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TITOLO TESI DI DOTTORATO DI RICERCA:

"IMMUNOLOGICAL STUDIES IN SEA BASS (*DICENTRARCHUS LABRAX*) VACCINATED AGAINST BETANODAVIRUS"

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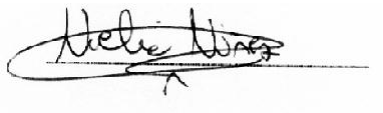

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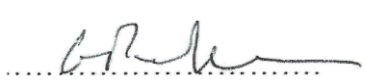

CONTENTS

RIASSUNTO

Lo scopo di questo lavoro di tesi è stato quello di fornire una migliore conoscenza sull'immunità mucosale nei Vertebrati utilizzando pesci Teleostei come modello, tramite lo studio delle risposte immunitarie in un modello di pesci marini (la spigola Europea) nei confronti di un virus patogeno (Betanodavirus), al fine di sviluppare possibili strategie di vaccinazione.

L'elaborato è costituito da 7 capitoli, dove, inizialmente viene introdotta e definita la terminologia di base utilizzata (**Chapter 1**). Sono stati anche descritti gli aspetti generali dell'immunologia dei Teleostei, le informazioni di base del modello animale (spigola europea) e la malattia dell'encefalopatia virale e della retinopatia (VER).

Per aumentare la conoscenza dell'immunità mucosale sono stati studiati alcuni aspetti cellulari di base tipici delle cellule T e l'espressione dei geni correlati alle cellule T durante la proliferazione in vitro indotta da lectine (**Chapter 2**). I leucociti delle branchie, in risposta alle lectine ConA e PHA, sono stati in grado di proliferare, come misurato dall'incorporazione di un colorante vitale. La proliferazione è stata superiore misurata a 48 ore e la PHA ha indotto una maggiore proliferazione rispetto alla ConA. Le percentuali medie di cellule riconosciute da entrambi gli anticorpi monoclonali contro le cellule pan-T (mAb DLT15) e le cellule CD45 (mAb DLT22) sono raddoppiate dopo 48 ore di stimolazione da parte di lectine. Le cellule T sono state indiciduate tramite immunoistochimica nell'epitelio dei filamenti branchiali e la trascrizione è stata particolarmente elevata per TRβ, TRγ e IL-10 seguita da CD8, CD45 e CD4, confermando così che le cellule che proliferano sono cellule T. Inoltre è stata effettuata la caratterizzazione cellulare di un nuovo anticorpo policlonale contro i leucociti IgT-specifici della spigola (**Chapter 3**). I leucociti IgT-specifici sono stati marcati mediante IIF ed rilevati mediante citometria di flusso e microscopia a fluorescenza. Queste cellule presentano le dimensioni e la morfologia tipiche dei leucociti e sono risultate più abbondanti a livello renale piuttosto che nella milza o nelle branchie. Inoltre, mentre nel siero è stato rilevato un basso contenuto di IgT solubili, nel muco è risultato elevato. Infine, l'immunoprecipitazione di leucociti IgT-specifici a livello renale con Dynabeads ci ha permesso di ottenere due frazioni, una delle quali

ricca di leucociti IgT-specifici e l'altra ricca di leucociti IgM specifici, come è stato verificato mediante RT-PCR.

Parallelamente, sono stati studiati gli effetti dell'immunizzazione del Betanodavirusv (VERv) sulle risposte immunitarie nelle spigole. Innanzitutto è stato sviluppato un sistema capture-based ELISA che può essere utilizzato come strumento per indagare e quantificare la presenza di VERv in campioni biologici di spigole adulte o giovani (**Chapter 4**). VERv è stato rilevato e quantificato in omogenati di cervello di spigola infettata *in vivo*. Questo sistema ELISA è stato in grado di rilevare diversi ceppi RGNNV e si può ipotizzare che sia in grado di rilevare altri genotipi a causa della conservazione dei residui proteici del capside e le somiglianze tra genotipi. Successivamente, è stato eseguito un approccio sperimentale che consiste nell'esaminare le possibilità di immunizzazione della spigola giovane contro VERv attraverso vaccinazione mucosale (immersione), impiegando un preparato di Betanodavirus inattivato con formalina. (**Chapter 5**). I risultati di questo ciclo di vaccinazioni hanno mostrato che pochissime IgM specifiche possono essere rilevate mediante saggi di ELISA indiretto; *in vitro*, l'induzione della proliferazione dei linfociti nelle branchie da parte di VERv non può essere indotta; dopo 24 ore è stata osservata una modulazione dei livelli di trascrizione di geni che codificano per Mx e ISG-12 e attraverso l'immunoistochimica sono stati rilevati nelle branchie gli antigeni VERv. Questi risultati hanno dimostrato che una singola immersione della vaccinazione, senza richiamo, induce nella spigola un'immunità sistemica debole.

Il passo successivo è stato quello di valutare la risposta immunitaria della spigola Europea dopo dell'immunizzazione intraperitoneale e per immersione con diverse preparazioni di Betanodavirus inattivato in seguito a formilazione, BPL e la temperatura (**Chapter 6**). È interessante notare che 30 giorni dopo l'immunizzazione intraperitoneale con VERv inattivato con la formalina, è stata rilevata una significativa quantità di IgM specifiche, a differenza degli altri protocolli di inattivazione. Inoltre, è stata analizzata l'espressione quantitativa dei geni antivirali nell'intestino e a livello renale ed è stato fatto un esperimento controllo utilizzando VERv vivo in il gruppo immunizzato intraperitonealmente con virus inattivato con formalina. Il gene Mx ha evidenziato una modulazione nella sua trascrizione nell'intestino dopo 48 ore e a livello renale dopo 24 ore dall'iniezione. Invece, il gene ISG-12 è stato quantitativamente modulato dopo 48 ore, nell'intestino e a livello renale. Infine, è stato osservato un aumento significativo

(81,9%) nella sopravvivenza dopo challenge di pesci immunizzati rispetto a pesci non immunizzati. I risultati mostrano che la vaccinazione intraperitoneale con VERv inattivato con formalina potrebbe essere un possibile candidato vaccino per l'encefalopatia virale e la retinopatia della spigola europea.

Nel **Chapter 7** sono esaminate le caratteristiche dell'immunità mucosale della spigola, focalizzandosi sulla proliferazione mucosale delle cellule T e dei leucociti IgT-specifici. Sono, inoltre, discusse le risposte immunitarie dopo l'immunizzazione mucosale e sistemica della spigola europea contro il Betanodavirus.

SUMMARY

The purpose of the work performed during the preparation of this thesis was to provide a better knowledge on fish mucosal immunity and immune responses in a marine fish model (European sea bass) against a pathogenic virus (Betanodavirus), with a view of developing possible strategies of vaccination.

The thesis is developed in seven chapters, the basic terminology has been introduced and defined in **Chapter 1**. The general aspects of Teleost immunology, basic information of the animal model (European sea bass) and viral encephalopathy and retinopathy (VER) disease were also described.

To increase the knowledge of mucosal immunity, some basic cellular aspects typical of T cells like *in vitro* induction of proliferation by lectins and expression of T cell-related genes during proliferation were investigated (**Chapter 2**). GIALT leukocytes were able to proliferate, as measured by CFSE incorporation, in response to ConA and PHA. The proliferation was higher at 48 h and PHA induced higher proliferation with respect to ConA. The mean percentages of cells recognized by both monoclonal antibodies against pan-Tcells (mAb DLT15) and CD45 cells (mAb DLT22) doubled after 48 h of stimulation by lectins. T cells were founded in the epithelium of branchial filaments and transcription was particularly high for TRβ, TRγ and IL-10 followed by CD8, CD45 and CD4, thus confirming that proliferating cells contained T cells. In addition, the cellular characterization of a new polyclonal antibody against sea bass IgT-specific leukocytes was dealt (**Chapter 3**). IgT-specific leukocytes were labelled by IIF and detected by flow cytometry and fluorescence microscopy. These cells presented dimensions and morphology of leukocytes-type cells and were more abundant in the head kidney than in the spleen or gills. Moreover, a very poor content of soluble IgT was obtained in serum and a high content in mucus. Finally, the immunoprecipitation of IgT-specific leukocytes from head kidney with Dynabeads allowed us to get two fractions, one of them rich in IgT-specific leukocytes and the other rich in IgM-specific leukocytes as verified by RT-PCR.

On the other hand, the effects of Betanodavirus immunization on immune responses in sea bass were investigated. First, a capture-based ELISA system that can be used as a

tool to investigate and quantify the presence of VERv in biological samples of adult or juveniles sea bass was developed (**Chapter 4**). In fact, VERv was detected and quantified in brain homogenates of *in vivo* infected sea bass. This capture-based ELISA system was able to detect different RGNNV strains and can be speculated that is able to detect other genotypes because of the conservation of capsid protein residues and the similarities between genotypes.

Subsequently, was performed an experimental approach that consisted of investigate the possibilites of immunizing young sea bass against VERv by means of mucosal (immersion) vaccination employing a formylated Betanodavirus preparation. (**Chapter 5**). The results of this round of immunisations showed that very few serum antigen-specific IgM can be detected by Indirect ELISA; no *in vitro* VERv-induced gills leukocyte proliferation can be induced; a modulation in transcription levels of genes coding for Mx and ISG-12 was observed after 24 hours and VERv antigens were detected in the gills by immunohistochemistry. These results shown that a single immersion vaccination without boosting induces a weakly systemic IgM-based immunity in sea bass.

The following was to evaluate immune response of sea bass after intraperitoneal and immersion immunization with different vaccines preparations inactivated by using formylation, BPL and temperature (**Chapter 6**). Interestingly, 30 days post-intraperitoneal immunization with formalin-inactivated VERv a significant antigen-specific IgM titer was detected, at difference with other inactivation protocols. Moreover, the quantitative expression of antiviral genes in gut and head kidney was analyzed and a challenge experiments using live VERv in the IP formalin inactivated group was performed. Mx gene showed a modulation of transcripts on gut after 48 hours and on head kidney after 24 hours post injection. Instead ISG-12 gene was quantitatively expressed after 48 hours on gut and head kidney. Finally, a significant increase (81.9 %) in the relative percentage survival of immunizated fish with respect to un-immunized fish was observed. This result suggest that intraperitoneally vaccination with formalin-inactivated VERv could be a possible candidate vaccine for viral encephalopathy and retinopathy of sea bass.

In **Chapter 7**, features of sea bass mucosal immunity focusing in proliferating mucosal T cells and IgT-specific leukocytes are examined. Also immune responses after mucosal and systemic immunization of sea bass against Betanodavirus are discussed.

CHAPTER 1:

1. General Introduction

Teleosts are considered to be an important model in comparative immunology studies because they are lower vertebrates presenting a morphological and functional immune system conserved until mammals. Thus, it represents a useful model for evolutionary studies, for study of human diseases and for studies on physiological activities of vertebrates. Zebrafish is the most used animal model to generate safe and effective medicines, adjuvant therapies and vaccines to be used in humans (Goody et al., 2014; Zhang et al., 2016).

Sea bass is one of the most important farmed species in the Mediterranean Sea and its importance and the necessity to control their health is related to its use as food of high quality.

Main problems of aquaculture are stress and severe pathologies without an effective therapeutic treatment. The phenomena of stress resulting from biotic and abiotic parameters makes farmed species more susceptible to infections, resulting in a loss of 100% of production. Antibiotics have been applied in farms as a bacterial control agents until evidences were presented on their risks to the consumer and environment (Hektoen et al., 1995; Cabello, 2006). The "European Medicines Agency" has banned the use of antibodies and has promoted the development of vaccination protocols and / or antigen preparations and use of immunostimulants and immunoadjuvants. There are tighter controls on the use of veterinary medicines, particularly for use in food animal species, in both Europe and USA. In this regards, the Biotechnology offers new perspectives for disease prevention, vaccination and health monitoring of breeding bony fish (Håstein, 2005).

Nowadays, there are vaccines against some viral and bacterial diseases, but there are no vaccines against parasites (Håstein et al., 2005; Biering et al., 2005; Sommerset et al., 2005). There are many different types of vaccines but the most common types are based on the administration of parts of the microorganism, inactivated pathogens or live attenuated pathogens. There is controversy surrounding the use of live attenuated pathogens because cannot be excluded the possibility that an attenuated vaccine strain will recombine with a wild-type virus to generate a fully virulent virus. In addition, when aquatic organisms are vaccinated, the confinement of the vaccine can not be

guaranteed. New kinds of vaccines are continuously under development as new vaccine preparations based on the recombinant protein technology, DNA vaccines, Virus-like Particles …

Teleost can be immunized by injection, immersion (for a short time, from 30 seconds to 2 minutes), and orally. Teleost vaccination is difficult because of their aquatic environment. Injection vaccination requires a labor-intensive and is stressful for fish because the animals must be removed from the water and anesthetized. Despite being stressful and difficult to implement on a large scale, injection immunization is currently, the most effective strategy and mostly performed by vaccination machines. Many commercial vaccines for salmonids are vaccines with oil adjuvants that greatly increase the duration of protection. However, these adjuvants cause local inflammation phenomena, a significant decrease in the growth, internal adhesions and melanization in the injection site. In order to improve the vaccines efficacy a new generation of adjuvants have been studied, wich are differents of classic adjuvants based on mineral oils or aluminum hydroxide (Anderson et al., 1997a). Recent examples are the use of interleukins (Staats et al., 1999) and CpG oligodeoxynucleotides whose effectiveness in improving the immune response is well documented (Allison et al., 2006).

Oral and / or immersion immunization are the most convenient administration method, because the treatment is simple and suitable for mass immunization and at the same time there is no fish handling so that stress is reduced. However very few oral vaccines have been commercialized so far. Some of the reasons is the need for large amounts of antigen to induce an immune respond and the lack of adaptive response after the immunization. The efficacy of oral administration depends on enzimatic hydrolysis of antigens during digestion and if the antigen will be captured in the lower gastrointestinal tracts in order to elicit an effective and adaptive immune response. Encapsulation technologies have been studies to protect the antigen but they are very expensive and not always effective strategies (Vandenberg et al., 2004; Joosten et al., 1995). PLGA-microparticles and Micromatrix are some new strategies used in the administration of oral vaccines.

The development of dietary formulations based on probiotics has been promoted in order to enhance endogenous protection mechanisms (Aly et al., 2009). Probiotics are cheaper than vaccination and protection is not specific to the pathogen. The

administration of probiotics actives not specifically the immune system, increasing significantly the parameters of innate and adaptive immunity. In bony fish, probiotics modulate the immune system acting at the systemic level (Irianto and Austin, 2002; 2003; Villamil et al., 2002; Nikoskelainen et al., 2003; Panigrahi et al., 2004; Salinas et al., 2005; Diaz-Rosales et al., 2006). It provides both adults and juveniles resistance against a wide variety of pathogens. Many authors have documented the increased capacity of defense against pathogens of Teleost whose diet was enriched with probiotics (Bly et al., 1997; Robertson et al., 2000; Spangard et al., 2001; Raida et al., 2003). Most studies on the use of probiotics in aquaculture refer to the evaluation of the effects of administering a single bacterium in one or more doses, while the comparative effects of multiple probiotic formulations have not been realized.

Immunostimulants (eg, beta-glucans, bacterial products derived from fungi and algae), always supplied as dietary supplements, can improve the innate defence mechanisms (Anderson et al., 1997a; Bonaldo et al., 2007), enabling increased resistance to pathogens and dramatically reduce the mortality rate, particularly in larval and post larval stage (Zapata et al., 1997; Mulero et al., 2008; Abelli et al., 2009). Oral vaccination and use of immunostimulating administered by immersion and / or orally are more sustainable for their easy and not stressful administration, although some of these vaccines may provide limited protection over time and require booster doses.

Fish mucosa is the first tissue invaded by the pathogens and the mucosal barriers are the first line of defence. The activation of mucosal immunity by bath and oral vaccinations will be helpful to antagonize pathogen attack. In fact, it has been demonstrated that fish mucosa is sensitive to stimulation with antigens (Zapata et al., 1996; Ototake et al., 1996; Moore et al., 1998; Vandenberg et al., 2004). It is essential to study mucosal immunity and in particular mucosal organs such as gills, skin and intestine, which are the sites directly exposed to the external environment and thus in direct contact with foreign antigens and, consequently, to potential targets.

On the other hand, sea bass (*Dicentrarchus labrax*) is one of the most important species farmed in European aquaculture industry and is very sensitive to the viral encefalopathy and retinopathy virus (VERv) (Frerichs et al., 1996, Péducasse et al., 1999). VERv is a severe pathogen that produce Viral encephalopathy and retinopathy (VER), also known

as viral nervous necrosis (VNN) disease. This infective disease is characterized by neuropathological changes associated with high mortality in several fish species. European sea bass are usually infected by RGNNV genotype because are more susceptible (Panzarin et al., 2012) and for being the most present in the Mediterranean region (Bovo et al., 1999, Olveira et al., 2009). Sea bass can be infected during young and adults ages (Breuil et al., 2001, Nakai et al., 2009). The mortality rate for this specie is higher when fish are smaller and the highest mortality rate has been reported during the larval stage, reaching 100% (Munday et al., 2002). Despite the huge losses that causes this disease to sea bass farming system, very few vaccines have been tested in this species and at present no commercially vaccines against Betanodavirus are available for sea bass.

1.1 Evolution of immune responses: The immune system of Teleosts

Fish is the largest group of vertebrates and can be find in nearly all aquatic environments. Fishes are divided into three basics groups: Teleost, also called bony fish; Elasmobranch, named Cartilaginous fishes because its skeleton is made of cartilage and Agnathans, known as jawless and represented by hagfishes (Myxiniformes) and lampreys (Petromyzontiformes).

The essential function of the immune system is the defense against pathogens that may cause infections. Some of these millions of potential pathogens are viruses, bacteria, fungi and parasites with whom there is a close coexistence. Fish present an efficiently developed immune system, that in the case of Teleosts show significant similarities with higher vertebrates. Early works on the presence of an immune system in fish appeared in the ‘40s (Duff, 1942). Subsequent studies have been focused on deeper understanding of the antibody response against viral antigen (Siegel and Clem, 1965). In 1969 it was supposed the presence of T cell-mediated activity (Leslie and Clem, 1969) and later the activity of functional T cells was shown (Cuchens and Clem, 1977).

Teleost immune system consists of two basic elements: innate or non specific and acquired or specific, both with humoral and cellular compartments (Olabuenaga, 2000). Initially it was presented as a clear division between two independent systems but today it is known that innate and acquired system are intimately linked, working together against pathogen infections and keeping homeostasis (Cooper et al., 2006; Boehm, 2012). Innate immune system is present in all the organisms and limits pathogen

invasion generating an inflammatory response through the production of generic receptors that recognize conserved patterns on different classes of pathogens (Cooper et al., 2006; Janeway and Medzhitov, 2002; Hoffmann, 2003; Akira et al., 2006). The adaptive immune system appeared about 500 million years ago during the Cambrian geological period and is unique to vertebrates. Two events seem to have played a key role in the birth of adaptive immune system. One was probably the acquisition of genes that activate the recombination or gene RAG. Thompson was the first to recognize that the cluster RAG 1 and RAG 2 seemed a separate transposon therefore suggesting a transposon origin for these genes (Thompson, 1995). The second event, which presumably had a role of equal importance in the emergence of the adaptive immune system, involve two waves of extensive gene duplication that occurred near the origin of vertebrates (Kasahara et al., 1997). To date, it is unclear whether the duplication has involved the entire genome. However we know that gene duplication and subsequent reorganization of exons have provided the conditions for the emergence of new genes, some of which have a key role in the adaptive immune system. The chromosomal duplication was responsible for the appearance of MHC paralogs (genes generated as a result of gene duplication) and also for creating essential genes to the adaptive immune system. One of the most popular hypothesis of genomic duplication seems to be the 2R hypothesis (round), which determines the possibility of two rounds of polyploidization. The first round of genome duplication after the onset of Urochordata (common ancestor of all vertebrates) and the second round before the diversification of jawed vertebrates from a common ancestor, after separation of jawless fish (Wolfe, 2001; Furlong and Holland, 2002). Actually the exact location of the second round of gene duplication is still controversial (Kasahara, 2007).

The immune system of vertebrates is characterized by its receptors of immunoglobulin superfamily (IgSF), lymphocytes and major histocompatibility complex. Moreover, it is unique in the production of a great variety of receptors for antigens, which ensures a great efficiency in preventing attacks and infections of external agents. These antigen receptors belonging to the immunoglobulin superfamily (IgSF) are composed of two polypeptide chains, a heavy chain (H) and light chain (L) for immunoglobulin and chains α/β e γ/δ for T-cell receptor (TR) (Lefranc et al., 2001; Du Pasquier, 2001). The variability of antigen receptors occurs through three important processes: variable,

diversity and joining region (V (D) J) recombination known as somatic recombination, somatic hypermutation and class-switch recombination.

T cells are directly involved in the destruction of cells expressing "danger signals". T cells are responsible of cell-cell immunity and plays an important role in the maintenance of immunological memory. T cells do not respond to soluble antigens such as nucleic acids, lipids and polysaccharides. The antigen receptor of T lymphocytes (membrane proteins, not secreted) recognize molecules of peptidic nature previously processed and presented in association with MHC membrane protein (MHC class I (cytotoxic T lymphocytes) or MHC class II (helper T lymphocytes). The family of T cells can be divided into cytotoxic T cells (Tc) responsible for cell-mediated cytotoxicity and helper T cells (Th), which modulates the immune response by secreting cytokines, in particular induce macrophage activation and other cells with immune function. The co-receptor CD4 is found on helper T cells and the co-receptor CD8 is found on cytotoxic T cells (Babbitt et al., 1985). Precursors of T cells express both CD4 and CD8 molecules and Lymphocytes lose the expression of one of them after maturing in the thymus. Other molecules are on the surface of T cells such as CD3 molecules that are involved in TR signaling. CD3 helps to transmit a signal from the TR after its interaction with MHC molecules.

DLT15 is a monoclonal antibody, "specific anti putative T-cell marker" (Scapigliati et al., 1995), which has allowed to determine T cells in lymphoid and non-lymphoid organs for the first time (Romano et al., 1997). DLT15 is the first specific marker of thymocytes and peripheral T cells discovered in fish. DLT15 antibody can be used with living cells, tissue sections and flow cytometry analysis. This has allowed, for the first time, to evaluate the content of T cells into a fish, consisting (approximated extimations) of 3% of peripheral blood leukocytes (PBL), 9% of splenocytes, 4% head kidney cells, 75% thymocytes and 51% of the leukocytes associated with GALT (Gut-associated lymphoid tissue) (Romano et al., 1997). Another works have also shown that gut-associated lymphoid tissue (GALT) of Teleost contains a large number of T cells (Abelli et al., 1997; Romano et al., 2007). This was confirmed by sequencing cDNA from RNA isolated from the intestinal cells. The cDNA was used as template in amplification reactions, using specific primers for the T-cell receptor beta-chain and resulted a αβ phenotype (Scapigliati et al., 2000). Another antibody directed against the

putative T cells is WCL38 "against carp intestinal T cells" for the carp (Rombout et al., 1998).

Distribution of TRβ+ T lymphocytes in the gut and in lymphoid tissues of sea bass have been determined by in situ hybridization and qPCR. The highest expression of TRβ+ has been found in the thymus and in the middle part of the intestine. The presence of this type of cell population might suggest a special role in antigen specific cellular immunity (Romano et al., 2007).

B cells produce antibodies upon stimulation by an antigen and in contrast to T cells can recognize antigens directly without require any processing by the intermediary cells (Coico et al., 2003). B cells are able to recognize an extremely large number of external agents of various chemical natures. At the same time they are responsible for the production of immunoglobulin (Ig) molecules that activates a wide range of immune responses including the neutralization of microbes and microbial toxins, opsonization and phagocytosis, Antibody-dependent Cellular Cytotoxicity and Complement activation. Two forms of Igs have been identified: B cell receptor (BCR), a membrane-bound molecule that acts as an antigen receptor on the B cell surface and the widely known antibody secreted by plasma cells, which is an important molecule mediating humoral immune responses (Zhu et al., 2013). A change in RNA splicing converts the membrane-bound receptor to a soluble product and is associated with the differentiation from receptor-expressing B cells to immunoglobulin-secreting plasma cells. Further modification of the primary function of immunoglobulins (that is, antigen recognition) is achieved through SHM or in some species by gene conversion (Litman et al., 2010). The Ig antibody is composed of four polypeptide chains (two identical heavy chains and two identical light chains), linked by disulfide bonds. Both chains, heavy and light, containing constant and variable regions. The variable regions of both chains form the binding site for the antigen. Ig classes are distinguished by the type of heavy chain they contain. The variation in heavy chain polypeptides allows each immunoglobulin class to function in a different type of immune response or during a different stage of the body's defense. In teleost, three classes of immunoglobulins (IgM, IgD and IgT/Z (for Teleost/Zebrafish) have been discovered. For a long time, it was believed that only IgM existed in fish, but posteriorly, IgD was discovered in channel catfish (*I. punctatus*) (Wilson et al., 1997) and IgT, similar to zebrafish IgZ, was identified in rainbow trout (*O. mykiss*) (Hansen et al., 2005) and pufferfish (*F. rubripes*) (Savan et al., 2005b). In

Teleost the antibodies have been found in many tissue fluids, such as plasma, lymph, bile, mucus skin and intestinal tract. Antibodies represent the 40-50% of total protein in serum and the main serum Ig in fish is IgM, although the concentration of IgM in serum varies according to the Teleost species (Uribe et al., 2011).

IgM is a complex polymeric molecule in Teleosts, typically is a tetramer (Warr, 1995) and has a conserved function in all jawed vertebrates (Flajnik, 2002). Fish IgM has common features with mammalian IgM. These features are hypersensitivity, complement activation via the classical and alternative pathway and acts in neutralizing the virus. Studies in some fish species, suggest that IgM may also has a role as an opsonin because of the presence of specific receptors on phagocytes that differs from mammals IgM (Galeotti, 1995).

B cells only expressing surface IgT and IgM + B cells lacking of expression of IgT were found in rainbow trout, indicating different functional roles of B cells in mucosal and non-mucosal tissues (Zhang et al., 2010; Scapigliati, 2013).

Immunoglobulins are also found in eggs and fry of different species which suggests the possibility of a transfer of maternal antibodies.

Immunoglobulin IgD has been found in all gnathostomes, except for birds, although its structure and function remains unclear (Flajnik and Kasahara, 2010).

IgT is specific to fish and is a mucosal immune-related Ig that has anti-pathogenic function only in the gut, similar to mammalian IgA and IgX in amphibians. In fact IgT has been reported to be an immunoglobulin specialized in gut mucosal immunity (Zhang et al., 2010). This is also confirmed by the higher presence of this immunoglobulin in the intestine than in serum. Secreted IgT is found in trout serum as a monomer, and in mucus as a tetramer (Zhang et al., 2010).

