). (In preparation).

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CHAPTER 7. Evaluation of waterborne exposition of heavy metals in skin mucus innate defence in gilthead seabream (*Sparus aurata*)

ABSTRACT

The aim of the present study was to investigate the constitutive innate immune repertoire functioning at the skin mucus of heavy metals waterborne [arsenic (As), cadmium (Cd) and mercury (Hg) 5, 5 and 0.04 µM, respectively] exposed gilthead seabream (*Sparus aurata* L.) specimens. Terminal carbohydrate composition, total immunoglobulin M, several enzymes and proteins, bactericidal activity as well as protein profiles and presence of fucose-binding lectin (DlFBL) were determined. The results corroborate a relationship between long-time exposure to Cd and As and the increase in the presence of various carbohydrates, as well as an increase of the concentration of Ig and most enzymes studied in the skin mucus of 10 days exposed fish. Nonetheless, the contrary effect was observed when fish were exposed for 30 days with As and Cd, while the activity of most of the enzymes continued to increase in fish exposed to Hg. Protein profiles by gel elcetrophoresis showed little variations in the protein bands excep in As and Cd exposed fish where a band of around 14 kDa disappeared. Western blot revealed the lack of DlFBL in control fish though it was detected in fish exposed to As and Cd for 2 or 10 days and in all cases when they were exposed to Hg. Finally, reversed phase chromatography analysis showed a similar pattern of peaks between skin mucus of control fish and exposed fish, however, this pattern varied in intensity depending on the exposure time and metal tested. The present results could be useful for better understanding the role and behaviour of the mucosal immunity in skin as a key component of the innate immune system against pollutants where there are many parameters that potentially could be employed as a good biomarkers of contaminant exposure.

1. INTRODUCTION

The mucosal immune system of vertebrates show an exclusive array of innate and adaptive immune cells and molecules (biologically actives) that act in concert to protect the host against pathogens and/or stressors [1]. Thus, an important component of the lymphoid tissue is the MALT (mucosa-associated lymphoid tissue). In fish, the MALT is present in skin (called SALT), gill (GIALT) and gastrointestinal tract (GALT) [2]. The MALT constitutes a very large surface for the possible pathogen invasion and contains defence mechanisms that constitute the first line of defence against a broad spectrum of pathogens present in the environment [3–7]. In the case of fish skin immunity, the mucus plays a major role [1].

Fish skin serves as the first line of defense against a wide variety of chemical, physical and biological stressors. Secretion of mucus is among the most leading characteristics of fish skin and this mucus plays a critical role in the animal defence acting as a natural, semipermeable, chemical and biological barrier where the immunological or protective function is the result of its mechanical and biochemical properties [8,9]. Thereby, fish epidermal mucus serves as a repository of numerous innate immune components of both innate and acquired immune system such as glycoproteins, lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein, flavoenzymes, proteolytic enzymes and antimicrobial peptides [10–14].

The epidermis of fish is a multifunctional tissue that is in constant and direct contact with the aquatic environment, which keeps metabolically active in all layers [15]. In fish populations, it has long been suspected a link between environmental contamination and disease [16–18]. In fact, many researchers have demonstrated that this connection could be due to the impairment of the innate immune system [19]. In the case of skin, both the structure and cellular composition of the epidermis can be affected by stressors, such as pathogens or environmental contaminants [20–24], including heavy metals [25–29]. Furthermore, changes in the composition and amount of the skin mucus produced have been also described, which could alter the proper functioning of the epidermis as a real barrier protecting against the possible hazards in the aquatic environment [29–31]. Mucus is mainly generated by mucous cells (also called goblet cells) and the first

response of these cells to adverse conditions (e.g. toxins, pollutants, pathogenic micro-organisms, irritating substances) is a great secretion of mucus [32,33]. This hypersecretion of mucus may have a protective role in reducing passive ion loss through damaged tissues [34]. Moreover, mucus binds toxic metals in exchange for primarily mucus Na^+ and K^+, though toxic metal binding will gradually deplete the mucus of essential ions. For this last reason, the mucus also prevent toxic ions access to the epithelial cells [35].

To date, there is a poor understanding of the interactions between heavy metals and skin mucus secreted of fish. It is not well known with whether determined processes of chemical sequestration of specific metals by mucus facilitates or retards metal uptake into the cell [36,37]. Mucus presents an overall negative charge and is polyanionic, meaning that it has a large metal binding capacity which could act as an ion trap to protect the body surface and gill membranes [35]. The accumulation of metals in the mucus of waterborne exposed fish was well reported [11], likewise, exposure to metals in the water increases mucus production in skin and gills [38,39], which facilitates the excretion through the detachment of the metal contaminated mucus [11]. Undoubtedly, mucus helps to keep metals away from epithelial surfaces [40].

It could be said that the sensitivity of an innate immune mechanism to a particular contaminant is similar among different species, which would make predicting the environmental impact of a toxicant easier [19]. In addition, sampling of the innate immune system is likely more easily and less invasive than some other systems, where peripheral blood, skin and skin mucus are potential sampling places [19]. Taking into account these previous considerations, the aim of the present study was to investigate the constitutive innate immune repertoire functioning at the skin mucus of heavy metals waterborne exposed gilthead seabream (*Sparus aurata* L.), as a result of the complexity of innate immunity, where there are many parameters that potentially could be employed as a good biomarkers of contaminant exposure.

2. MATERIAL AND METHODS

2.1. Fish care and maintenance

Hundred eight adult specimens (60 ± 35 g weight and 16 ± 3.2 cm length) of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.) obtained from Doramenor Acuicultura S.L. (Murcia, Spain), were kept in re-circulating seawater aquaria (250 L) in the Marine Fish Facilities at the University of Murcia. The water temperature was maintained at 20 ± 2°C with a flow rate of 900 L h^{-1} and 28‰ salinity. The photoperiod was of 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting, Spain) at a rate of 2% body weight day^{-1}. Fish were allowed to acclimatise for 15 days before sampling. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.2. Experimental design

Fish were randomly assigned and divided into six identical tanks (18 fish per tank): three as unexposed (control groups), one exposed to 5 µM of arsenic trioxide (As_2O_3, Fluka) (As group), one exposed to 5 µM of cadmium chloride ($CdCl_2$, Sigma) (Cd group) and another exposed to 0.04 µM of methylmercury chloride (CH_3HgCl, Sigma) (Hg group). The exact quantity of three heavy metals (previously dissolved in a small volume of water) was administered directly into the aquarium water. Six fish per tank and group were sampled after 2, 10 or 30 days.

2.3. Skin mucus collection

Fish were anesthetized prior to sampling with 100 mg L^{-1} MS222 (Sandoz). Skin mucus samples were collected from naïve specimens using the method of Palaksha et al. [41] with some modifications. Briefly, skin mucus were collected by gentle scraping the dorso-lateral surface of naïve seabream specimens using a cell scraper with enough care to avoid contamination with blood and urino-genital and intestinal excretions. In order to get sufficient mucus, equal samples of mucus were pooled (2 pools of 6 fish) and homogenized with 1 volume of Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl). The homogenate was vigorously shaken and centrifuged (1,500 rpm, 10 min, 4ºC) being the supernatant lyophilized (Christ) following freezing at -80ºC.

Lyophilized skin mucus powder was dissolved in Milli-Q water, being the undissolved mucus portion isolated by centrifugation (1,500 rpm, 10 min, 4ºC). Protein concentration present in each sample was determined by the dye binding method of Bradford [42] using bovine serum albumin (BSA, Sigma) as the standard. The samples were adjusted to 500 µg protein ml^{-1} of skin mucus with Milli-Q water, aliquoted and stored at -20ºC until use.

2.4. Haemagglutination assay

Haemagglutination activity (HA) of two-fold serial dilutions of the samples was assayed in a 96-well microtitre U plate by using a 1% rabbit erythrocyte suspension (RBC) in TRIS-buffered saline (TBS: 50 mM Tris HCl, 0.15 M NaCl, pH 7.4) containing 0.1% (w/v) gelatin, which were previously washed with PBS (PBS-E: 6 mM KH_2PO_4, 0.11 mM Na_2HPO_4, 30 mM NaCl, pH 7.4). Erythrocytes were supplied by the *Istituto Zooprofilattico della Sicilia* (Palermo, Italy) and maintained in sterile Alsever's solution (27 mM sodium citrate, 115 mM D-glucose, 18 mM EDTA, 336 mM NaCl in distilled water, pH 7.2). Aliquots of 25 µl of serially diluted skin mucus samples were mixed with an equal volume of RBC suspension and incubated at 37°C for 1 h. To examine divalent cation requirements for HA, $CaCl_2$ or $MgCl_2$ was added in the HA medium to obtain a 5-10 mM final concentration. The haemagglutination titre (HT) was recorded as the reciprocal of the highest dilution showing complete agglutination. Skin mucus was replaced by TBS (containing 0.1% (w/v) gelatin) as negative controls.

2.5. Terminal glycosylation pattern determination

Glycosylation pattern in the skin mucus of gilthead seabream was determined by lectin ELISA as described previously [43]. Thus, 10 µg $well^{-1}$ of skin mucus samples were placed in flat-bottomed 96-well plates in triplicate and coated by overnight incubation at 4ºC with 100 µl of 50 mM carbonate-bicarbonate buffer, pH 9.6. After three rinses with 100 µl per well of PBS-T (0.1 M phosphate buffer (PBS) and 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% BSA in PBS-T, followed by three new rinses with PBS-T. Samples were then incubated for 1 h with 20 µg per well of biotinylated lectins (Table 1), washed and incubated with streptavidin horseradish-peroxidase (1:1,000; Life

Technologies) for 1 h. After exhaustive rinsing with PBS-T the plates were developed using 100 µl of a 0.42 mM solution of 3,3',5,5'- tetramethylbenzidine hydrochloride (TMB, Sigma), prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µl of 2M H_2SO_4 and the plates were read at 450 nm in a plate reader (FLUOstar Omega, BMG Labtech). Negative controls consisted of samples without skin mucus or without lectins, whose optical density (OD) values were subtracted for each sample value. Data are presented as the OD at 450 nm for each fish species and lectin used.

Table 1. Lectins used in ELISA, their acronym, and sugar binding

Acronym	Lectin source	Sugar binding specificity
BSL I	*Bandeiraea simplicifolia*	α-D-galactose, N-acetyl-α-D-galactosamine
PNA	*Arachis hypogaea*	β-D-galactose
UEA I	*Ulex europeaus*	α-L-Fucose
Con A	*Canavalia ensiformis*	α-D-mannose, α-D-glucose
WFA	*Wisteria floribunda*	N-acetyl-D-galactosamine
WGA	*Triticum vulgaris*	N-acetyl-β-D-glucosamine, N-acetylneuraminic acid
LEA	*Lycopersicon esculentum*	N-acetyl-β-D-glucosamine

2.6. Total immunoglobulin M levels

Total IgM levels were analyzed using the enzyme-linked immunosorbent assay (ELISA) [44]. Thus, mucus proteins were coated to wells, washed and blocked as described in section 2.5. The plates were then incubated for 1 h with 100 µl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1,000 in blocking buffer, Sigma). Washing, development and reading was carried out as above. Negative controls consisted of samples without skin

mucus or without primary antibody, whose optical density (OD) values were subtracted for each sample value.

2.7. Evaluation of enzyme activities

2.7.1. Lysozyme

Lysozyme activity was measured according to the turbidimetric method described by Parry et al. [45] with some modifications. One hundred µl of skin mucus samples diluted 1/2 with 10 mM PBS, pH 6.2, were placed in flat-bottomed 96-well plates in triplicate. To each well, 100 µl of freeze-dried *Micrococcus lysodeikticus* in the above buffer (0.3 mg ml^{-1}, Sigma) was added as lysozyme substrate. The reduction in absorbance at 450 nm was measured after 0 and 15 min at 22ºC in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. The units of lysozyme present in skin mucus were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) and the results were expressed as U mg^{-1} mucus proteins.

2.7.2. Alkaline phosphatase

Alkaline phosphatase activity was measured by incubating an equal volume of skin mucus sample with 4 mM p-nitrophenyl liquid phosphate (Sigma) in 100 mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$ (pH 7.8, 30ºC) as described by Ross et al. [46]. The OD was continuously measured at 1-min intervals over 3 h at 405 nm in a plate reader. The initial rate of the reaction was used to calculate the activity. One unit of activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol product in 1 min.

2.7.3. Esterase

Esterase activity was determined according to the method of Ross et al. [46]. An equal volume of skin mucus sample was incubated with 0.4 mM p-nitrophenyl myristate substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30ºC). The OD and activity was determined as above.

2.7.4. Peroxidase

The peroxidase activity in skin mucus samples was measured according to Quade and Roth [47]. Briefly, 30 µl of skin mucus sample were diluted with 120 µl of Hank's buffer (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. As substrates, 50 µl of 20 mM TMB and 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid and the OD was read at 450 nm in a plate reader. Standard samples without skin mucus samples were used as blanks. One unit was defined as the amount producing an absorbance change of 1 and the activity expressed as U mg^{-1} mucus proteins.

2.7.5. Ceruloplasmin

The ceruloplasmin oxidase activity was measured according to Dunier et al. [48]. Briefly, equal volume of skin mucus was incubated with 0.1% para-phenylenediamine in acetate buffer, pH 5.2, and 0.02 % sodium azide. The kinetic of increase of absorbance was followed at 550 nm for 15 min and 1 unit was defined as an increase of OD of 0.001 min^{-1}.

2.7.6. Protease

Protease activity was quantified using the azocasein hydrolysis assay according to the method of Ross et al. [46]. Briefly, equal volume of skin mucus sample was incubated with 100 mM ammonium bicarbonate buffer containing 0.7% azocasein (Sigma) for 19 h at 30ºC. The reaction was stopped by adding 4.6% trichloro acetic acid (TCA) and the mixture centrifuged (10,000 rpm, 10 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 µl $well^{-1}$ of 0.5 N NaOH, and the OD read at 450 nm using a plate reader. Skin mucus was replaced by trypsin (5 mg ml^{-1}, Sigma), as positive control (100% of protease activity), or by buffer, as negative controls (0% activity).

2.7.7. Antiprotease

Total antiprotease activity was determined by the ability of skin mucus to inhibit trypsin activity [49]. Antiprotease activity in skin mucus was very low and for this assay we used samples adjusted to 2 mg ml^{-1} of protein [50]. Briefly, 10 µl of skin mucus

samples were incubated (10 min, 22ºC) with the same volume of standard trypsin solution (5 mg ml^{-1}). After adding 100 µl of 100 mM ammonium bicarbonate buffer and 125 µl of 0.7% azocasein, samples were incubated (2 h, 30ºC) and, following the addition of 250 µl of 4.6% TCA, a new incubation (30 min, 30ºC) was done. The mixture was then centrifuged (10,000 rpm, 10 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 µl $well^{-1}$ of 0.5 N NaOH, and the OD read at 450 nm using a plate reader. For a positive control, buffer replaced skin mucus and trypsin, and for a negative control, buffer replaced the skin mucus. The antiprotease activity was expressed in terms of percentage trypsin inhibition according to the formula: % Trypsin inhibition = (Trypsin OD – Sample OD) Trypsin OD^{-1} x 100.

2.8. Bactericidal activity

Three opportunistic marine pathogenic bacteria (*Vibrio harveyi, V. angillarum and Photobacterium damselae* subsp. *piscicida*) and two non-pathogenic bacteria (*Escherichia coli and Bacillus subtilis*) were used in the bactericidal assays. Bacteria were grown in agar plates at 25ºC in the adequate media: tryptic soy (TSB, Sigma) for *V. harveyi, V. angillarum and P. damselae*, Luria (LB, Sigma) for *E. coli* and nutrient broth (NB, Conda) for *B. subtilis*. Then, fresh single colonies of 1-2 mm were diluted in 5 ml of appropriate liquid culture medium and cultured for 16 h at 25°C on an orbital incubator at 200-250 rpm.

The skin mucus bactericidal activity was determined by evaluating their effects on the bacterial growth curves using the method of Sunyer and Tort [51] with some modifications. Aliquots of 100 µl of each one of the bacterial dilutions (1/10) were placed in flat-bottomed 96-well plates and cultured with equal volumes of gilthead seabream skin mucus samples. The OD of the samples was measured at 620 nm at 30 min intervals during 24 h at 25ºC. Samples without bacteria were used as blanks (negative control). Samples without mucus were used as positive controls (100% growth or 0% bactericidal activity).