Teleost innate immunity and adaptive immunity show similarities and differences with mammals (Table 1) (Sunyer, 2013). The immunoglobulins IgM have the same functions as in humans but they are structurally different. IgT shows similarities with IgA of Mammals being involved in the mucosal immune system (Savan et al., 2005a; Salinas et al., 2011). Moreover, most primary and secondary lymphoid organs found in mammals are also found in fish, except lymph nodes and bone marrow (Press and Evensen, 1999). Lymphoid organs in marine Teleosts are kidney, spleen and thymus. In fish, the head kidney assumes hematopoietic functions (Zapata et al., 2006) and, unlike higher

vertebrates, is the main immune organ responsible for phagocytosis, antigen processing and the formation of IgM. Moreover immune memory is carried out in head kidney, through melanomacrophage centers (Tsujii and Seno, 1990; Tort et al., 2003). Gut, skin and gills of teleosts contain mucosa-associated lymphoid tissue that developed a mucosal immunity response (Salinas et al., 2011; Sunyer, 2013).

The immune system cells of fish present the same main characteristics of the immune cells of all vertebrates. RAG genes which promotes somatic recombination producing specific receptors of B and T lymphocytes (T cell receptors TR) were identified only in jawed vertebrates, and until date, no antibody to RAG 1 and RAG 2 are available (Randelli et al., 2008). Many sequences of RAG from different fish species are present in GenBank, such as trout (Hansen and Kaattari, 1995) and sea bass (Chen et al., 2006; Cuesta et al., 2007).

Features	**Teleots**	**Mammals**
Immunoglobulin isotypes	IgM, IgD and IgT (or IgZ)	IgM, IgG, IgA, IgD and IgE
AID	Yes	Yes
Class-switch recombination	No	Yes
Somatic hypermutation	Yes	Yes
Affinity maturation	Yes	Yes
Memory responses	Yes	Yes
TR, CD4, CD8	Yes	Yes
MHC class I and II	Yes	Yes
CD28, CD40, CD80, CD86, ICOS	Yes	Yes
Th1, Th2 and Th17 cytokines	Yes	Yes
Lymphoid organs	Spleen and thymus but no true bone marrow	Spleen and thymus and bone marrow
Lymphoid nodes	No	Yes
Mucosa-associated lymphoid tissue	Yes	Yes
Germinal centers and lymph nodes	No	Yes

Table 1. Similarities and differences in the immune system between Teleosts and mammals. AID, activation-induced deaminase; MHC, major histocompatibility complex; ICOS, inducible costimulator; Th1, Th2, Th17, subsets of helper T cells.

Thymus is the first lymphoid organ in vertebrates, where takes place T cell differentiation. Thymus is present in cartilaginous fish but not in Agnatha. However both present the genetic program that specifies the distribution of cells from the neural crest (essential for construction of the thymus) (Mayer et al., 2002).

Although Agnathans Myxiniformes do not present classical hematopoietic tissues, they have cells similar to lymphocytes which are particularly abundant in the intestine,

suggesting that this intestine works as a primary spleen (Zapata et al., 2000). In Agnathans Lamprey, cells similar to lymphocyte were found in the intestinal epithelium, in pronephros and gonads, but true lymphoid organs are anatomically indistinguishable (Mayer et al., 2002).

1.2 Mucosa-associated immune system

Teleost are constantly exposed to aggressions coming from the environment around them, such as microbes and stressors. Mucosal surfaces have been developed as a strategy to protect themselves from the external environment. Mucosal surfaces are the interface between each animal and the external environment and act as a real physical barrier and active immunological sites. Each epithelium is coated with a layer of mucus secreted by goblet cells and has the function of not allowing the adhesion of fungi, bacteria and parasites (Pickering and Richards, 1980). The secretions coating external and internal epithelial surfaces of fish incorporate lectins, pentraxin, growth inhibitors, agglutinins, precipitins, lysozyme, complement proteins, antibacterial peptides and immunoglobulins, among others. Mucus is an excellent first line of defense that completely covers the skin, gills and intestines, therefore it constitutes a barrier to inhibit tegument colonization by microorganisms (Lieschke and Trede, 2009; Uribe et al., 2011). Mucus contains various enzymes such as trypsin, lysozyme, antibodies and antimicrobial peptides that act directly or indirectly on the bacterial cell wall, causing metabolic dysfunction and cell lysis besides pore formation *per se* (Pag et al., 2008; Zhang et al., 2010).

The epidermis integrity is essential to maintain osmotic balance and to prevent the entry of external agents such as pathogenic microorganisms. In the case where this type of physical and chimical barriers are no longer effective, specialized defense cells, such as monocytes, macrophages, granulocytes and nonspecific cytotoxic cells, act against pathogens developing inflammatory, phagocytic or nonspecific cytotoxicity responses. These cells are recruited to the site of infection by inflammatory cytokines in the first place (Fischer et al., 2006).

Temperature, stress and density have been found to influence the mucosal immune system in many species, having suppressive effects. On the contrary, immunostimulants and food additive have stimulatory effects (Magnadóttir, 2010).

The mucosa-associated lymphoid tissue (MALT) contains immunoglobulins (IgM, IgD and IgT) and a variety of leukocytes including T cells, B cells, plasma cells, macrophages and granulocytes. Plasma cells in the mucosal surfaces secrete immunoglobulins which mainly play a role in maintaining the mucosal system. Four different MALTs have been described in teleosts according to the anatomic location: gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT) (Salinas et al., 2011). IgT+ B cells are the preponderant B cell subset in GALT, SALT and NALT. Instead IgM+ B cells are the main subset in spleen or head kidney (Zhang et al., 2010; Xu et al., 2013; Tacchi et al., 2014). B cells in the gut and skin of trout comprise ~4%–5% of the total cells (Zhang et al., 2010; Xu et al., 2013), whereas in the olfactory organ is ~40% (Tacchi et al., 2014). Out of this total number, in all MALT approximately half of the B cells are IgT+ and the other half are IgM+ (Zhang et al., 2010; Xu et al., 2013; Tacchi et al., 2014), although in the skin the proportion can be up to 60%/40% (Xu et al., 2013). This is in agreement with results from other species, for instance carp, where the percentage of B cells was estimated to be 5%–10% in the intraepithelial lymphocyte (IEL) compartment and the same in the skin and the gills (Rombout et al., 1998).

Gills are composed of four gill arches and every arch contains filaments, both with cores of cartilage (wich may ossify), connective tissue, blood vessels, nerves and immune cells. The gill arch is covered with epidermal tissue and presents abundant lymphatic tissue below. The filaments, however, are covered with mucosa epidermis.

Gills are large multifunctional surfaces involved in osmoregulation, nitrogenous waste excretion, pH regulation and hormone production (Evans et al., 2005). Futhermore, gills are in constant contact with water and therefore with circulating particles, microbial agents…

Presence of small and large lymphocytes have been observed in GIALT of different fish species (Grove et al., 2006a), also macrophages (Mulero et al., 2008b) eosinophil granulocytes (Mulero et al., 2007) and antibody-secreting cells (Davidson et al., 1997; Dos Santos et al., 2001). Pathogens have been found to induce innate and adaptive immune responses when entering the fish through the gills (Koppang et al., 2015).

In some salmon gills have been demonstrated significant expression of the molecules belonging to the β chain of the major histocompatibility complex type II (MHC) (Koppang et al., 1998). Moreover, intraepithelial aggregations of T cells are located at the terminal (caudolateral) end of the interbranchial septum of salmonids (Koppang et al., 2003).

1.3 The sea bass (*Dicentrarchus labrax* L.)

Dicentrarchus labrax (Linnaeus, 1758) (sea bass) is an eurythermic (5-28 °C) and euryhaline (3‰ to full strength sea water) Teleost from the family Moronidae (Cultured Aquatic Species Information Programme FAO, 2005). Sea bass is one of the farm fish species of major economic importance and is an excellent animal model with B cell and T cell markers available. Sea bass presents homologies with the adaptive and innate defense systems of vertebrates superiors like the presence of lymphocytes, MHC and cytokines.

European sea bass has a slimb and elongate body and can reach and even exceed the meter in length and 10-14 kg in weight. Sea bass are silvery grey colour to bluish on the back and silvery on the sides with a white belly sometimes tinged with yellow (Cultured Aquatic Species Information Programme FAO, 2005). The differences in colouring are usually due to different habitats and stress. During youth presents some dark spots on upper part of body, never seen on adults. Sea bass mouth is terminal and moderately protractile. It has vomerine teeth in a crescentic band, without a backward extension on midline of roof of mouth. The two separate dorsal fins are characteristic of the specie; the first with 8 to 10 spines; the second with 1 spine and 12 or 13 soft rays. The caudal fin is moderately forked and anal fin have 3 spines and 10 to 12 soft rays. Sea bass presents small scales and the lateral line complete with 62 to 74 (mode 70), but not extending onto caudal fin. The Opercle has 2 flat spines; preopercle with large, forward-directed spines on its lower margin and with a diffuse spot on the edge of opercle.

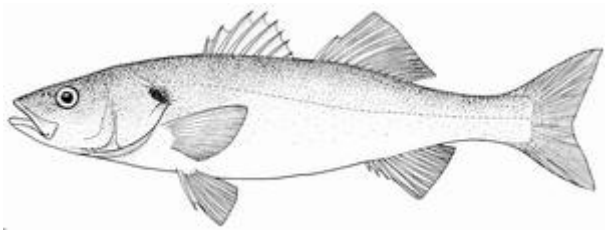

Figure 1. (Source Cultured Aquatic Species Information Programme FAO [online]). *Dicentrarchus labrax*. Kingdom Animalia, Subkingdom Bilateria, Infrakingdom Deuterostomia, Phylum Chordata, Subphylum Vertebrata, Infraphylum Gnathostomata, Superclass Osteichthyes, Class Actinopterygii, Subclass Neopterygii, Infraclass Teleostei, Superorder Acanthopterygii Order Perciformes, Suborder Percoidei, Family Moronidae, Genus Dicentrarchus, Species *Dicentrarchus labrax* (Linnaeus, 1758), known as European seabass, sea bass, white salmon.

The European (or common) sea bass, besides being spread throughout the Mediterranean is also present in the Atlantic Ocean east, the Black Sea to the Canary Islands (Fig. 2). Their preferred environment is the coastal area or near the mouth of the rivers lakes (Corbera et al., 2000). Fish farming mainly consist in sea cage farming but also can be in seawater ponds and lagoons.

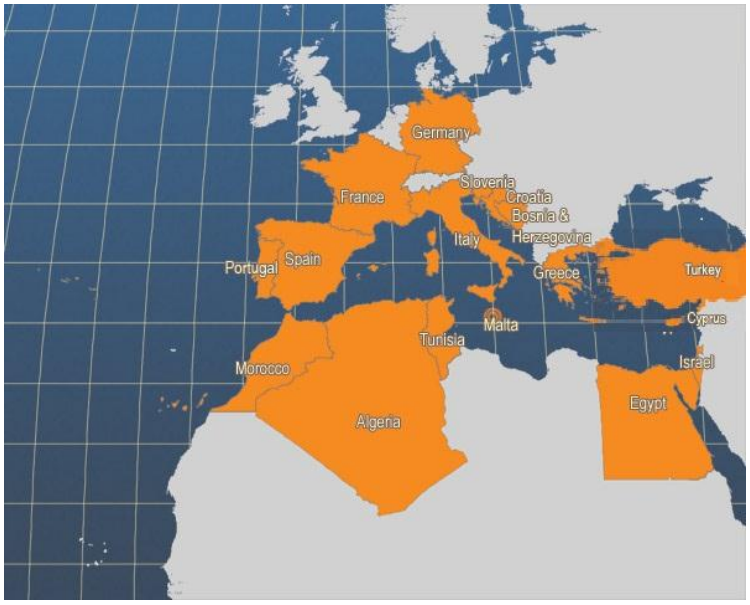

Figure 2. (Source FAO Fisheries and Aquaculture Department [online]). Main producer countries of *Dicentrarchus labrax* (FAO Fishery Statistics, 2006).

During youth are gregarious but adults become more solitary. Occasionally, adults come together to attack small fish banks. European sea bass are predators and its usual food consist mainly of shrimp, small fish, prawns, crabs and cuttlefish. Sexual maturity depends on the age and size. Sexual maturity corresponds to about 23-28 cm and 2-4 years for males and 32-43 cm and 3-5 years for females. Breading season takes place from December to March and usually every female carries 3 to 4 depositions during the breeding season, spawning floating pelagic eggs with a measure from 1.1 to 1.2 mm in diameter. Usually the eggs laid by each female are fertilized by several males; from the eggs after 3 days of incubation, depending on the temperature, they hatch small larvae.
The larvae of 3.5-4 mm are planktonic and present a voluminous yolk sac to ensure the nutrition, because newborns have closed eyes and mouth. The pectoral fins are developed two days after hatching. In the following days, from three to six, depending on the water temperature, the larvae increase the pigmentation of the body, open their mouth and consume the most part of the yolk sac. Between the fifth and fifteenth days, the eyes become functional, and they are recognizable by their dark color. Between the fifteenth and thirty-fifth day develop caudal fins, anal and bladder that expands progressively up to its final form. Between the fortieth and forty-fifth day appear the dorsal and ventral fins.

1.4 The Betanodavirus: pathogenicity, detection, vaccination

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) is a severe pathology produced by Betanodavirus (VERv) that affects marine and fresh water fish (Munday et al., 2002; Vendramin et al., 2012).
Betanodavirus is a non-enveloped icosahedral RNA virus with a diameter of about 30 nm that belongs to the family Nodaviridae. Its genome consists of two single-stranded positive sense RNA molecules, RNA1 (3.1 Kb) that encodes the RNA-dependent RNA polymerase (RdRp) and RNA2 (1.4 Kb) that encodes the viral capsid protein (Mori et al., 1992; Frerichs et al., 1996). Also presents RNA3 (0.4 Kb), a subgenomic transcript originated from the RNA1 that encodes two non-structual proteins named B1 (11 kDa) and B2 (8.4 kDa) (Souto et al., 2015).
The Betanodavirus infection can be transmitted vertically (Yanong 2010), that is from broodstock to offspring (Breuil et al., 2002; Kai et al., 2010), potentially within or on the outside of fertilized eggs, in fact the virus has been detected in gonads and in

association with eggs. It has also been shown to be transmitted horizontally (from infected fish through the water) or by exposure to virus-contaminated water. Betanodaviruses may also be transmitted through feeding of contaminated live foods, including *Artemia* (brine shrimp), *Acetesinte medius* (shrimp), *Tigriopus japonicas* (copepod) or through feeding with VNN-contaminated fish. Trash fish, such as Japanese jack mackerel (*Trachurus japonicus*), and molluscs, such as Japanese common squid (*Todarodes pacificus*), can be vectors of Betanodavirus and thus may pose a risk for outbreaks of VNN in susceptible species (Gomez et al., 2010).

Betanodavirus infection is either charaterized by a high pathogenicity and is associated with extensive mortality in fish farms (Chen et al., 2014). VERv main target is nervous system causing extensive tissue vacuolation in retina, brain and spinal cord (Aranguren et al., 2002; Lopez-Jimena et al., 2011; Chaves-Pozo et al., 2012). Generally reaches 100% of mortality in larval and juvenile stages but also can infects adults fish of more than 50 fish species worldwide (Munday et al., 2002; Castric et al., 2001; Vendramin et al., 2013). Principal symptoms are hyperinflation of the swim bladder, abnormal swimming behaviour that may cause traumatic injuries, flexing of the body, muscle tremors, changes in skin pigmentation (darkening) and encephalopathies and vacuolating retinopathy (Maltese and Bovo, 2007). VERv infection is detected by microscopic examination of vacuoles in the nervous tissue of brain, retina, and spinal cord. Also by PCR analysis of brain tissue (Arimoto et al., 1992; Lopez-Jimena et al., 2011; Chaves-Pozo et al., 2012) employing amplification primers specific to RNA1 or RNA2 (Baud et al., 2015) and enzyme-linked immunosorbent assays (ELISAs) (Arimoto et al., 1992; Romestand and Bonami, 2003; Fenner et al., 2006; Shieh and Chi, 2005; Breuil et al., 2001).

So far four differents genotypes of Betanodavirus have been determined based on the phylogenetic analysis of T4 region, within the RNA2 segment: Barfin flounder nervous necrosis virus (BFNNV), Red spotted grouper nervous necrosis virus (RGNNV), Striped jack nervous necrosis virus (SJNNV) and Tiger puffer nervous necrosis virus (TPNNV) (Nishizawa et al., 1997). A fifth genotype isolated from *Scophthalmus maximus* named as Turbot nervous necrosis virus (TNNV) has been described by Johansen et al., 2004.

RGNNV has been found in warm water fish, TPNNV and BFNNV have been found in cold water fish, and SJNNV has been found in warm and cold water fish. The optimal

temperatures for viral replication varies in the different strains (RGNNV: 25–30 °C, TPNNV: 20 °C, BFNNV: 15–20 °C, and SJNNV: 20–25 °C) and the temperature also affects their infection rate (Chen et al., 2014; Iwamoto et al., 2000).

Genotype of betanodavirus[a]	Optimal temperature for replication	Susceptible host	Serotype[b]	Key reference
Striped jack nervous necrosis virus (SJNNV)	20–25 °C	Japanese striped jack (*Pseudocaranx dentex*) Gilthead sea bream (*Sparus aurata*) Solea senegalensis (*Senegalese sole*) Greasy grouper (*Epinephelus tauvina*)	A	Nishizawa et al. (1997), Munday et al. (2002), Iwamoto et al. (2000)
Tiger puffer nervous necrosis virus (TPNNV)	20 °C	Japanese striped jack (*Pseudocaranx dentex*) Tiger puffer (*Takifugu rublipes*)	B	Munday et al. (2002)
Barfin flounder nervous necrosis virus (BFNNV)	15–20 °C	Atlantic cod (*Gadus morhua*) Barfin flounder (*Verasper moseri*) Haddock (*Melanogrammus aeglefinus*) Atlantic halibut (*Hippoglossus hippoglossus*)	C	Munday et al. (2002)
Redspotted grouper nervous necrosis virus (RGNNV)	25–30 °C	Sevenband grouper (*Epinephelus septemfasciatus*) Redspotted grouper (*Epinephelus akaara*) Kelp grouper (*Epinephelus moara*) Orange spotted grouper (*Epinephelus coioides*) Dragon grouper (*Epinephelus lanceolatus*) Greasy grouper (*Epinephelus tauvina*) Humpback grouper (*Cromileptes altivelis*) Barramundi (*Lates calcarifer*) Japanese sea bass (*Lateolabrax japonicus*) European sea bass (*Dicentrarchus labrax*)	C	Chi et al. (2003)

[a] Striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV) and redspotted grouper nervous necrosis virus (RGNNV).
[b] Antisera test against 4 genotypes of betanodavirus, serotype A: only neutralize SJNNV; serotype B: only neutralize TPNNV; seratype C: both neutralize RGNNV and BFNNV.

Table 2. Characteristics of Betanodavirus genotypes (Chen et al., 2014).

Different vaccines have been developed using formalin-inactivated virus (Kai and Chi, 2008; Pakingking et al., 2009), binary ethylenimine (BEI)-inactivated virus (Dos Santos et al., 2005; Kai and Chi, 2008), recombinant proteins (Dos Santos et al., 2005; Husgard et al., 2001; Lin et al., 2007; Sommerset et al., 2005; Tanaka et al., 2001; Vimal et al., 2014a) and DNA based vaccines (Sommerset et al., 2005; Vimal et al., 2014b).

2. Aims and Outline of the thesis

In order to study the evolution of immune responses in fish against pathogens, the European sea bass was employed as experimental model, being this the most important farmed species in Mediterranean area and a specie for which molecular and cellular reagents are available. The sea bass is very sensitive to the infection of Encephalopathy and Retinopathy Virus (VERv), or Betanodavirus that causes massive mortalities in this species. Thus the selected pathogen model has been the Betanodavirus, also considering that effective vaccines to fight the pathology are not yet available. Nowadays the new

vaccination strategies for VER are focusing on developing methods mucosa (mass) of delivery to avoid problems due intraperitoneal vaccination as stress. Main objectives of the thesis were to improve knowledge of mucosal immunity and develop strategies of vaccination against Betanodavirus.

Research on mucosal immunity can give insight into the active immunological mechanisms against VERv infection, being mucosal tissues the entry site of VERv. In order to improve knowledge of mucosal immunity basic molecular and cellular signatures of T cells in the gills of sea bass have been investigated by transcriptome profiling and lectin-induced T cell proliferation (**Chapter 2**). A whole transcriptome was obtained by deep sequencing of RNA from unstimulated gills that has been analyzed for the presence of T cell-related transcripts. Futhermore the number of T cells in the gills of sea bass has been measured and analysed their abundance and distribution in the epithelium. In addition lectin-proliferation assays of leukocytes from gills against ConA and PHA have been performed, controlling the expression of T cell-related genes TRβ, TRγ, CD4, CD8α, CD45 and IL-10.

Immunoglobulin T (IgT) is one of the key effector molecules of jawed vertebrate's mucosal immune system and should play a role in anti-viral immunity. Until now no antibodies to sea bass IgT were available as in trout (Zhang et al., 2010). Taking advance of the whole gill RNA transcriptome that was obtained, a polyclonal antibody against sea bass IgT was developed and has been used it to perform a characterization of sea bass (*Dicentrarchus labrax*) IgT-specific leukocytes (**Chapter 3**).

The investigations on humoral responses of European sea bass against VERv have allowed the development of a capture-based ELISA system to facilitate the diagnosis and detection of VERv in biological samples of adult or juveniles sea bass (**Chapter 4**). In fact, the assay resulted reliable and useful to detect VERv in brain homogenates of *in vivo* infected sea bass.

The efficacy of a formalin-inactivated vaccine against viral encephalopathy and retinopathy to be administered by immersion has been studied (**Chapter 5**). Immunological responses have been monitored through quantitative expression of antiviral genes as well as determination of an effective uptake of VERv antigens after

immersion and protection in experimentally infected European sea bass were checked. Moreover, different methods for viral inactivation have been tested for the production of a prototype vaccine that could be administered by immersion or intraperitoneally (**Chapter 6**).

CHAPTER 2: Mucosal responses: T cell transcripts and T cell activities in the gills of the teleost fish sea bass (*Dicentrarchus labrax*).

This Chapter describes cellular and molecular features of mucosal T cells that contributes to the thesis for the acquisition of a wider knowledge of mucosal immune responses. T cells (and their respective receptors and signaling molecules) play an essential role in regulating mucosal immune responses, responding to mucosal infection or vaccination.

2.1 A B S T R A C T

The gills of fish are a mucosal tissue that contains T cells involved in the recognition of non-self and pathogens, and in this work some features of gill-associated T cells of European sea bass, a marine model species, were described. A whole transcriptome was obtained by deep sequencing of RNA from unstimulated gills that have been analyzed for the presence of T cell-related transcripts. Of the putative expressed sequences identified in the transcriptome, around 30 were related to main functions related to T cells including Th1/Th2/Th17/Treg cell subpopulations, thus suggesting their possible presence in the branchial epithelium. The number of T cells in the gills of European sea bass, measured with the specific T cell mAb DLT15 range from 10% to 20%, and IHC analysis shows their abundance and distribution in the epithelium. Leukocytes from gills are able to proliferate in the presence of lectins ConA and PHA, as measured by flow cytometry using CFSE fluorescence incorporation, and during proliferation the number of T cells counted by immunofluorescence increased. In lectin-proliferating cells the expression of T cell-related genes TRβ, TRγ, CD4, CD8α, CD45 and IL-10 increased dramatically. These data represent a first analysis on T cell genes and on basic T cell activities of fish gills, and suggest the presence of functionally active subpopulations of T lymphocytes in this tissue.

2.2 INTRODUCTION

The gills of fish are, in terms of exposed surface, the biggest tissue of most Teleost species (e.g. 1 m^2/kg in carp; Oikawa and Itazawa, 1985), they serve to maintain fish homeostasis by the uptake of nutrients and substances, and by forming an active barrier against the entry of pathogens. The thin gill epithelium is a mucosal tissue at direct

contact with the water environment, and contains a gill-associated lymphoid tissue (GIALT) with macrophages/granulocytes (Barnett et al., 1996; Lin et al., 1998; Mulero et al., 2008b), B cells (Davidson et al., 1997; Dos Santos et al., 2001; Salinas et al., 2011), T cells (Scapigliati et al., 1999), and with high expression levels of T cell-related genes (Boschi et al., 2011). The GIALT is a first-line of defense against the entry of pathogens from the microbe-rich water environment and thus must be armed with either a fast nonself recognition and elimination system, together with an antigen recognition/antigen memory asset.

For these features of innate and acquired immune defenses, the GIALT is regarded as a target tissue for mucosal vaccination and, effectively, vaccination of some fish species is achieved by brief immersion of fish in antigenic mixtures diluted in water, from which the antigen enters the animal through the gills and other mucosal tissues (Salinas et al., 2011).

Of particular importance are lymphocytes of mucosal tissues, where T cells may be present in percentages up to 60% in gut-associated lymphoid tissue (GALT) and of 25% in GIALT (Randelli et al., 2008) and, as in mammals, are considered to be involved in a first line of defense against pathogen entry. However, despite the obvious importance of GIALT in maintaining fish health, the knowledge on its cellular and molecular components is still meagre.

In order to extend our previous observations on T cells and T cell transcripts (Boschi et al., 2011; Pallavicini et al., 2010; Randelli et al., 2008), and to achieve a more comprehensive knowledge of GIALT asset and function in fish my group in collaboration with the University or Trieste employed the European sea bass (*Dicentrarchus labrax*) as a model to produce a whole gill RNA transcriptome and analyze it for the presence of genes coding for T lymphocyte-related peptides. Moreover, some basical cellular aspects typical of T cells like *in vitro* induction of proliferation by lectins and expression of T cell-related genes during proliferation were investigated as part of this thesis, an argument poorly known in fish.

2.3 MATERIAL AND METHODS

Fish and leucocytes

Healthy juveniles of sea bass were obtained from a local fish farm (Civitaittica SrL, Civitavecchia, Italy). The fish were lethally anesthetized with tricaine methanesulfonate (Sigma) and blood was drawn from caudal vein with a syringe. The gills were then removed and immersed in cold HBSS, branchial arches were gently cleaned with filter paper to remove external mucus and gill filaments cutted out with small scissors in a Petri dish. The fragments were finely minced in HBSS with the aid of a scalpel and pushed through a 100 μm nylon mesh. Obtained cells were collected in HBSS, washed in HBSS at 490 × *g* for 10 min, passed through a 40 μm nylon mesh and loaded over Percoll gradients at 1.02 and 1.07 g/ cm^3. After centrifugation at 600 × *g* for 20 min, leukocytes were collected at the interface, washed with HBSS and counted in a hemocytometer.

Leukocyte cultures were employed immediately and were maintained at 22 °C in L15 complete medium (CM) containing 10% fetal bovine serum (FBS) and penicillin–streptomycin (reagents from Gibco). All culture media and solutions were adjusted to 350 mOsm/ kg by adding 2M NaCl.

RNA sequencing and* de novo *transcriptome assembly

The RNA was obtained from gill filaments of a healthy juvenile (cells obtained as described earlier) immediately placed in 1 mL of denaturing solution (Tripure, Roche). Total RNA was isolated from leukocytes following the manufacturer's instructions. The quality of RNA was checked carefully by a denaturing electrophoresis gel and quantified by spectrophotometry (Picodrop) before cDNA preparation procedures.

The cDNA libraries were prepared and subjected to massive sequencing at the Institute for Applied Genomics (Dr. M. Gerdol, Udine, Italy), using an Illumina HiSeq2000 sequencing platform, with a 100 cycles paired-end strategy. The output sequencing reads were trimmed for the removal of low quality bases (the base calling quality threshold was set at 0.5), ambiguous nucleotides and short reads (the length threshold was set at 75 bp) with the "trim sequences" tool included in the CLC Genomics Wokbench v. 7.0.3 (CLC bio, Aarhus, Denmark). The resulting sequences were assembled with Trinity (Grabherr et al., 2011) using the default options and setting the minimum allowed contig length to 201 bp (Grabherr et al., 2011).

Only the longest transcript generated per each gene model was kept for further analysis, in order to eliminate sequence redundancy in the transcriptome assembly. Contigs

resulting from the assembly of mitochondrial RNAs were detected by BLASTn, using the *Morone saxatilis* full mitochondrial genome as a query (HM447585.1), with an e-value cutoff of 1×10^{-30}.

Details on contigs annotation, quality assessment, and digital gene expression analysis are available in Nuñez Ortiz et al. (2014).

Leukocyte proliferation

Leukocytes obtained from the gills (n = 5) as described earlier were resuspended at 2.5 × 106/mL in serum free L15 medium and incubated for 15 min with 5 μg/mL of carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) as previously reported (Hohn and Petrie-Hanson, 2012; Toda et al., 2009), then washed with PBS by centrifugation at 450 × g for 10 min and incubated in 1 mL of CM (in a well of a 24-well plate) containing 10 μg/mL of phytohaemoagglutinin-P (PHA, Sigma) or same amount of concanavalin-A (ConA, Sigma). At the end of incubation (48 h), cells in the wells were mixed extensively by gentle pipetting, 200 μl was removed and the CFSE fluorescence was read in a Beckman-Coulter Epics LS-200 Flow cytometer. Analysis of acquired 10.000 events was done with Expo32 system software.

Immunofluorescence and flow cytometry

For immunofluorescence (IIF) analysis, leukocytes from the gills (n = 3) were cultured and stimulated with lectins as described earlier for proliferation, but omitting CFSE labeling. At the end of incubation, the wells containing leukocytes were mixed gently and 300 μl of cell suspension was removed, dispensed in three tubes and incubated for 60 min on ice with 300 μl of the mAb DLT15 (likely specific for pan-T cell) (Scapigliati et al., 1995), or with mAb DLT22 (specific for CD45 cells) (Marozzi et al., 2012) as culture supernatants. For background staining the cells were incubated with CM. After washing with PBS at 450 × g for 10 min the cells were incubated with a 1:250 dilution of a FITC-labeled antimouse antibody (Cappel) for 30 min, washed again and the fluorescence read by flow cytometry as earlier.

Real-time quantitative PCR

Leukocytes incubated with PHA and ConA as earlier were pelleted by centrifugation at 450 × *g* at 4 °C for 10 min, washed with PBS by centrifugation and dissolved in 1 mL of Tripure (Roche).

Total RNA was isolated from leukocytes following the manufacturer's instructions. RNA was suspended in DEPC-treated water and used for quantitative PCR (qPCR). Controls for the presence of DNA contamination were performed using β-actin primers that bracket an intron. For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used following manufacturer's instructions.

The transcription levels of the genes TRβ, TRγ, CD4, CD8α, CD45, and IL-10 were determined with an Mx3000P™ real-time PCR system (Stratagene) equipped with version 2.02 software and using the Brilliant SYBR Green Q-PCR Master Mix (Stratagene) following the manufacturer's instructions. ROX was used as internal passive reference dye since it is not reactive during real-time PCR and therefore can be used to normalize slight differences in the volume of the added real-time PCR reaction, transparency of the plastic caps and other sources of well-to-well differences. Specific PCR primers (Table 1) were designed for the amplification of products (ca. 200 bp) from the conserved region of all analyzed genes and ribosomal 18S rRNA was used as house-keeping gene. A 10 ng of cDNA template was used in each PCR reaction. The PCR conditions were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. Reactions were performed in triplicate for each template cDNA, which was replaced with water in all blank control reactions. Each run was terminated with a melting curve analysis which resulted in a melting peak profile specific to the amplified target DNA. The analysis was carried out using the endpoint method option of the Mx3000P™ software that allows the fluorescence data at the end of each extension stage of amplification to be collected.

A relative quantification was performed comparing the levels of the target transcript to the reference transcript (18S rRNA) and the sample control was used as calibrator and defined as 1.0 value. Quantitative assessment was based on determination of threshold cycle (Ct), defined as the cycle at which a statistically significant increase in fluorescence above the background signal was detected. The specificity and size of the real-time PCR products were checked by agarose gel electrophoresis.

Gene	Primer sequence	EMBL accession
18S rRNA	FW: 5′-CCAACGAGCTGCTGACC-3′ RW: 5′-CCGTTACCCGTGGTCC-3′	AY831388
CD8-α	FW: 5′-CTAAGATTCGGCAAAATAACTCGA-3′ RW:5′-GATGAGGAGTAGAAGAAGAAGGCC-3′	AJ846849
TRβ	FW: 5′-GACGGACGAAGCTGCCCA-3′ RW: 5′-TGGCAGCCTGTGTGATCTTCA-3′	AY831387
IL-10	FW: 5′-ACCCCGTTCGCTTGCCA-3′ RW: 5′-CATCTGGTGACATCACTC-3′	AM268529
CD4	FW: 5′-GTGATAACGCTGAAGATCGAGCC-3′ RW: 5′-GAGGTGTGTCATCTTCCGTTG-3′	AM849812
TRγ	FW: 5′-CTGCTGTGTGTGGCCTCAGAC-3′ RW: 5′-GTGCTGGACGGAGCAGTGGAT-3′	FR745889
CD45	FW: 5′-GTCTGGAGGCAGAAGG-3′ RW: 5′-CTCTCAAACTCCTCCTCC-3′	HE585247

Table 1. Sequence of primers for qPCR.

Immunohistochemistry

Gills from three different fishes were fixed for 7 h at 4 °C in Bouin's fixative. After embedding in paraplast, blocks were serially sectioned at a thickness of 7 μm. Some sections were stained with May–Grümwald Giemsa or hematoxylin and eosin, while others were used for the immunohistochemistry (IHC).

IHC was performed by ABC-peroxidase with nickel enhancement as previously described (Scapigliati et al., 1995). In summary, serial sections were incubated for 18 h at room temperature with the mAb DLT15 (diluted 1:10 in PBS containing 5% normal horse serum and 0.1% sodium azide). Hybridoma medium substituted the primary antibody in negative controls. Thereafter, sections were incubated for 60 min with biotinylated horse anti-mouse IgG serum (Vector Labs., Burlingame, USA) diluted 1:1000 with PBS containing 0.1% sodium azide and 1% BSA, followed by incubation for 60 min with avidin-biotinylated peroxidase complex (ABC, Vectastain Elite, Vector). After rinses and staining (diaminobenzidine and nickel enhancement), sections

were dehydrated, mounted and examined under a Zeiss microscope equipped with a colour video camera (Axio Cam MRC, Arese, Milano Italy) and a software package (KS 300 and AxioVision).

Statistical analysis

The data from different experiments have been presented as the mean ± SD of five individual fish for proliferation, and three individual fish for IIF and quantitative PCR. For proliferation and IIF the significance with respect to controls was for $P < 0.05$. The statistics for transcriptome sequences readings and for quantitative PCR are given in the description of Fig. 4 and in Appendix: Table S1b.

2.4 RESULTS

Transcriptome sequencing

The Illumina sequencing of *Dicentrarchus labrax* gills transcriptome produced 68,643,808 raw nucleotide paired-end reads. The average read length was 100 bp, corresponding to a complete dataset of 6.86 GB of sequence data. This set was reduced to 6.56 GB following the trimming procedure, which removed short reads and low quality or ambiguous nucleotides (the average reads length was thus reduced to 98.7 bp). A total of 66,423,874 paired-end reads were used for the *de novo* assembly, that generated a total of 133.270 contigs (length of the assembled contigs from 201 to 28.088 bp), including redundant entries from alternatively spliced transcripts originated from the same gene.

Overall, even though our assembly just represents the transcriptome of a single tissue, it appears to be quite complete, if compared to the *G. aculeatus* genome. In fact, the 90.65% of all the proteins predicted from the stickleback genome found a significant match within the sea bass transcriptome.

The statistics of the annotation performed with Trinotate are detailed in Appendix: Table S1d. The BLASTx against the UniProtKB/SwissProt protein database identified 22,585 contigs with at least one positive hit. PFAM domains (Punta et al., 2012) could be annotated in 14,461 of the proteins predicted from the assembled contigs. Gene ontology mappings assigned 3662 contigs to at least a biological process, molecular function or cellular component category and 1990 contigs were assigned to an eggNOG

term (Powell et al., 2014). In these contigs T cell-specific expressed genes were identified and were reported in Table 2.

The most abundant annotated gene ontology, PFAM domains and eggNOG terms are shown in Table 3 and Appendix: Tables S2–S3.

List of T cell-related genes in sea bass gills transcriptome.

Gene product	Accession number	Function	
TRα	AY831387	T cell receptor	
TRβ	FN667953	T cell receptor	
TRγ	FR745889	T cell receptor	
CD3γ/δ	FN667954	Pan-T cell coreceptor	
CD4	AM849811	T helper cell coreceptor	
CD6	KJ818340	T cell and thymocyte marker	
CD8	AJ846849	T killer cell coreceptor	
CD28	KJ818341	T cells activation coreceptor	
IFN-γ	KJ818329	T cell-activated product	Cytokine markers of T cell subpopulations (Th1, Th2, Th17)
IL-2	KJ818330	T cell growth factor	
IL-4 (three isoforms)	KJ818331-3	$CD4^+$ T cell product	
IL-10	AM268529	$CD4^+$ T cell product	
IL-12 (α-subunit)	KJ866951	$CD4^+$ T cell product	
IL-12 (β-subunits)	KJ818334	$CD4^+$ T cell product	
IL-17 (five isoforms)	KJ818335-39	$CD4^+$ T cell product	
IL-21	KM217411	$CD4^+$ T cell product	
IL-22	KJ818327	$CD4^+$ T cell product	
Lck	KJ818349	T cell-associated tyrosine kinase	
Zap70	KJ818342	T cell-associated protein kinase	
FoxP3 (many isoforms)	KJ818328	$CD4^+$ T cell-associated molecule	Master regulators of Treg cells
T-bet	KJ818346	$CD4^+$ T cell-associated molecule	
GATA-3	KJ818348	T cell-associated transcription factor	
Granzyme A	KJ818347	$CD8^+$ T cell product	
CCR3	KJ818345	$CD4^+$ T cell-associated chemokine receptor	
CCR5	KJ818343	T cell trophic factor	
CXCL13	KJ818344	T cell-associated chemokine	

Table 2. List of T cell-related genes in sea bass gills transcriptome. Master genes for all T cell subset are expressed as for example: T cell receptors; co-receptors; key cytokines of the adaptive immune response; set of cytokine gene markers of T cell subpopulations, master regulators for T regulatory (Treg) cells,... Matches with RAG-1 gene sequences were not found and thus can be speculated that in unstimulated gills the expression of genes for somatic recombination of Ig-domains is below the detection threshold.

Top 20 most abundant PFAM domains

PFAM ID	Description	Abundance
PF13912.1	C2H2-type zinc finger	1883
PF13894.1	C2H2-type zinc finger	1764
PF00096.21	Zinc finger, C2H2 type	1343
PF13465.1	Zinc-finger double domain	1114
PF13504.1	Leucine rich repeat	1044
PF00400.27	WD domain, G-beta repeat	828
PF00023.25	Ankyrin repeat	699
PF13895.1	Immunoglobulin domain	529
PF00069.20	Protein kinase domain	500
PF12796.2	Ankyrin repeats (3 copies)	487
PF13637.1	Ankyrin repeats (many copies)	485
PF07714.12	Protein tyrosine kinase	471
PF13606.1	Ankyrin repeat	404
PF13857.1	Ankyrin repeats (many copies)	343
PF12874.2	Zinc-finger of C2H2 type	277
PF00560.28	Leucine Rich Repeat	270
PF13927.1	Immunoglobulin domain	257
PF14259.1	RNA recognition motif (a.k.a. RRM, RBD, or RNP domain)	255
PF00076.17	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)	241
PF07686.12	Immunoglobulin V-set domain	225

TLRs

Ig, TCR

TLRs

Ig, TCR

Ig, TCR

Table 3. Top 20 most abundant PFAM domains. Interestingly, in most abundant PFAM domains are repeatedly present leucine-rich repeats (LRR) and immunoglobulin (Ig) domains. Very likely, the contigs annotated as bearing LRR should contain Toll-like receptors (TLR) related to the presence of pathogen specific TLRs, which have been described in fish. On the other hand, Ig domains should correspond to T cell receptors, co-receptors and immunoglobulins, all of which have been already described to be present in gills.

Leukocyte proliferation

The results of lectin-induced proliferation experiments of GIALT leukocytes monitored by CFSE fluorescence are shown in Fig. 1. The upper panel presents the mean ± SD of five diverse experiments after 24 and 48 h of lectin incubation, the fluorescence percent values in selected M1 gate (see lower panel) for control cultures at 24 and 48 h were 13 ± 5 and 10 ± 4, the values raised at 53 ± 9 and 58 ± 9 for PHA-stimulated cells, and 30 ± 5 and 25 ± 4 for ConA-stimulated cells, thus showing a clear CFSE increase for both lectins.

The flow cytometric pattern of a typical lectin-induced proliferation of GIALT leukocytes is shown in lower panel of Fig. 1. Morphological analysis shows three main leukocyte populations distinguishable by different FSC (Fig. 1a), the two populations at lower FSC values were selected for the analysis, and the third population was excluded. After 48 h of incubation the CFSE fluorescence in control leukocytes is shown in Fig. 1b with the M1 marker region considered for the analysis, having a value of 9.4%. In ConA-stimulated and PHA-stimulated cells (Fig. 1c and d, respectively), the percent values of positivity in the M1 region were 37.5% in ConA-stimulated cells, and 45.8% in PHA-stimulated cells.

Three lectin-induced proliferation experiments were monitored by IIF and flow cytometry using an anti-T cell mAb (DLT15) and an anti-CD45 T cell mAb (DLT22). A summary of DLT15 and DLT22 IIF staining data after 24 and 48 h, as the mean ± SD of four diverse experiments, is shown in upper panel of Fig. 2. From the obtained data a statistically significant increase in the number of DLT15 and DLT22 immunoreactive cells for both lectins at 24 and 48 h is evident. The percentages of staining are reported in each bar of Fig. 2 and reached maximal increase values at 48 h of 32.8% for DLT15 and 30% for DLT22.

The flow cytometric results of a typical lectin-induced IIF experiment are shown for mAb DLT15 in the lower panel of Fig. 2, with the FSC and SSC gate employed for analysis (Fig. 2a). Control GIALT leukocytes show a DLT15 positivity in the M1 region of 19.1% (Fig. 2b) that increased to 28.8% with ConA (Fig. 2c) and to 27.2% with PHA (Fig. 3d). When using the DLT22 mAb, a similar pattern of IIF staining was observed, with an increase in the M1 region to 34.6% for ConA and to 38.3% for PHA (Appendix: Fig. S1).

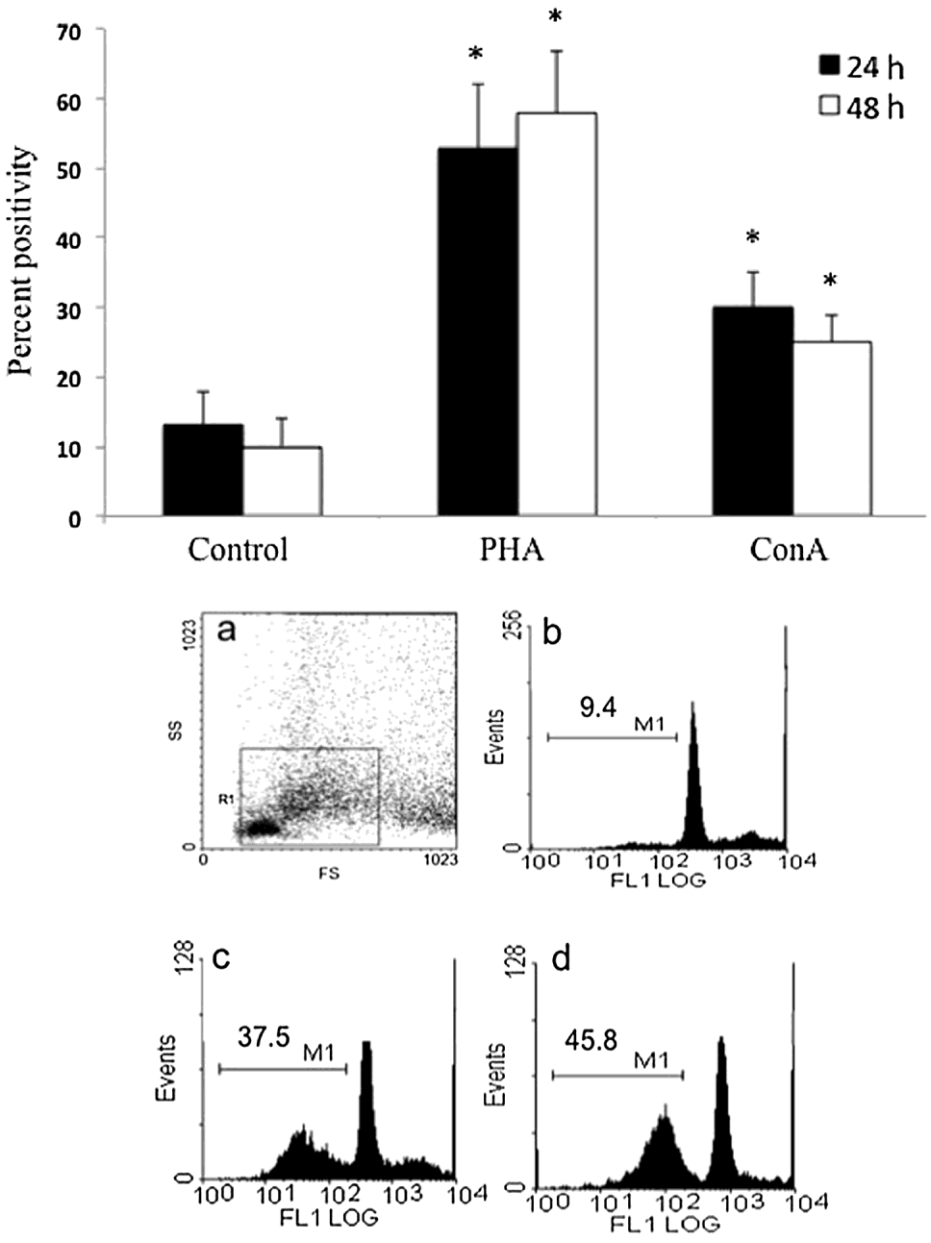

Figure 1. Lectin-induced proliferation. The upper panel shows mean percentages of CFSE positivity in control gill leukocytes, and in cells treated with 10 μg/mL of PHA and ConA after 24 h (black bars) and 48 h (empty bars). The percentages, evaluated from the M1 regions (lower panel) come from five independent experiments and are shown as mean value ± SD. Asterisks indicate significant difference ($P < 0.05$) with controls. The lower flow cytometric panel shows a typical lectin-induced proliferation experiment of gill leukocytes, with the FS and SS morphology of cell populations in (a) and the square gate employed for analysis. The CFSE fluorescence in control cells after 48 h is shown in (b) together with the M1 region considered for positivity, and in cells

treated with ConA (c) or PHA (D). Numbers are the percent of cell positivity of M1 region.

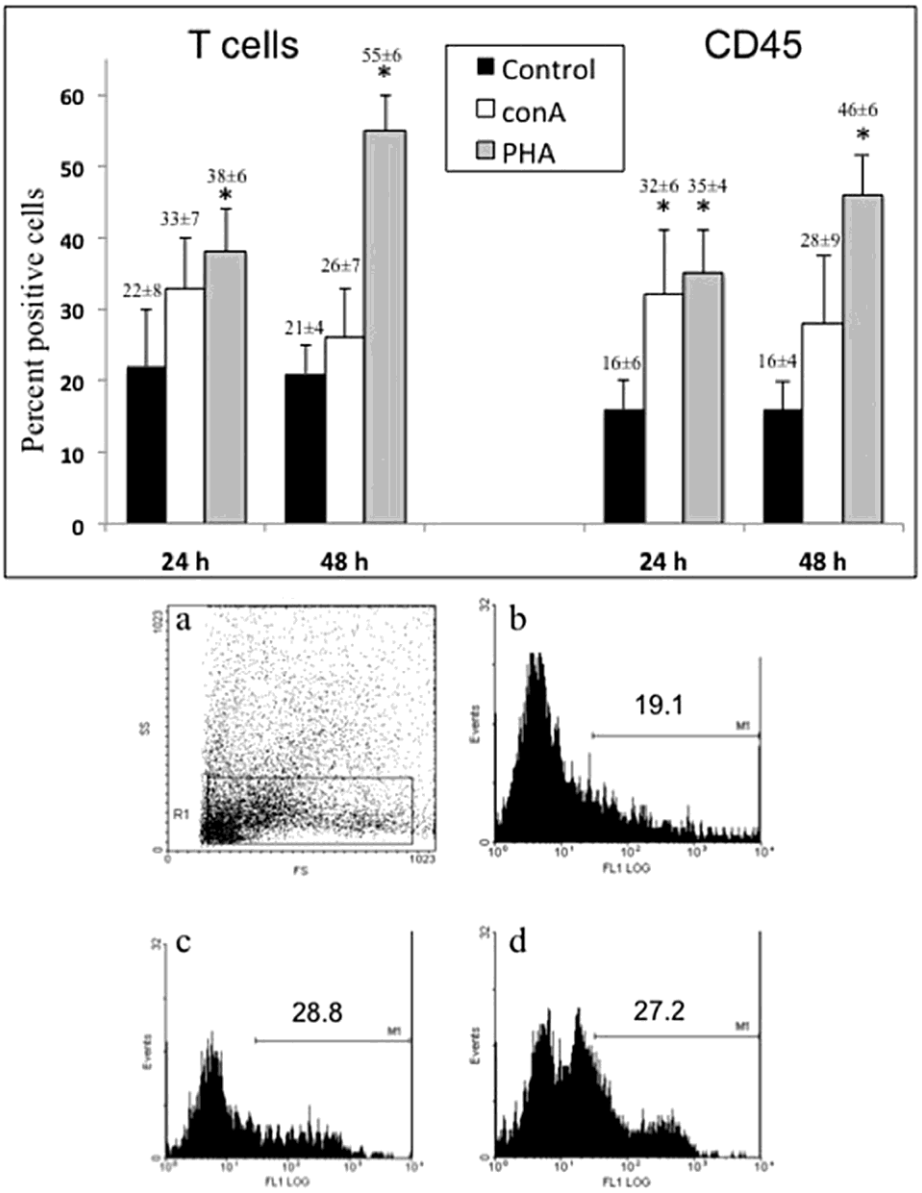

Figure 2. Immunofluorescence with anti-T cell mAbs. The upper panel shows mean percentages of gill leukocytes positive to mAbs DLT15 (T cells) and DLT22 (CD45-T cells) in control cells (black bars) and in cells treated with 10 μg/mL of ConA (empty bars) and PHA (gray bars) after 24 and 48 h. The percentages of positive cells come from four independent experiments and are shown as mean value ± SD on each bar. Asterisks indicate significant difference ($P < 0.05$) with controls. The lower flow cytometric panel shows a typical lectin-induced proliferation experiment of gill

leukocytes, with the FS and SS morphology of cell populations in (A) and the square gate employed for analysis. The DLT15 mAb fluorescence in control cells after 48 h is shown in (B) together with the M1 region considered for positivity, and in cells treated with ConA (C) or PHA (D). Numbers are the percent of positive cells in the M1 region.

Immunohistochemistry and histology

The gill system of sea bass was confined within two interconnected gill chambers. Each gill was displayed semilunar in shape consisting of a gill arch that carried gill rakers on its concave border and gill filaments on its convex border. The filaments appeared long in the middle and shortened toward the extremities. Lamellae, parallel to each other, were located perpendicularly on the filaments. The filaments were covered by an epithelial layer with some mucous cells and immune cells interspersed in it. Few chloride cells were generally located in the filaments, mostly at the base of the lamellae. The epithelial layer was extended to cover the contra lateral lamellae, while in the center of each lamella, numerous vascular spaces were located (data not shown).

The in situ distribution of T cells in the branchial epithelium was performed using the DLT15 mAb (Fig. 3A–C) that recognized numerous T cells localized in the epithelium of the filaments while rare T lymphocytes were found in the epithelium of the branchial lamellae. No reaction was observed in the control sections (Fig. 3D).

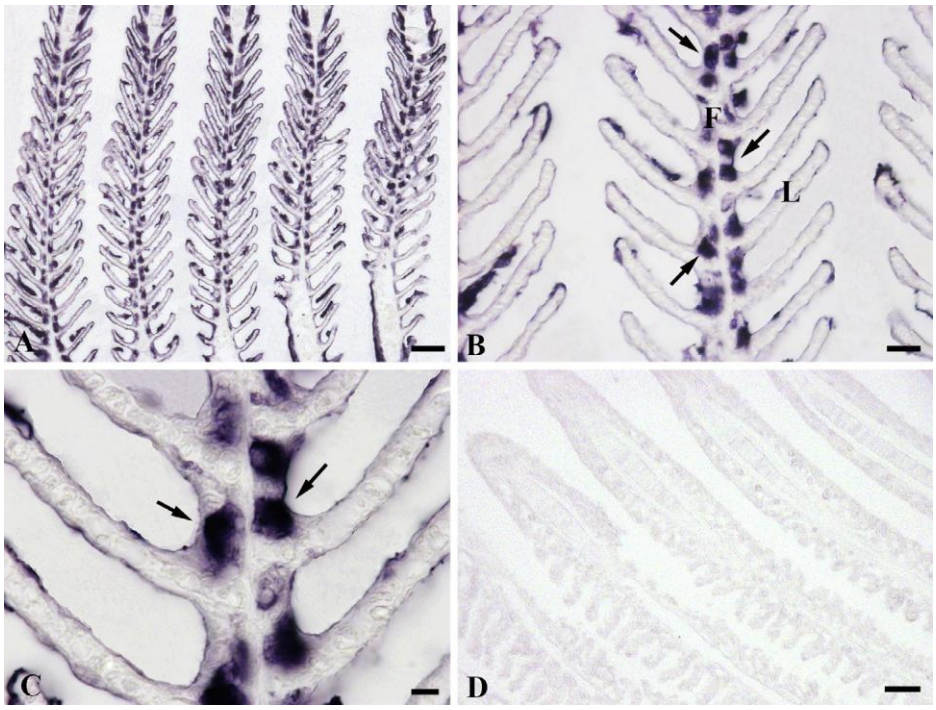

Figure 3. Immunohistochemical localization of T cells in the gills of sea bass. (A) DLT 15 immunoreactive cells localized within the branchial filaments. Scale bar = 50 μm. (B) Higher magnification of (A) showing T cells within the filament epithelium (arrows). Scale bar = 10 μm. (C) DLT 15 positive cells are shown at higher resolution in (C). Scale bar = 5 μm. (D) Negative control showing the absence of immunoreactivity in gill tissue. Scale bar = 50 μm. F: filament; L: lamella.