2.9. SDS-PAGE and western blot

SDS-PAGE (polyacrylamide gel electrophoresis under denaturing conditions) was performed on 7.5% acrylamide-bisacrylamide gel by the method of Laemmli [52].

Samples were diluted 1/2 with Milli-Q water and reduced conditions were obtained by treating the sample with 5% mercaptoethanol. After electrophoresis, protein bands were stained with Coomassie Brilliant Blue R250 (Sigma) and silver (Plus Silver Stain Kit, Sigma). To evaluate the molecular size, gels were calibrated with low range SDS-PAGE standard proteins (Sigma): aprotinin, bovine lung (6.5 kDa), α-Lactalbumin, bovine milk (14.2 kDa), trypsin inhibitor, soybean (20.0 kDa), trypsinogen, bovine pancreas (24.0 kDa), carbonic anhydrase, bovine erythrocytes (29.0 kDa), glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36.0 kDa), ovalbumin, chicken egg (45.0 kDa), glutamic dehydrogenase, bovine liver (55.0 kDa), albumin, bovine serum (66.0 kDa), phosphorylase B, rabbit muscle (97.0 kDa), β-galactosidase, *E. coli* (116.0 kDa), myosin, porcine heart (200.0 kDa).

Western blot was used to detect the presence of fucose-binding lectin (DlFBL) in the skin mucus. Thus, SDS–PAGE gels were soaked in transfer buffer (20 mM Tris, 192 mM glycine, 10% methanol, pH 8.8) for 10 min and proteins transferred for 60 min at 0.8 mA cm^{-2} to nitrocellulose sheet in a semi-dry blotting bath (Biorad, USA). The filter was soaked in blocking buffer PBS-T (PBS: 6 mM KH_2PO_4, 0.11 mM Na_2HPO_4, 30 mM NaCl, 1% Tween 20, pH 7.4) containing 3% BSA for 1 h. After washing with PBS-T, the nitrocellulose membranes were incubated with anti-DlFBL-antiserum (1:400 in PBS-T with 0.1% BSA) for 1 h, then washed 3 times in PBS-T and incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit sheep IgG (Sigma; 1:10.000 in PBS-T with 0.1% BSA). After washing 3 times with PBS-T the membranes were treated with 3 ml of 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT) liquid substrate system. Nitrocellulose membranes were scanned for image processing.

2.10. Reversed phase chromatography

Skin mucus samples were submitted to reversed phase chromatography (or reversed phase HPLC) on a silica column C18 Interchrom UP5ODB-25QS 250x4.6 mm (a hydrophobic chain of 18 carbon atoms is suitable for the separation of small proteins or peptides). Two hundred µl of each sample, diluted 1/4 with Milli-Q water, were loaded for analysis with a manual injector reodine and the reading was performed at 280 nm (mAU). Elution was achieved with a mixture of 0.05% trifluoroacetic acid in Milli-Q over 35 min at a flow rate of 1 ml min^{-1}.

2.11. Statistical analysis

Data are expressed as mean ± standard error (SE). Data were statistically analysed by one-way analysis of variance (ANOVA) to determine differences between groups. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal-Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPSS 19 and differences were considered statistically significant at a 95% of confidence level when the calculated F value for 5 degrees of freedom was exceed the theoretical value (F = 5.05).

3. RESULTS

3.1. Haemagglutination assay (HA) of skin mucus exposed to metals

The diluted skin mucus of gilthead seabream, exposed to different heavy metals, agglutinated rabbit erythrocytes at different concentrations. Thus, skin mucus of fish exposed to heavy metals showed decreased agglutination compared to the control samples (data not shown).

3.2. Glycosilation of skin mucus proteins exposed to heavy metals

All the tested terminal monosaccharide residues were present in the skin mucus samples on the studied fish species unexposed and exposed to heavy metals that differed within the sugars, exposure time and heavy metals studied (Fig. 1). In general, the lectin binding to skin mucus exposed to arsenic, cadmium and mercury was WGA>Con A>WFA> BSL I>PNA>LEA>UEA I as evidenced by the OD readings. This binding pattern suggests that terminal carbohydrates abundance in skin mucus is N-acetylneuraminic acid, glucose, N-acetyl-glucosamine, N-acetyl-galactosamine, galactose and fucose residues in descendent order or presence.

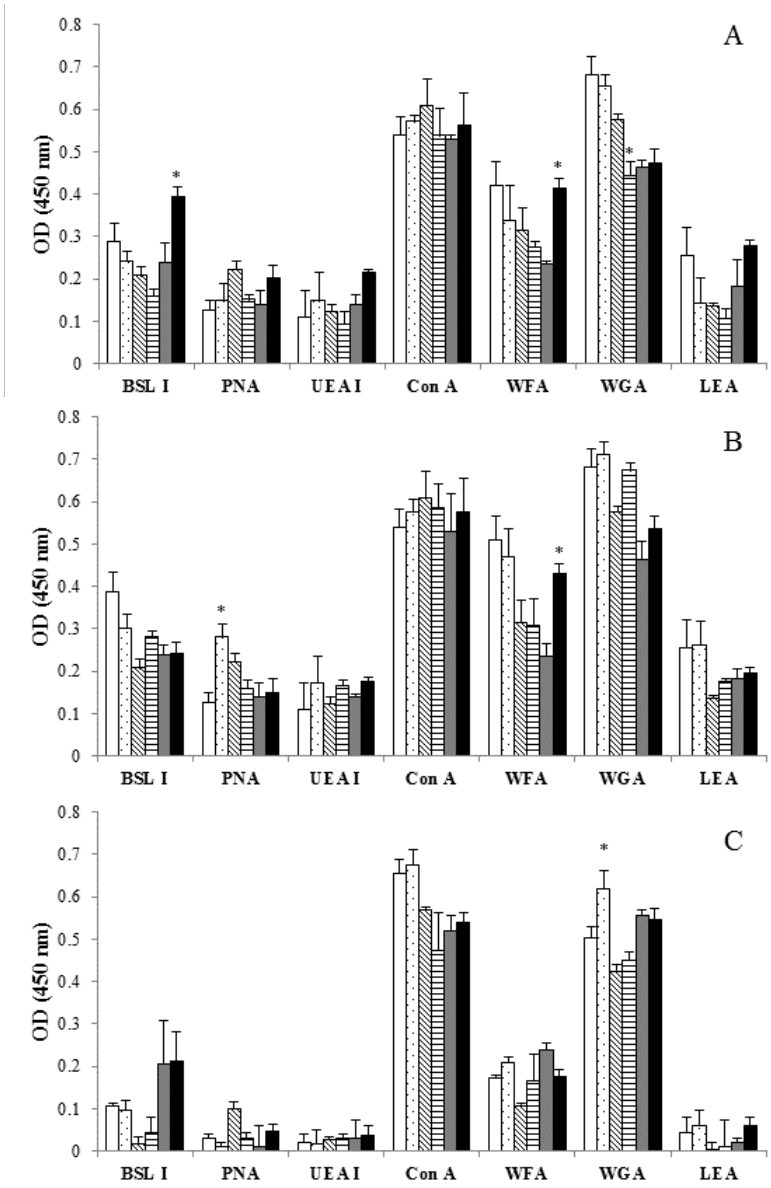

Fig. 1. Lectin binding (OD 450 nm) to carbohydrates present in skin mucus of gilthead seabream specimens unexposed (control) and exposed to arsenic (A), cadmium (B) and mercury (C). Data represent the mean ± SE (n=6). Asterisks denote significant differences between unexposed and exposed groups ($P \leq 0.05$). See Table 1 for lectin specificity.

When the treated fish were compared, all exposed-fish showed an increase of carbohydrate levels after 30 days of exposure with respect the values found in unexposed fish, being statistically significant in WFA after arsenic and cadmium exposure and BSL I only after arsenic exposure. Highlight an increase of carbohydrate levels after short exposure time (2 days) to PNA and WGA in fish exposed to cadmium and mercury, respectively (Fig. 1B and C). Therefore, it was observed a statistically

significant decrease of N-acetyl-glucosamine levels (WGA binding) after 10 days of exposure with arsenic (Fig. 1A).

3.3. IgM type natural antibody levels of skin mucus exposed to heavy metals

Mucus skin IgM levels were always slightly affected in fish exposed to any of the heavy metal assayed (Table 2). Only the exposure to methylmercury for 30 days produced an increase in the IgM levels.

Table 2. IgM level (OD 450 nm) and protease (%) and antiprotease (%) activities in the skin mucus of gilthead seabream specimens unexposed (control) or after waterborne exposure to arsenic (5 μM), cadmium (5 μM) and mercury (0.04 μM). Data represent the mean ± S.E. (n=6). Asterisks denote significant differences between unexposed and exposed groups (P≤0.05).

	Days of exposition	**Experimental groups**					
		Arsenic (5 μM)		Cadmium (5 μM)		Mercury (0.04 μM)	
		Unexposed	Exposed	Unexposed	Exposed	Unexposed	Exposed
IgM levels (OD 450 nm)	**2**	0.127 ± 0.011	0.123 ± 0.011	0.126 ± 0.011	0.117 ± 0.014	0.131 ± 0.009	0.120 ± 0.008
	10	0.121 ± 0.012	0.144 ± 0.027	0.122 ± 0.012	0.139 ± 0.004	0.126 ± 0.004	0.146 ± 0.011
	30	0.130 ± 0.015	0.132 ± 0.006	0.131 ± 0.015	0.157 ± 0.005	0.134 ± 0.006	0.188± 0.005*
Protease (%)	**2**	28.58 ± 1.81	25.81 ± 2.73*	28.98 ± 2.12	22.36 ± 2.87*	31.93 ± 3.45	59.21 ± 9.28*
	10	28.60 ± 0.86	33.94 ± 3.31*	29.08 ± 1.53	35.23 ± 3.21*	30.50 ± 6.07	41.40 ± 8.16*
	30	28.28 ± 1.93	28.03 ± 6.12	28.73 ± 2.42	22.22 ± 2.18*	31.76 ± 4.35	32.57 ± 9.12
Antiprotease (%)	**2**	9.07 ± 0.41	10.89 ± 0.31	8.70 ± 0.19	11.53 ± 0.29*	7.74 ± 0.42	8.07 ± 0.41
	10	9.11 ± 0.23	7.69 ± 0.17	8.44 ± 0.40	6.27 ± 0.50*	8.38 ± 0.33	7.14 ± 0.23
	30	8.97 ± 0.95	9.51 ± 1.01	8.97 ± 0.26	9.01 ± 0.16	7.58 ± 0.43	12.91 ± 0.53*

3.4. Enzyme activities in skin mucus

Protease, antiprotease, lysozyme, alkaline phosphatase, esterase, peroxidase and ceruloplasmin, activities found in skin mucus varied depending on the metals and exposure time (Tables 2 and 3). Protease activity decreased in fish mucus from specimens exposed to As or Cd for 2 days and also to Cd for 30 days, respect to the samples from control group. However, this activity increased in specimens exposed to As or Cd for 10 days. Furthermore, in those specimens exposed to As, protease activity increased after 2 and 10 days of exposure (Table 2). Regarding antiprotease activity, this increased in fish exposed to Cd and to As for 2 or 30 days, respectively.

Lysozyme activity increased in a statistically significant extent in samples of mucus from specimens exposed to As for 10 days, Cd for 10 and 30 days and Hg for 30 days, respect to the samples from control fish. In the case of alkaline phosphatase the activity was increased in As exposed fish for 10 and Hg exposed fish for 2 and 10 days. However this activity was reduced in mucus from fish exposed to As and Cd for 30 days compared to values from control or unexposed fish (Table 3).

Esterase activity was increased in fish exposed to As or Hg for 2 days and mainly for 10 days, or incubated with Cd for 2 and 30 days. Peroxidase activity present in mucus was slightly affected by the presence of heavy metals in the water. More concretely, this activity was increased by Hg after 10 days and decreased by As after 10 days of exposure. Finally, ceruloplasmin activity was significantly increased in As-exposed fish for 10 days and in Cd-exposed fish for 2 days. However, the activity decreased in Cd-exposed fish for 30 days. On the other han, Hg always provoked an increase in the activity that never reached significance (Table 3).

Table 3. Enzyme activities, expressed as U mg^{-1} protein, in the skin mucus of gilthead seabream specimens unexposed (control) or after exposure to arsenic (5 μM), cadmium (5 μM) and mercury (0.04 μM). Data represent the mean ± S.E. (n=6). Asterisks denote significant differences between unexposed and exposed groups (P≤0.05).

Heavy metal and days of exposition		Groups	Enzyme activities (U mg^{-1} protein)				
			Lysozyme	Phosphatase alkaline	Esterase	Peroxidase	Ceruloplasmin
As	2	Unexposed	36.71 ± 7.67	36.56 ± 2.75	36.76 ± 3.74	4.68 ± 0.24	29.32 ± 0.53
		Exposed	28.81 ± 3.76	48.86 ± 3.13	81.54 ± 4.25*	5.04 ± 0.39	37.31 ± 0.83
	10	Unexposed	36.66 ± 3.86	36.24 ± 2.15	40.77 ± 3.85	4.32 ± 0.49	45.31 ± 2.13
		Exposed	43.64 ± 4.12*	54.39 ± 2.43*	93.55 ± 4.95*	5.48 ± 0.28	59.17 ± 1.07*
	30	Unexposed	34.38 ± 6.46	38.53 ± 1.74	33.85 ± 2.33	5.04 ± 0.64	32.24 ± 0.63
		Exposed	32.71 ± 3.16	18.82 ± 1.97*	36.40 ± 2.89	3.28 ± 0.68*	22.92 ± 0.53
Cd	2	Unexposed	37.11 ± 6.69	36.12 ± 3.14	36.16 ± 2.91	4.76 ± 0.48	28.35 ± 0.53
		Exposed	35.56 ± 6.11	34.94 ± 2.73	62.97 ± 3.55*	4.96 ± 0.60	65.03 ± 5.33*
	10	Unexposed	35.26 ± 4.96	36.58 ± 2.58	40.32 ± 3.13	4.68 ± 0.24	44.32 ± 2.13
		Exposed	42.78 ± 4.84*	36.56 ± 2.86	52.05 ± 3.86	5.88 ± 0.27	54.90 ± 1.60
	30	Unexposed	35.98 ± 4.36	37.96 ± 3.67	34.21 ± 2.54	4.44 ± 0.65	31.23 ± 0.63
		Exposed	27.58 ± 3.04*	28.53 ± 1.84*	61.88 ± 3.52*	4.12 ± 0.36	17.46 ± 1.20*
Hg	2	Unexposed	23.68 ± 3.76	36.49 ± 1.92	44.59 ± 3.12	5.48 ± 0.77	69.83 ± 5.46
		Exposed	28.53 ± 3.16	58.33 ± 4.17*	79.32 ± 4.34*	5.88 ± 0.83	79.96 ± 5.99
	10	Unexposed	24.52 ± 3.76	30.87 ± 2.15	53.50 ± 3.18	4.86 ± 0.68	62.37 ± 1.61
		Exposed	28.04 ± 3.26	52.78 ± 3.68*	73.11 ± 3.78*	7.58 ± 0.80*	68.23 ± 3.22
	30	Unexposed	23.91 ± 4.51	38.17 ± 2.72	56.39 ± 2.35	5.28 ± 0.35	75.69 ± 2.25
		Exposed	40.94 ± 3.81*	28.21 ± 2.32	57.53 ± 2.92	6.61 ± 0.61	85.82 ± 4.82

3.5. Bactericidal activity

Bactericidal activity of skin mucus from gilthead seabream exposed to heavy metals against both pathogenic and non-pathogenic bacteria was determined (Table 4). The bacterial growth showed slight differences depending of the metal, time of exposure and bacteria. *V. anguillarum* growth was significantly reduced in skin mucus of specimens exposed to Hg for 2 and 10 days whilst the growth of *P. damseale* was reduced in the skin mucus of fish exposed to Hg for 10 days, respect to the values found in mucus of fish from control (unexposed) group. The bactericidal activity of seabream mucus

against *V. harveyi*, *E. coli*, *B. subtilis* and *S. putrefaciens* was not affected by heavy metals in a significant way, compared to the values from control fish.

Table 4. Bacterial growth in skin mucus of gilthead seabream specimens unexposed (control) or after exposure to arsenic (5 μM), cadmium (5 μM) and mercury (0.04 μM). Data represent the mean ± S.E. (n=6). Asterisks denote significant differences between unexposed and exposed groups (P≤0.05).

Bacteria	Days of exposition	Experimental groups					
		Arsenic (5 μM)		Cadmium (5 μM)		Mercury (0.04 μM)	
		Unexposed	Exposed	Unexposed	Exposed	Unexposed	Exposed
Vibrio harveyi	2	43.51 ± 2.01	44.46 ± 0.82	42.91 ± 2.81	44.70 ± 3.30	49.24 ± 1.27	46.10 ± 1.12
	10	43.23 ± 3.24	43.77 ± 2.66	42.58 ± 3.04	43.54 ± 1.75	43.38 ± 1.82	39.72 ± 2.54
	30	42.93 ± 3.73	45.28 ± 2.30	43.23 ± 2.78	42.60 ± 0.76	41.21 ± 2.61	44.90 ± 1.45
Vibrio angillarum	2	68.88 ± 4.54	63.85 ± 1.91	67.28 ± 4.74	65.67 ± 3.35	69.50 ± 1.36	53.92 ± 1.23*
	10	66.77 ± 1.84	64.88 ± 2.39	67.87 ± 1.24	72.65 ± 6.34	66.51 ± 8.53	50.48 ± 1.34*
	30	63.77 ± 2.26	66.61 ± 1.25	62.33 ± 2.66	61.08 ± 2.35	70.67 ± 6.15	67.21 ± 6.31
Photobacterium damselae	2	65.82 ± 5.48	62.74 ± 2.60	66.12 ± 4.58	65.42 ± 4.65	66.34 ± 3.01	69.33 ± 2.35
	10	62.61 ± 3.84	64.31 ± 3.90	63.11 ± 2.14	70.32 ± 8.62	67.97 ± 2.07	47.26 ± 1.27*
	30	60.65 ± 3.24	67.36 ± 2.33	61.35 ± 4.54	57.63 ± 0.77	64.01 ± 0.32	57.01 ± 0.98
Escherichia coli	2	105.51 ± 3.03	107.64 ± 0.66	104.61 ± 3.33	106.04 ± 4.79	122.84 ± 2.10	113.32 ± 1.38
	10	103.52 ± 4.09	99.91 ± 1.75	101.12 ± 3.34	106.28 ± 1.64	116.87 ± 4.35	109.68 ± 2.38
	30	103.59±2.02	101.84 ± 1.62	104.89 ± 2.54	102.67 ± 2.28	118.97 ± 1.94	117.73 ± 2.11
Bacillus subtilis	2	119.93 ± 7.32	125.99 ± 2.69	118.83 ± 5.66	112.33 ± 3.12	107.86 ± 2.09	98.14 ± 5.12
	10	125.81 ± 4.08	112.67 ± 1.22	124.61 ± 4.78	122.26 ± 3.32	109.97 ± 1.65	100.26 ± 3.02
	30	124.22 ± 0.98	123.36 ± 4.51	125.66 ± 2.78	110.33 ± 8.54	101.05 ± 6.68	100.29 ± 5.38
Shewanella putrefaciens	2	83.68 ± 2.12	81.14 ± 1.26	84.58 ± 2.76	83.45 ± 1.47	102.89 ± 1.98	102.94 ± 3.12
	10	80.21 ± 1.07	82.61 ±1.34	79.51 ± 1.65	83.39 ± 2.93	102.21 ± 1.34	98.42 ± 2.43
	30	84.61 ± 0.96	84.36 ± 0.21	86.67 ± 1.53	81.19 ± 2.17	100.58 ± 2.69	101.95 ± 7.02

3.6. SDS-PAGE and western blot

In the skin mucus from gilthead seabream exposed to heavy metals, by using gel electrophoresis, different bands of proteins were clearly seen (Fig. 2). Interestingly, all the samples followed a very similar pattern, independent of the waterborne heavy metal

used. Nevertheless, a band around 14 kDa was absent in fish exposed to As and Cd for 30 days (Fig. 2A and B, lanes 5 and 6).

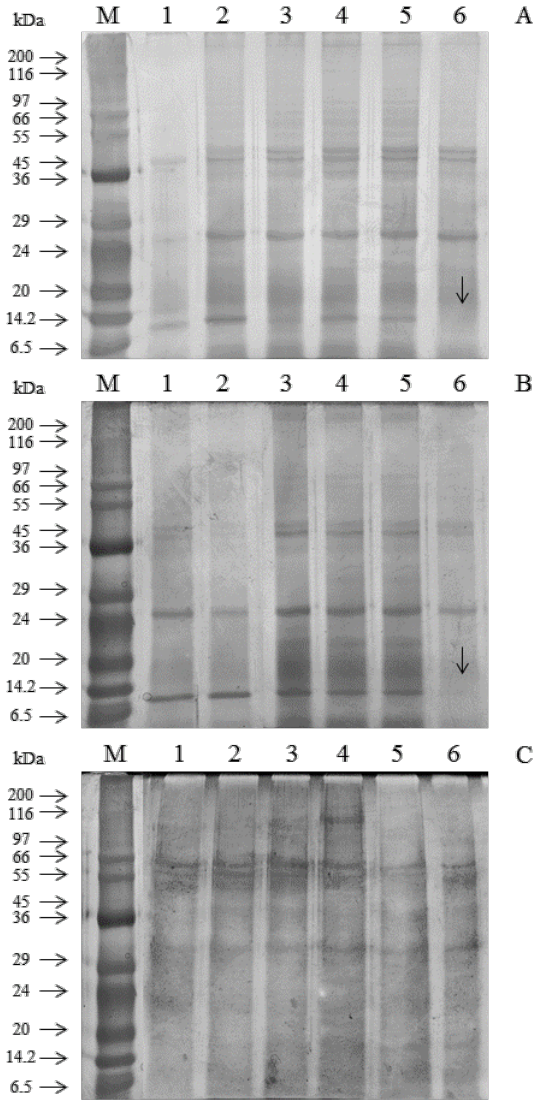

Fig. 2. SDS-PAGE of skin mucus of gilthead seabream specimens unexposed (control: lanes 1, 3 and 5) and exposed to arsenic (A), cadmium (B) and mercury (C) for 2 (lane 2), 10 (lane 4) and 30 days (lane 6). Gels were stained with silver nitrate. Arrowheads mark the position of the standards; molecular weights are given in kilodaltons (kDa).

Western blot revealed the absence of DlFBL in the skin mucus of control seabream specimens (Fig 3).. Interestingly, the fucose-binding lectin was present in the skin from all the exposed fish in samples of skin mucus from fish exposed for 30 days to As or Cd

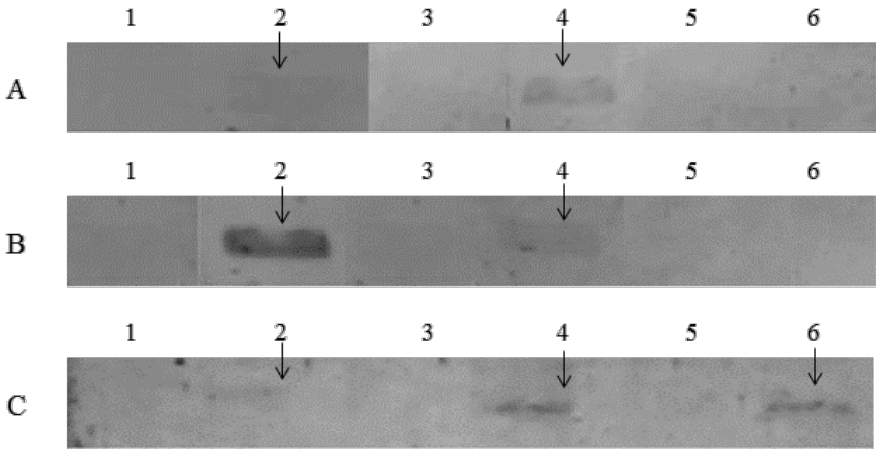

Fig. 3. Western blot to detect the presence of DlFBL in the skin mucus of gilthead seabream specimens unexposed (control: lanes 1, 3 and 5) and exposed to arsenic (A), cadmium (B) and mercury (C) for 2 (lane 2), 10 (lane 4) and 30 days (lane 6) in reducing conditions.

3.7. Reversed phase chromatography

Chromatograms showed a similar pattern of peaks between skin mucus of control fish (unexposed) and heavy metal exposed fish, however, this pattern varied in some peaks and absorbance intensity, depending on exposure time and type of metal (Fig. 4). In general, the peaks showing more variability were found at retention times around 200 min. Moreover, Cd exposure for 10 days as well Hg for 30 days produced an increase in the intensity of many peaks.

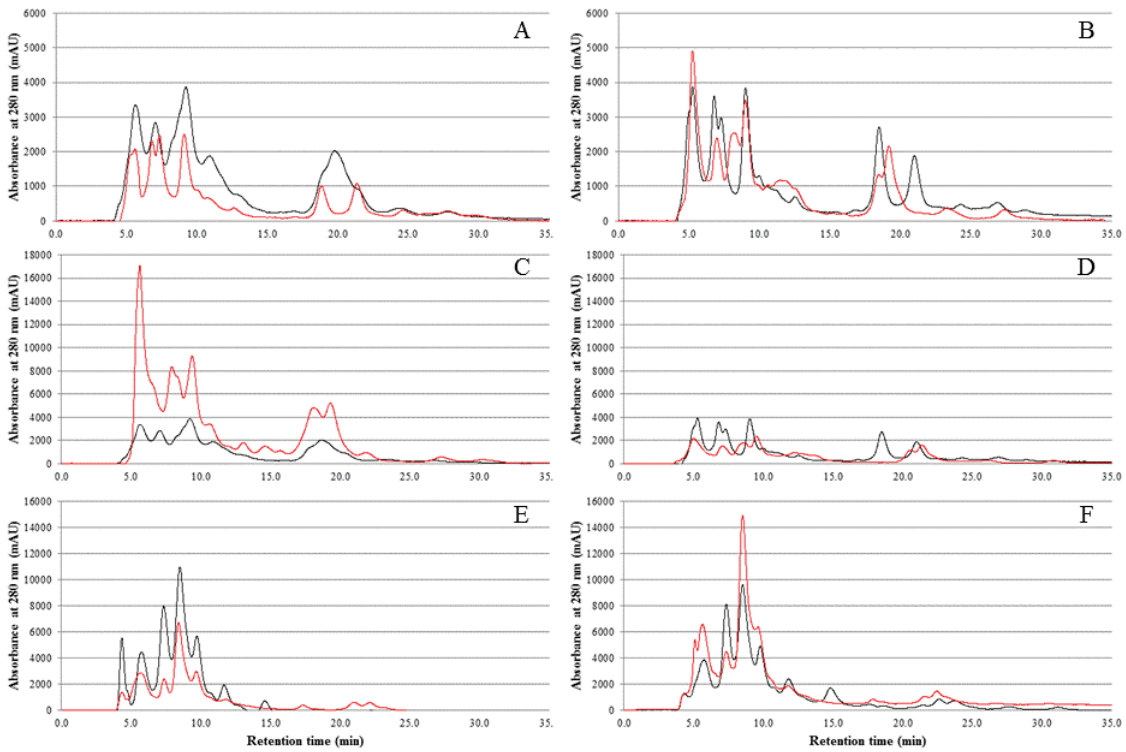

Fig. 4. Chromatographic profile of skin mucus from gilthead seabream specimens unexposed (black line) and exposed (red line) to arsenic (after 10 (A) and 30 (B) days), cadmium (after 10 (C) and 30 (D) days) (5 μM) and mercury (after 10 (E) and 30 (F) days). The profiles were determined by measuring absorbance at 280 nm.

4. DISCUSSION AND CONCLUSIONS

External and internal epithelial surfaces of fish are covered with a mucus layer providing protection against environmental factors like microorganisms, toxins, pollutants, acidic pH and hydrolytic enzymes [53]. Fish diseases may result from increased numbers of bacterial pathogens, from immune depression of fish due to pollution stress, or a combination of both [17]. Thus, the importance of fish skin is appreciated if it is considered as the interface between the external and internal of the organism.

The fish skin and mucosal surfaces are a key component of the innate immunity and they are considered the first line of defense. They are into direct contact with all

waterborne toxic chemicals, parasites, non-pathogenic and pathogenic microorganisms present in the aquatic environment [34]. In the case of the tegument, the barrier consists of an ephitelium coated by a mucus layer containing many kinds of biologically active (including defensive) molecules [54–57]. In this sense, skin mucus plays an important role in protecting fish skin against waterborne heavy metals [58]. One of the first responses of the skin epithelium to a stressful situation is the increase in the production and release of mucus by goblet cells (also called mucous cells and present in the epithelium), where the maturing cells differentiate to cells producing either acidic or neutral mucins [34]. Thereby, all the surfaces of the fish likely to be in direct contact with the environment would have a greater mucus layer during toxic episodes [59]. Nonetheless, the lethal and sublethal toxicity of chemicals to fish skin or any other target organ, with the exception of fish gills, has not been extensively studied. Previous studies carried out in gilthead seabream (one of the most cultured fish in the Mediterranean area) have revealed the main innate immune parameters present in the skin mucus pointing to a central role for the skin in the mucosal immunity [7,50]. Thus, we carried out the present study knowing the importance of the skin mucus in fish defence and taking into account that, to our knowledge, this is the first study that focus on the effects of heavy metal waterborne exposure in skin mucus of gilthead seabream. Furthermore, there is only one similar previous paper although they focus on the effect of waterborne zinc on the alteration of the epidermal response to a Monogenea (*Gyrodactylus turnbulli)* of the guppy (*Poecilia reticulata)* [58], but not in the skin mucus.

The innate immune system protects the organism against the invading pathogens individually and cooperatively being the recognition of foreign microorganisms the first step in the initiation of infectious diseases [60,61]. This recognition is mediated by host lectins, which have effector functions, such as agglutination, immobilization, and complement-mediated opsonization and killing of potential pathogens [62]. Many of these organisms use sugar-binding proteins as lectins to recognize and bind to host terminal carbohydrates [61,63]. The carbohydrate residues tested in this study are present in mammals and also have been observed in mucosal surfaces of fish such as skin, digestive tract and gills [64–67]. In our study, haemagglutination of rabbit erythrocytes was decreased at different degree by incubation with skin mucus from gilthead seabream exposed to heavy metals for short exposure time (2 and 10 days)

while after 30 days of exposure it was observed a decrease in agglutination in all the cases. This indicates that mucus lectins are also decreased by heavy metals. On the other hand, the terminal carbohydrates abundance in skin mucus is N-acetylneuraminic acid >glucose> N-acetyl-glucosamine> N-acetyl-galactosamine> galactose> fucose residues. Comparing the exposition to metals, an increase of carbohydrates was observed in all fish exposed to heavy metals for 30 days, being statistically significant to WFA (N-acetylneuraminic acid binding) for As and Cd exposure and BSL I (N-acetyl-galactosamine binding) only for As exposure. The increase of N- acetylneuraminic acid, the predominant sialic acid, may be associated with involvement in preventing infections hence it may be a response to exposure of these metals. In like manner, N-acetyl-galactosamine is related with the correct cell-to-cell communication, which is important for both normal systemic function and disease processes as inflammation and immunity [68]. In addition, an increase in PNA and WGA binding to carbohydrates was also observed after short time (2 days) in fish exposed to Cd and Hg, respectively, while it was observed a statistically significant decrease of N-acetylneuraminic acid after 10 days of exposure to As. The decline of sialic acid detectable by WGA in As-exposed fish could aid to explain the higher susceptibility to infections. This was corroborated in a previous study where it was observed a remarkable decrease in the expression of sialic acid in the digestive tract, detectable by SNA lectin (*Sambucus nigra*), in gilthead seabream parasitized by *Enteromyxum leei* [69]. Thus, sialic acid could protect initially the intestine of this fish from invasion and dispersion of parasites, nevertheless, a dramatic decrease in the expression of N-acetylneuraminic acid occurred with the infection progression. Therefore, further studies are needed in the characterization of mucus carbohydrates in order to understand their precise role in pathogen adhesion and invasion, as well as, if their presence is related to disease resistance or not.