Quantitative PCR

The GIALT leukocytes stimulated with ConA and PHA for 24 and 48 h were employed for transcriptomic analysis of some marker genes for T cell-related activities, and obtained results for TRγ, TRβ, CD4, CD8, CD45, IL-10 are shown in Fig. 4.

All tested genes showed an increase of transcription after 24 h: in the case of TRβ, TRγ and IL-10 the increases of expression were about 2500, 750, and 800 times, respectively, compared to control cells. Transcription levels of CD8 and CD45 were also increased, with values of 110 times for CD8, and 17 times for CD45 with respect to the control. The increasing of CD4 was of five times and with the exception of CD4 in cells stimulated with ConA, all transcription levels of tested genes were much lower at 48 h, as can be seen in Fig. 4. These data confirm the effects of lectin stimulation in the expression of T cell-related genes in GIALT leukocytes.

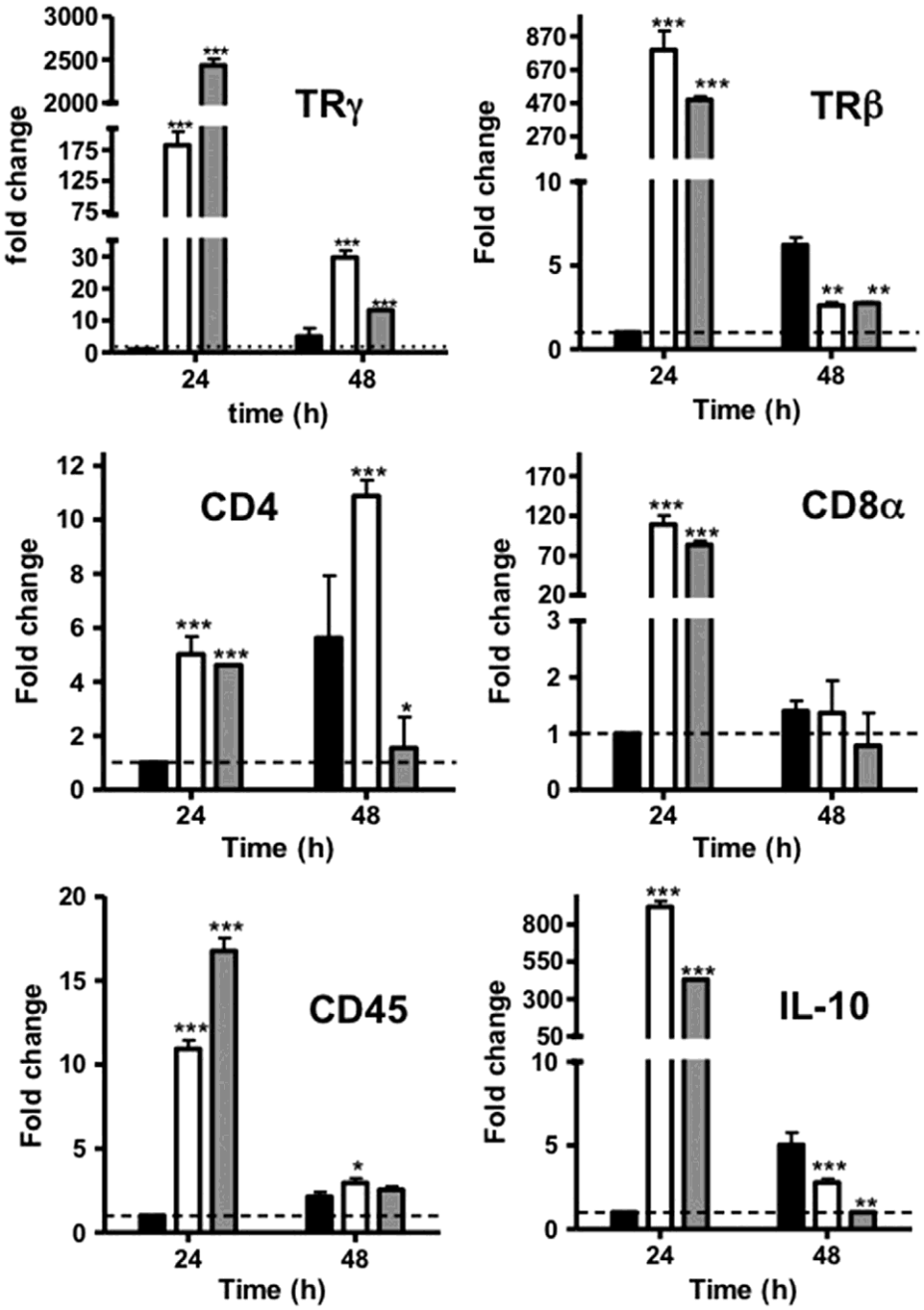

Figure 4. Quantitative PCR. The quantitative expression of indicated genes (normalized respect to 18S rRNA) is shown in control cells (black bars) and in cells treated with 10 μg/mL of ConA (empty bars) and PHA (gray bars) after 24 and 48 h. Control cells after 24 h are used as calibrator. Results are expressed as the mean ± SD of three diverse GIALT cell cultures. Two-way ANOVA followed by Bonferroni's multiple comparison tests was used to determine differences from the control at each time point. Data analysis was performed using the GraphPad Prism 3.0 software

statistical package. The level for accepted statistical significance was $P < 0.05$. One asterisk represents a $P < 0.05$, two asterisks a $P < 0.01$ and three asterisks a $P < 0.001$.

2.5 DISCUSSION

The gills of fish have an obvious importance for the maintaining of homeostasis by regulating exchanges with the outside water microbiome, but despite this fundamental feature the knowledge on cellular and molecular asset of mucosal immune system in this tissue is limited. Considering the importance of T lymphocytes in mucosal immune defenses of vertebrates, the aim of our work was to investigate basic molecular and cellular signatures of T cells in the gills of sea bass by transcriptome profiling and lectin-induced T cell proliferation.

Transcriptome profiling in the gills of some fish species have been already investigated to detect possible expression responses to environmental changes (Pinto et al., 2010; Rebl et al., 2013a), and to pathogenic conditions induced by bacteria (Rebl et al., 2013b) or viruses (Aquilino et al., 2014). In these works the attention was paid to display the presence of total expressed genes grouped on the base of physiological processes, or changes of families of genes upon treatment.

Taking advantage from our previous works on fish T cells (Buonocore et al., 2012; Rombout et al., 2011; Scapigliati, 2013), and of deep-sequencing technology of RNA that have been already employed to study physiological features of fish (Amemiya et al., 2013; Boudinot et al., 2014; Pallavicini et al., 2013), a whole RNA transcriptome was obtained from the gills of a healthy sea bass. The transcriptome has been analyzed and assembled as described in Nuñez Ortiz et al., 2014 by Dr. Gerdol, generating 84,370 non-redundant contigs from which putative sequences of interest have been sorted.

The contigs expressed in the gills of sea bass can be collected in several clusters related to physiological functions, among which the more represented gene ontology (GO) terms are listed in Appendix: Table S2, and PFAM domains in Table 3 and Appendix: Table S3. Interestingly, in most abundant PFAM domains are repeatedly present leucine-rich repeats (LRR) and immunoglobulin (Ig) domains.

Very likely, the contigs annotated as bearing LRR should contain Toll-like receptors (TLR) related to the presence of pathogen- specific TLRs, which have been described in fish (Purcell et al., 2006; Ribeiro et al., 2010; Sundaram et al., 2012). On the other hand,

Ig- domains should correspond to T cell receptors, co-receptors, and immunoglobulins, all of which have been already described to be present in gills (Salinas et al., 2011).

The obtained transcriptome was mined for the search of the main T cell-related expressed gene sequences, and results have been presented in Table 2. In addition to T cell receptors, identified CD coreceptors are CD3 as marker of pan-T cells and the associated protein-kinases Lck and Zap-70, CD4/8 defining helper and killer T cell subpopulations, CD6 as a co-stimulatory molecule in T-cell activation, and CD28 involved in the NF-κB signaling cascade during CD4 T cell activation (Park et al., 2014). Among the cytokine genes it is interesting to observe the presence of key cytokines of the adaptive immune response (IFN-γ, IL-10 and IL-22) and of IL-2, that was previously reported to be expressed in unstimulated trout kidney (Díaz-Rosales et al., 2009) whereas expressed IL-2 was found in stimulated Fugu (Bird et al., 2005) and trout (Secombes et al., 2011; Wang et al., 2011). It is also interesting to observe that the set of cytokine gene markers of T cell subpopulations are present, namely IFN-γ, IL-2, and CCR5 for Th1, IL-4 and IL-10 for Th2, IL-17, IL-21 and IL-22 for Th17 and, in addition, Foxp3 and Gata3 gene transcripts as master regulators for T regulatory (Treg) cells.

Overall, from the transcriptomic data can be speculated that in agreement with previous fish studies, the repertoire of genes found within the gills (namely, Th1, Th2, Th17, and Treg) indicates that subsets of T-cells may exist, but further characterization of these cell types is required to determine if they are the same or different to existing mammalian subsets. Our observations find confirmations from a previous work on trout where constitutive high expression of interleukin-4/13A and GATA-3 was detected in the gills (Takizawa et al., 2011), and from a recent paper on zebrafish, where immersion vaccination against a *Vibrio* species induced Th17-like immune responses (Zhang et al., 2014).

Notably, in the transcriptome were not found matches with RAG-1 gene sequences and thus can be speculated that in unstimulated gills the expression of genes for somatic recombination of Ig-domains is below our detection threshold. This observation should be compared with the situation in the thymus and intestine of sea bass, where RAG-1 is highly expressed (Randelli et al., 2009) and somatic recombination of TRγ-chain occurs (Buonocore et al., 2012). Moreover, the very low expression of RAG enzymes raises important speculations on the presence of differentiating and differentiated T cells in

mucosal tissues, with the intestine acting as a "producer" of T cells and the gills working as an "effector" tissue. This hypothesis is reinforced by the observation that intestinal IEL do not show evident *in vitro* lectin-induced proliferation (Scapigliati G., unpublished), whereas GIALT leukocytes do, as shown in this work.

In order to investigate basic functional features of GIALT leukocytes, *in vitro* proliferation of cells with lectins was attempted, known to induce T cell proliferation in vertebrates, and the results of these experiments (shown in Fig. 1) showed that GIALT leukocytes are able to proliferate, as measured by CFSE incorporation, in response to ConA and PHA. The proliferation was higher at 48 h and PHA induced higher proliferation with respect to ConA.

To verify that proliferating leukocytes contained T cells specific reagents to quantify sea bass T cells were employed, namely the mAb DLT15 likely specific for pan-T cells (Scapigliati et al., 1999) and mAb DLT22 specific for CD45-T cells (Marozzi et al., 2012). Using these mAbs in proliferation experiments revealed that the number of T cells increased, as can be appreciated from Fig. 2 (lower panel), where the mean percentages of cells recognized by either DLT15 and DLT22 doubled after 48 h of stimulation by lectins. These data are, to my knowledge, the first report on leukocyte and T cell proliferation from fish gills.

A view on the tissue distribution of T cells in the gills, as shown in Fig. 3, shows the richness of lymphocytes stained by IHC with DLT15 in the epithelium of branchial filaments. Differently, rare T cells were found in the lamellae epithelium and at difference with Atlantic salmon (Haugarvoll et al., 2008), it is dificult to evidence a T cell-containing inter branchial lymphoid tissue in sea bass.

A further verification of the presence of T cells in GIALT leukocytes comes from qPCR experiments, where the RNA obtained by cells treated with lectins was employed to measure transcription of some marker genes of T cells, namely TRβ, TRγ, CD4, CD8, CD45, and IL-10, as shown in Fig. 4. The results showed consistent increases in transcription for tested genes in proliferating cells, and transcription was particularly high for TRβ, TRγ and IL-10 followed by CD8, CD45 and CD4, thus confirming that proliferating cells contained T cells.

In conclusion, the gills of sea bass are a mucosal tissue containing an entire set of T cell-specific expressed genes that define T cell subpopulations as they are known in other vertebrates, together with the presence of *in vitro* proliferating T cells. Future

work will investigate the functional involvement of T cells during mucosal (immersion) immunization of sea bass with viral and bacterial antigens.

2.6 APPENDIX:

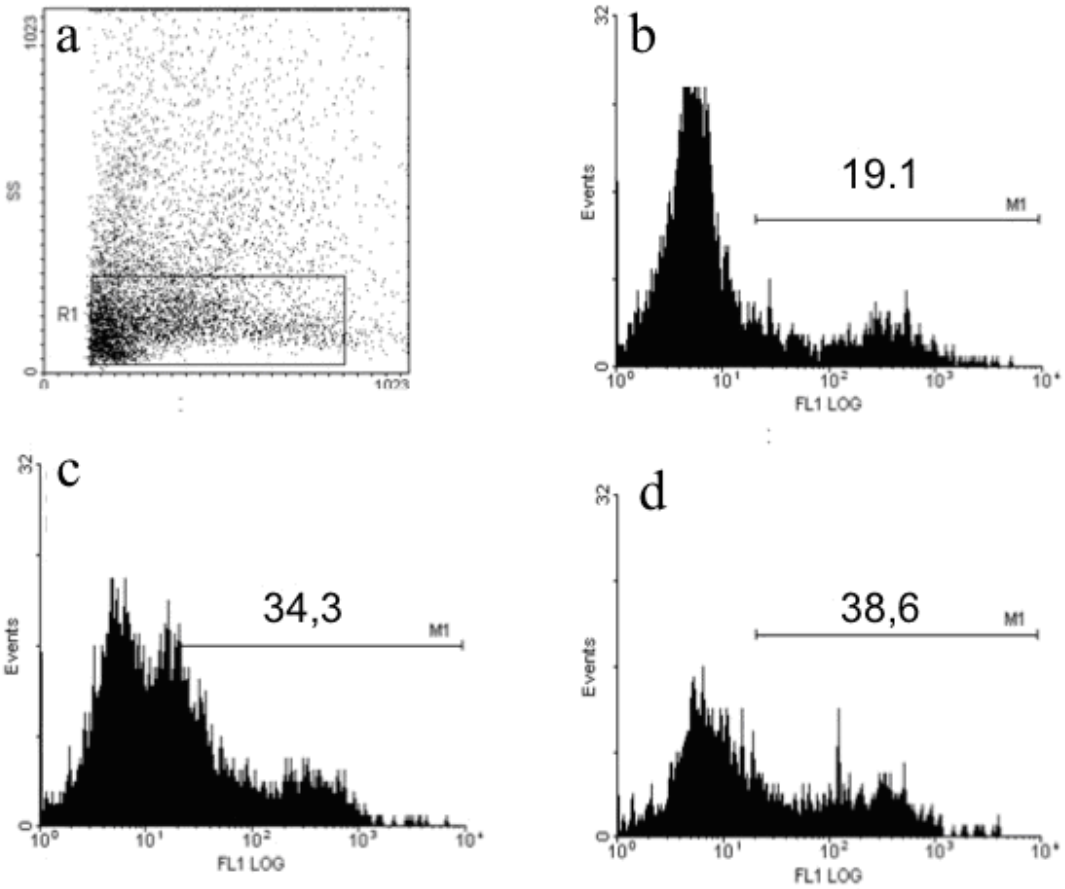

Figure S1. Immunofluorescence with anti-CD45 T cell mAb.

The flow cytometric panel shows a typical lectin-induced proliferation experiment of gill leukocytes, with the FS and SS morphology of cell populations in (a) and the square gate employed for analysis. The DLT22 mAb fluorescence in control cells after 48 hors is shown in (b) together with the M1 region considered for positivity, and in cells treated with ConA (c) or PHA (D). Numbers are the percent of positive cells in the M1 region.

a: trimming report

Number of reads before trimming	68,643,808
Number of reads after trimming	66,423,874
Percentage of discarded reads	3.23%
Average read length before trimming	100 bp
Average read length after trimming	98.7 bp

b: assembly statistics

	redundant assembly	non-redundant assembly
number of contigs	133,270	84,370
average length	1,357	799
shortest contig	201	201
longest contig	28,088	28,088
contigs longer than 5 Kb	5,411	1,163
N50	2,776	1,526

c: mapping statistics

Reads mapped in pairs	77.28%
Reads mapped in broken pairs	7.18%
Unmapped reads	15.54%
Reads mapped specifically	99.2%
Reads mapped non-specifically	0.8%

d: annotation statistics

Contigs with a BLASTx hit (UniProtKB/Swiss-Prot)	22,585
Contigs with a PFAM domain	14,461
Contigs annotated with Gene Ontology terms	3,662
Contigs annotated with eggNOG	1,99

Table S1. (a) Trimming report. (b) Assembly statistics. (c) Mapping statistics. (d) Annotation statistics.

GO ID	Type	Description	Abundance
GO:0016021	CC	integral component of membrane	747
GO:0046872	MF	metal ion binding	726
GO:0005634	CC	Nucleus	717
GO:0005737	CC	Cytoplasm	650
GO:0005886	CC	plasma membrane	483
GO:0005524	MF	ATP binding	471
GO:0008270	MF	zinc ion binding	382
GO:0006351	BP	transcription, DNA-templated	316
GO:0003677	MF	DNA binding	305
GO:0005829	CC	Cytosol	225
GO:0003723	MF	RNA binding	200
GO:0006355	BP	regulation of transcription, DNA-templated	175
GO:0005509	MF	calcium ion binding	171
GO:0005576	CC	extracellular region	155
GO:0005887	CC	integral component of plasma membrane	146
GO:0003964	MF	RNA-directed DNA polymerase activity	132
GO:0006278	BP	RNA-dependent DNA replication	132
GO:0005739	CC	Mitochondrion	124
GO:0003700	MF	sequence-specific DNA binding transcription factor activity	119
GO:0007155	MF	cell adhesion	118

Table S2. Top 20 most abundant gene ontology terms.

eggNOG ID	**Description**	**Abundance**
COG0515	Serine/threonine protein kinase	132
NOG12793	Calcium ion binding protein	108
COG5048	Zn-finger	53
COG2319	WD40 repeat	44
COG1100	GTPase SAR1 and related small G proteins	40
COG5022	Myosin heavy chain	31
COG0666	Guanosine polyphosphate pyrophosphohydrolases/synthetases	30
COG2124	Cytochrome P450	25
COG4886	**Leucine-rich repeat (LRR) protein**	**23**
COG5069	Ca2+-binding actin-bundling protein fimbrin/plastin (EF-Hand superfamily)	20

Table S3. Top 10 most abundant eggNOG terms.

CHAPTER 3: Mucosal responses: A polyclonal antiserum for characterization of sea bass (*Dicentrarchus labrax*) IgT-specific leukocytes.

This Chapter describes studies on the mucosal immunoglobulin IgT in the sea bass by employing a polyclonal antiserum specific for sea bass (*Dicentrarchus labrax*) IgT. The antiserum has been obtained by immunization with synthetic peptides deduced from the IgT sequence and employed to reveal and quantitate IgT-producing cells in mucosal and non mucosal tissues.

3.1 ABSTRACT

Immunoglobulin T (IgT) is one of the key effector molecules of jawed vertebrate's adaptive immune system. We are investigating immune responses in sea bass after mucosal (immersion) vaccination against inactivated Betanodavirus, and we believe that IgT should play a role in anti-viral immunity.

A necessary step to investigate by ELISA the presence of IgT in serum and mucus, and the presence of IgT-bearing cells in tissues is to have an antibody against sea bass IgT. In this respect, rabbit was immunized with synthetic peptides that have been deduced from the full length cDNA sequence and located in the surface-exposed sequence of sea bass IgT peptide. The obtained antisera, named RAIgT, resulted able to stain in Western blot of lysates splenocytes a polypeptide at 75 kDa and at 25 kDa in reducing conditions and at 150 kDa in non-reducing conditions.

By IIF and flow cytometry of leukocytes, RAIgT stained 32±12% in head kidney, 23±10% in spleen and 14±6% in gills. At the fluorescence microscope, live cells from these tissues showed a typical membrane-associated positivity. Moreover, Dynabeads Immunoprecipitation of head kidney cells allowed to obtain two fractions with different expression of IgT and IgM genes as have been detected by RT-PCR.

In addition, by using RAIgT an Indirect ELISA assay platform was performed to evaluate the presence of IgT in sera and intestinal mucus of wild sea bass. Preliminary results showed a very poor content of IgT in serum and a high content of IgT in mucus, in line data obtained in rainbow trout (*Onchorhynchus mykiss*).

3.2 INTRODUCTION

Fish mucosal immune system was detected by Duff in 1942 for the first time in rainbow trout orally immunized with *Aeromonas salmonicida* antigens (Duff, 1942). The immunoglobulin isotype IgT was not discovered until 2005 in rainbow trout (IgT) and zebrafish (IgZ) after analysis of genomes of several teleost fish species (Hansen et al., 2005; Danilova et al., 2005). IgT plays an important role in mucosal defences and is monomeric in serum and polymeric in the gut mucus.This immunoglobulin does not exist in other vertebrates. Several B cell subsets can be distinguished according to their expression of distinct Ig class combinations. In some fish species two subsets of B cells can be identified by their expression of both IgM and D, or IgT only. In adult fish, B cells reside in the anterior and posterior kidney, spleen, gut lamina propria, and blood (Rombout et al., 1993; Abelli et al., 1997). The distribution of IgT+ B cells in different organs in any species of fish and their gene expression in response to pathogenic infection have seldom been reported.

Polyclonal and monoclonal antibodies have been generated to detect trout IgT. These have allowed in-depth study of function and distribution of IgT in rainbow trout (Zhang et al., 2010; Olsen et al., 2011; von Gersdorff Jorgensen et al., 2011). So far an antisera against sea bass IgT was not available. Taking advance of the full-length amino acid sequence of Ig heavy-chain (IgT) sea bass (accession number KM410929) that has been determined by transcriptomic analysis of sea bass gill (Chapter 2) a polyclonal antiserum for sea bass (*Dicentrarchus labrax*) IgT was produced and named RAIgT. It was obtained by immunizing a rabbit with a mix of 3 synthetic peptides located in the surface-exposed sequence of sea bass IgT peptide. Then functional characterization of this antibody was required. In this chapter obtained results after functional characterization of this antibody have been presented and at the same time these are the first data of B cell expressing Ig T in sea bass.

3.3 MATERIALS AND METHODS

Polyclonal antiserum for sea bass (Dicentrarchus labrax) IgT

The rabbit polyclonal antiserum recognizing sea bass IgT was obtained by immunizing a New Zealand rabbit subcutaneously in the back with a mix of three synthetic peptides conjugated to KLH:

Peptide 1 NH_2-SRVSSDGDQHSKTSTMSC-COOH;

Peptide 2 NH_2-KEIFSNNEAKLEC-COOH;

Peptide 3 NH_2-RNRTEWQGVNKVRC-COOH.

The initial injection was followed by four administrations of the prepared mix in the lateral ear vein at 7-day intervals. Several bleedings from the saphenous vein were performed and was confirmed the peptides detection by Indirect ELISA assay. Then, the rabbit was bled to death from heart after anaesthesia (pre-anaesthesia with 2 mg kg^{-1} of acepromazine, then xylazine at 7 mg kg^{-1} and ketamine at 50 mg kg^{-1}).

The serum concentration was determined spectrophotometrically at 280 nm using 1.2 as the molar extinction value to correct for IgG protein.

Indirect ELISA

The recognition of synthetic peptides was verified by developing an Indirect ELISA. Lyophilized peptides were resuspended in 2 mL of Water Molecular Biology Reagent (Sigma-Aldrich) to be used as antigen in ELISA assay. The polystyrene wells were coated with 100 µL/well of a final concentration of 115 ng/well (diluted with 0.05 M carbonate-bicarbonate buffer pH 9.4). The wells were then washed three times with Tris-HCl 50 mM pH 7.4 containing 0.05% Tween-20 and 0.15 M NaCl (TTN). After blocking remaining sites with 3% BSA in TTN (TBT), 100 µL/well of antiserum RAIgT diluted in TBT (1:300) was added, incubating the plates overnight at 4°C. Every peptide was analysed in triplicate wells and control wells without any peptide coating were used for each peptide. The wells were carefully washed with TTN and then incubated for 90 min with a HRP-conjugated secondary antibody solution (Cappel) in TBT. The wells were then washed with 50mM phosphate-citrate buffer (pH 5.0) and the reaction was visualised using o-phenylenediamine (Sigma) as substrate. The absorbance values were read at 450 nm with an automatic plate reader (Labsystems Multiskan MS). Optical density values (OD 450nm) of control wells were automatically subtracted from positive wells and the ELISA assays data are presented as the mean absorbance ± SD of triplicate wells.

IgT detection in sera and intestinal mucus was determined by another Indirect ELISA. The blood from wild sea bass (n=7) was collected from the caudal vein and sera were obtained by centrifugation at 1000 g for 5 min. Intestinal mucus was sampled from the same animals, cutting the gut, cleaning with PBS 0.01 M and scraping with a slide. The intestinal mucus was weighed and was added the same amount of volume of PBS 0.01

M (1mM DTT, 1.5 mM EDTA). After 15 min of incubation, the supernatant was obtained by centrifugation at 20000g at 4C. Sera and intestinal mucus samples were diluted 1:100 with 0.05 M carbonate-bicarbonate buffer pH 9.4 and used to coat the polystyrene wells (100 µL/well). Next steps were as explained above. Optical density values (OD 450nm) of control wells were automatically subtracted from positive wells and the ELISA assays data are presented as the mean absorbance ± SD.

Immunofluorescence and flow cytometry

Healthy juveniles sea bass were obtained from a local fish farm (Civitaittica SrL, Civitavecchia, Italy). Blood was drawn from caudal vein of lethally anesthetized fish with tricaine methanesulfonate (Sigma). Head kidney (n=3), spleen (n=3) and gills (n=3) were then removed and immersed in cold HBSS. All culture media and solutions were adjusted to 350 mOsm/ kg by adding 2M NaCl. The leukocytes isolation protocol was as previously described (Chapter 2: Materials and Methods). Head kidney, spleen and gills were cutted with small scissors and pushed through a 40 µm nylon mesh using cold HBSS. Obtained cells were washed two times in HBSS at 490×g for 10 min and then loaded over Percoll gradients at 1.02 and 1.07 g/cm3. After centrifugation at 600 × *g* for 20 min, leukocytes were collected at the interface and washed with HBSS.

For Immunofluorescence (IIF) analysis, leukocytes obtained were resuspended in HBSS and every preparation was divided in control cells, cells incubated with preinmune serum and cells incubated for 1h with dilution 1:300 of RAIgT (containing 10 µg/mL of anti serum RAIgT). After washing with PBS at 450 × *g* for 10 min the cells were incubated at 4°C with a 1:250 dilution of FITC-labeled antirabbit antibody (Cappel) for 30 min. Cells were again washed and then fixed using Formaldehyde Solution in PBS (2%). The fluorescence was read by flow cytometry in a Beckman-Coulter Epics LS-200 Flow cytometer and analysis of acquired 10.000 events was done with Expo32 system software. The same preparations were examined under a Zeiss fluorescence microscope equipped with a colour video camera (Axio Cam MRC, Milano Italy) and a software package (KS 300 and AxioVision).