Regarding to IgM, antibodies, they play a crucial role in protecting fish from invading agents in the habitat inasmuch as their level and evolutionarily important specificities are sufficient to protect against a relevant infection [41,70]. It is known that both adaptive and innate immune factors are present in the fish skin mucus. Fish from polluted waters are subject to increased prevalence of diseases therefore they respond to bacterial pathogens by producing serum antibodies [71]. In agreement with this, in the present study, the concentration of Ig detected in the skin mucus of exposed fish was decreased at short exposure time (2 days) while it was increased for the rest of

experimental groups and exposure times with respect to unexposed (control) fish. The waterborne Hg exposure showed the most significant increase in the level of IgM antibodies after 30 days of exposure. When comparing data from a short exposure time, they correlated with the results obtained in serum of seabream exposed to Cd and As [72,73]. Though, after long exposure time, the values of IgM present in serum and skin mucus were only correlated in the Cd-exposed fish [72] but not in fish exposed to As [73]. On the contrary, Sánchez-Dardon et al. [74] did not observe any variation on Ig serum level in rainbow trout exposed for 30 days to Cd or Hg. However, Tellez-Bañuelos et al. [75] demonstrated a significantly increased secretion of IgM serum in Nile tilapia (*Oreochromis niloticus*) exposed to endosulfan and challenged with *Aeromona hydrophila*. Therefore, we could state that the increase of these immunoglobulins in the skin mucus could be a good indicator of contaminated waters; however more studies would be needed to confirm the apparent interespecific variations observed till now.

The presence of extracellular plasma proteins in the mucus could be a result of a number of mechanisms. One of these mechanisms is the direct expression of the proteins by the cells of the epidermis. A second possibility is leakage from the plasma into the mucus. Extracellular proteins may also enter the mucus by a “shuttling” effect of the secondary circulatory system that bridges the fish vascular system, blood and mucus [76]. Distribution of the secondary circulatory system throughout the gills, fins and skin of fish identify a convenient mechanism for transfer and exchange between mucus and plasma that may “filter” plasma proteins [77]. Thus, the epidermal mucus enzymes may play an important role in the fish immune functions, including the role of proteases, antibacterial agents and other compounds related to the immune system. Therefore, lysozyme, alkaline phosphatase, esterase, peroxidase, ceruloplasmin, proteases and antiproteases were measured in the present study because there are very few available studies where some of these enzymes have been studied in the mucus from fish exposed to metals by bath [78], or by diet [79]; to parasites [80] or other pathogens [81]. The basal levels of lysozyme and proteases in the mucus protect the organism from bacteria living in the same environment [78,82]. These enzymes, the most studied immune activities in fish and two of the most important against bacteria, had different levels in all the exposed-fish. Consequently, the role of lysozyme, proteases and antiproteases has been related with the defence against bacterial or

parasite infections. Proteases are essential for activation of both the innate and adaptive immune systems. Furthermore, they may play a protective role against pathogens by: i) directly degrading pathogens [8], ii) hampering their colonization and invasion due to modifications in the consistency of mucus surfaces and/or increasing the sloughing of these mucus layers [83], and iii) activating and enhancing the production of other innate immune components present in fish mucus such as complement, immunoglobulins or antibacterial peptides [82,84,85]. In our study, lysozyme and protease activities showed an increase in all fish exposed to Hg, while in fish exposed to As and Cd groups did it only after 10 days of exposure; furthermore, these enzymes were diminished in skin mucus of fish exposed to such metals for 30 days. Contrarily, antiproteases activity increased in most of exposed-fish (except after 10 days). Interestingly, the production of antiproteases was negatively correlated with proteases activity in fish exposed to As and Cd, perhaps in order to compensate the variations the levels of active mucus proteases. The correlation between lysozyme and protease activity in our study was not found in the response of fish mucus from different salmonids after been incubated with *Lepeophtheirus salmonis* [86]. Stabili and Pagliara [78] had demonstrated an inhibition of the lysozyme-like activity in mucus of the seastar *Marthasterias glacialis* exposed to zinc.

Other studied enzymes were the alkaline phosphatase and esterase, which are also present in skin mucus but their role in mucosal immunity is not well understood [14]. Alkaline phosphatase has been shown to act as an antibacterial agent in mucus, due to its hydrolytic activity, and to increase in fish after physical or chemical stress, skin regeneration, immunostimulation and bacterial and parasitic infections [46,87–90]. In our study, alkaline phosphatase activity was reduced after long time exposure with metals, while esterase activity was increased in all exposed fish independently of the exposure time. In some studies, increases of alkaline phosphatase activity in skin mucus of Atlantic salmon after *L. salmonis* infection were observed but the source of this variation is not known [46,80,91]. According to our results, we could advance that the source of the variations found is the exposure to these heavy metals. Regarding peroxidases, they are important microbicidal agents that effectively eliminate H_2O_2 and maintain the redox balance of immune system [92]. Thus, it could be considered that peroxidase in mucus will be essential for mucosal immunity and skin defense. Thus, in our study, it was observed inhibition of peroxidase activity after long exposure time to

As and Cd while this activity increased in fish exposed to Hg, which presupposes an important role in skin defence against metals as Hg. Regarding the last enzyme studied, ceruloplasmin, it is a major antioxidant protein being an acute-phase protein, released in response to infection and inflammation and playing an important role as anti-inflammatory agent, copper transport from hepatocytes to other tissues, regulator for hepatic iron mobilization as well as an eliminator of free radicals and superoxide [93,94]. Metal-binding proteins such as ceruloplasmin has special functions in the detoxification of toxic metals, and also play a role in the metabolism and homeostasis of essential metals [95]. In addition, this enzyme is believed to function in a variety of defence related activities which include limiting the dispersal of infectious agents, repairing tissue damage, killing microbes and other potential pathogens [96]. Equally to peroxidase, ceruloplasmin activity was decreased after long exposure time to As and Cd, while this activity was always increased after Hg-exposure. Similarly, ceruloplasmin levels were elevated after infection with pathogens in rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprius carpio*) [97,98]. In the same way, catfish ceruloplasmin gene was significantly up-regulated in the liver after bacterial infection [99]. Moreover, the level of this enzyme in plasma was increased in rainbow trout fed with different doses of sulfamerazine [100]. Therefore, most of the enzymes measured were affected by the exposure to metals, which could serve as good evaluators of adverse situations caused by contamination.

The prevention of colonization by aquatic parasites, bacteria and fungi is mediated both by immune system compounds (IgM, lysozyme, protease, etc.) and by antibacterial peptides and polypeptides. Independently of the effector substances and the mechanisms involved in the bacterial killing, the evaluation of the parameter of bactericidal activity of the skin mucus could be more important than single enzymatic activities. In this sense, several articles have studied the antimicrobial activity of skin mucus extracts in a numerous fish species, revealing a strong anti-bacterial and anti-fungal activity against a broad range of microbial pathogens and fungi [101–106]. It is now well established that the epidermal mucus of fish plays a significant role in the mechanical and physiological protection of fish against unfavourable environmental conditions and pathogenic infections [101]. Unfortunately, few reports have studied whether marine bacteria inhabiting environmental seawater can easily attach to the fish and/or grow in the mucus after the exposure to stressors such as contaminants or heavy metals. Our results

showed slight differences in bacterial growth dependent on metals exposure, time of exposition or bacteria. However, we could remark that generally the growth of bacteria was reduced in skin mucus of Hg exposed fish. Several studies have reported reduced fish resistance against bacterial challenges after exposure to contaminants [107–110]. Further, the results obtained by Song et al. [111] suggested that immune suppression of the fish occurred due to heavy oil stressor, and bacteria could invade the mucus, resulting in the increasing blood leukocyte number. Changes observed in these studies could result from direct effects of the metals on the bacteria or indirect effects mediated through the host. In the case of seabream exposed to Hg during 30 days, the skin mucus of exposed fish increased the bactericidal activity against the pathogenic *V. anguillarum* and *P. damselae* though As and Cd failed to change it. Unfortunately, no clear correlations between the bactericidal activity and the rest of determined enzymes and proteins could be made. In addition, it has been hypothesised that pathogenic bacteria may coat in sialic acid, providing resistance to components of the host's innate immune response or use it as a nutrient [112] but these factors have not related herein. Moreover, lectins that exhibit agglutination activities, as those described in pufferfish mucus, may play an antibacterial function, though this has been described against a limited number of bacterial species [113,114]. Hence, further studies on skin mucus could be developed in order to understand the implication of other antimicrobial compounds, or the sum of many factors together, in the bactericidal activity in the light of the data obtained.

By using SDS-PAGE the protein profiles were identified in fish skin mucus. Nevertheless, the most relevant difference consequence of the exposure to heavy metals was the absence of a band around 14 kDa in fish exposed for 30 days to As and Cd. Moreover, immunoblotting analysis revealed the appearance of a fucose-binding lectin, previously identified in the serum of *Dicentrarchus labrax* (DlFBL) [115], in skin mucus of fish exposed to waterborne metals; however, this fuco-lectin was absent in control fish and disappeared after 30 days of exposition to As and Cd. These results could support the hypothesis that the exposition of fish to As and Cd for longer time, could cause a reduction in fish defence, contrary to what might happen when the fish are exposed to Hg. To further confirm whether exposure to metals could alter protein pattern, chromatography analysis was also performed. A similar pattern of peaks was found between skin mucus of unexposed and exposed fish, nevertheless, it was observed different intensities depending on exposure time and metal. Thus, seabream

exposure to Cd for 10 days and to Hg for 30 days resulted in the greatest differences compared to control fish. Therefore, we could say that long-time exposure to As and Cd could alters the defences of fish, while the concentration of Hg used in this work does not seem to weaken the defense system of gilthead seabream. Nevertheless more studies are needed to confirm this hypothesis in relation to time and concentrations of exposure.

In conclusion, it was found that the skin mucus of seabream exposed to heavy metals showed little changes in the carbohydrate profiles and most of the immune responses determined were increased, mainly by Hg. This might correlate with the lower bacterial growth (higher bactericidal activity) in the skin mucus of Hg-exposed seabream specimens. Moreover, some differences in the protein profiles determined by SDS-PAGE and reversed phase chromatography are attributable to the exposure to heavy metals though whether they are related to the immune functions determined deserves further characterization. Therefore, further characterization of the fish mucus enzymes and their precise role in the mucosal immunity would be necessary to understand the effects of certain contaminants in the innate immune system of fish.

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CONCLUSIONS

Heavy metal immunotoxicology and skin mucus in fish

FA Guardiola 2014

CONCLUSIONS

1. Gilthead seabream exposure to waterborne arsenic increases the hepato-somatic index, enhances the innate immune parameters and produces histopathological alterations in liver after short time, though it is only bioaccumulated in the liver of gilthead seabream, but not in the muscle, after long exposure.

2. Waterborne exposure to cadmium produces acute toxicity in gilthead seabream provoking immunotoxicological effects at humoral and cellular levels, histopathological alterations in liver and pancreas, and it is accumulated in the liver ant in the muscle.

3. Gilthead seabream exposed to waterborne mercury causes acute toxicological effects such as histopathological alterations in liver and skin and increments in the hepatosomatic index, antioxidant enzymes and innate immune parameters. Moreover, the expression of genes related to toxic metabolism, cellular stress and apoptosis are up-regulated in the skin but not in the head-kidney.

4. Gilthead seabream skin mucus contains lower levels of IgM, similar activities of lysozyme, alkaline phosphatase and proteases, and higher esterase, peroxidase and antiprotease activities than serum. Interestingly, skin mucus reveals stronger bactericidal activity against fish pathogenic bacteria compared to the serum, while human bacteria can even grow better in the presence of mucus.

5. Physico-chemical parameters in skin mucus of several species of marine teleosts show clear correlations, which could have an important role in pathogen adhesion and invasion.

6. Skin mucus of the marine teleosts seabream, sea bass, common dentex, shi drum and dusky grouper showed significant variations in the carbohydrate profile, innate immune enzymes and bactericidal activity, which could be related with differential resistance to pathogens.

7. Exposure of gilthead seabream to waterborne heavy metals provoked little variations in the carbohydrate and protein profiles of skin mucus, whilst most of its immune parameters were increased, being more evident in the case of mercury. Some of these parameters could be useful as potential biomarkers for fish toxicology.

RESUMEN EN CASTELLANO

Inmunotoxicología producida por metales pesados y caracterización del moco de piel en peces

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RESUMEN EN CASTELLANO. Inmunotoxicología producida por metales pesados y caracterización del moco de piel en peces

1. RESUMEN

Durante la realización de la presente Tesis Doctoral se han estudiado los efectos inmunotoxicológicos de la exposición mediante baño a arsénico, cadmio y mercurio en la dorada (*Sparus aurata* L.), se han identificado y caracterizado diferentes parámetros inmunológicos y físico-químicos del moco de la piel de cinco peces teleósteos marinos y, en el caso de la dorada se han comparado con la inmunidad presente en el suero, y finalmente se evaluaron los efectos de estos metales pesados en la inmunidad de la mucosa de la piel de la dorada, una especie que posee la mayor tasa de producción en la acuicultura Mediterránea. Esta Tesis Doctoral se presenta en tres bloques con un total de siete capítulos.

El primer bloque de esta Tesis consta de tres capítulos. En primer lugar, hemos evaluado los efectos de la exposición en baño a concentraciones sub-letales de arsénico (As), cadmio (Cd) y mercurio (Hg) en la dorada, con especial atención en la respuesta inmunitaria innata. Cada uno de los estudios realizados con estos metales se corresponde con un capítulo de la presente Tesis.

1. En primer lugar demostramos que la exposición de doradas a As hace que dicho metal se bioacumule en el hígado, pero no en el músculo, y produzca un aumento del índice hepato-somático y alteraciones histopatológicas hepáticas tales como hipertrofia, vacuolización y muerte celular. Con respecto a la respuesta inmunitaria, los parámetros inmunitarios humorales (niveles de inmunoglobulina M (IgM) y actividades hemolítica del complemento y peroxidasa) no se vieron afectados de manera estadísticamente significativa. Por otro lado, los parámetros inmunitarios innatos celulares de los leucocitos de riñón cefálico (RC), tales como la actividad peroxidasa, la explosión respiratoria y la fagocitosis, se incrementaron significativamente después de 10 días de exposición, en comparación con los valores encontrados en los peces control. En general, la exposición a As en la dorada afecta al sistema inmunitario y podría interferir con la biología de los peces, la gestión de la acuicultura o con sus consumidores, incluyendo a los humanos.

2. En el segundo capítulo, encontramos que la exposición de doradas a Cd produce su acumulación en el hígado y en el músculo y alteraciones histopatológicas en hígado y páncreas exocrino, pero sin alterar los índices hepato- o espleno-somáticos de dichos ejemplares. Con respecto al potencial inmunotoxicológico, la exposición a Cd produce una reducción en la actividad hemolítica del complemento en el suero y en la explosión respiratoria de los leucocitos de RC de una manera significativa después de 10 y 30 días de exposición, mientras que la actividad de la peroxidasa sérica y la fagocitosis de los leucocitos aumentaron en diferentes momentos del experimento. Por otro lado, los niveles de IgM en el suero y la actividad peroxidasa de leucocitos resultaron inalterados. Los presentes resultados parecen indicar que los ejemplares de dorada expuestos a Cd en las presentes condiciones sufren una toxicidad aguda y podrían presentar un cierto riesgo para los humanos.

3. En el último capítulo de este primer bloque, se han evaluado los efectos del Hg en la dorada. En primer lugar, se confirmaron los efectos toxicológicos del metilmercurio (MeHg) porque los ejemplares de dorada expuestos en baño a este metal muestran un aumento de las enzimas antioxidantes del hígado (superóxido dismutasa, catalasa y glutatión reductasa) después de 2 días, un aumento en el índice hepatosomático después de 10 días, alteraciones histopatológicas en el hígado y en la piel, así como una sobre-expresión de genes relacionados con el metabolismo de xenobióticos (CYP1A1), el estrés celular (HSP-70 y HSP-90) y la apoptosis (CASP-3) en la piel, pero no en el RC, lo que indicaría la importancia de este tejido por estar en contacto directo y prologando con el tóxico. En cuanto al sistema inmunitario, la actividad hemolítica del complemento y peroxidasa en el suero se incrementaron en los ejemplares expuestos a MeHg, pero sólo se observaron diferencias estadísticamente significativas para la primera actividad después de 30 días de tratamiento. Por otro lado, la actividad peroxidasa, la explosión respiratoria y la actividad fagocítica de los leucocitos de RC se incrementaron aunque sólo la fagocitosis y la peroxidasa de los leucocitos mostraron un aumento significativo después de 10 y 30 días, respectivamente. En general, nuestros datos demuestran que la exposición a una concentración subletal en baño de MeHg produce efectos tóxicos agudos y un aumento de los parámetros inmunitarios en la dorada.