Dynabeads Immunoprecipitation

Head kidney form a wild sea bass was obtained from a local fish farm (Civitaittica SrL, Civitavecchia, Italy). Head kidney was removed and immersed in cold HBSS containing

heparin. Then it was cutted with small scissors and pushed through a 40 μm nylon mesh using cold HBSS. Obtained cells were rotated incubated for 2 hours at 4°C with RAIgT previously incubated overnight by rotation at 4°C with Dynabeads® M-280 Sheep Anti-Rabbit IgG (Thermofisher) following the manufacturers' instructions.

After magnetic separation both fractions were washed with PBS by centrifugation at 2000g 4°C 10 minutes and pellets were resuspended with Tripure (Roche).

RT-PCR

Total RNA was isolated from both fractions with Tripure (Roche) following the manufacturer's instructions and were suspended in DEPC treated water (Sigma). For reverse transcription the protocol described by Scapigliati et al., 2010 was followed.

The presence of IgT and IgM was investigated by RT-PCR using specific primers (Table 1) and the same quantity of cDNA. The RT-PCR conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s, and final extension of 72 °C for 10 min. RT-PCR products (15 μL) were visualized on 1% (w/v) agarose gels containing GelRed using a 100-bp DNA Marker (Antagene) as size marker.

Gene	Primer sequence	EMBL accession
ß-actin	FW: 5′-ATGTACGTTGCCATCC-3′ RW: 5′-GAGATGCCACGCTCTC-3′	AJ493428
IgM	FW: 5′- GAGCTGCAGAAGGACAGTG -3′ RW: 5′- TCAGACTGGCCTCACAGCT -3′	AJ400233
IgT	FW: 5′-CGGACTTCATTCAGTACTCTG -3′ RW: 5′-CAACTGTACACATCAGGGCC -3′	KM410929

Table 1. List of primers used in RT-PCR.

3.4 RESULTS

Peptides detection

In Fig. 1 is shown the affinity detection of peptides 1, 2 and 3 by RAIgT. RAIgT presented a high-affinity detection of peptide 2 (OD 450 nm of ca. 0.900). Peptide 1 and 3 were recognized but with a lower-affinity (OD 450 nm of ca. 0.015 and 0.086 respectively).

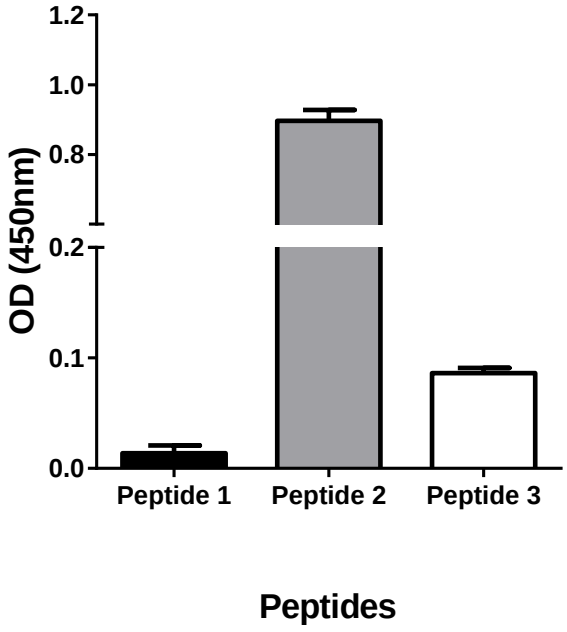

Figure 1. RAIgT peptides detection by ELISA. The figure shows mean ± SD optical density values (OD 450 nm) of triplicate wells. Background values (no adsorbed synthetic peptides) were automatically subtracted from OD 450 nm values of samples.

IgT detection in biological samples

Soluble IgT was detected by Indirect ELISA in sera and intestinal mucus samples, coating the antisera IgT in polystyrene plates. A high amount of soluble IgT was detected in intestinal mucus (mean OD 450 nm of 0.171 +/-0.037) unlike sera samples which presents an OD 450 nm of 0.003 +/-0.008 (Fig. 2).

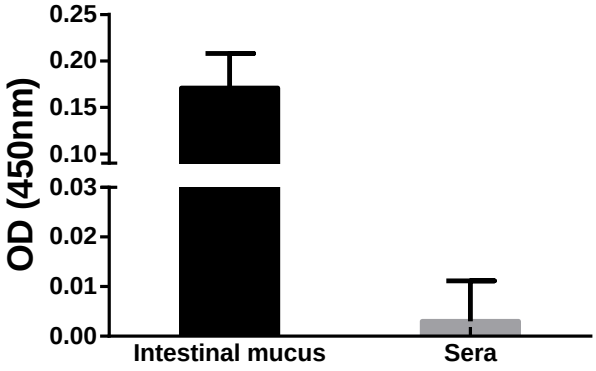

Figure 2. IgT detection by ELISA in biological samples. The figure shows mean ±SD optical density values (OD 450 nm) of triplicate wells. Background values were automatically subtracted from OD 450 nm values of samples.

Dynabeads Immunoprecipitation of IgT+ B cells from head kidney

Two fractions of head kidney cells were generated using Dynabeads. The fractions were named: 1, corresponding to the eluted fraction during magnetic separation (not immuno-purificated) and fraction 2 that corresponds to eluted cells after magnetic separation (immuno-purificated). RT-PCR of IgT and IgM genes were developed and differences of expression were get in both fractions.

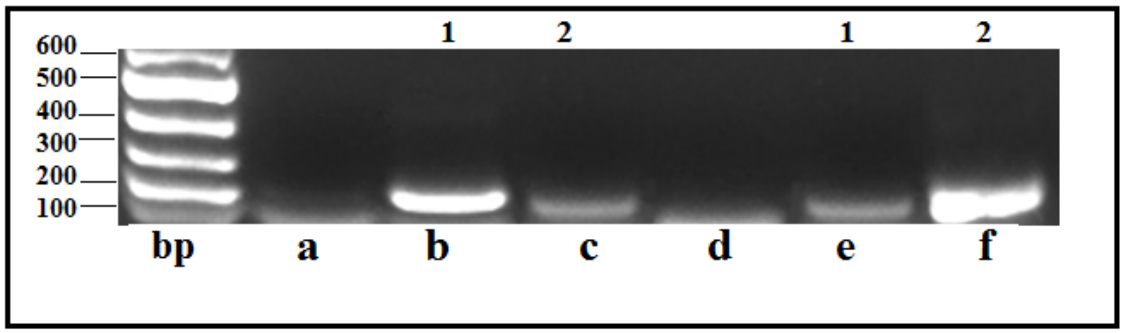

Figure 3. RT-PCR of head kidney cells after Immunoprecipitation with Dynabeads. The expression of genes coding for sea bass IgT and IgM are shown after 30 cycles of RT-PCR amplification in a 1% agarose gel. RT-PCR of IgT gene is shown in lane a, b and c (a= RT-PCR control; b=fraction 1, immunopurificated fraction; c=fraction 2, no immunopurificated). RT-PCR of IgM gene is shown in lane d, e and f. (d= RT-PCR control; e=fraction 1; f=fraction 2).

Immunofluorescence and flow cytometry

Head kidney, spleen and GIALT leukocytes were labelled with RAIgT and monitored by flow cytometry and results are shown in Fig. 4 as the mean ± SD of five diverse experiments and Fig. 5. Head kidney was the organ with a higher content of IgT+ cells, follow by spleen and gills. All the labelled cells presented dimensions and morphology of leukocytes-type cells.

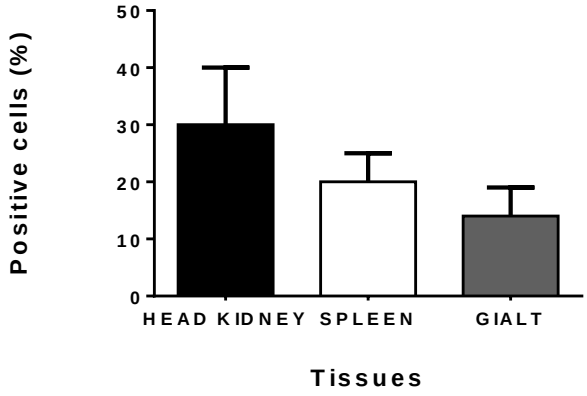

Figure 4. Immunofluorescence (IIF) and flow cytometry. Positive cells (%) from head kidney, spleen and GIALT leukocytes after incubation with RAIgT (1:300). The staining values are subtracted of the background staining of the secondary FITC-labeled antibody.

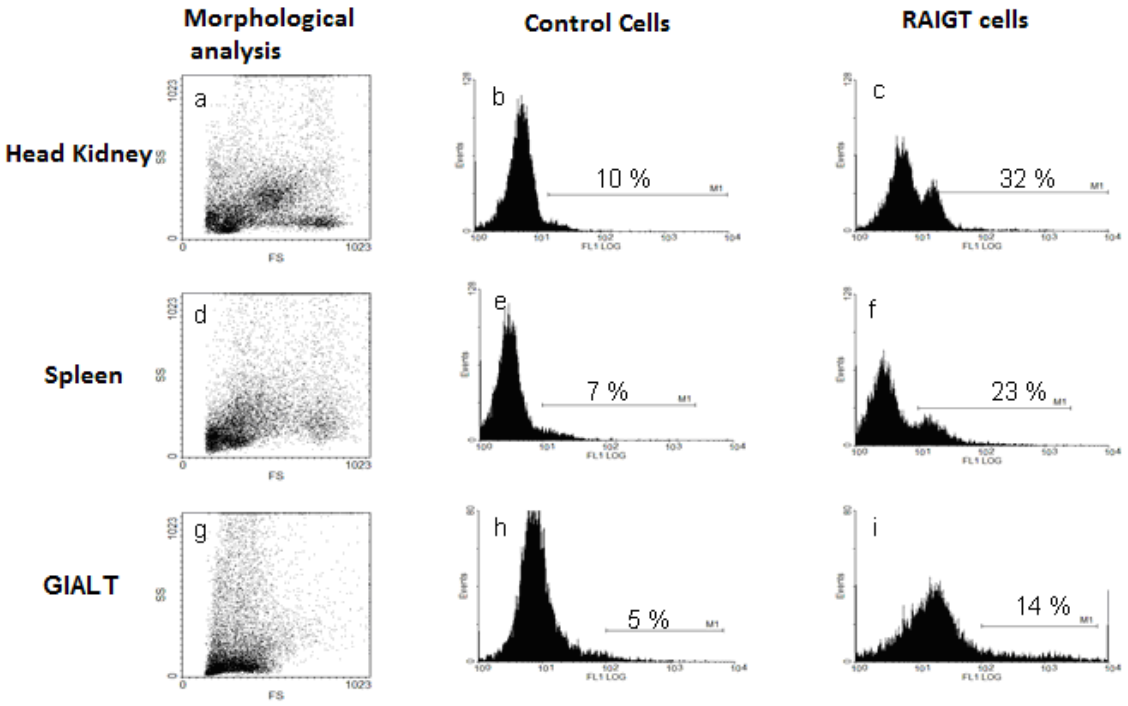

Figure 5. Flow cytometric immunohistograms of head kidney, spleen and GIALT leukocytes. The fluorescence percent values in selected M1 gate are shown in flow cytometric histograms. Upper panels presents results of head kidney analysis; morphological analysis of leukocyte populations from head kidney (Fig. 5a), flow cytometric pattern of control cells (Fig. 5b) and flow cytometric pattern of cells incubated with RAIgT (1:300) (Fig. 5c). Middle panels correspond to spleen leukocytes analysis; morphological analysis of splenocytes (Fig. 5d), flow cytometric pattern of control cells (Fig. 5e) and flow cytometric pattern of cells incubated with RAIgT (1:300) (Fig. 5f). Bellow panels correspond to GIALT leukocytes analysis; morphological analysis of leukocytes (Fig. 5g), flow cytometric pattern of control cells (Fig. 5h) and flow cytometric pattern of cells incubated with RAIgT (1:300) (Fig. 5i).

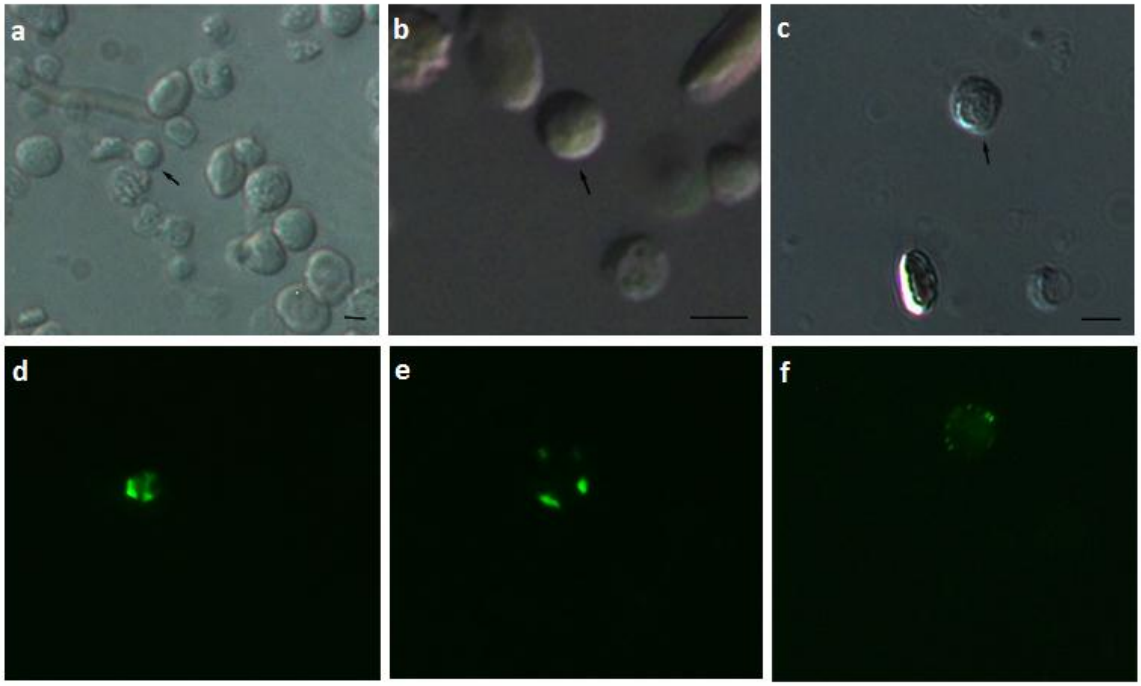

Figure 6. Fluorescence microscopy of head kidney, spleen and GIALT leukocytes labelleb by IIF.

3.5 DISCUSSION

Since the discovery of the immunoglobulin IgT, lots of information have been obtained of trout IgT thanks to the developed polyclonal and monoclonal antibodies that have allowed a better knowledge of molecular features and functions of IgT in rainbow trout (Zhang et al., 2010; Olsen et al., 2011; von Gersdorff Jorgensen et al., 2011). However, the knowledge in another species as sea bass is still meagre.

Then, a polyclonal antiserum against sea bass IgT has been produced using three synthetic peptides designed from the full-length amino acid sequence of Ig heavy-chain (IgT). The sequences have been obtained from transcriptomic analysis of sea bass gill, as described in Chapter 2. These synthetic peptides belong to a repeated sequence in the constant and conserved region CH3 in the heavy chain of IgT, which was founded in known isoforms of IgT, but not in others immunoglobulins. The antiserum recognized all the synthetic peptides, being peptide 2, the shorter peptide sequence, the most detected as proved by Indirect ELISA (Fig. 1). The obtained antiserum has been also characterized (data presented at the 2nd ISFSI congress, 2016) by Western blot analysis after immunopurification with protein A-sepharose of a spleen leukocytes lysate. The Western blot was performed under reducing conditions and the antibody

immunostained two sharp bands at ca. 75 kDa and ca. 25 kDa corresponding to the immunoglobulin heavy chain and light chain, repectively, as previously shown in trout (Zhang et al., 2010).

Interestingly, IgT+ leukocytes from head kidney, spleen and gills have been successfully detected and quantify by IIF and flow cytometry. Labelled cells presented dimensions and morphology of leukocytes-type cells (Romano et al., 1997; Mulero et al., 2001). The antibody RAIgT is able to recognize the antigen both in live cells and in tissue sections (data presented at the 2nd ISFSI congress, 2016) and the flow cytometry analysis has allowed, for the first time, to evaluate the content of the IgT-specific leukocytes into a fish, consisting of 32 % of head kidney cells, 23% of splenocytes and 14% of leukocytes in the lymphoid tissue associated to the gills. IgT expression is especially high in spleen and head kidney of rainbow trout (Hansen et al., 2005) and head kidney is a major hematopoietic organ and site of production of antibodies and other immune cells in Teleost fish (Rombout and Joosten, 1998; Press and Evensen, 1999).

The importance of having an antibody that can be used in Immunofluorescence assay comes from the fact that can be used for comparative immunology studies and functional studies. Although fish IgT presents different structural features and evolutionary origin compared to mammalian IgA, its implication in the B-cell response evokes these mouse and human counterparts.

Although initially it was thought that IgT presented only a mucosal role, different functional studies in rainbow trout have proved that it is also involved in systemic responses. The first work describing the mucosal role of IgT was a study of cell mediated immunity in the rainbow trout gut against a gut parasite, *Ceratomyxa shasta*, where the IgT+ cells were the main responders, whereas the IgM response was restricted to the serum (Zhang et al., 2010). Moreover, in rainbow trout, the oral vaccination with an alginate encapsulated DNA vaccine against IPNV resulted in increase of IgM+ and IgT+ B cell populations, an indication that both B cells populations are important for mucosal responses (Ballesteros et al., 2013). Subsequents works have demonstrated its involvement in systemic response, for example in trout spleen was observed that a complex clonal IgM and IgT responses can be developed upon systemic viral infection (Castro et al., 2013). Also Castro et al., 2013 has confermed a significant IgT response in spleen of infected fish.

Histochemical observations on the digestive tract of rainbow trout have revealed the presence of IgM+ cells in the lamina propria and IgT+ cells in the epithelium (Ballesteros et al., 2013). As presented in the accepted Abstract of 2nd ISFSI congress Portland (ME), 2016 two populations of B cells in head kidney and spleen were detected using RAIgT and a monoclonal antibody that recognizes sea bass IgM light chain (DLIg3) (Scapigliati et al., 1996). Moreover, the immunoprecipitation of IgT+ B cells from head kidney with Dynabeads allowed us to get two fractions, fraction 1 with immunoputificated IgT+ B cells and fraction 2 with non immunopurificated head kidney cells. RT-PCR of IgT and IgM genes resulted in a high expression of IgT and a low expression of IgM in fraction 1(RAIgT-positive) and the opposite in the fraction 2 (RAIgT-negative).

Significantly, the new capability of measuring not only IgM but also IgT responses will greatly facilitate the evaluation and understanding of fish immune responses as well as the protective effects of fish vaccines. Of particular interest to us is the study of mucosal responses after mucosal immunization of sea bass. Previously studies have demonstrated that high IgT levels can be detected after bath or immersion vaccination unlike IgM, which is highly presented when vaccines are administered by injection (Kai et al., 2014; Munang'andu et al., 2013).

CHAPTER 4: Betanodavirus detection: Quantitative immunoenzimatic detection of viral encephalopathy and retinopathy virus (Betanodavirus) in sea bass *Dicentrarchus labrax*.

This Chapter describes a by-product obtained studying antiviral responses in European sea bass, namely a capture-based ELISA that can be used as a tool to investigate and quantify the presence of Betanodavirus in biological samples when RT-PCR cannot be employed.

4.1 ABSTRACT

Viral encephalopathy and retinopathy disease caused by Betanodavirus, genus of the family Nodaviridae, affects marine, wild and farmed species including sea bass, one of the most important farmed species in Europe. This chapter describes a reliable and sensitive Indirect ELISA assay to detect Betanodavirus in biological samples using a polyclonal antiserum (pAb 283) against the isolate 283.2009 RGNNV, belonging to red-spotted grouper nervous necrosis virus (RGNNV) genotype the most common in the Mediterranean area, and a capture-based ELISA using a monoclonal antibody (mAb 4C3) specific to a common epitope present on the capsid protein. Using adsorbed, purified VERv preparation, the detection limit of Indirect ELISA was 2 µg mL^{-1} (3 x 10^5 TCID50 per mL), whereas for capture-based ELISA, the sensitivity for the antigen in solution was 17 µg mL^{-1} (35 x 10^5 TCID50 per mL). The capture-based ELISA was employed to detect VERv in brain homogenates of *in vivo* infected sea bass and resulted positive in 22 of 32 samples, some of these with a high viral load estimates (about 1.1 x 10^8 TCID50 per mL). The proposed ELISA system may be helpful in investigations where coupling of viral content in fish tissues with the presence of circulating VERv-specific IgM is required, or for use in samples where PCR is difficult to perform.

4.2 INTRODUCTION

Betanodavirus is the aetiological agent of viral nervous necrosis (VNN), it replicates in the central nervous system of fish (Aranguren et al., 2002; Lopez-Jimena et al., 2011; Chaves-Pozo et al., 2012), and affects more than 50 marine wild and farmed species and thus causes high losses in aquaculture production (Castric et al., 2001; Munday, Kwang and Moody, 2002; Vendramin et al., 2013). Sea bass is quite sensitive to VERv

(Frerichs, Rodger and Peric, 1996; Péducasse et al., 1999) and can become infected during youth and adulthood (Breuil et al., 2001; Nakai et al., 2009).

The VERv infection can be exhibited by clinical signs and requires confirmation by microscopic examination of vacuoles in the nervous tissue of brain, retina and spinal cord for diagnosis. Alternatively, detection of the virus can be made by immunohistochemistry when antibodies are available, and by PCR analysis of brain tissue (Arimoto et al., 1992; Lopez-Jimena et al., 2011; Chaves-Pozo et al., 2012) employing amplification primers specific to RNA1 or RNA2 (Baud et al., 2015). By taking advantage from the virus binding to polystyrene, some attempts have been made to develop enzyme-linked immunosorbent assays (ELISAs) to detect VERv in biological fluids or other samples, as it is an easy, inexpensive and an accessible test. For instance, ELISAs have been employed to detect Betanodavirus in striped jack (Arimoto et al., 1992), in a shrimp species (Romestand and Bonami, 2003), in juvenile barramundi *Lates calcarifer* (Fenner et al., 2006) and using purified Betanodavirus and supernatant of VERv-infected GF-1 cells as in Shieh and Chi (2005). However, after a work performed in experimentally infected sea bass larvae (Breuil et al., 2001), other ELISA assays to reveal and quantitate the presence of virus in biological fluids of sea bass have not been described.

To develop a sensitive ELISA assay for the RGNNV genotype by indirect and capture-based technology, a polyclonal antiserum against VERv and a monoclonal antibody against the VERv capsid were employed for the purpose of gaining knowledge of immune responses of sea bass against VERv. The possibility of using the ELISA assay as a tool to investigate and quantify the presence of the virus in biological samples from infected fish or in samples where the running of RT-PCR analysis is difficult has been also discussed.

4.3 MATERIALS AND METHODS

Antibodies and virus preparation

The rabbit polyclonal antiserum recognizing VERv (pAb 283), raised against strain 283/I09–RGNNV, was obtained by immunizing two New Zealand rabbits subcutaneously in the back and shoulder with 0.3 mL of formalin-inactivated ultracentrifuge-purified virus emulsified with 0.7 mL of adjuvant Montanide

ISA763VG (Seppic). The initial injection was followed by five administrations of 0.5 mL each of the antigen prepared without adjuvant in the lateral ear vein at 20-day intervals. Several bleedings from the saphenous vein were performed during the trial to verify the seroconversion level by serum neutralization assay (as described by Mori et al., 2003) until a satisfactory level was reached. Then, rabbits were bled to death from heart after anaesthesia (pre-anaesthesia with 2 mg kg^{-1} of acepromazine, then xylazine at 7 mg kg^{-1} and ketamine at 50 mg kg^{-1}).

The pAb 283 serum concentration was determined spectrophotometrically at 280 nm using 1.2 as the molar extinction value to correct for IgG protein.

The mouse monoclonal antibody specific to VERv capsid protein (mAb 4C3) has been previously described (Costa et al., 2007) and was available through the doctor K.D. Thompson as a hybridoma culture supernatant at a concentration of 10 µg mL^{-1}. The mAb 4C3 is an IgG2a and recognizes peptides corresponding to residues 141–162 and 181–202 in the capsid protein.

The Betanodavirus isolate 283/I09-RGGNV (VERv 283) (GenBank accession number JN189865 and JN189992, Panzarin et al., 2012) was prepared from serum-free culture supernatants of infected E-11 cell monolayers as previously described (Iwamoto et al., 2000; Vendramin et al., 2014). The E-11 cell medium containing VERv was centrifuged at 2000g for 10 min to remove cell debris, and then, the virus was inactivated by adding 1% buffered formalin at room temperature for 12 h.

Before formalin treatment, the virus yield was determined by titration on 96-well plates by the 50% tissue culture infective dose method per mL (TCID50), as previously described (Cunningham, 1973). A 100 mL batch of the serum-free culture supernatant containing inactivated virus was centrifuged at 150 000 g for 2 h at 4 °C in an ultracentrifuge. The pellet was washed with 0.05 M NaCl by ultracentrifugation as described above and resuspended in 2 mL of the same solution. Total protein content of resuspended ultracentrifugates was determined at 280 nm with a Picodrop spectrophotometer. For electron microscopy, 3 µL of resuspended pellet or centrifugation supernatant was layered onto a copper grid, stained with uranyl acetate and lead citrate and observed at 120 kV using a Jeol JEM EX II TEM, equipped with a Veleta CCD camera (Olympus). The VERv isolates 378/I02-RGGNV (VERv 378/I02) (GenBank accession number JX290515 and JX290517) (Vendramin et al., 2013) and 475/I98-RGNNV (VERv 475/I98) (Baud et al., 2015), viral haemorrhagic septicaemia

(VHSv F-1) virus (Jørgensen 1972), and infectious pancreatic necrosis virus (IPNv sp) (Smail, Grierson and Munro, 1986) were produced and inactivated as described above.

Indirect ELISA

The working dilution of pAb 283 was determined using a fixed concentration of concentrated viral preparation and various dilutions of the pAb 283. The wells of 96 well polystyrene plates (Maxisorp; Nunc) were coated with 5 µL of 10^7 TCID50 of virus preparation diluted in 95 µL of 0.05 M carbonate–bicarbonate buffer (pH 9.4) and incubated overnight at 4 °C. The plates were then washed three times for 5 min with 100 µL of Tris-HCl 50 mM (pH 7.4) containing 0.05% Tween-20 and 0.15 M NaCl (TTN) to remove unbound VERv. Each well was incubated for 30 min at room temperature with 100 µL of 3% BSA in TTN (TBT) to block non-specific sites. The plates were then washed as above and coated wells and control wells incubated for 3 h at 4 °C with various dilutions of pAb 283 diluted in TBT. The wells were carefully washed as above and then incubated for 2 h at 4 °C with a 100 µL dilution of horseradish peroxidase (HRP)-conjugated goat IgG fraction to rabbit IgG (GAR-HRP) (Cappel, catalog number: 55676) diluted 1:1500 in TBT. After washing as above, the wells were washed twice with 100 µL of 50 mM phosphate–citrate buffer (pH 5.0). The reaction was then developed with 100 µL per well of 0.04% o-phenylenediamine (Sigma) in phosphate–citrate buffer containing 0.001% H_2O_2. The reaction was allowed to proceed for 20 min at room temperature and stopped with 50 µL of 3 N sulphuric acid. The absorbance of duplicate wells was read at 450 nm with an Automatic Plate Reader (Labsystems Multiskan MS). To normalize the results, optical density values (OD 450 nm) of control wells were automatically subtracted from OD 450 nm values of samples.

Having determined an optimal working concentration of pAb 283 (20 µg mL^{-1}), its recognition sensitivity was tested by adsorbing in wells with different dilutions of concentrated VERv preparation in carbonate–bicarbonate buffer, starting from 67 µg mL^{-1} (10^7 to 9 x 10^3 TCID50). After absorbing the virus, all subsequent steps were as described above.

Capture-based ELISA

The capture antibody pAb 283 was diluted in duplicate wells at a concentration of 20 µg mL^{-1} in 0.05 M carbonate–bicarbonate buffer (pH 9.4) and adsorbed overnight at 4 °C. After washing three times with TTN, 100 µL of TBT was added to the wells to block non-specific binding for 30 min at room temperature. The plates were then washed with TTN as above and incubated for 5 h at 4 °C with various dilutions of concentrated viral preparation in TBT. After washing as above, the capture mAb 4C3 diluted 1:10 in TBT was added and incubated overnight at 4 °C. The plates were then washed with TTN as above; then, a peroxidase-conjugated goat IgG fraction to mouse IgG (GAM-HRP) (Cappel, catalog number: 55550) diluted 1:1000 in TBT was added and incubated for 3 h at 4 °C. The wells were washed with TTN and processed as described above.

To test the specificity of the developed capture-based ELISA, the assay was performed with different strains of RGNNV (VERv 283, VERv 378/ I02, VERv 475/I98) and two viruses, not belonging to the family Nodaviridae (VHSv F-1 and IPNv sp), each with a concentration of $10^{7.5}$ TCID50, diluted 1:4 in TBT.

For the analysis of VERv-infected brains, sea bass previously tested by PCR to be VERv free (data not shown) from the Istituto Zooprofilattico Sperimentale delle Venezie were received. Fish with an average weight of 25m ± 9 g were kept in a UV-treated recirculation system with artificial salt water (35 ‰) pH 7.5 ±0.3 at 20 °C, 8 +/- 1 mg L^{-1} of dissolved oxygen, and acclimatized for 15 days before the infection. The fish were fed daily ad libitum with standard commercial food. Fish were divided into two groups: a control group with 10 unmanipulated fish and an infected group with 32 fish anesthetized by immersion in salt water with 100 ppm of anaesthetic ethylenglycolmonophenylether (Merck) for about 5 min and then infected by intramuscular injection with 100 µL of 10^8 TCID50 per mL of Betanodavirus isolate 283/I09-RGGNV.

After 15–20 days was recorded ca. 70% mortality, and all fish showed signs of VERv. Brains were collected randomly from dying fish that were frozen at -20 °C until use. Each sample was homogenized with 10 passages in a glass Dounce homogenizer with 1 mL of serum-free DMEM on ice, transferred in an Eppendorf tube and centrifuged at 14.000 g for 5 min; the supernatant was carefully removed and transferred to a new tube. The supernatant diluted 1/15 in TBT was added to duplicate wells previously coated with pAb 283 and processed for capture-based ELISA as above.

Western blot

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 10% polyacrylamide slab minigels loaded with 8 µL of (540 µg mL^{-1}) denatured VERv 378/I02 preparation per lane. PageRulerTM prestained protein ladder ranging from 10 kDa to 170 kDa was employed as molecular marker. After electrophoresis, the gel was electroblotted onto nitrocellulose (BA85 Schleicher and Schuell) at 100 mA for 90 min on ice. The nitrocellulose was treated with PBS containing 3% bovine serum albumin and 0.1% Triton X-100 (PBT) for 30 min and then incubated overnight at 20 °C with pAb 283 diluted 1:500 in DMEM containing 10% FBS. Immunoreactive bands were detected with a peroxidase-labelled goat anti-rabbit antibody (Cappel) diluted 1:1500 in PBT for 2 h and revealed by adding 50 mM Tris-HCl pH 6.8 containing 0.3% 4-chloronaphthol and 0.002% H2O2. As a control, a VERv-loaded blotted nitro- cellulose lane was incubated with DMEM containing 10% FBS instead of pAb 283.

RT-PCR

Brain samples were obtained by handling homogenization of tissue fragments dissolved in 1 mL of Tripure (Roche). Total RNA was isolated following the manufacturer's instructions and resuspended in DEPC-treated water. The presence of VERv in brain samples was investigated by RT-PCR using the capsid protein specific primers: VERv-F1 (5'-TCGGCTGATACTCCTGTGTG-3') and VERv-R1 (5'-GTCATGATGGGAGCGGTAGT-3') (Scapigliati et al., 2010). The RT-PCR conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 57 °C for 45 s and 72 °C for 45 s, and a final extension of 72 °C for 10 min. RT-PCR products (15 µL) were visualized on 1% (w/v) agarose gels containing GelRed using a 100-bp DNA Marker (Antagene) as size marker. Controls for the presence of DNA contamination were performed using the RNA samples as template in the RT- PCR cycle.

Statistical analysis

In ELISA assays, each sample was measured in duplicate wells, and all tests were repeated at least three times with data presented as the mean absorbance SD. The statistical significance among groups was determined using one-way ANOVA. The analysis was performed using the GraphPad Prism 3.0 software statistical package, and the level for accepted statistical significance was $P < 0.05$.

4.4 RESULTS

Indirect ELISA

The ultracentrifuged virus preparation was monitored by electron microscopy, and observations confirmed the presence of a clean preparation of icosahedral-shaped virus particles with a size of ca. 40 nm (Fig. 1d, e). The virus preparation was tested in Western blotting with pAb 283 (Fig. 1). The antibody immunostained a sharp band at ca. 110 kDa and a broader one at 38 kDa (Fig. 1b), corresponding to the RNA-dependent RNA polymerase (protein A) and the capsid protein, respectively.

The antibody reactivity for VERv particles was determined by adding various dilutions of pAb 283. Results are shown in Fig. 2 as mean values SD of three different determinations. The results produced a typical S-shaped curve showing a direct relationship between the amount of antibody added per well and the detected optical density. The curve is linear in the range from 100 to 1 µg mL^{-1} of pAb 283, and a 50% binding efficiency was achieved at about 10 µg mL^{-1}. From these data, a working dilution of 20 µg mL^{-1} of pAb 283 was chosen for subsequent determinations.

To test the antibody recognition sensitivity, ultracentrifuged VERv dilutions from 10^7 to 9 x 10^3 TCID50 were adsorbed and assayed with 20 µg mL^{-1} of pAb 283, as shown in Fig. 3. Obtained data showed a linear correspondence between OD 450 nm values and the concentration of adsorbed VERv. The lower detection limit was around 0.25 µg mL^{-1} (3 x 10^4 TCID50 per mL), corresponding to an OD 450 nm of 0.01. Considering a possible variation between different experiments, a reliable detection limit was established for the indirect ELISA at about 2 µg mL^{-1} (3 x 10^5 TCID50 per mL), corresponding to an OD 450 nm of 0.1.

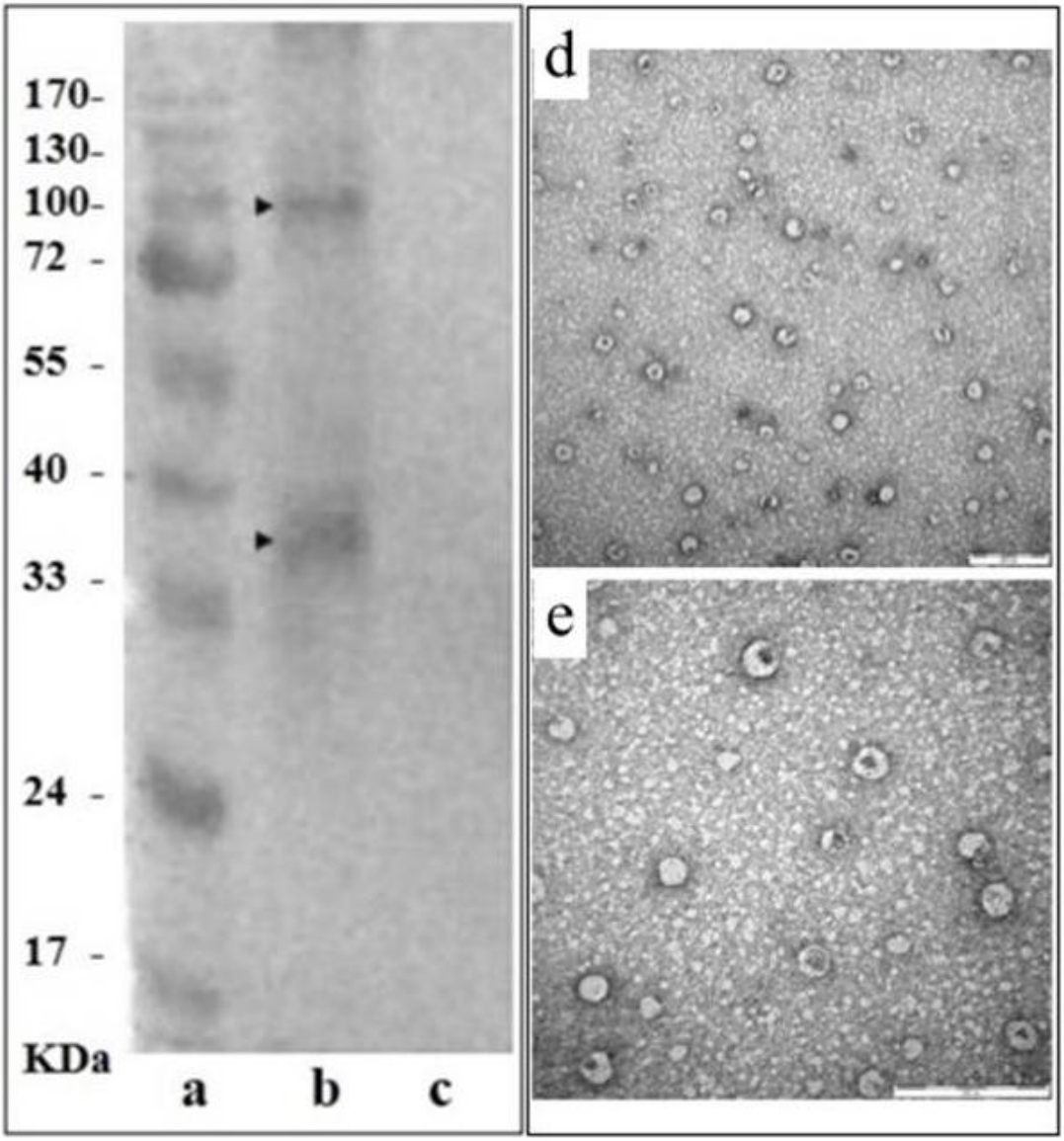

Figure 1. Western blot and Electron Microscopy. The figure shows a blotted 10% SDS–PAGE loaded with 8 lL of virus preparation (3.9 10^7 TCID50) (b) incubated with anti-VERv antiserum pAb 283 and (c) incubated with DMEM containing 10% FBS as control. Bound antibody was revealed with a peroxidase-bound secondary antibody. Reference MW markers are shown in (a) and immunoreactive bands are indicated with an arrow corresponding to 110 and 38 kDa. The quality of virus ultracentrifuged preparations is shown by electron microscopy at lower (d) and higher (e) magnifications. Scale bar is 200 nm.

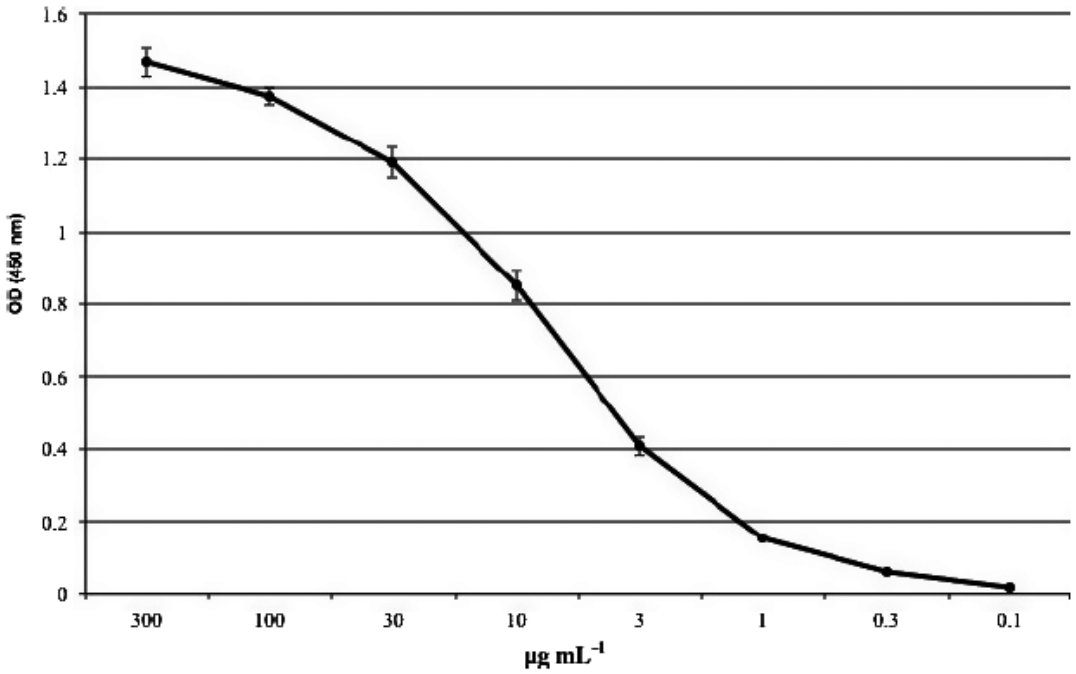

Figure 2. Determination of optimal dilution of capture antibody pAb 283 by Indirect ELISA. The plates were coated with 2.5 x 10^7 TCID50 per mL of virus preparation and probed with indicated dilutions of pAb 283 antibody. The figure shows mean +/- SD optical density values (OD 450 nm) of duplicate wells from three diverse determinations. Background values (no pAb 283 added) were automatically subtracted from OD 450 nm values of samples.

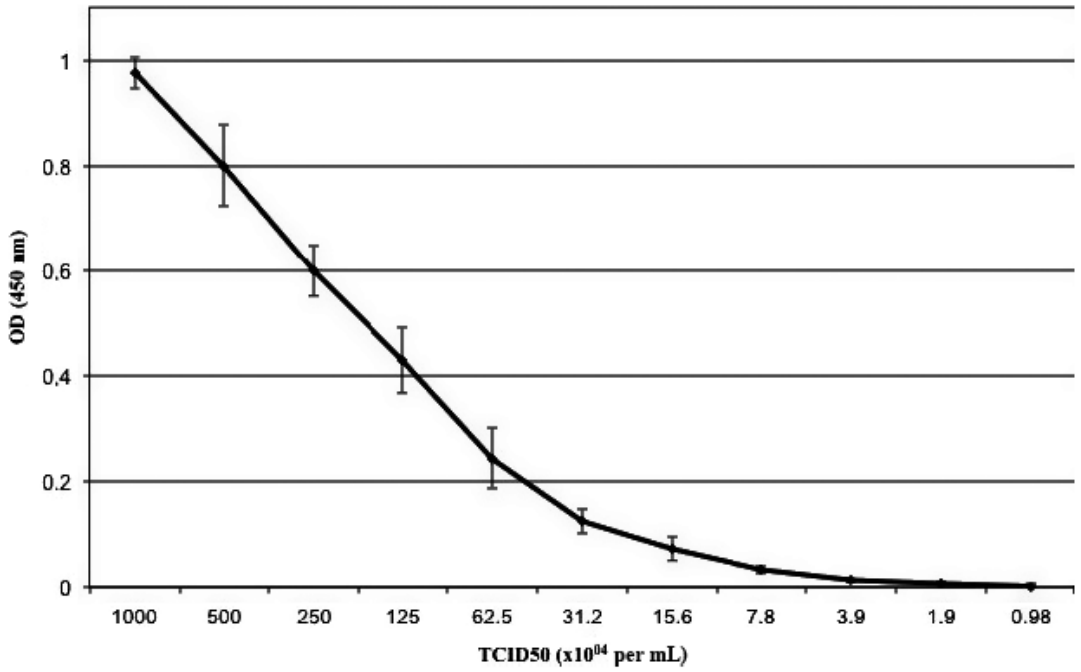

Figure 3. Determination of sensitivity of pAb 283 developing an Indirect ELISA. The plates were coated with indicated dilutions of VERv and probed with 20 µg mL^{-1} of pAb 283 antibody. The figure shows mean +/- SD optical density values (OD 450 nm) of duplicate wells from three diverse determinations. Background values (no pAb 283 added) were automatically subtracted from OD 450 nm values of samples.

Capture-based ELISA

To measure the presence of VERv in solutions, the plates were coated with 20 µg mL^{-1} of capture antibody pAb 283 and then added a series of virus dilutions from 540 µg mL^{-1} (11 x 10^7 TCID50) to 0.52 µg mL^{-1} (1.1 x 10^5 TCID50 per mL). To reveal bound VERv, it was employed a 1:10 dilution of the mouse monoclonal antibody mAb 4C3. The mAb 4C3 recognizes a capsid protein region corresponding to residues 141–162 and 181–212 (Costa et al., 2007). The results of this experiment are summarized in Fig. 4 as mean values +/- SD of three different determinations. The figure shows a clear and linear correlation between the amount of added virus and OD 450 nm values from 540 µg mL^{-1} (11 x 10^7 TCID50 per mL, corresponding to an OD 450 nm of ca. 0.27) to 8 µg mL^{-1} (17 x 10^5 TCID50 per mL, corresponding to an OD 450 nm of ca. 0.019).

The specificity of the developed capture-based ELISA is shown in Fig. 6, where different strains of RGNNV Betanodavirus (VERv 378/I02; 475/I98; 475/I98) were

clearly recognized, whereas other species of viruses (VHSv; IPNv) were not positive. To measure the presence of VERv in sea bass brain, the homogenates were tested for the presence of VERv through the capture-based ELISA, and the results are shown in Fig. 5. Negative controls showed OD 450 nm values of 0.007 +/- 0.001. The infected fish were grouped in three arbitrary groups, namely responders, low responders and negatives. The responders group consisted of 13 samples with a mean OD 450 nm of 0.274 +/- 0.158, the low responders group consisted of nine samples with a mean OD 450 nm of 0.049 +/- 0.025, and the rest of samples, under the limits of detection, were grouped in the negatives groups with a mean OD 450 nm of 0.011 +/- 0.007. Statistical analysis of data showed a significance of $P < 0.001$ (ANOVA) for the responders group. The obtained OD 450 nm values of each sample of infected fishes are shown in Table 1, from these values and OD 450 nm data from capture-based ELISA, can be extrapolated the presence of more than 540 µg mL^{-1} (11.2 x 10^7 TCID50 per mL) of VERv in the mean of responders and of 3.5 x 10^6 TCID50 per mL in the mean of low responders group. RT- PCR performed in sampled brains showed the presence of an 800 bp VERv-amplified product in all infected individuals, but not in non-infected control fish (Supplementary material).

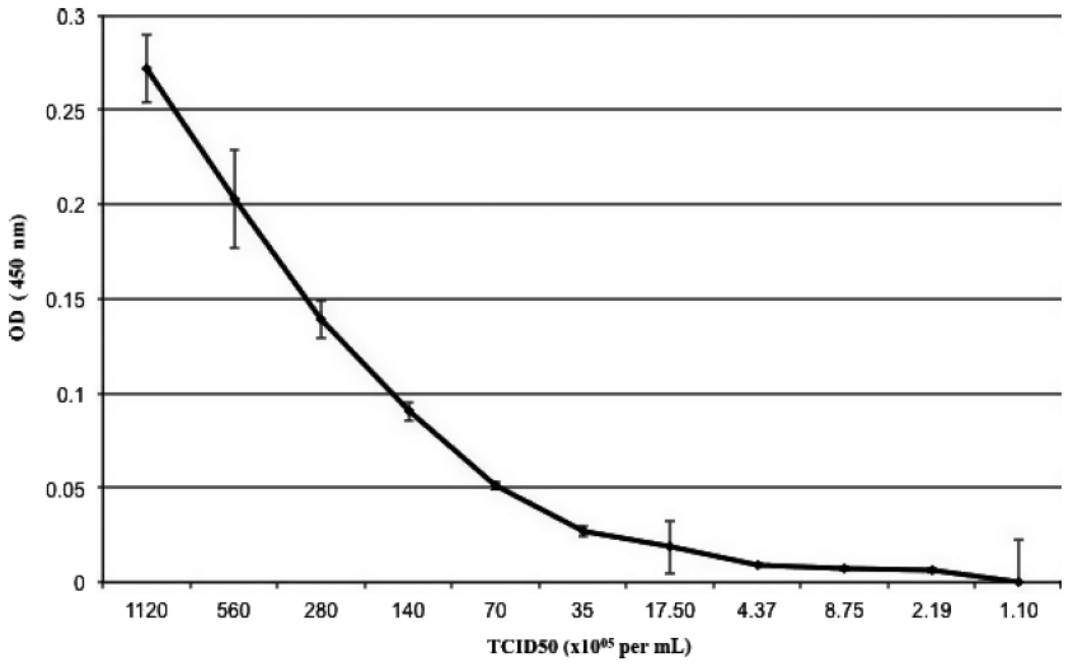

Figure 4. Standard curve of capture-based ELISA. The plates were coated with 20 µg mL^{-1} of pAb 283 antibody, added with indicated dilutions of VERv and with a 1:10

dilution of mAb 4C3. The figure shows mean +/- SD optical density values (OD 450 nm) of duplicate wells from three diverse determinations.

Brain sample	**RT-PCR**	**OD 450nm**	**TCID50(x10^5/mL)**	**Grouped as**
1	+	0.030	35	Low responders
2	+	0.030	35	Low responders
3	+	0.008	ND	Negatives
4	+	0.002	ND	Negatives
5	+	0.006	ND	Negatives
6	+	0.347	>1120	Responders
7	+	0.020	17	Negatives
8	+	0.030	35	Low responders
9	+	0.002	ND	Negatives
10	+	0.017	ND	Negatives
11	+	0.030	35	Low responders
12	+	0.011	>8.75	Negatives
13	+	0.114	280	Responders
14	+	0.020	17	Negatives
15	+	0.070	105	Low responders
16	+	0.272	1120	Responders
17	+	0.020	17	Negatives
18	+	0.137	280	Responders
19	+	0.030	35	Low responders
20	+	0.008	ND	Negatives
21	+	0.098	140	Responders
22	+	0.239	>560	Responders
23	+	0.198	560	Responders
24	+	0.332	>1120	Responders
25	+	0.174	280	Responders
26	+	0.301	>1120	Responders
27	+	0.592	>1120	Responders
28	+	0.572	>1120	Responders
29	+	0.180	280	Responders
30	+	0.084	140	Low responders
31	+	0.072	105	Low responders
32	+	0.076	105	Low responders

Table 1. The table shows OD values at 450 nm from 32 brain homogenates of sea bass experimentally infected with VERv, the corresponding extrapolated TCID50 per mL values and the RT-PCR positivity to VERv. (ND = undetected).

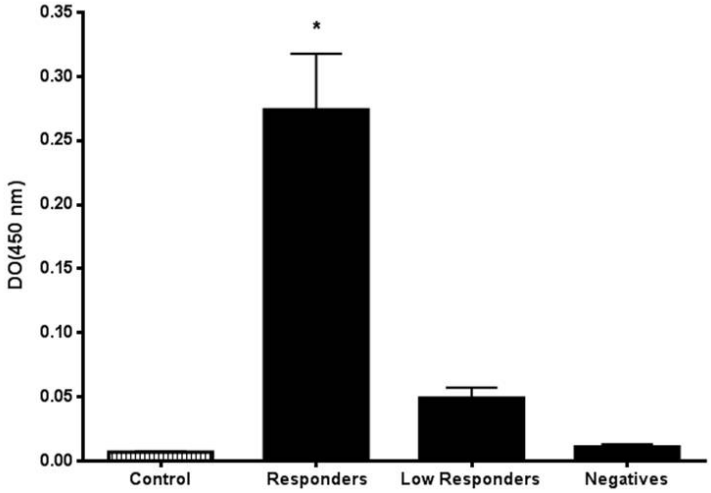

Figure 5 Detection of VER virus (VERv) in brain homogenates by capture-based ELISA assays using a capture antibody (pAb 283) and detection antibody (mAb 4C3). The figure shows mean +/- SD optical density values (OD 450 nm) of duplicate wells of control samples and infected samples grouped in responders, low responders and negatives. Background values (no pAb 283 added) were automatically subtracted from OD 450 nm values of samples.

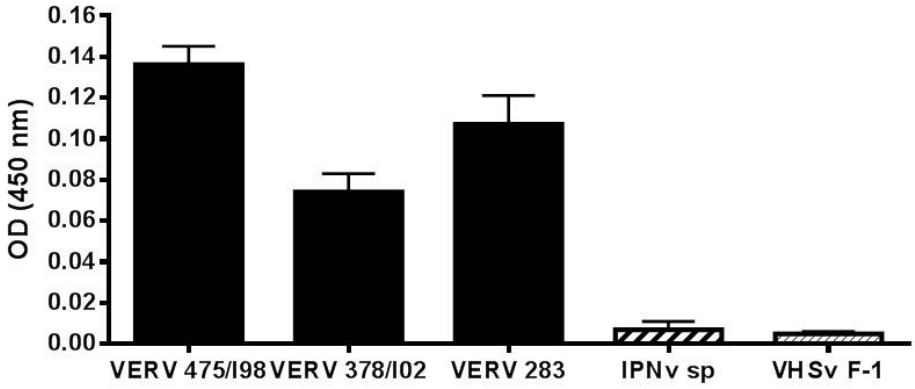

Figure 6. Detection of different types of virus through the developed capture-based ELISA. The plates were coated with 20 µg mL^{-1} of pAb 283, capture antibody, adding

$10^{7.5}$ TCID50 for each sample: Betanodavirus 378/I02-RGGNV (VERv 378/ I02); Betanodavirus 475/I98-red-spotted grouper nervous necrosis virus (RGNNV; VERv 475/I98); Betanodavirus 283/I09-RGGNV (VERv 283); viral haemorrhagic septicaemia (VHSv F-1); and infectious pancreatic necrosis virus (IPNv sp). The detection antibody, mAb 4C3, was diluted 1:10 until 1 µg mL^{-1}. The figure shows mean +/- SD optical density values (OD 450 nm) of triplicate wells. Background values (no pAb 283 added) were automatically subtracted from OD 450 nm values of samples.