El segundo bloque de esta Tesis Doctoral también contiene tres capítulos. En ellos, se han estudiado diferentes factores humorales del sistema inmunitario en el moco de dorada, los cuáles han sido comparados con los mismos parámetros presentes en suero. Además, se ha realizado una caracterización físico-química del moco de la piel de dorada. Estos resultados se han comparado con otras especies de teleósteos marinos: la lubina (*Dicentrarchus labrax* L.), el verrugato (*Umbrina cirrosa* L.), el dentón (*Dentex dentex* L.) y el mero (*Epinephelus marginatus* L.).

4. En este primer capítulo sobre la caracterización del moco de la piel de peces nos propusimos identificar y caracterizar los diferentes mecanismos de defensa humorales constitutivos de la mucosa de la piel de la dorada. Para ello, los niveles totales de inmunoglobulina M, varias enzimas y proteínas (peroxidasa, fosfatasa alcalina, lisozima, esterasas, proteasas y antiproteasas), así como la actividad bactericida frente a patógenos de peces (*Vibrio harveyi*, *V. anguillarum* y *Photobacterium damselae*) y bacterias no patógenas (*Escherichia coli* y *Bacillus subtilis*) se midieron en el moco de la piel. Además, dichas actividades se compararon con las encontradas en el suero. Este estudio demuestra que el moco de la piel de la dorada contiene, con respecto al suero, niveles más bajos de IgM, niveles similares de lisozima, fosfatasa alcalina y proteasas, y niveles más altos de esterasas, peroxidasas y antiproteasas. Además, se observó que el moco de la piel posee una fuerte actividad bactericida contra bacterias patógenas de dorada en comparación con la actividad presente en el suero, mientras que las bacterias no patógenas de peces pueden incluso crecer mejor en presencia de moco. Estos resultados pueden ser útiles para comprender mejor la función de la mucosa de la piel como un componente clave del sistema inmunitario innato, el cuál es de vital importancia para la salud de los peces, y por lo tanto para la gestión de la acuicultura.

5. En este capítulo se evaluaron las propiedades físico-químicas, tales como la concentración de proteínas, el pH, la conductividad, el potencial redox, la osmolaridad, la densidad y la viscosidad, así como un barrido diferencial de calorimetría (DSC), del moco de la piel de las cinco especies de teleósteos marinos ya citadas anteriormente. Se observa una correlación entre el pH, la conductividad y el potencial redox en el moco de la piel de todas las especies estudiadas. Por otra parte, generalmente, se observó una

interrelación clara entre la densidad y la osmolaridad así como entre la densidad y la temperatura. La viscosidad mostró una relación indirecta entre la velocidad de cizalla y la temperatura. Finalmente, las mediciones microcalorimétricas confirmaron la presencia de sustancias con diferentes estructuras en el moco de las diferentes especies estudiadas, las cuáles son más estables en *S. aurata* y *D. labrax* que en el resto de especies. Los resultados se han discutido con el fin de elucidar la posible relación entre los parámetros físico-químicos de la mucosa de la piel con la susceptibilidad a enfermedades debido a la presencia y actividad de los diversos factores antibacterianos encontrados.

6. En el último capítulo de este segundo bloque, el sexto, la presencia de hidratos de carbono terminales, los niveles totales de IgM y varias enzimas relacionadas con la inmunidad (lisozima, peroxidasa, fosfatasa alcalina, esterasas, proteasas y antiproteasas), así como la actividad bactericida (contra los patógenos de peces *V. harveyi*, *V. angillarum* o *P. damselae* y bacterias no patógenas como *E. coli*, *B. subtilis* y *S. putrefaciens*) fueron identificadas, medidas y comparadas en el moco de la piel de los cinco teleósteos marinos ya mencionados. En primer lugar, los resultados de unión a lectinas sugieren que las glicoproteínas de las mucosas de la piel contienen, en su parte glucídica, en orden de abundancia, ácido N-acetilneuramínico, glucosa, N-acetil-glucosamina, N-acetil-galactosamina, galactosa y residuos de fucosa. En segundo lugar, los resultados mostraron que, si bien algunas de las actividades inmunitarias fueron muy similares en todos los peces estudiados (por ejemplo, la actividad de la lisozima y los niveles de IgM) otras, tales como las actividades de la proteasa, antiproteasa, fosfatasa alcalina, esterasa y peroxidasa, varian dependiendo de las especies de peces. Se encontraron los niveles más altos de actividad peroxidasa y proteasa en *U. cirrosa* mientras que *E. marginatus* y *S. aurata* mostraron los mayores niveles de actividad de fosfatasa alcalina y esterasa, respectivamente. Por otra parte, el moco de la piel de *S. aurata* mostró la actividad bactericida más alta contra las bacterias patógenas, contrariamente a lo que ocurrió con las bacterias no patógenas (*E. coli* y *B. subtilis*). Así, el estudio de las variaciones en el perfil de hidratos de carbono y los componentes relacionados con la inmunidad de la mucosa de la piel podrían ayudar a comprender la resistencia a enfermedades de los peces, así como la presencia y distribución de los

patógenos y la magnitud de las infecciones, aspectos que son de gran importancia para la industria de la acuicultura.

El tercer y último bloque de esta Tesis Doctoral contiene un único capítulo.

7. Finalmente, en el capítulo siete se investigó si los parámetros inmunitarios innatos de la mucosa de la piel de la dorada se veían afectados por la presencia en baño de As, Cd y Hg. Por otra parte, se estudiaron el perfil proteico mediante cromatografía líquida de alta presión (HPLC) y electroforesis desnaturalizante en geles de acrilamida (SDS-PAGE) así como la detección de la lectina que une a fucosa (FBL) mediante *western blot*. Este estudio demuestra algunos cambios en la composición del moco y en los parámetros inmunitarios después de la exposición a metales pesados. En general, el perfil de carbohidratos sufrió pequeños cambios mientras que la mayoría de las actividades enzimáticas se incrementaron después de la exposición. Curiosamente, el Hg provocó los incrementos más importantes (los niveles de IgM, la presencia de FBL y la actividad bactericida) en el moco de la piel. Los perfiles de proteínas obtenidos a través de SDS-PAGE y HPLC mostraron pocas variaciones aparentes en el moco de la piel dorada después de la exposición a metales pesados. Los resultados podrían ser útiles para una mejor comprensión de la función y el comportamiento de la inmunidad de la mucosa en la piel como un componente clave del sistema inmune innato, y algunos de estos parámetros podrían ser considerados como biomarcadores en la toxicología de los peces.

2. INTRODUCCIÓN

La demanda mundial de alimentos de origen marino, en particular los productos de pescado se ha triplicado entre 1961 y 2001, debido al crecimiento demográfico y el aumento del consumo *per cápita* de pescado. De acuerdo con la FAO (Organización para la Agricultura y la Alimentación de las Naciones Unidas), la pesca extractiva sólo cubre el 60% de la producción mundial de pescado anual [1], una situación en la que la acuicultura es vista como la única manera de satisfacer la demanda en un futuro más cercano. En concreto, España es el tercer país miembro de la Unión Europea (UE) con una mayor producción de pescado procedente de la acuicultura, así como en la producción de dorada, emergiendo como un área de actividad económica de gran importancia estratégica.

El éxito de la acuicultura moderna se basa en el control de la reproducción, un buen conocimiento de la biología de los peces de cultivo, en la innovación tecnológica y en el desarrollo de un alimento específico. Sin embargo, hay algunos desafíos importantes para el desarrollo de la acuicultura productiva, factible y sostenible en los sistemas intensivos actuales. Uno de estos retos es que en las instalaciones de producción a gran escala, donde los animales acuáticos están expuestos a condiciones de estrés, los problemas relacionados con las enfermedades y el deterioro de las condiciones ambientales a menudo resulta en pérdidas económicas [2]. En la agricultura intensiva, los animales son sometidos a condiciones de estrés que debilitan su sistema inmunitario, lo que aumenta la susceptibilidad a los patógenos, favoreciendo así la aparición de enfermedades. Por otra parte, desde hace tiempo se sospecha de un vínculo entre la contaminación ambiental y las enfermedades en las poblaciones de peces [3]. Esta conexión podría ser debido a la deficiencia del sistema inmune innato [4], donde la estructura y la composición celular de la epidermis (una de las principales barreras inmunitarias innatas), así como superficies mucosas, pueden verse afectados por factores de estrés, tales como patógenos y contaminantes ambientales [5–7].

Muchos de los contaminantes del medio ambiente afectan a los diferentes animales acuáticos en cierto grado dependiendo de la sustancia tóxica, su concentración, vida media, así como el comportamiento de los animales y la biología de los mismos [8].

Entre las cuestiones importantes a considerar en el sector de la acuicultura, encontramos el impacto de los contaminantes ambientales en las especies producidas por y para el ser humano, que debe ser controlado por el acuicultor [8]. En este campo específico, la mayoría de los estudios disponibles han evaluado los efectos tóxicos en términos de viabilidad o la inducción de tumores utilizando diferentes modelos de peces. Sin embargo, las especies de peces pertinentes para la acuicultura han sido menos utilizadas en estos experimentos. Por otra parte, el impacto de los contaminantes ambientales en la respuesta inmunitaria de estos peces, y por consiguiente en la resistencia a las enfermedades, han recibido mucha menos atención [8].

Además de estos aspectos aplicados, los peces tienen una posición filogenética clave representando el primer grupo animal que posee un sistema inmunitario innato, que está constituido por barreras físicas y químicas, efectores celulares (leucocitos) y humorales, y un sistema inmunitario adaptativo, que está constituido por linfocitos y los anticuerpos que producen. Por ello, el estudio de la inmunología de este grupo de vertebrados tiene un interés científico básico. Así, el sistema inmunitario de vertebrados presenta un patrón común aunque esto no excluye la existencia de diferencias importantes entre ejemplares de una misma especie o entre especies diferentes de vertebrados. Quizás la diferencia más importante sea el desarrollo y la preponderancia de los mecanismos de defensa innatos de peces, en contraste con la respuesta adaptativa potente y bien desarrollada de vertebrados superiores [9]. Los teleósteos son el primer grupo de animales que tienen un sistema inmunitario innato y adaptativo bien estructurado y diferenciado. Por lo tanto, diferentes órganos y tejidos están implicados en la inmunidad de los peces teleósteos. Otro punto a destacar es la ausencia de médula ósea en los peces, siendo el riñón el órgano hematopoyético por excelencia, el cual, está constituido por células precursoras y por tres poblaciones de leucocitos: linfocitos, macrófagos y granulocitos, siendo estos últimos las principales células que participan en la respuesta inmunitaria innata. En la dorada (*S. aurata* L), principal especie estudiada en la presente Tesis Doctoral, representa la mitad de la producción de peces marinos que, junto con la lubina (*D. labrax* L.), y también estudiada en este trabajo, son consideradas especies de excelencia en la acuicultura del Atlántico Sur y la zona mediterránea de España.

En cuanto a la respuesta inmunitaria innata, esta incluye las barreras físicas (epitelios y las membranas mucosas), efectores celulares (células fagocíticas y células citotóxicas

no específicas) y los factores humorales (complemento y otras proteínas de fase aguda), que actúan como primera línea de defensa contra la infección hasta que la respuesta específica se activa, y en la cual se incluyen componentes celulares (linfocitos) y humorales (anticuerpos secretados) que aparecen exclusivamente en los vertebrados [10]. Las respuestas inmunitarias innata y adaptativa actúan en un procedimiento integrado y coordinado en el cual se desarrollan un gran número de interacciones entre ellas. El resultado de estas interacciones es que la mayoría de las respuestas inmunitarias frente a patógenos consisten en una amplia variedad de componentes innatos y adaptativos y no se restringen a un único mecanismo. Aunque, en las primeras etapas de la infección predomina la respuesta innata, posteriormente los linfocitos comienzan a generar la respuesta adaptativa.

En los peces, los tejidos y órganos inmunitarios muestran capacidad hematopoyética y linfoide, por lo que se llaman tejidos y órganos linfomieloides [11]. Así, como órganos primarios nos encontramos con el riñón y el timo y como secundarios el bazo. El riñón se estructura en tres partes: la anterior o cefálica (RC), con función linfoide y hematopoyética; posterior o caudal, con función renal; y el intermedio, que comparte ambas funciones. El timo, que aparece cerca de las branquias, se compone principalmente de linfocitos T, y es considerado como la principal fuente de linfocitos T maduros. Por otra parte, el bazo, aunque presenta algunos linfocitos, después de la administración de antígenos su número aumenta considerablemente, encontrando linfocitos T y B [11].

Un componente importante del tejido linfoide está asociado con las superficies mucosas, formando el denominado MALT (tejido linfoide asociado a mucosas). En los peces, el tejido linfoide asociado a las mucosas se compone de poblaciones de células incluyendo linfocitos T y B, macrófagos, células plasmáticas, granulocitos y mastocitos. De acuerdo con la ubicación anatómica, el MALT en peces teleósteos se subdivide en tejido linfoide asociado al intestino (GALT), tejido linfoide asociado a las branquias (GIALT) y tejido linfoide asociado a la piel (SALT) [12]. En general, el MALT de los peces constituye un área muy grande para la posible invasión microbiana [13] por tanto contiene mecanismos de defensa (tanto innata y adaptativa) que constituyen la primera línea de defensa frente a un amplio espectro de patógenos presentes en el medio acuático [14,15]. Además de ser barreras físicas, la superficie de estos epitelios mucosos

está recubierta por una capa variable de moco y constituye sitios inmunitariamente muy activos armados con defensas celulares y humorales. Por lo tanto, las funciones del MALT parecen estar relacionadas con la capacidad de atrapar antígenos y liberar inmunoglobulinas (Ig) y otros péptidos y enzimas que participan en la defensa frente a patógenos [12] y que desempeñan un papel fundamental en el mantenimiento de la homeostasis de la mucosa (revisado por [16]).

En el caso del SALT de peces, el moco forma una capa en forma de gel adherente que cubre las células vivas epiteliales [17], la cual es secretada por las células caliciformes localizadas en la epidermis [18]. De esta manera, la superficie mucosa de la piel de los peces actúa como una barrera natural física, bioquímica, dinámica y semipermeable que permite el intercambio de nutrientes, agua, gases, olores, hormonas y gametos [19]. El moco de la piel se compone principalmente de agua y glicoproteínas [20,21], con un alto contenido en oligosacáridos de alto peso molecular, llamadas mucinas [22–24]. Entre sus funciones, el moco de la piel está implicado en la respiración de los peces, la regulación iónica y osmótica, la reproducción, la locomoción, la defensa contra las infecciones microbianas, la resistencia a enfermedades y la protección, así como en la excreción o la comunicación [25,26]. Al mismo tiempo, el moco juega un papel crítico en los mecanismos de defensa de los peces porque también actúa como una barrera biológica [27–29]. El moco de la piel ha evolucionado para tener mecanismos que pueden atrapar e inmovilizar a los patógenos antes de que puedan ponerse en contacto con las superficies epiteliales, ya que es impermeable a la mayoría de las bacterias y muchos otros patógenos [30]. Esto se produce porque en esta capa de moco, partículas, bacterias o virus quedan atrapados y se eliminan de la mucosa por la corriente de agua [31]. Además, el moco de la epidermis se sustituye continuamente y el espesor de la capa de moco y su composición evitan la adherencia de patógenos a los tejidos subyacentes y proporciona un medio en el que los mecanismos antibacterianos pueden actuar más eficientemente [30,32,33].