4.5 DISCUSSION

Betanodaviruses represent a great threat to the health of many fish species including farmed sea bass. It affects larvae, juveniles and adults, causing serious economic losses. Diagnosis of the disease can be performed through qualitative assays, as reported in the introduction or, more recently, through the use of VERv-specific antibodies employed in immunohistochemistry of fixed tissues (Lopez-Jimena et al., 2012).

A great help to VERv detection and diagnosis in fish tissues have been achieved by RT-PCR through amplification of RNA1 and/or RNA2 genes (Arimoto et al., 1992; Lopez-Jimena et al., 2011; Chaves-Pozo et al., 2012; Panzarin et al., 2012) and at an higher degree of sensitivity by quantitative real-time PCR (Hodneland et al., 2011). Recently, a one-step RT-PCR method targeting the RNA1 of most VERv genotypes and characterized by a very high sensitivity has been reported (Baud et al., 2015). However, despite the great advances achieved in the detection of VERv by molecular techniques, little attention has been paid to the development of immunoenzymatic methods for the quantitative determination of virus in biological fluids. In this chapter, an ELISA-based assay is proposed to evaluate VERv in biological samples and in brain homogenates of infected sea bass.

The principal reagents of the ELISA assay were two antibodies: the rabbit polyclonal antiserum pAb 283 against RGNNV genotype and the mouse monoclonal antibody 4C3 specific to capsid protein. The combined employment of these reagents permitted the use of pAb 283 as a capture antibody for antigen in solution and of mAb 4C3 to reveal bound antigen.

The evidence that pAb 283 recognized VERv comes from the Western blot shown in Fig. 1, where the antibody stained virus RNA1 product (polymerase) at around 110 kDa and RNA2 product (capsid protein) at ca. 38 kDa. The high quality of ultracentrifuged

virus preparations can be appreciated by electron microscopy at lower and higher magnifications (scale bar is 200 nm).

Subsequently, the pAb 283 was tested for its capability to recognize VERv immobilized on plastic wells, and the results (Fig. 2) showed the binding as dose-dependent and very sensitive, with a lower detection limit at antibody dilutions around 1 µg mL^{-1}. Once time established by Western blot the antigen recognition, and obtained a working dilution of pAb 283 (20 µg mL^{-1}), the subsequent step was to establish the sensitivity/detection limit of pAb 283 for its antigen. As can be appreciated from Fig. 3, decreasing concentrations of virus corresponded to decreasing OD 450 nm values in a linear fashion; and thus, it can be evaluated an Indirect ELISA sensitivity of about 2 µg mL^{-1} (3 x 10^5 adsorbed TCID50 per mL).

To use pAb 283 as a capture antibody for the binding of VERv in solutions, pAb 283 was coated in plastic wells with the mAb 4C3 to reveal antibody bound virus. The results, shown in Fig. 4, show that the capture-based ELISA system is working, with a sensitivity of about 17 µg mL^{-1} (35 x 10^5 TCID50 per mL). It should be noted that the capture-based ELISA sensitivity was much lower than that of Indirect ELISA (shown in Fig. 3), and this can be reasonably attributed to the higher number of steps necessary to run a capture-based ELISA. However, with a TCID50-titrated VERv preparation as a reference, with the assay it could be possible to make an estimation of the amount of TCID50 in a biological sample. To test this hypothesis, 42 brain homogenates were assayed, 32 corresponding to fish experimentally infected with VERv and 10 to control uninfected fish by capture-based ELISA. Obtained results (shown in Fig. 5) suggested the presence of detectable virus in 22 brain homogenates out of 32 infected fish.

Infected brain samples were grouped in three arbitrary groups, namely responders, low responders and negatives. The responders group consisted of 13 samples with an OD 450 nm higher of 0.1, the low responders group were nine samples with an OD 450 nm between 0.09 and 0.03, and the remaining 10 samples that were under the limit of detection, called negatives, with an OD 450 nm lower of 0.03. With the data obtained by assays that were run, it can be assumed that OD values higher than 0.01 may indicate the presence of VERv. It should be noted that all 32 infected brain samples tested positive for the presence of VERv by RTPCR (data not shown); and thus, an apparent discrepancy with our ELISA data is present. This discrepancy can be attributed to the higher sensitivity of RT-PCR amplification and possible differences in measuring, by

RT-PCR, the presence of viral RNA rather than the actual viral capsid protein measured with antibodies by ELISA.

Other ELISA-based systems have been previously described for types of Betanodavirus. First, an Indirect ELISA was performed for the detection of Betanodavirus in striped jack using a rabbit polyclonal serum raised against the SJNNV and had a sensitivity of 25 µg mL^{-1} of purified virus (Arimoto et al., 1992). In invertebrates, a capture-based ELISA for a shrimp nodavirus type was developed to detect the virus in tissue extracts or whole larvae (Romestand and Bonami, 2003). Shieh and Chi (2005) developed a capture assay using purified Betanodavirus and supernatant of VERv infected GF-1 cells. Fenner et al. (2006) developed a capture-based ELISA to detect and quantify nodavirus in juvenile barramundi *L. calcarifer*. In sea bass, VERv belonging to RGNNV genotype was previously detected by capture-based ELISA in infected larvae homogenates with a sensitivity of 6 µg mL^{-1} (Breuil et al., 2001), but ELISA tests to reveal and quantitate the presence of VERv in biological fluids in infected juveniles and adult sea bass are not available to date. Different from previous works, possible relationships between ELISA data (absorbance values) and infective capability (TCID50 per mL) of RGNNV-type nodavirus were evaluated.

The presented data showed that the capture-based ELISA system may detect other RGNNV strains, but viruses not belonging to the family Nodaviridae were not detected (Fig. 6), thus suggesting the assay is specific for RGNNV. Despite the differences between RGNNV isolates, it is possible to affirm that the capture-based ELISA with pAb 283 may give positive detection of RGNNV isolates. Further work is necessary to verify whether our ELISA system might be also reactive with other genotypes. This can be speculated for the conservation of capsid protein residues 193–212 (Nishizawa et al., 1995) and for the similarities between SJNNV/TPNNV and BFNNV/RGNNV, sharing 76% and 81% homology in nucleotide and amino acid sequences, respectively (Munday et al., 2002).

In conclusion, the described capture-based ELISA may be used as a method to facilitate the diagnosis and detection of VERv in biological samples of adult or juveniles sea bass, to extrapolate a relation between ELISA absorbance values with a relative number of TCID50 and to detect viral contamination in samples difficult to be analysed by PCR. The assay is simple and sensitive and could potentially be employed by end-users (e.g. in fish farms), to detect Betanodavirus in samples such as egg homogenates, or in

samples where PCR assays are difficult to be performed such as fish food. The ELISA could be used in addition to RT-PCR as a tool to measure viral presence and, together with an ELISA assay previously shown to detect VERv specific IgM in infected fish (Scapigliati et al., 2010), can be employed as a comprehensive 'immune kit' to investigate viral presence and antiviral immune response in sea bass.

4.6 APPENDIX:

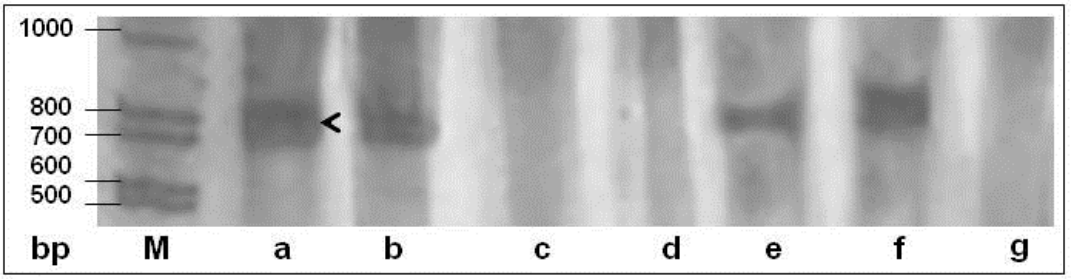

Figure S1. RT-PCR of brain homogenates. The figure shows a RT-PCR (30 cycles) amplification products using specific primers for VERV capsid protein, loaded in a 1 % agarose gel. Lane M contains 100 bp DNA Marker. An amplification product corresponding to a VERV positive control is in (a), and a control for DNA contamination as negative control in (g). Control fish samples are in (c, d), and infected fish samples are in (b, f). The expected RT-PCR product (800 bp) is indicated with an arrow.

CHAPTER 5: Betanodavirus mucosal immunization: The Encefalopathy and Retinopathy virus of European sea bass *Dicentrarchus labrax*: immunization of juveniles by immersion.

This Chapter describes an original approach to investigate the effects of Betanodavirus immunization of European sea bass. The experimental approach investigated cellular and molecular immune responses of fry sea bass immunized by immersion with a formylated Betanodavirus preparation.

5.1 ABSTRACT

Viral encefalopathy and retinopathy virus (VERv) represent a major threat for many wild and farmed fish species including the European sea bass, wich is preferentially infected during young life stages, and the present work investigates the possibilites of immunizing young sea bass against VERv by means of mucosal (immersion) vaccination employing inactivated VERv. VERv-free sea bass of 2-10 grams were immunized by immersion in virus solution for 2 minutes and then sampled after 24, 48 hours and after 30 days. The results of this round of immunisations showed that very few serum antigen-specific IgM can be detected by Indirect ELISA; no *in vitro* VERv-induced gills leukocyte proliferation can be induced; a modulation in transcription levels of genes coding for Mx and ISG-12 was observed after 24 hours and VERv antigens were detected in the gills by immunohistochemistry. These results shown that a single immersion vaccination without boosting do not induces systemic IgM-based immunity in sea bass, and represent a first comprehensive approach to investigate the effects of VERv immunization of sea bass.

5.2 INTRODUCTION

Viral encefalophaty and retinopathy (VER) is a fateful disease that affects several marine fish species as sea bass. The sea bass (*Dicentrarchus labrax*) is the second farmed specie in the Mediterranean Sea (FEAP 2005-2014) and is a highly susceptible to VER. Notably, the VER introduction in the Mediterranean Sea dates back to the early ‘90s in Europe and was detected in sea bass hatcheries (Bovo et al., 1999).

Betanodavirus is a highly pathogenic virus able to evade the host’s protective systems and can either replicate and transmit progeny to other cells or remain in a latent

condition (Vossen et al., 2002). In general, the clinical signs relate to the lesions present in the brain and retina, i.e. there are abnormalities of movement, swim bladder control, sight and colouration (Munday et al., 2002) but the main outcome is mass mortality, often reaching 100%, especially of larvae (Munday and Nakai, 1997). In this species, mortality can vary depending on the fish age and water temperature: outbreaks in hatcheries can be devastating with a mortality rate reaching 80-100%, but also elder fish may be seriously affected (Munday et al., 2002; Chérif et al., 2009). To prevent viral encephalopathy and retinopathy disease in aquaculture industry, it is necessary to find strategies of mass vaccination, possibly through mucosal routes that can be used with fry sea bass. So far very few vaccines have been tested in this species.

In order to develop strategies for the control of virus infection, the possibilities of vaccinate sea bass at young age through mucosal immunization have been studied, namely immersion, employing formalin-inactivated VERv and monitoring cellular and molecular immune responses.

5.3 MATERIALS AND METHODS

Fish

Juveniles sea bass tested by RT-PCR to be VERv-free (data not shown), with an average weight of 2 ± 9 g were transferred from a commercial farm and were divided according to the different experiments to be performed. On arrival, one hundred fish were sampled in order to be checked for the most common pathogens (parasites, bacteria and viruses). Preliminary analyses confirmed that all the fish were pathogens-free. Fish were kept in a UV-treated recirculation system with artificial salt water 25‰ of salinity, at a temperature of 22±1 °C, pH 7.5 ± 0.3, oxygen 6±0.5 ppm, an artificial photoperiod of 8 hours of light and 16 of darkness and were fed daily ad libitum with standard commercial food.

Vaccine preparation, immunization and challenge

The Betanodavirus isolate 283/I09–RGGNV (VERv 283) (GenBank accesion number JN189865 and JN189992, Panzarin et al., 2012) was prepared from serum-free culture supernatants of infected E-11 cell monolayers as previously described (Iwamoto et al., 2000; Vendramin et al., 2014), in 150 cm^2 tissue culture flasks with L-15 medium

(Leibovitz) (Sigma-Aldrich) without fetal calf serum. This isolated has been previously used in the IZSVe experimental aquarium, showing a mortality rate of >35% by bath exposure in sea bass (Vendramin et al., 2014; Pascoli et al., 2016).
The collected virus was subjected to titration by endpoint dilutions assays. Titres were calculated according to the Spearman-Karber formula (Spearman-Karber and Finney, 1978) and expressed as TCID50 mL^{-1}.

The viral solution (initial titre $10^{7,80}$ TCID50 mL^{-1}) was inactivated adding buffered formalin 10% 1:100 at room temperature for one week. The inactivated preparation was checked after treatment using virological analyses according to a standard procedure (Pascoli et al., 2016), doing three sequential serial blind passages to ensure the complete inactivation of the pathogen.
One batch of virus was keep untreated and used for live virus immersion trial.

After a period of acclimation of 10 days, an immersion immunization (IM) with 10^6 TCID50 mL^{-1} of formalin-inactivated virus solution for 2 minutes was performed. Fish were sedated by immersion in salt water containing 100 ppm of anaesthetic Ethylenglycolmono-phenylether (MERCK-Schuchardt) and then they were divided in 2 homogeneus groups: control group (CTR) with 100 immersed fish in sea water and formalin-inactivated VERv group (NNV) with 100 immersed fish in sea water containing formalin-inactivated VERv.
Sampling consisted of different types of samples and were taken at different times during the trial. Gill samples were taken from 20 fish (n=4 pools) per group to perform a Real-time quantitative PCR (qPCR) at 0, 24 and 48 hours post immersion. Also muscles of control fish were collected at time 0.
Sampling of gills of control fish and immunizated fish were collected at 48 h post-immersion to be analyzed by IHC.
Thirty days post vaccination, blood and brain samples were collected from fish (n=10) from all groups. Also five gill and spleen samples per group were collected to perform a proliferation assay as explain in Chapter 2: Materials and methods.
After 30 days post vaccination (dpv), fish vaccinated during 2 min were challenged with VERv 283 by immersion (decision based on Immunological results). For immersion challenge, each group of 50 fish was transferred to a 30-L water tank with aerator and

infected for 4 h by adding 30 mL of virus solution (approximately $10^{7.80}$ TCID50 mL^{-1}) to the water. The "negative control" group was mock infected by immersion, adding minimum essential medium (MEM-10) without virus to the water. Fish were checked twice a day and the dead ones were removed. Samples of brain were collected individually from each dead fish and stored at -80°C. The experiment ended on day 30 and the relative percent survival (RPS) was calculated. All the remaining fish were euthanized by an overdose of MS-222, sampled and stored at -80 °C until analyses.

Immunohistochemistry

For IHC, gills from sea bass immersed with formalin-inactivated VERv were sampled at 48 h post-immersion. Gills were fixed in ice-cold Bouin's fixative for 7 h at 4 °C, and obtained paraffin sections processed as previously described in Chapter 2: Materials and Methods.

The immunohistochemical reaction was performed by ABC-peroxidase with nickel enhancement as previously described in Abelli et al., 1997. Serial sections were incubated with the mAb 4C3 (that recognizes VERv capsid protein) (Costa et al., 2007) diluted 1:10 in PBS 0.1 M, pH 7.3 containing 5% normal horse serum and 0.1% sodium azide, or with pAb 283 (that recognizes the whole virus) (Chapter 4: Results) diluted 1:3000 using as diluent PBS 0.1 M, pH 7.3 containing containing 5% normal sea bass serum, 5% normal goat serum and 0.1% sodium azide. Controls were done by omitting the primary antibody. Thereafter, sections were incubated for 60 min with biotinylated horse anti-mouse or anti rabbit IgG sera (Vector Labs., Burlingame, USA) diluted 1:1000 with PBS containing 0.1% sodium azide and 1% BSA, followed by incubation for 60 min with avidin-biotinylated peroxidase complex (ABC, Vectastain Elite, Vector). Following rinses and staining (diaminobenzidine and nickel enhancement), sections were dehydrated, mounted and examined under a Zeiss microscope equipped with a colour video camera (Axio Cam MRC, Arese, Milano Italy) and a software package (KS 300 and AxioVision).

Immunological analyses

Thirty days after immunization blood from fry sea bass (n=10) was collected from the caudal vein of lethally anaesthetized fish (10 mg/L of MS-222). Sera were obtained by centrifugation at 1000 g for 5 min and were tested by Indirect ELISA previously shown

to detect VERv-specific IgM in infected fish (Scapigliati et al., 2010) using as antigen formalin-inactivated VERv preparation (10^8 TCID50 mL^{-1}).

A 100 mL batch of the serum-free culture supernatant containing formalin-inactivated virus was ultracentrifuged to be used as antigen in ELISA assay as explain in Chapter 4: Materials and methods. A final concentration of 10^8 TCID50 mL^{-1} of inactivated VERv preparation was diluted with 0.05 M carbonate-bicarbonate buffer pH 9.4 and was used it to coat polystyrene wells (100 µL/well). The wells were then washed three times with Tris-HCl 50 mM pH 7.4 containing 0.05% Tween-20 and 0.15 M NaCl (TTN). After blocking remaining sites with 3% BSA in TTN (TBT), 100 µL of sera diluted in TBT (1:60) were incubated overnight at 4°C. The wells were carefully washed with TTN and then incubated for 90 min with 100 µL of an anti-sea bass IgM antiserum (Scapigliati et al., 2010) diluted 1:10.000 in TBT and, after washing with TTN, with a HRP-conjugated secondary antibody solution in TBT. The wells were then washed with 50mM phosphate-citrate buffer (pH 5.0) and the reaction was visualised using o-phenylenediamine as substrate. The absorbance values were read at 450 nm with an automatic plate reader (Labsystems Multiskan MS). The ELISA assays data are presented as the mean absorbance ± SD. Each sample was measured in duplicate wells and optical density values (OD 450nm) of control wells were automatically subtracted from samples values

Leukocytes from gills and spleen were obtained as explained in Chapter 2 and incubated for 15 min with 5 µg/mL of carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) following the manufacturer's instructions. After 24 and 48 hours post-stimulation with Phytohaemagglutinin (PHA) and formalin-inactivated VERv (10^6 TCID50 mL^{-1}), leukocytes proliferation was controlled by flow cytometry as explain in Chapter 2: Materials and Methods.

The transcriptional activity of antiviral genes Mx and ISG-12 was observed in four pools of five gills by group after 24 and 48 hours by Real-time quantitative PCR (qPCR) (Table 1). Samples were immediately placed in 1 ml of denaturing solution (Tripure, Roche), total RNA was isolated following the manufacturer's instructions and subsequently suspended in DEPC-treated water. For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used following the manufacturer's instructions. The absence of DNA contamination was checked using sea bass β-actin primers that

bracketed an intron [20]. The transcripts relative to Mx and ISG-12 genes were determined with an Mx3000P™ Real-time quantitative PCR system (Stratagene) equipped with version 2.02 software, using the Brilliant SYBR Green Q-PCR Master Mix (Stratagene) following the manufacturer's instructions. ROX was used as internal passive reference dye.

Specific PCR primers (Table 1) were designed for the amplification of products (ca. 200 bp) from the conserved region of all the analyzed genes and ribosomal 18S rRNA was used as house-keeping gene. A 10 ng of cDNA template was used in each PCR reaction. PCR conditions were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. Reactions were performed in triplicate for each template cDNA, which was replaced with water in all blank control reactions. Each run was terminated with a melting curve analysis, which resulted in a melting peak profile specific to the amplified target DNA. The analysis was carried out using the endpoint method option of the Mx3000P™ software, allowing the fluorescence data to be collected at the end of each extension stage of amplification. A relative quantification was performed comparing the levels of the target transcript to the reference transcript (18S rRNA). Quantitative assessment was based on determination of the threshold cycle (Ct), defined as the cycle at which a statistically significant increase in fluorescence above the background signal was detected. The calibrator, defined as 1.0 value, was the average of 3 muscle samples from control fish due to the low expression of these antiviral genes in muscle.

The specificity and size of the qPCR products were checked by agarose gel electrophoresis.

Gene	Primer sequence	EMBL accession
18S rRNA	FW: 5′-CCAACGAGCTGCTGACC-3′ RW: 5′-CCGTTACCCGTGGTCC-3′	AY831388
ß-actin	FW: 5′-ATGTACGTTGCCATCC-3′ RW: 5′-GAGATGCCACGCTCTC-3′	AJ493428
Mx	FW: 5′-GTCTGGAGATCGCCTCT-3′ RW: 5′-GTGGATCCTGATGGAGA-3′	AM228977
ISG12	FW: 5′-CCTGGTACAGCTGCTGT-3′ RW: 5′-AGCTGCTCCTGCTGACT-3′	FN665389

Table 1. Specific Real-time quantitative PCR primers for the amplification of products (ca. 200 bp).

Virological analysis

Virus isolation in cell culture: samples (cell culture supernatant and fish brains) were examined by standard virological techniques (Pascoli et al., 2016).

Brain samples were homogenized in a mortar with a pestle and sterile sand. The samples were diluted 1:10 in transport medium (Eagle's minimum essential medium supplemented with 10% foetal bovine serum, 2% of antibiotic/antimycotic solution, 10.000 IU mL^{-1} penicillin G, 10 mg mL^{-1} streptomycin sulphate, 25 lg mL^{-1} amphotericin B and 0.4% of 50 mg mL^{-1} kanamycin solution) and centrifuged at 4000 g at 4 °C for 15 min.

Cell culture supernatant of E11 cells incubated during one week with the formylated preparation was processed as explain above.

Then, 10% of antibiotic/antimycotic solution was added to the supernatant and the samples incubated at 4 °C overnight.

The 24-well plates with 24-h-old E11 cell line were inoculated with samples in final dilutions 1:100 and 1:1000. The inoculated cell cultures were incubated at 15 °C for 1 week, with regular examination for cytopathic effect (CPE). After 1 week, samples with no CPE were re-inoculated into a new culture of E11 cells. If no CPE was observed at the end of the 2nd week, the samples were tested negative for the virus.

Real Time RT-PCR analyses for detection of Betanodavirus RNA2 were performed using a slightly modified version of the published protocol (Panzarin et al., 2010). Briefly, total RNA was extracted from 100 µL of the sample using the NucleoSpin® RNA kit (Macherey-Nagel) and according to the manufacturer's instructions. Real Time RT-PCR was conducted with RotorGene Q (Qiagen), Rotor Gene 6000 (Corbett, Australia) using the OneStep RT-PCR Kit (Qiagen) following the manufacturer's recommendations, adding Random primers (Applied Biosystems) and RNase Inhibitor (Promega). Primers and probe were used at a final concentration of 0.9 µM and 0.75 µM, respectively, random primers at concentration 1X, RNase Inhibitor at final concentration of 8U μL^{-1} and thermal cycling was performed at 50°C for 30 min and denaturation at 95° C for 10 min followed by 45 cycles of 95° C for 10 s,54° C for 35 s and 72° C for 1 s, with a final incubation at 40° C for 30 s. Data analyses were performed with the Rotor Gene 6000/RotorGene Q series software.

Statistical analysis

The number of fish per experimental group was calculated according to the expected mortality rate (35%) and 0.05 as α error (one-side) and power 80% (1-β=0.80).

Each fish in the study was followed over time and the event "death" was recorded and verified by Real Time RT-PCR.

The Kaplan–Meier method was used to estimate the survival function from lifetime data, which allowed to draw the survival curve for each group as a step curve and to measure the length of time the fish would survive the infection. In the graph, the y-axis plot indicates the cumulative probability of the surviving fish at each time (Van Belle et al., 2004). To compare the different survival curves, the non-parametric Wilcoxon–Breslow–Gehan test was used for equality of survivor functions. One-sided tests with a p-value<0.05 were considered as significant. Statistical analyses for potency test were performed using the STATA 12.1 software.

Statistical analysis for immunological parameters was performed by Graph Pad Prism 6.0 software statistical package. The data from different experiments have been presented as the mean ± SD of four pools of five organs in the gills in qPCR and the average of ten single samples in Indirect ELISA. The statistical significance qPCR was determined using One-way ANOVA followed by Bonferroni's multiple comparison test

and among groups for Indirect ELISA by Student's t test, the level for accepted statistical significance was $p<0.05$.

5.4 RESULTS

Vaccines safety

The inactivation method proved to completely inactivate Betanodavirus with no viral growth in cell culture after 3 blind passages and no mortality was observed in fish during and after immunization procedures. Thirty days post vaccination, brains from vaccinated fish were collected and checked for VERv positivity by Real Time RT-PCR and virological analysis and no positivity was found.

Immunological analyses

Mucosal vaccination has induced a very low specific IgM titer in immunizated fish (NNV group). The high variability bewteen individuals resulted in a poor statistical significance of data. Furthermore, no significant differences between the different immersion times were found, and only the results from two minutes of immersion are presented.

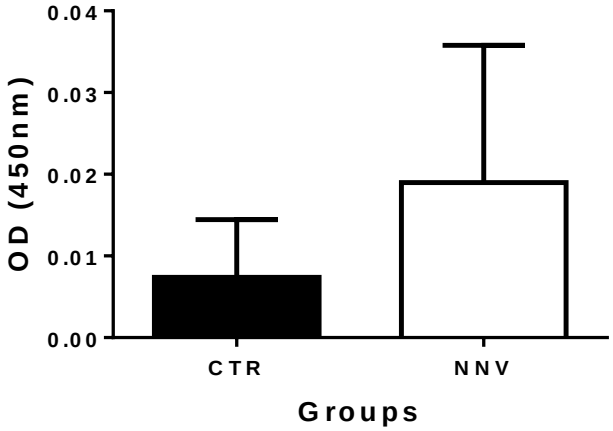

Figure 1. Detection of specific VERv IgM in sera samples 30 days after immunization. Dilutions 1/60 of sera from differents groups were tested by Indirect ELISA using as antigen 10^6 TCID50 per well of formalin-inactivated viral preparation and Pab IgM (diluted 1:10000). Background values (no viral preparation added) were automatically subtracted from OD 450 nm of samples values.

Proliferation of gills and spleen leukocytes stimulated with Phytohaemagglutinin (PHA) at 24 and 48 hours of incubation was detected by flow cytometer as expected (see Chapter 2: Results) but none was obtained after formalin-inactivated VERv stimulation (Data not shown).

Formalin-inactivated VERv uptake was detected in the gills of immersed sea bass by employing IHC. In particular, the mAb 4C3 (Fig. 2b) recognised scattered cells, with strong positive signal, distributed in the gill filaments and parallel secondary lamellae. In addition a weak reaction was homogeneously detected in the gill mucosal tissue. To notice, the control specimens (no IM vaccinated animals) did not evidenced any mAb 4C3 staining (Fig. 2a).