Hasta la fecha, existe un conocimiento limitado acerca de los mecanismos de defensa de la mucosa epidérmica de peces, aunque mecanismos de defensa tanto innatos como específicos han sido identificados [34]. Las moléculas inmunitarias en la mucosidad de los peces incluyen lisozima, inmunoglobulinas, proteínas del complemento, lectinas, aglutinina, calmodulina, interferón, proteína C-reactiva, flavoenzimas, enzimas

proteolíticas y péptidos antimicrobianos [25,33,35–39], que ejercen una actividad inhibidora o lítica contra diferentes tipos de patógenos [40,41]. Esta composición del moco determina su adhesividad, viscoelasticidad, el transporte y la capacidad protectora [42]. Desafortunadamente, el repertorio completo de factores inmunitarios presentes en el moco de la piel y su papel exacto en la inmunología de peces y en la defensa es poco conocida [43] y se limita a unas pocas especies de peces, principalmente de agua dulce.

La respuesta inmunitaria de los peces, y la resistencia a enfermedades, puede verse afectada por multitud de factores, de entre los cuales uno que afecta no sólo a los peces sino que puede tener una incidencia importante en los consumidores de pescado es la contaminación. Los contaminantes ambientales están ampliamente distribuidos en el medio acuático, y aunque muchos de ellos están prohibidos o restringidos, la mayoría de ellos son muy persistentes en la naturaleza [8] y son capaces de alterar la inmunocompetencia de los peces [44,45]. Las situaciones de estrés impuestas sobre el sistema inmunitario de los peces por los contaminantes ambientales no siempre se manifiestan abiertamente puesto que estos pueden actuar directamente matando a los peces o indirectamente pueden agravar los estados de enfermedad mediante la reducción de la resistencia y permitiendo la invasión de patógenos ambientales [46]. Además, la exposición química tiene el potencial de interferir con las fases críticas de la respuesta inmunitaria de peces, mediante la destrucción, la sensibilización o alteración de la función celular (por ejemplo, bloqueo de la actividad fagocítica, inducción o inhibición de la proliferación celular, o la ausencia de la formación de células precursoras). Aunque la relación exacta entre la contaminación del medio ambiente y las enfermedades en los organismos acuáticos es todavía incierta, la inmunosupresión es una hipótesis fuertemente apoyada ya que se piensa que los contaminantes acuáticos aumentan la prevalencia de la enfermedad en los peces expuestos [46,47].

La contaminación de los hábitats acuáticos con metales pesados provenientes de diversas fuentes industriales y mineras ha sido un problema desde hace muchos siglos. Los intereses en la extracción de minerales y el desarrollo y uso de energía, sin duda, se ha traducido, y por desgracia proseguirá, en una mayor contaminación de los ambientes acuáticos por metales como el arsénico (As), cadmio (Cd), plomo (Pb), mercurio (Hg) y zinc (Zn). Los múltiples impactos en los ecosistemas acuáticos incluyen la contaminación de los sedimentos y la columna de agua, la acumulación de

contaminantes en la biota y aparente aumento en las anomalías en las especies que allí residen [48]. Por lo tanto, los metales pesados en estos ecosistemas están recibiendo cada vez más atención. Entre los efectos adversos, estos pueden producir mortalidad, alteraciones en los parámetros hematológicos, el metabolismo y el desarrollo, así como la alteración de la maduración sexual o inmunodeficiencia [8]. Además, los estudios de laboratorio y de campo han demostrado la capacidad de los metales pesados de perturbar la respuesta inmunitaria innata y específica en una variedad de especies de peces, así como interferir en la resistencia del huésped contra los patógenos infecciosos. Algunos metales pesados se pueden transformar en compuestos metálicos persistentes con mayor toxicidad, que pueden ser bioacumulados en los organismos y magnificados en la cadena alimentaria, poniendo así en peligro la salud humana [49].

Por último, teniendo en cuenta que se sabe poco sobre el impacto de la exposición mediante baño a metales pesados en la biología de los peces, y concretamente en el sistema inmunitario, así como en la presencia y caracterización de los componentes del moco de la piel de los peces, hemos desarrollado la presente Tesis Doctoral con el objetivo de arrojar algo de luz en el riesgo potencial de la contaminación por metales pesados en la biología de peces de cultivo, y a su vez para el consumo humano.

3. OBJETIVOS

Este trabajo tiene los siguientes objetivos específicos:

1. Evaluar algunos efectos toxicológicos de la exposición en baño a arsénico, cadmio y mercurio en la dorada (*Sparus aurata* L.) con especial énfasis en la respuesta inmunitaria.

2. Identificar y caracterizar los parámetros inmunológicos y físico-químicos del moco de la piel de varios peces teleósteos marinos y, en el caso de la dorada compararlos con la inmunidad presente en el suero.

3. Analizar el repertorio inmunitario innato presente en el moco de la piel de doradas expuestas a arsénico, cadmio y mercurio mediante baño.

4. PRINCIPALES RESULTADOS Y DISCUSIÓN

4.1 Efectos inmunotoxicológicos causados por exposición a As mediante baño en la dorada

En este primer estudio, nuestros resultados confirman que el As se acumula en el hígado de la dorada después de 30 días de exposición, pero no en músculo, lo que es muy importante, ya que esta especie está destinada al consumo humano.

Además, el índice espleno-somático de la dorada no se vio afectado significativamente por la exposición a arsénico. Sin embargo, la exposición a arsénico se tradujo en un aumento del índice hepato-somático dependiente del tiempo después de 2 y 10 días, siendo estadísticamente significativo después de 10 días de exposición, retornando a valores similares al control al final del ensayo. Se ha sugerido que este aumento en el tamaño del hígado podría deberse a fenómenos de hiperplasia o hipertrofia de los hepatocitos [50] ya que en la dorada hemos observado hipertrofia de los hepatocitos, vacuolización de sus citoplasmas, la deposición de glucógeno y la aparición de células con el núcleo desplazado y picnótico. Además, estudios previos identifican una disminución del índice hepato-somático de los peces tras una larga y crónica exposición que fue asociado con un aumento en la apoptosis de los hepatocitos, una reducción en su contenido de proteínas y/o una proliferación debido a que estas células son potenciales dianas de este metal [51–53]. Además, nuestros datos después de 30 días de exposición, también demuestran alteraciones histopatológicas con aumento de la vacuolización y muerte celular en los hepatocitos, aunque el índice hepato-somático no cambió.

Respecto a la respuesta inmunitaria, los parámetros humorales no muestran variaciones estadísticamente significativas en los ejemplares expuestos a As. La actividad hemolítica del complemento en el suero muestra una ligera disminución después de 2 días de exposición a arsénico, pero un aumento después de 10 y 30 días, con respecto a los peces en el grupo de control. La mayoría de los componentes del complemento se sintetizan en el hígado, pero los leucocitos también los producen, aunque a menor escala [54]. Dado que el daño hepático y la acumulación de arsénico

coinciden, en parte, con ligeros incrementos de las actividades del complemento y peroxidasa séricos, podría sugerirse que los leucocitos ahora podrían ser la principal fuente de dichas proteínas y que han sido activados para producirlos. Este es el primer trabajo donde se estudia la actividad peroxidasa sérica en los peces expuestos a contaminantes y los resultados obtenidos sugieren que la peroxidasa podría incluirse como un nuevo bioindicador de contaminación, sin embargo, se deberían realizar más estudios para confirmar esta afirmación. En cuanto a los niveles de IgM en suero, se encontró que las doradas mantenidas en presencia de arsénico muestran una ligera disminución a los 2 y 30 días de exposición con respecto a los ejemplares no expuestos, lo que sugiere una interferencia del arsénico en el número y la viabilidad de los linfocitos B de la dorada. Esto está de acuerdo con estudios previos que muestran que el trióxido de arsénico puede inducir la apoptosis de los linfocitos a través de estrés oxidativo y que conlleva una linfocitopenia en humanos [55,56] y la reducción de la producción de Ig en los peces [57–60].

En cuanto a los parámetros inmunitarios celulares innatos, se observa un aumento de la capacidad fagocítica, explosión respiratoria y actividad peroxidasa de los leucocitos de RC de la dorada a los 10 días de exposición con arsénico. En cuanto a la actividad fagocítica de los peces, disminuyó tras una exposición a arsénico en *Clarias batrachus*, a una concentración de 42,42 µM durante 21 días o de 0,5 µM durante 1 o 30 días [59,61]. Por otro lado, en ratas, la actividad fagocítica se incrementa significativamente después de la administración oral de arsénico (20 ppm) durante 4 semanas, pero disminuye después de 12 semanas [62]. Estos datos están en consonancia con nuestros resultados en la dorada e indican que el As, en exposiciones de corta duración o dosis bajas, podría aumentar algunas de las respuestas inmunes. En este sentido, también hay varios estudios que documentan alteraciones inducidas por el arsénico en la producción de ROS (especies reactivas de oxigeno) [63,64]. Nuestros resultados también muestran un aumento en la capacidad de los leucocitos de dorada de producir ROS después de 10 días de exposición a arsénico. Estos resultados corroboran datos previos obtenidos después de la exposición a 0,5 µM de As tanto *in vitro* como *in vivo* [61]. Así mismo, contrastan con un estudio de Hermann y Kim [65] que observaron una reducción en la producción de ROS en embriones de pez cebra después de la exposición *in vivo* al arsénico aunque tal vez, estas diferencias aparentes son debidas a la maduración del sistema inmunitario.

En conclusión, los presentes resultados demuestran que la exposición de la dorada al arsénico inorgánico a una concentración de 5 µM produce una bioacumulación en el hígado después de 30 días de exposición. Por otra parte, las exposiciones breves (10 días) producen un aumento en el índice hepato-somático, alteraciones histopatológicas en el hígado y el aumento de las actividades de los fagocitos en la respuesta inmunitaria innata. Sorprendentemente, cuando los peces están realmente acumulando As y muestran efectos hepáticos adversos, la respuesta inmunitaria celular no disminuye. Nuevos estudios deberán aclarar los riesgos potenciales para la salud de los peces en tiempos de exposición más largos y si esto podría contribuir a efectos significativos sobre la bioacumulación en las cadenas alimentarias.

4.2. Efectos histopatológicos e inmunotoxicológicos causados por la exposición a Cd en la dorada

El hígado es un órgano frecuentemente recomendado como indicador ambiental de la contaminación más que otros órganos de peces [66]. En este estudio, la acumulación de Cd en el hígado y el músculo de los ejemplares de dorada expuestos a Cd aumenta considerablemente, hasta 12 veces, con el tiempo de exposición, lo que demuestra que el Cd es bioacumulable y puede transmitirse al siguiente eslabón de la cadena alimentaria. También se sabe que esta acumulación puede verse afectada por la dieta, la edad, la salinidad, el tamaño, la temporada, el pH, la temperatura, etc. En trabajos anteriores sobre la dorada, la acumulación de Cd en el hígado también se produjo durante exposiciones por inyección (hasta 160 veces) o en baño (4 a 5 veces) [67].

Los índices órgano-somáticos en el bazo y en el hígado de ejemplares de dorada no sufren ninguna alteración aunque se observa una acumulación de Cd en el hígado, como se acaba de citar. Sin embargo, el estudio microscópico del hígado demuestra alteraciones importantes y progresivas tanto en el tejido hepático propiamente dicho como en el páncreas exocrino. Los cambios histopatológicos en el hígado de doradas expuestas a Cd, así como en otros peces, incluyen la pérdida de la organización típica celular, alteraciones en las células hepáticas, aumento en el tejido conectivo, infiltración de células inmunitarias, formación de granulomas o congestión en los vasos sanguíneos [68–71].

A nivel fisiológico, algunos estudios han evaluado el impacto del Cd sobre el sistema inmunitario de los peces y ésta es la primera vez que se realiza en la dorada, uno de los peces de cultivo de mayor importancia económica en las aguas del Mediterráneo. Nuestros resultados parecen contradictorios a los datos generales que se encuentran en la literatura. A nivel humoral, no se encuentran variaciones en los niveles séricos totales de IgM, mientras que los niveles de anticuerpos específicos después de la exposición a Cd tienden a reducirse significativamente en bergall (*Tautogolabrus adspersus*) y carpa común (*Cyprinus carpio*), mientras que se encontró un aumento y una disminución en la trucha arco iris (*Oncorhynchus mykiss*) y no se encontraron alteraciones en medaka común (*Oryzias latipes*) [72–76]. En cuanto a la actividad hemolítica del complemento, las muestras de dorada muestran una disminución significativa a lo largo del tiempo de exposición, que coincide con los resultados encontrados en la tilapia híbrida (*Oreochromis niloticus* × *O. aureus*) [77]. Esto podría estar relacionado con la histopatología de hígado ya que la mayoría de los componentes del complemento se sintetizan en él, aunque también, a niveles bajos, en leucocitos [54]. Por lo tanto, es tentador especular que las alteraciones en el hígado están relacionadas con una disminución de la actividad del complemento en el suero de los ejemplares de este estudio. Por el contrario, la actividad de la peroxidasa en el suero aumenta después de tiempos de exposición cortos (2 y 10 días). De manera similar, la exposición a arsénico también produjo un aumento de la actividad peroxidasa en suero en la dorada [78] y sugiere que esta enzima podría incluirse como un nuevo bioindicador de contaminación, sin embargo se deberían hacer más estudios para confirmar esto.

A nivel celular, también encontramos un patrón dual de la inmunotoxicologia del Cd como con otros contaminantes [8]. Así, la explosión respiratoria se ve afectada, la fagocitosis aumenta, mientras que la actividad de la peroxidasa no se ve afectada en los leucocitos del RC de los especímenes de dorada expuestos al cadmio, lo que concuerda con la literatura existente. Por ejemplo, la fagocitosis y la producción de ROS se vieron afectados en la trucha arco iris, en la limanda (*Limanda limanda*) y en la lubina (*D. labrax*) [72,79–82], pero mejoraron en medaka [75] después de la exposición *in vivo* a una dosis subletal de cadmio. Por otra parte, los estudios *in vitro* revelaron que el tratamiento de leucocitos con Cd produce un aumento o una disminución de las funciones relacionadas con los fagocitos [79,83].

Para concluir, nuestros resultados corroboran que la dorada expuesta en baño a cadmio acumula en gran medida este metal en el hígado. Los índices órgano-somático en el bazo y en el hígado no se vieron modificados, aunque se observaron alteraciones histopatológicas en el hígado y el páncreas que aumentaron con el tiempo de exposición. Por otra parte, se produjeron diferentes efectos en la respuesta inmunitaria. Mientras que la actividad hemolítica del complemento del suero y la explosión respiratoria de los leucocitos de RC disminuyeron significativamente, la actividad peroxidasa en el suero y las actividades fagocíticas de los leucocitos se incrementaron por la exposición a Cd. Estos resultados parecen indicar que los ejemplares de dorada expuestos a Cd en las presentes condiciones sufren toxicidad aguda y bioacumulan dicho Cd, lo cual puede llegar a suponer un riesgo potencial para el consumo humano.

4.3. Daños estructurales y en el estatus inmunitario y antioxidante de la dorada en respuesta a la exposición en baño de Hg

En este estudio, los ejemplares de dorada fueron expuestos a una concentración subletal de mercurio (en forma de metilmercurio, MeHg; 10 µg L^{-1}) mediante baño con el fin de comprender mejor los efectos de este compuesto organometálico en el sistema inmunitario innato. Al mismo tiempo, también se analizaron otros aspectos referentes a la toxicología como histopatología, enzimas antioxidantes del hígado o el metabolismo de detoxificación, para confirmar la toxicidad de este compuesto.

El índice hepato-somático de las doradas expuestas muestra un aumento después de 10 días de exposición, mientras que el índice somático en el bazo permanece inalterado. Estos resultados están de acuerdo con nuestros resultados obtenidos para el As y Cd. Al mismo tiempo, el factor de condición no se ve afectado por la exposición a MeHg como también se describe en la literatura, como por ejemplo, después de la ingesta mediante dieta en *Salmo salar* [84], *Orthodon microlepidotus* [85] u *Hoplias malabaricus* [86]. En nuestro trabajo, también hemos encontrado cambios histopatológicos en el hígado de doradas expuestas a MeHg, los cuales aumentan drásticamente con la prolongación del tiempo de exposición. Del mismo modo, De Oliveira Ribeiro y col. [87] documentaron cambios importantes en el hígado de trucha alpina (*Salvelinus alpinus*) después de la exposición a MeHg mediante dieta, pero no se produjeron cambios cuando fue

administrado por baño. Otros estudios que utilizaron la exposición de MeHg en dieta han encontrado algunos cambios histológicos en el tejido diana, el intestino [86]. Sin embargo, hasta donde sabemos, ningún estudio ha realizado esta exposición por baño y ha estudiado la piel, el primer tejido en contacto con los compuestos tóxicos, el MeHg en este caso. De esta manera, hemos encontrado en la epidermis de los especímenes de dorada expuestos a MeHg una disposición más desorganizada de las células (más evidente en la parte basal del epitelio) de la epidermis de los peces con respecto a la epidermis de los peces control (no expuestos). Las alteraciones morfológicas observadas en la piel de los peces expuestos a MeHg están correlacionadas con la degeneración epidérmica caracterizada por la inflamación de las células epidérmicas (edema intracelular) y la aparición de núcleos picnóticos (condensados).

Otra determinación importante para evaluar la toxicidad fue realizar un perfil de expresión génica. En este sentido, se sabe muy poco en el caso de la toxicidad del MeHg en los peces. Curiosamente, se encuentra que la exposición a MeHg no altera la expresión de los genes relacionados con el metabolismo tóxico (CYP1A1 o MTA), estrés celular (HSP-70 y HSP-90) o la muerte celular (CASP-3) en el RC, mientras que la mayoría de ellos están sobre-expresados en la piel, en correlación con los cambios histopatológicos observados en este tejido. En la dorada, la enzima CYP1A1 se ha detectado en las células epidérmicas de la piel [88], sin embargo, este es el primer estudio en el que se observa una sobre-expresión de este gen después de la exposición a MeHg en baño en la piel. Genes relacionados con el estrés celular (HSP-70, HSP-90) y la muerte celular (CASP-3) son también sobre-expresados después de la exposición a MeHg, lo que indica que este tejido está dañado, tal y como lo demuestra el estudio histopatológico. Además, si la falta de inducción del gen de protección MTA es responsable del aumento de estrés celular y la muerte en la piel merece un posterior análisis. En este sentido, González y col. [89] no revelaron ninguna relación directa entre los niveles de bioacumulación de MeHg y las respuestas genéticas en el hígado, el músculo esquelético y el cerebro del pez cebra. Del mismo modo, un *microarray* realizado por Liu y col. [90] en juveniles de pez cebra (*Danio rerio*) y la trucha arco iris, expuestos a una dieta suplementada con MeHg, no ofreció evidencias suficientes para seleccionar buenos biomarcadores en teleósteos para la exposición de este metal, lo que sugiere que se necesitarán buscar otros potenciales biomarcadores.

Se sabe que el Hg inorgánico induce estrés oxidativo en los peces, provocando alteraciones en la actividad de las enzimas antioxidantes en varios tejidos [84,86,91–94]. En general, los mecanismos de defensa antioxidantes que actúan en el hígado son más fuertes en comparación con otros tejidos como el riñón y el cerebro. Por lo tanto, en nuestro estudio, las enzimas antioxidantes SOD (superóxido dismutasa), CAT (catalasa) y GR (glutatión reductasa) fueron evaluadas en el hígado de la dorada. Así, las actividades SOD y CAT se incrementaron significativamente después de 2 días de exposición a MeHg pero volvieron a los niveles observados en los peces control posteriormente. Sin embargo, la actividad GR en el hígado estuvo siempre aumentada aunque no alcanza niveles significativos. Esto sugiere que la producción de ROS ocurre tras tiempos de exposición cortos y que pueden ser eliminados directamente por el aumento de las enzimas SOD y CAT. De manera similar a nuestro estudio, en el hígado del sargo picudo (*Diplodus puntazzo*) expuesto por baño al MeHg (2 µg L^{-1}), la actividad de GR aumentó después de 28 días, mientras que la actividad de GPx (glutatión peroxidada) resultó inhibida durante todo el ensayo [95].

Hasta la fecha, hay muy pocos estudios sobre el efecto del MeHg en el sistema inmunitario de los peces. En este trabajo, se encontró que el MeHg aumenta los parámetros humorales evaluados. La mayoría de los componentes del complemento se sintetizan en el hígado, pero los leucocitos también participan en su producción a menor escala [54]. Puesto que el daño hepático coincide en parte con ligeros incrementos de las actividades del complemento del suero y la peroxidasa esto podría sugerir que los hepatocitos, antes de ser completamente dañados, aumentan la producción de proteínas importantes que intervienen en la homeostasis general o defensa en determinadas situaciones de estrés celular. Este patrón también se observó en el trabajo realizado con As [78], pero fue opuesto en el caso de Cd o deltametrina [96,97]. En el caso de la IgM, producida por los linfocitos B, no se ve afectada en las dorada mientras que fue inhibida en la trucha arco iris, expuestas a cloruro de mercurio, y en el gourami azul (*Trichogaster trichopterus*) [98,99].

En cuanto a la respuesta inmunitaria celular, el RC es también un tejido diana para la acumulación de MeHg [100] lo que sugiere que las funciones de los leucocitos pueden verse afectadas negativamente. Sorprendentemente, la exposición a MeHg induce la actividad de fagocitosis y peroxidasa de los leucocitos de RC en diferentes momentos

como también sucedió en las doradas expuestas a Cd o As. Estos datos indican que el RC de dorada no se altera significativamente como se piensa, lo cual se corrobora por la falta de regulación de genes de estrés (HSP-70 y 90) y muerte celular (CASP-3) en este órgano. Sin embargo, mientras que algunos autores han documentado que la exposición de MeHg provocó inmunosupresión, determinada como la reducción del número de macrófagos en el RC y células inflamatorias y la expresión alterada de genes relacionados con la respuesta inflamatoria [101,102], otros han demostrado que la exposición por baño a MeHg produce la acumulación de este metal en los riñones y en gran medida altera la morfología de los túbulos renales [86,103], pero no del tejido hematopoyético. Esto podría estar ocurriendo en el RC de la dorada ya que las actividades de leucocitos se incrementaron y la expresión de genes no se alteró significativamente.

En conclusión, los presentes resultados demuestran que la dorada expuesta por baño a MeHg (10 µg L^{-1}) produce alteraciones histopatológicas en el hígado y la piel, así como incrementos en el índice hepato-somático, enzimas antioxidantes y los parámetros de inmunidad innata. Por otra parte, la expresión de genes relacionados con el metabolismo de tóxicos, el estrés celular y la apoptosis fueron sobre-expresados en la piel de doradas expuestas al metilmercurio, pero no en el RC. En general, nuestros datos demuestran que la exposición a una concentración subletal de MeHg produce efectos toxicológicos agudos y el aumento de los parámetros inmunitarios en la dorada.

4.4. Comparación de los mecanismos de defensa humorales presentes en el moco de la piel y en el suero de la dorada

Varios estudios han demostrado que la presencia y la actividad de factores inmunitarios en el moco de la piel depende de las condiciones ambientales y las especies de peces [29,33,104–106]. Hemos llevado a cabo este estudio teniendo en cuenta la importancia de la mucosa de la piel como la primera línea de defensa y la falta de conocimiento en el caso de la dorada. Todas las moléculas estudiadas en el presente trabajo están constitutivamente presentes tanto en el moco de la piel como en el suero de la dorada. Curiosamente, algunas de ellas están presentes en el moco de la piel en

niveles mayores que en el suero, lo cual indica la relevancia inmunológica de la mucosa de la piel.

En relación a la IgM de dorada, su presencia en el moco de la piel es significativamente menor que en el suero, lo cual se correlaciona con los resultados obtenidos en otras especies de peces [107–112]. Diferentes enzimas con un importante papel en las funciones inmunitarias se han identificado en varias especies de peces y se incluyen en nuestro estudio, como por ejemplo, peroxidasa, lisozima, fosfatasa alcalina, esterasa, proteasa y antiproteasa. En primer lugar, con respecto a la fosfatasa alcalina, el moco de dorada muestra una actividad muy similar a la observada en suero. En el caso de la trucha arco iris, salmón plateado (*Oncorhynchus kisutch*) y salmón Atlántico (*Salmo salar*) esta enzima no fue detectada en el moco de la piel a menos que los peces fueran transferidos del agua dulce al agua marina [104]. Otra enzima estudiada fue la esterasa, siendo los valores obtenidos para el moco de la dorada dos veces superior a los encontrados en el suero. Aunque no hay fuentes comparativas, estudios recientes confirman que ambas enzimas (fosfatasa alcalina y esterasa) aumentan en el moco de la piel de los peces después de la administración de inmunoestimulantes [113,114]. Todos estos datos parecen sugerir que ambas enzimas podrían desempeñar un papel importante defensivo en la piel de la dorada. Por otro lado, las proteasas en el moco de la piel pueden desempeñar un papel protector contra los agentes patógenos tanto de forma directa, mediante la escisión de sus proteínas [28], o indirecta, al obstaculizar los mecanismos de colonización e invasión [115]. En nuestro estudio, la actividad de las proteasas también es ligeramente mayor en el moco que en el suero. Loganathan y col. [116] han sugerido que la importancia relativa de las proteasas es mayor en la mucosa de los peces que las otras enzimas, por ejemplo, lisozima, fosfatasa alcalina o esterasa. Curiosamente, la producción de antiproteasas es baja con el fin de mantener estos altos niveles de proteasas activas en las mucosas. Sin embargo, hay muy pocos datos disponibles pero algunos compuestos con actividad antiproteasa se han identificado y caracterizado en el salmón Atlántico [117] y fugu pantera (*Takifugu pardalis*) [118]. En cuanto a la última enzima estudiada, la actividad de la peroxidasa en el moco de la piel de la dorada fue significativamente mayor que en el suero. Dado que las peroxidasas son importantes agentes microbicidas, es tentador considerar que la peroxidasa en el moco será esencial para la inmunidad de la mucosa y defensa de la piel.

Una de las funciones inmunitarias más práctica es la capacidad directa para matar las bacterias y esto también es importante en el moco de la piel. Esta actividad bactericida puede llevarse a cabo por muchos compuestos diferentes pero desafortunadamente la mayoría de ellos no son conocidos. Entre ellos, uno de los más caracterizados es la lisozima. La actividad de la lisozima presente en el moco de la piel y en el suero de dorada muestra valores similares. Se ha documentado una mayor actividad de la lisozima en el moco de la piel de los peces criados en agua dulce que las especies criadas en agua de mar [104] y lo contrario [28]. Por lo tanto, la actividad de la lisozima en el moco de la piel no muestra correlación significativa con otras sustancias inmunes, lo que sugiere que la lisozima es secretada constitutivamente en el moco de la piel de estas especies de peces [37].

Pero independientemente de las moléculas efectoras y los mecanismos implicados en la destrucción bacteriana, la medición de la actividad bactericida es una aproximación muy realista. En este sentido, numerosos informes han estudiado la actividad antimicrobiana de extractos de moco de la piel en varias especies de peces contra una amplia gama de patógenos y hongos [41,119–125]. Las tres bacterias seleccionadas en el presente estudio tienen un gran impacto económico, debido a las pérdidas ocasionadas en las explotaciones de cultivo de la dorada. La actividad bactericida en el moco de la piel es significativamente mayor que en el suero y las bacterias más susceptibles han sido *V. harveyi* seguida por *V. anguillarum* y *P. damselae*. Estos datos sugieren que estos agentes patógenos son refractados en gran medida por el moco y no pueden entrar por la piel. Por otra parte, también se evaluó la actividad contra bacterias no patógenas para los peces, tales como *E. coli* y *B. subtilis*. Sorprendentemente, la viabilidad de *E. coli* no se ve afectada por el suero o moco, mientras que *B. subtilis* incubada con el moco de la piel presenta incluso un mejor crecimiento.

En conclusión, el estudio revela que el moco de la piel de dorada contiene actividades de la proteasa, fosfatasa alcalina y lisozima muy similares al suero, pero niveles de IgM inferiores. Por el contrario, el moco de la piel de la dorada mostró una mayor actividad de la peroxidasa, antiproteasa, esterasa y actividad bactericida que el suero. Estos resultados podrían ser útiles para entender mejor el papel de la mucosa de la piel como un componente clave del sistema inmune innato, lo que podría ser beneficioso en la acuicultura para la salud de los peces.

4.5. Caracterización de diferentes parámetros físico-químicos del moco de la piel de cinco especies de teleósteos marinos

El estudio de las propiedades del moco de la piel en especies de peces teleósteos y su caracterización debe beneficiar la comprensión de la biología, la función de la mucosa como barrera y su posible relación con patógenos y enfermedades. Por tanto, resultó muy interesante investigar los parámetros físico-químicos que posee el moco de la piel de cinco especies diferentes de teleósteos marinos [dorada (*S. aurata* L.), lubina (*D. labrax* L.), verrugato (*Umbrina cirrosa* L.), dentón (*Dentex dentex* L.) y mero (*Epinephelus marginatus* L.)], todos ellos con consagrado o potencial interés comercial en la acuicultura de la zona del Mediterráneo.

De esta manera, la concentración de proteínas es similar en todas las especies de peces evaluados, aunque fue más alta en *D. labrax* y más baja en *S. aurata*. Sin embargo estas concentraciones de proteína son mayores que en salmónidos [126]. Estas pequeñas variaciones pueden atribuirse a la diferencia en las especies, ya que todas ellas se habían mantenido en las mismas condiciones previas (composición del agua, el volumen y la circulación, la temperatura, la salinidad, el fotoperiodo, etc.).

El pH, la conductividad, el potencial redox y la temperatura están claramente relacionadas entre sí, por la cantidad de iones que típicamente tiene una solución acuosa. Nuestros resultados muestran una correlación positiva entre los valores de pH, potencial redox y conductividad a excepción de algunos casos: por ejemplo, en *U. cirrosa*, la cual presenta valores bajos de conductividad mientras que el pH y el potencial redox son altos. Del mismo modo, *D. labrax* muestra bajo el potencial redox con respecto a sus valores de pH y conductividad. Las implicaciones prácticas de estos parámetros son la demostración de que la adhesión bacteriana a las superficies de los peces es dependiente del pH [127,128] aunque nunca se ha estudiado con respecto a los demás parámetros.

En el caso de la osmolaridad, este parámetro en la mucosa de la piel sigue un patrón muy similar a los parámetros previamente medidos (pH y potencial redox) siendo mayor en *S. aurata* y menor en *E. marginatus*. Estas diferencias pueden indicar grandes gradientes iónicos en el moco de la piel. Gradientes de iones entre el agua circundante y moco ofrecerían un gradiente de iones reducido para el plasma, reduciendo así el coste

de transporte de iones [129]. Por el contrario, Roberts y Powell [126] observaron leves diferencias entre la osmolaridad de tres especies marinas de peces, lo que puede indicar pequeños gradientes iónicos. Teniendo en cuenta que la osmolaridad del agua de mar medida por nosotros fue 1106 ± 3,2 mmol kg^{-1}, podríamos considerar que el moco de *S. aurata*, *U. cirrosa* y *D. dentex* es iso-osmótico, mientras que el moco de la piel de *D. labrax* y *E. marginatus* podría ser categorizado como hipo-osmótico, con respecto al agua circundante.

La densidad es la relación entre la masa de una solución y el volumen que ocupa. Por lo tanto, a diferencia de la osmolaridad que depende de la concentración total de partículas y es independiente de su masa, la densidad depende del número de partículas de soluto y su masa. En nuestro estudio, las mediciones de densidad siguen un patrón similar entre las diferentes temperaturas ensayadas, donde se encuentra una disminución con el aumento de las temperaturas. Por otra parte, se observa en general una correlación positiva entre la densidad y osmolaridad. Sin embargo, ningún estudio ha relacionado estos parámetros con las funciones biológicas.

Por otra parte, la viscosidad del moco para las cinco especies estudiadas demuestra generalmente un comportamiento no-Newtoniano, donde el moco exhibe una mayor viscosidad a velocidades de cizallamiento bajas que a altas velocidades. Interesantemente, se ha demostrado el comportamiento no-Newtoniano en la mucosa de la piel de varios peces marinos cultivados como el salmón, la trucha marrón (*Salmo trutta*), la trucha arco iris y la solla (*Pleuronectes platessa*), mientras que no se observó claramente este comportamiento en el salmón Atlántico y la trucha arco iris cuando fueron mantenidos en agua dulce [126,130]. Además, la viscosidad puede estar relacionada con el aumento en la cantidad de proteína presente en el moco, donde las muestras con mayor viscosidad se corresponden con las que tienen una concentración de proteína más alta, y viceversa. Por otra parte, la viscosidad puede estar relacionada con la osmolaridad. Por ejemplo, Roberts y Powell [126] observaron una correlación positiva entre la viscosidad y la osmolaridad, con una viscosidad más baja en los peces criados en agua dulce que en agua de mar. Sin embargo, nosotros solo hemos encontrado esta correlación para *U. cirrosa*, *D. dentex* y *E. marginatus*, pero no para *S. aurata* y *D. labrax*.

Finalmente, las mediciones microcalorimetricas no muestran diferencias entre los tres barridos de temperatura ensayados, con excepción de *S. aurata* y *D. labrax*, donde se observaron una o dos sustancias que soportan los rangos de temperatura establecidos. De este modo, podríamos decir que las muestras de moco tienen una composición diferente y una cantidad de sustancias en cada muestra, así como unas poblaciones de sustancias diferentes, sin embargo, se podría confirmar que las estructuras que se encuentran en *S. aurata* y *D. labrax* serían más estables que en el resto de los peces estudiados.

En conclusión, se han determinado y comparado los parámetros físico-químicos de la mucosa de la piel de cinco especies de teleósteos marinos. De este modo, se ha observado una correlación entre el pH, la conductividad y el potencial redox en el moco de la piel de todos los peces testados. Por otra parte, ha sido generalmente observada una clara interrelación entre la densidad y la osmolaridad, así como, entre la densidad y la temperatura. En el caso de la viscosidad del moco, se ha demostrado que tiene un comportamiento no-Newtoniano en todas las muestras, donde el moco mostró una mayor viscosidad a bajas velocidades de cizallamiento que a altas velocidades. Sin embargo, la correlación entre la viscosidad y la osmolaridad sólo fue observada para *U. cirrosa*, *D. dentex* y *E. marginatus* pero no para *S. aurata* y *D. labrax*. Sin embargo, las implicaciones de estos parámetros del moco de la piel con la susceptibilidad a enfermedades merecen un análisis mucho más profundo.

4.6. Comparación de los parámetros de defensa innatos presentes en la mucosa de la piel de cinco especies de teleósteos marinos

Tomando en consideración la importancia de la mucosa de la piel en la inmunidad de los peces y la pobre caracterización de las moléculas inmunes presentes en ella, identificamos, cuantificamos y comparamos el perfil de los hidratos de carbono terminales, así como, algunos de los principales parámetros de inmunidad innata en la mucosa de la piel de las cinco especies nombradas con anterioridad.

Con respecto a los residuos de carbohidratos probados en este estudio, los niveles de de unión a lectinas en la mucosa de la piel son WGA> Con A> WFA> BSL I> PNA>

UEA I> LEA lo que sugiere que la abundancia de los carbohidratos terminales en el moco de la piel son el ácido N-acetilneuramínico, la glucosa, la N-acetil-glucosamina, la N -acetil-galactosamina, la galactosa y, como el menos abundante, la fucosa. Por ejemplo, el ácido N-acetilneuramínico proporciona carga negativa a las moléculas de mucina y reduce la unión bacteriana [131] y se ha demostrado que se reduce en el moco de la piel de la carpa común después de una infección bacteriana [17]. Por lo tanto, este hecho merece una caracterización adicional con el fin de entender el papel preciso de los hidratos de carbono del moco y su papel en la adhesión y la invasión de patógenos.

Las enzimas en el moco epidérmico pueden desempeñar un papel importante en las funciones inmunitarias en el pez, así, lisozima, peroxidasa, fosfatasa alcalina, esterasa, antiproteasa y proteasas se han estudiado en estas especies. La actividad de la lisozima, tiene los mismos niveles en todos los peces a pesar de que parecen tener diferente susceptibilidad a los brotes bacterianos. Nuestros datos muestran que el verrugato y el dentón poseen una alta actividad proteasa y antiproteasa, y además, se ha demostrado que son más propensos a sufrir enfermedades producidas por parásitos que por bacterias [132,133]. Otras enzimas estudiadas fueron la fosfatasa alcalina y la esterasa, cuyo papel en la inmunidad de la mucosa no está muy bien estudiado. En nuestro estudio encontramos un patrón similar de ambas enzimas en *S. aurata, D. labrax, U. cirrosa* y *D. dentex*, mientras que los niveles en *E. marginatus* para la fosfatasa y la esterasa son altos y bajos, respectivamente. Por último, la actividad de la peroxidasa, muestra niveles similares en *S. aurata*, *D. labrax* y *E. marginatus* y superiores para *U. cirrosa* y *D. dentex*. En general, nuestros datos muestran que cada especie de pez tiene una o más actividades enzimáticas elevadas pero nunca todas las actividades son altas o bajas al mismo tiempo.

Algunos estudios han revelado que el moco de la piel de varias especies de peces tiene una fuerte actividad anti-bacteriana y anti-fúngica contra una amplia gama de patógenos microbianos y hongos [41,120–125]. Nuestros datos también confirman esto y evidencian que el moco de la piel de las cinco especies de peces marinos muestra actividad bactericida contra las bacterias patógenas y no patógenas con diferencias sustanciales entre las especies de peces y las cepas bacterianas.

En conclusión, todas las especies de peces mostraron una o más actividades en niveles altos lo que indica que los peces están siempre alerta y la respuesta inmunitaria

global no se basa en componentes individuales. El moco de todas las especies también ejerce actividad bactericida pero esto es difícil de correlacionar con las actividades enzimáticas individuales. No obstante, los resultados podrían ser útiles para entender mejor el papel de estas sustancias en la mucosa de la piel como un componente clave del sistema inmunitario innato.

4.7. Efectos en los parámetros del sistema inmunitario presentes en moco de la dorada en respuesta a la exposición a metales pesados (As, Cd y Hg) en baño

La piel de los peces y las mucosas están en contacto directo con todos los productos químicos tóxicos así como con los organismos no patógenos y patógenos presentes en el medio acuático [134]. Por lo tanto, se realizó este estudio sabiendo la importancia de la mucosa de la piel en la defensa de los peces.

De esta manera, el sistema inmunitario innato protege al organismo contra los patógenos invasores siendo el reconocimiento de microorganismos extraños el primer paso en la iniciación de las enfermedades infecciosas [135,136]. Este reconocimiento está mediado por los residuos de carbohidratos y las proteínas que los reconocen, las lectinas. Así, la unión a lectinas en la mucosa de la piel expuesta al As, Cd y Hg es WGA> Con A> WFA> BSL I> PNA> LEA> UEA lo que sugiere que los carbohidratos terminales abundantes en el moco de la piel son el ácido N-acetilneuramínico > la glucosa > la N-acetil-glucosamina > la N-acetil-galactosamina > la galactosa > la fucosa. El aumento de ácido N-acetilneuramínico, el ácido siálico predominante, puede estar asociado con la participación en la prevención de infecciones, por lo tanto, puede ser una respuesta a la exposición de estos metales. De la misma manera, la N-acetil-galactosamina está relacionado con la correcta comunicación célula a célula, que es importante para los procesos de funcionamiento y las enfermedades sistémicas normales como la inflamación y la inmunidad [137].

En cuanto a los anticuerpos tipo IgM, que desempeñan un papel crucial en la protección de los peces contra los agentes invasores en el hábitat en la medida en que su nivel y especificidades son suficientes para proteger contra una infección relevante [138,139]. De acuerdo con esto, la concentración de Ig detectada en el moco de la piel

de los peces expuestos es reducida durante tiempos de exposición cortos (2 días) mientras que aumenta para el resto de los grupos experimentales y con el tiempo de exposición.

Por otro lado, la lisozima, fosfatasa alcalina, esterasa, peroxidasa, ceruloplasmina, proteasas y antiproteasas fueron evaluadas, ya que hay muy pocos estudios disponibles donde algunas de estas enzimas han sido estudiadas en el moco de los peces expuestos a metales por baño [140] o por la dieta [141]. En el caso de la lisozima y la proteasa, éstas muestran un aumento en todos los peces expuestos a Hg, mientras que en los peces expuestos a As y Cd sólo aumentan después de 10 días de exposición. Además, estas enzimas disminuyen en el moco de la piel de los peces expuestos a tales metales después de 30 días de exposición. Por el contrario, la actividad de las antiproteasas aumenta en la mayoría de los peces expuestos (excepto después de 10 días de exposición). Es interesante señalar que la producción de antiproteasas se correlaciona negativamente con la actividad de las proteasas, en los peces expuestos a As y Cd, tal vez con el fin de compensar las variaciones de estos niveles de proteasas activas en las mucosas. La actividad de la fosfatasa alcalina se reduce después de la exposición durante un largo tiempo con los metales, mientras que la actividad esterasa se incrementa en todos los peces expuestos, independientemente del tiempo de exposición. En algunos estudios, se observaron aumentos de actividad de la fosfatasa alcalina en el moco de la piel de salmón Atlántico después de la infección con *Lepeophtheirus salmonis*, pero la fuente de esta variación no se conoce [142–144]. De acuerdo con nuestros resultados, podríamos decir que el origen de las variaciones encontradas es la exposición a estos metales. En cuanto a la actividad peroxidasa, se observa que dicha actividad se inhibe después de un largo tiempo de exposición para los peces expuestos a As y Cd, mientras que esta actividad se incrementa en los peces expuestos a Hg, lo cual presupone un papel importante en la defensa de la piel contra los metales como el Hg. Para la ceruloplasmina, una de las principales proteínas antioxidantes de la fase aguda, liberada en respuesta a procesos de infección e inflamación, se reduce después de un largo tiempo de exposición al As y Cd, mientras que esta actividad siempre se mantiene elevada en los peces expuestos al Hg. Del mismo modo, los niveles de ceruloplasmina fueron elevados después de la infección con patógenos en la trucha arco iris y en la carpa [145,146]. Además, el nivel de esta enzima en el plasma también aumentó durante

todo el experimento en la trucha arco iris cuando fue alimentada con diferentes dosis de sulfamerazina [147].

Por desgracia, pocos estudios se han realizado sobre si las bacterias marinas que habitan en el agua pueden atacar fácilmente a los peces y/o crecer en el moco después de la exposición a unos agentes estresantes como los contaminantes o metales pesados. Nuestros resultados muestran ligeras diferencias en el crecimiento bacteriano dependientes de la exposición a metales, el tiempo de exposición o las bacterias. Sin embargo, podríamos señalar que en general la proliferación de bacterias se reduce en el moco de la piel de ejemplares expuestos a Hg y en el caso de *B. subtilis* donde se encuentra una reducción del crecimiento de esta bacteria en todos los peces expuestos independientemente del tiempo de exposición. Por otra parte, el aumento y la reducción del crecimiento bacteriano que se encuentra en el moco de peces expuestos durante 30 días a arsénico y cadmio, respectivamente, sugiere un efecto después de un tiempo largo de exposición dependiente de los metales. Varios estudios han mostrado la disminución de la resistencia de los peces frente a bacterias después de la exposición a contaminantes [44,148–150]. Además, los resultados obtenidos por Song y col. [151] sugirieron que la supresión inmunitaria de los peces se produjo debido a una contaminación estresante, y las bacterias pudieron invadir las superficies mucosas, lo que resultó también en un aumento de leucocitos en la sangre para prevenir la enfermedad infecciosa.

Además, mediante el uso de ensayos de electroforesis en gel se observa la presencia de varias bandas de proteínas en el moco de los peces tanto expuestos como no. Sin embargo, no se observa la presencia de una banda alrededor de 14 kDa en los peces expuestos durante 30 días a As y Cd. Esto podría estar correlacionado con la disminución de la actividad en la mayoría de las enzimas medidas después de 30 días de exposición a estos dos metales. Igualmente, la presencia de esta banda en muestras de moco de la piel expuestas a Hg se correlaciona con el incremento observado de las enzimas evaluadas.

Por otra parte, el análisis mediante *western blot* revela la presencia de una lectina de unión a fucosa, aislada a partir del suero de *D. labrax* (DlFBL) [152] en el moco de la piel de los peces expuestos a los metales por baño pero no en los peces control. Sin

embargo, la existencia de esta fuco-lectina vuleve a estar ausente después de 30 días de exposición a As y Cd.

Para confirmar si la exposición a los metales podría alterar el patrón de proteínas, también se realizó un análisis de cromatografía. En el cromatograma del HPLC se encuentra un patrón similar de picos entre el moco de la piel de los peces no expuestos y expuestos, sin embargo, se observan diferentes intensidades en función del tiempo de exposición y del metal. Por lo tanto, en tiempos de exposición cortos, no se observan grandes variaciones en la intensidad de los picos, mientras que para largos tiempos de exposición, se observa un aumento en la pauta de los peces expuestos a Hg pero no a As y Cd. Por lo tanto, podríamos decir que la exposición de largo plazo a As y Cd podría reducir las defensas de los peces, mientras que la concentración de Hg utilizada en este trabajo no parece debilitar el sistema de defensa de la dorada. Sin embargo se necesitan más estudios para confirmar esta hipótesis en relación con el tiempo y las concentraciones de exposición.

En conclusión, se encontró una relación entre la exposición a largo plazo a cadmio y arsénico con el aumento de la presencia de diversos hidratos de carbono como el N-acetilneuramínico. Por otra parte, se observó un aumento de la mayoría de las enzimas estudiadas y la concentración de Ig en el moco de la piel después de 10 días de exposición, mientras que se produjo la disminución de las actividades de dichas enzimas cuando los peces fueron expuestos durante 30 días con As y Cd, pero no para los peces expuestos a Hg. Esto se podría correlacionar con la facilidad que presentaron las bacterias patógenas para crecer en el moco de los ejemplares expuestos al arsénico y la dificultad de hacerlo en las muestras expuestas a Hg. Del mismo modo, la banda de proteínas (~14 kDa) encontrada en los peces expuestos a arsénico y cadmio, se mostró ausente después de 30 días de exposición, pero no para los peces expuestos Hg. Por lo tanto, los resultados han sido discutidos a la luz de aclarar la posible relación entre los contaminantes ambientales y el aumento de la susceptibilidad a enfermedades en dorada debido a la disminución de la protección antibacteriana.

5. CONCLUSIONES

1. La exposición de doradas mediante baño a arsénico aumenta el índice hepato-somático, incrementa los parámetros de inmunidad innata y produce alteraciones histopatológicas en el hígado después de un tiempo corto de exposición, a pesar de que sólo se bioacumula en el hígado de la dorada, pero no en el músculo, después de 30 días.

2. La exposición de doradas mediante baño a cadmio produce toxicidad aguda, provoca efectos inmunotoxicológicos, alteraciones histopatológicas en el hígado y en el páncreas, y se acumula en el hígado y en el músculo.

3. Las doradas expuestas a mercurio mediante baño sufren efectos toxicológicos agudos tales como alteraciones histopatológicas en hígado y piel, así como incrementos en el índice hepato-somático, enzimas antioxidantes y parámetros de la inmunidad innata. Por otra parte, la expresión de genes relacionados con el metabolismo de los tóxicos, el estrés celular y la apoptosis están sobre-expresados en la piel, pero no en el riñón cefálico.

4. El moco de la piel de la dorada contiene niveles más bajos de IgM, niveles similares de lisozima, fosfatasa alcalina y proteasas, y más altos de esterasa, peroxidasa y antiproteasa que el suero. Curiosamente, el moco de la piel presenta una alta actividad bactericida contra bacterias patógenas de peces en comparación con el suero.

5. Los parámetros físico-químicos del moco de la piel de varias especies de teleósteos marinos revela claramente relaciones entre ellos. Dichos parámetros podrían tener un papel importante en la adhesión y la invasión de patógenos.

6. El moco de la piel de teleósteos marinos (dorada, lubina, verrugato, dentón y mero) muestra variaciones significativas en el perfil de sus carbohidratos, en las enzimas relacionadas con el sistema inmunitario innato y en la actividad bactericida presentes en él, lo que podría estar también relacionado con diferencias en la resistencia a los patógenos.

7. La exposición de doradas a metales pesados mediante baño provocó pequeñas variaciones en los perfiles de hidratos de carbono y proteínas en el moco de la piel, mientras que la mayoría de sus parámetros inmunitarios se vieron incrementados, siendo más evidente en el caso del baño con mercurio. Algunos de estos parámetros podrían ser considerados como potenciales biomarcadores para la toxicología de los peces.

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