Sections incubated with pAb 283 (Figs. 3 a-b) showed a strong reaction in the thin epithelium covering the secondary lamellae and in the basal cells localized between the secondary lamellae on gill filaments. No reaction was found in control sections without primary antibody (Figs. 3c).

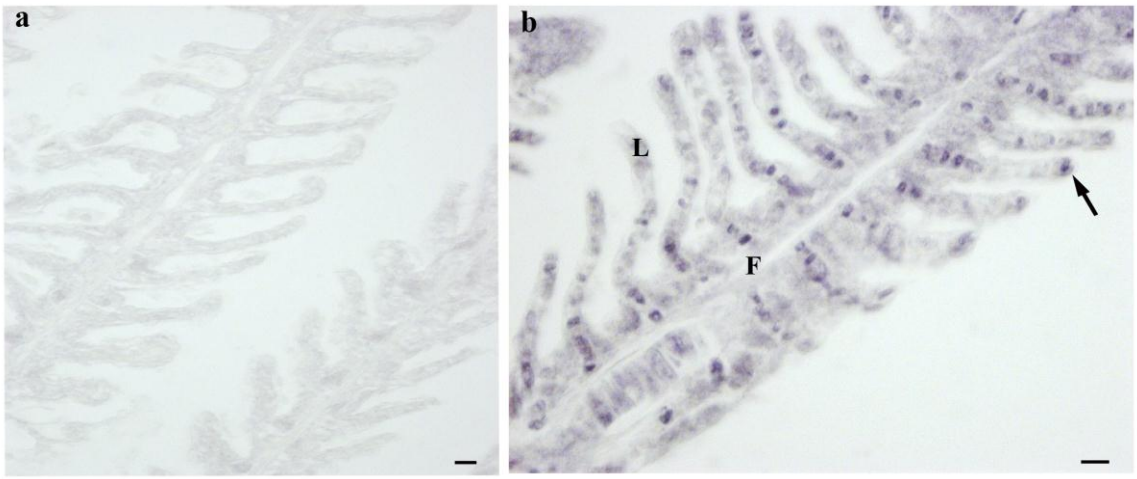

Figure 2. Immunoreactive uptake within the branchial filaments using Mab 4C3, an antibody that recognizes VERv capsid protein. (a) Control fish, scale bar = 10 μm. (b) Immunized fish, immunoreactivity is represented with an arrow, scale bar = 10 μm. F: filament; L: lamella.

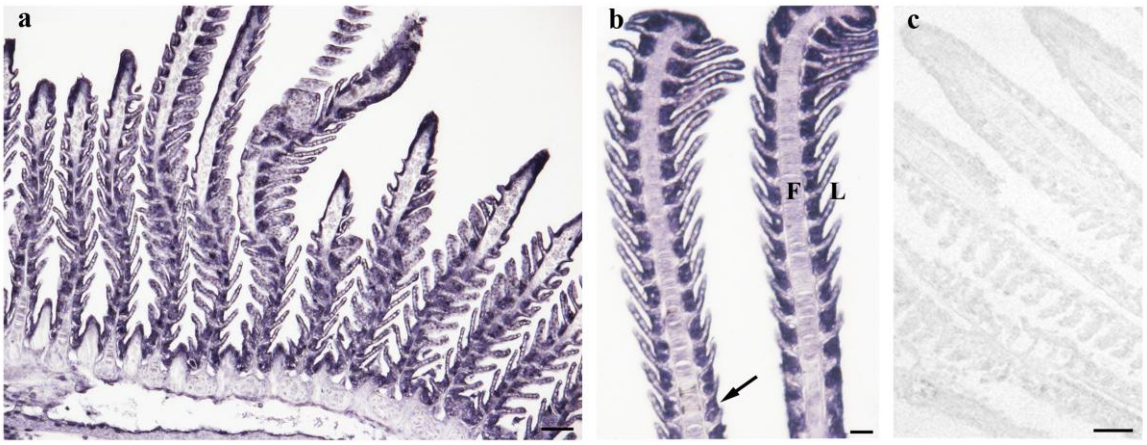

Figure 3. Immunoreactive uptake within the branchial filaments using pAb 283, an antibody that recognizes the whole virus. (a) Gill epithelium showing the presence of immunoreactivity, scale bar = 50 μm. (b) Higher magnification of immunoreactivity (arrow), scale bar = 20 μm. (c) Negative control showing the absence of immunoreactivity in gill tissue. Scale bar = 50 μm. F: filament; L: lamella.

QPCR was performed to analyse the expression of antiviral immune response genes in gills after immersion immunization. Transcripts levels of Mx and ISG12 genes are shown in Figure 4. Results are represented as a scatter plot of pooled samples, with each dot as a pool of 5 fry samples at 24 and 48 hours after immersion, using the average of 3 muscle samples of control fish as calibrator and comparing the expression with mock-immunized fish. A significant modulation of Mx gene ($p<0.05$) has been observed at 24 hours (Fig. 4a), as well an increase at the same time for ISG12 gene (Fig. 4b), although not statistically significant due to the high fish individual variability.

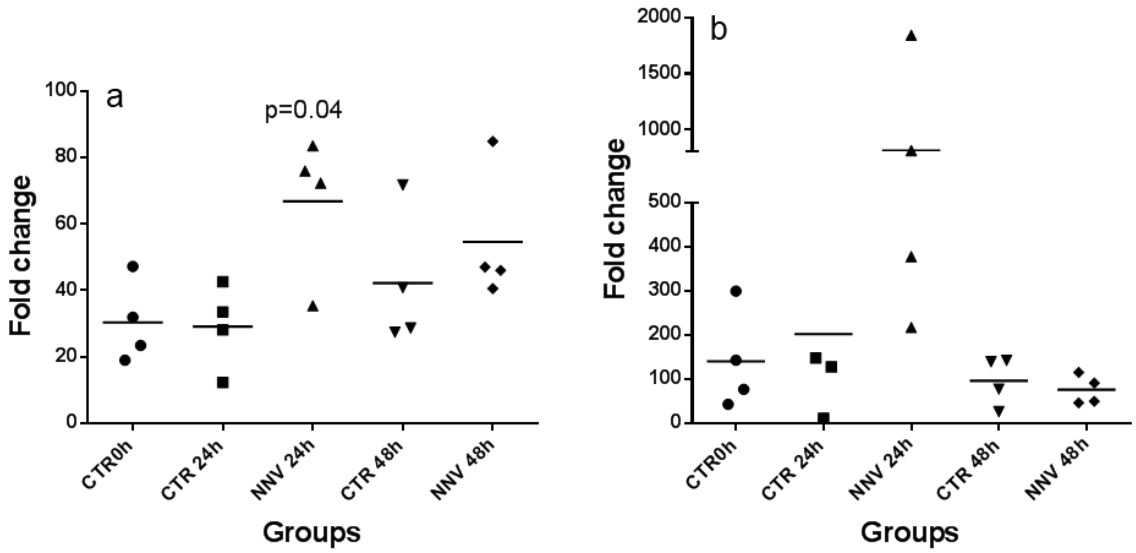

Figure 4. Real-time quantitative PCR in gills of sea bass immunizated by immersion with formalin-inactivated VERv. The quantitative expression of Mx (4a) and ISG12 (4b) genes (normalized respect to 18S rRNA and using the average of 3 muscle sample from control fish as calibrator (data not shown) are shown in control fish (CTR) and in immersed immunizated fish (NNV) at differents sampling points. Results are expressed as the mean ± SD of four pools. One-way ANOVA followed by Bonferroni's multiple comparison tests was used to determine differences from the control at each time point. Data analysis was performed using the GraphPad Prism 6.0 software statistical package.

Moreover, bath vaccinated fish showed a cumulative mortality of 50%, whereas in the positive control group it was 52%, with no differences between groups ($p>0.05$), and a RPS of 1.6 %. Then immersion immunization with formalin-inactivated virus has not provided fish protection.

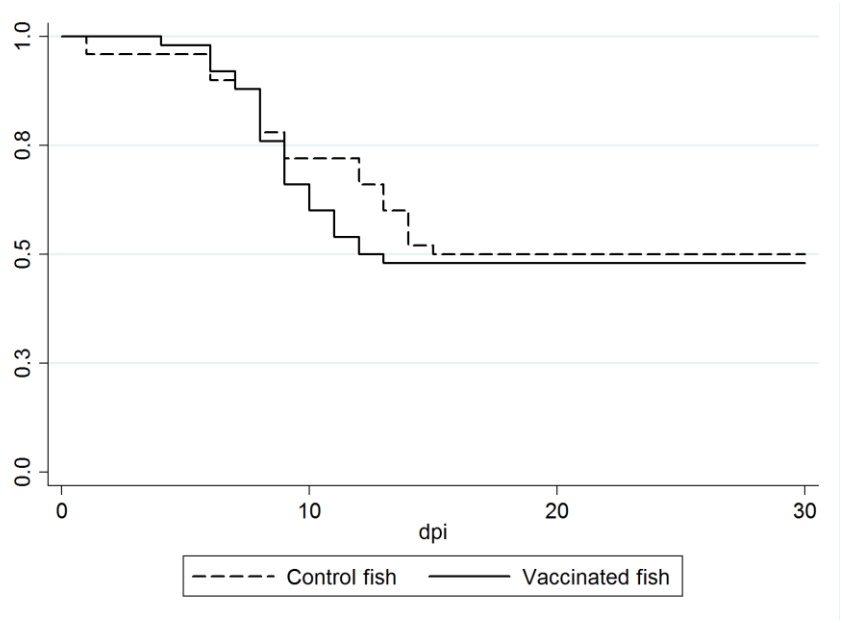

Figure 5. Kaplan-Meier survival stimates. dpi=days post infection

5.5 DISCUSSION

Betanodaviruses commonly cause disease and mortality in larval and juvenile sea bass stages. As mentioned in the introduction, the highest mortality rate has been reported during the larval stage. Althought causes great losses to the fish farms, mucosal vaccines to fight the pathology are not yet available (Munday et al., 2002). Sea bass present a high sensitivity to stress and it is necessary to develop strategies of mass vaccination, possibly through mucosal routes because are easy handling, quick, not stressful for the fish and is an effective and practical method to induce humoral and cell-mediated immune responses (Angelidis et al., 2006; Villumsen and Raida, 2013). This method, for example, could be used as a prevention method in fish farms.

Promising results have been obtained using different approaches to inactivate Betanodavirus. For example the efficacy of nano-encapsulated formalin-inactivated or BEI-inactivated Betanodavirus preparations given by immersion immunization have been shown in protecting grouper larvae (*E. coioides*) from NNV disease (Kai and Chi, 2008).

In this work were evaluated the sea bass immune responses after immersion immunization during 2 minutes with a preparation of formalin-inactivated VERv. By analyzing ELISA results of antigens administered by immersion low, but detectable, specific serum IgM was observed in fish treated with formylated virus, suggesting that a single immersion induced a weak humoral response in sea bass. The use of

nanoparticles or other encapsulating materials could be useful to improve the delivery and uptake of the inactivated antigen also in *D. labrax*, but this assumption needs further investigation. No proliferation of *in vivo* assay measured by flow cytometry using CFSE fluorescence incorporation was detected (data not shown).

Being the gills directly involved in antigen uptake during immersion vaccination, the transcription of Mx and ISG-12 genes were analyzed at 24 and 48 hours post immersion with formylated antigen, and was observed a significantly upregulation of these genes at 24 hours, likely suggesting a possible antiviral response in the tissue. Both are interferon-inducible genes and are known to play an active role in antiviral immune responses (Verrier et al., 2011; Collet, 2014); in particular, the Mx gene has already proved to be modulated in Betanodavirus-infected sea bass (Scapigliati et al., 2010). The ISG-12 gene is activated by the interferon, which exerts its biological function downstream mainly through the activation of ISGs genes (Boudinot et al., 2014; Pallavicini et al., 2010; Matsuyama et al., 2012; Caipang et al., 2003).

Importantly, the presence of viral particles was demonstrated by IHC in the gills epithelium of immersion-treated fish by using antibodies against the virus capsid protein (mAb 4C3) and the whole virus (pAb 283) (Fig. 2 and 3).

Finally, the cumulative mortality reached the 50% whereas in the positive control group was 52% (Fig. 5).

Another times of immersion were checked and same results were obtained. These data suggest that a single immersion vaccination without boosting do not induces systemic IgM-based immunity in sea bass. Must be considered a possible denaturation of antigenic sites during the inactivation process, the possible detection limit of the ELISA assay on sera of very young fish and the individual variability.

In summary, data obtained from immersion-vaccinated fish, including the lack of protection in challenge trials, suggests that this way of administration needs better investigation before being effective. Further studies will be focused in improving this type of vaccination (i.e. with adjuvants, a second booster, etc.) due to the importance of this delivery route for protecting young sea bass against VERv disease.

CHAPTER 6: Betanodavirus vaccination: Efficacy of intraperitoneal vaccination using inactivated viral encephalopathy and retinopathy virus (VERv).

This Chapter describes an approach to evaluate the efficacy of different antigenic preparations to be used as tentative vaccines, and the antiviral immune responses of sea bass immunized through systemic (intraperitoneal) and mucosal (immersion) against VERv.

6.1 ABSTRACT

The European sea bass is the most important farmed species in Mediterranean area and is very sensitive to the infection of Encephalopathy and Retinopathy Virus (VERv), or Betanodavirus that causes massive mortalities. Effective vaccines to fight the pathology are not yet available, and here is described a promising intraperitoneal immunization route against VERv in juveniles. After inactivation of VERv (isolate 283.2009 RGNNV) by formalin, β-propiolactone and temperature treatment, intraperitoneal and immersion vaccination trials were performed. The antigen-specific IgM production was monitored 30 days post-immunization and, interestingly, the intraperitoneal immunization with formalin-inactivated VERv produced a significant titer, at difference with other inactivation protocols. However, the same formalin-inactivated antigen did not elicited detectable IgM antibody when administered by immersion. Quantitative expression of antiviral Mx gene after intraperitoneal injection with formalin-inactivated virus showed a modulation of transcripts on gut after 48 hours and on head kidney after 24 hours, whereas ISG-12 gene was quantitatively expressed after 48 hours on gut and head kidney.

Finally, in challenge experiments using live VERv after intraperitoneal immunization with formalin-inactivated VERv was observed a significant increase (81.9 %) in the relative percentage survival with respect to un-immunized fish. Our result suggest that intraperitoneally vaccination with formalin-inactivated VERv could be a safe and effective strategy to fight Betanodavirus infection in European sea bass.

6.2 INTRODUCTION

Betanodavirus is considered one of the most significant viral pathogens of finfish, and a bottleneck for mariculture development in several countries (Crane and Hyatt, 2011;

Terlizzi et al., 2012). Betanodaviruses are naked RNA viruses characterized by an extremely high resistance to chemical and physical agents (Tofollo et al., 2007). Several experimental vaccines against VERv have been tested so far (Yuasa et al., 2002; Thiéry et al., 2006; Kai et al., 2014; Pakingking et al., 2009), however the majority of them have been tested on grouper (*Epinephelus* spp.), which is probably the most important and valuable fish amongst VER susceptible species (Gomez-Casado et al., 2011). An inactivated RGNNV vaccine against VER of sevenband grouper has been produced and marketed (in Japan only). Despite the huge losses that causes this disease to the Mediterranean aquaculture and in particular to the sea bass farms, very few vaccines have been tested in this species. Only few experimental products based on recombinant proteins or synthetic peptides have been tested injecting intramuscularly 20-66 g sea bass (Coeurdacier et al., 2003; Thiéry et al., 2006). The use of recombinant capsid protein gave interesting results but, at present, no commercially vaccines against Betanodavirus for sea bass are available.

The aims of the present work were therefore to investigate the best inactivation system for the production of inactivated vaccines that could be administered by immersion or intraperitoneally, test the efficacy of the best product to protect experimentally infected fish and evaluate the immune responses of vaccinated sea bass.

6.3 MATERIALS AND METHODS

Fish

Eight hundred European sea bass (400 with an average weight of 2.10±0.25 g and 400 with an average weight of 6.30±0.45 g) were transferred from a commercial farm, known to be VER-free by RT-PCR (data not shown), to the experimental aquarium. Fish were treated as previously described in Chapter 5: Materials and methods and kept on the same premises.

Virus and Vaccine preparation

The Betanodavirus isolate 283/I09–RGGNV (VERv 283) (GenBank accesion number JN189865 and JN189992, Panzarin et al., 2012) was prepared as explained in Materials and methods of Chapter 5.

The viral solution (initial titre $10^{7,80}$ $TCID_{50}$ mL^{-1}) was inactivated by three different protocols. Formalin inactivation was carried out adding buffered formalin 10% 1:100 at room temperature for one week. β-propiolactone (BPL) inactivation was performed by adding BPL 1:500 for 3 hours at 37 °C. Inactivation by temperature treatment was carried out by bath at 70 °C for 1 hour. All vaccine preparations were checked after treatment using virological analyses as explained in Materials and methods of Chapter 5. One batch of virus was kept untreated to be used for live virus immersion trial.

Fish vaccination, samplings and challenge

After a period of acclimation of 10 days, fish were vaccinated as follows. Fish (mean weight of 6.30±0.45 g), after sedation with 30 ppm tricaine methasulfonate (MS222), were vaccinated by intraperitoneal injection (IP) of 0.1 mL/fish of the three different vaccine preparations described above (one per tank); whereas smaller fish (mean weight of 2.10±0.25 g) were vaccinated by immersion for 2 minutes with the same vaccine formulations plus live virus (final titre $10^{7,80}$ $TCID_{50}$ mL^{-1}) by adding 30 mL of it into the water. Two tanks were mock-vaccinated by IP and immersion, respectively, using PBS 0.01 M to act as positive controls. One tank was left untreated, to act as negative control.

Thirty days post vaccination, blood and brain samples were collected from fish (n=10) from all vaccinated groups.

Basing on the results of immunological analyses (see below), was decided to study in depth the group intraperitoneally immunized with formalin-inactivated virus for which qPCR and challenge were performed. Single samples of muscle, head kidney and gut were collected at 0, 24 and 48 hours after administration for further investigations. After 30 days post vaccination (dpv), fish were challenged with live virus by intramuscular injection. After anesthesia, 50 fish were injected with 0.1 mL of virus solution (approximately $10^{6.80}$ $TCID_{50}$ $fish^{-1}$). After this period, the fish were transferred back to the original tanks and kept at a temperature of 25±1°C, oxygen 6±0.5 ppm and artificial photoperiod of 8 hours of light and 16 of darkness. The "negative control" group was mock injected with 0.1 ml of MEM-10 without virus. Fish were checked twice a day and the dead ones were removed. Samples of brain were collected individually from each dead fish and stored at -80 °C. The experiment was ended on day 30 and the

relative percent survival (RPS) calculated. All remaining fish were euthanized by an overdose of MS-222, sampled and stored at -80 °C until analyses.

Immunological analyses

The blood from fry sea bass was collected from the caudal vein of lethally anaestetized fish (10 mg/L of MS-222). Sera were obtained by centrifugation at 1000 g for 5 min.

VERv-specific IgM detection was performed using a previously developed Indirect ELISA (Scapigliati et al., 2010; in Materials and methods of Chapter 5). The ELISA assay data are presented as the mean absorbance ± SD. Each sample was measured in duplicate wells and optical density values (OD 450nm) of control wells were automatically subtracted from samples values.

Real-time quantitative PCR of formalin-inactivated VERv group consisted of three single samples of head kidney and gut at 0, 24 and 48 hours post immunization. Samples were immediately placed in 1 mL of denaturing solution (Tripure, Roche), total RNA was isolated following the manufacturer's instructions and successively suspended in DEPC-treated water. For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used following manufacturer's instructions. The absence of DNA contamination was checked using sea bass β-actin primers that bracketed an intron. The transcripts of Mx and ISG-12 genes were determined with an Mx3000P™ Real-Time PCR system (Stratagene) equipped with version 2.02 software, using the Brilliant SYBR Green Q-PCR Master Mix (Stratagene) and following the manufacturer's instructions. ROX was used as internal passive reference dye. Specific PCR primers (Table 1) were designed for the amplification of products (ca. 200 bp) from the conserved region of all analyzed genes and ribosomal 18S rRNA was used as house-keeping gene. A 10 ng of cDNA template was used in each PCR reaction. The PCR conditions were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. Reactions were performed in triplicate for each template cDNA, which was replaced with water in all blank control reactions. Each run was terminated with a melting curve analysis which resulted in a melting peak profile specific to the amplified target DNA. The analysis was carried out using the endpoint method option of the Mx3000P™ software that allows the fluorescence data at the end of each extension stage of amplification to be collected.

A relative quantification was performed comparing the levels of the target transcript to the reference transcript (18S rRNA) and the average of 3 muscle samples from control fish was used as calibrator and defined as 1.0 value. Quantitative assessment was based on determination of threshold cycle (Ct), defined as the cycle at which a statistically significant increase in fluorescence above the background signal was detected.

Gene	**Primer sequence**	**EMBL accession**
18S rRNA	FW: 5′-CCAACGAGCTGCTGACC-3′ RW: 5′-CCGTTACCCGTGGTCC-3′	AY831388
ß-actin	FW: 5′-ATGTACGTTGCCATCC-3′ RW: 5′-GAGATGCCACGCTCTC-3′	AJ493428
Mx	FW: 5′-GTCTGGAGATCGCCTCT-3′ RW: 5′-GTGGATCCTGATGGAGA-3′	AM228977
ISG12	FW: 5′-CCTGGTACAGCTGCTGT-3′ RW: 5′-AGCTGCTCCTGCTGACT-3′	FN665389

Table 1. Sequence of primers for qPCR

Serum neutralization tests were carried out doing Serial 2-fold dilutions (1:20 to 1:2560) of temperature inactivated serum in a 96 well plate (Corning) with MEM Leibovitz medium without FBS. Diluted sera were incubated with a defined amount of infectious virus (100 $TCID_{50}$/50uL), 4 wells were used for each sample. After incubation over-night at +4°C, the virus-serum mixture was added to confluent E-11 cell line and incubated for 10 days at 25°C. Plates were observed every 3 days for appearance of cytopathic effects (CPE). Neutralization value of a virus is defined as the reciprocal of the highest dilution of serum that completely inhibits CPE.

Virological analysis

Virological analysis were performed through virus isolation in cell culture and RT-PCR of Betanodavirus RNA2 as explain in Chapter 5: Materials and methods.

Statistical analysis

The number of fish per experimental group was calculated according to the expected mortality rate (35%) and 0.05 as α error (one-side) and power 80% (1-β=0.80).

Each fish in the study was followed over time and the event "death" was recorded and verified by Real Time RT-PCR.

The Kaplan–Meier method was used to estimate the survival function from lifetime data, which allowed to draw the survival curve for each group as a step curve and to measure the length of time the fish would survive the infection. In the graph, the y-axis plots indicates the cumulative probability of the surviving fish at each time (Van Belle et al., 2004). To compare the different survival curves, the non-parametric Wilcoxon–Breslow–Gehan test was used for equality of survivor functions. The mortality percentage at the end of the study and the relative binomial exact 95% confidence interval were calculated in each experimental group. One-sided tests with a p-value<0.05 were considered as significant. Statistical analyses for potency test were performed using the STATA 12.1 software.

Statistical analysis for qPCR for immunological parameters was performed by Graph Pad Prism 6.0 software statistical package. The data from different experiments have been presented as the mean ± SD of three single samples in the head kidney and gut in qPCR and ten single samples of sera in Indirect ELISA.

The statistical significance among groups for qPCR and Indirect ELISA was determined using One-way ANOVA followed by Bonferroni's multiple comparison test and the level for accepted statistical significance was $p<0.05$.

6.4 RESULTS

Vaccines safety

All three inactivation methods proved to completely inactivate Betanodavirus with no viral growth in cell culture after 3 blind passages. The vaccine were then suitable to be used for animal experiments. No mortality was observed in fish during and after vaccination procedures in all treated groups. Thirty days post vaccination, brains from vaccinated fish were collected and checked for VERv positivity by Real Time PCR and virological analysis. Positivity was found only in the live-virus bath vaccinated group (average ct 20.88±4.29), whereas all other group gave negative results.

Immunological analyses

Anti-VERv specific IgM was detected by Indirect ELISA in sera samples after immersion and intraperitoneal immunization with virus inactivated by different ways. In Fig. 1 it is shown the presence of anti-VERv specific antibodies in all the samples intraperitoneally injected. Fish immunized with formalin-inactivated VERv presented a high specific IgM titer (OD 450 nm of ca. 0.650). Fish immunized with BPL-inactivated VERv presented a high specific IgM titer too (OD 450 nm of ca. 0.500) but in comparison with a high standard deviation. Moreover, fish immunized with temperature-inactivated VERv (IP Heat) presented a high standard deviation, four of ten samples have produced a high level of specific IgM and the rest just low but detectable IgM (OD 450 nm of ca. 0.350).

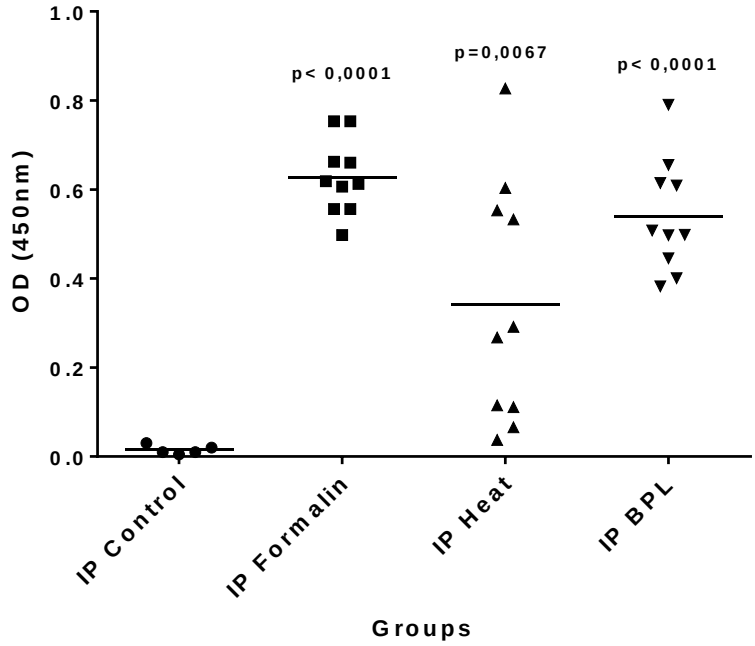

Figure 1. Detection of VERv specific IgM after intraperitoneal immunization with different viral preparations. Dilutions 1/60 of sera from different groups (n=10 fish by group) were tested by Indirect ELISA using as antigen 10^6 TCID50 per well of viral preparation and Pab IgM (diluted 1:10000). Background values (no viral preparation added) were automatically subtracted from OD 450 nm values of samples.

The results of immersed sea bass in different preparations are shown in Fig. 1. Sea bass immersed with live virus presented high quantity of specific IgM (OD 450 nm of ca.

0.450) in contrast to the formalin–inactivated VERv group which has a lower antibody titer. Temperature-inactivated VERv and BPL-inactivated VERv immunized groups both presented a higher standard deviation compared with formalin-inactivated one due to the uneven responses among examined samples.

As negative control, samples from mock-immunized fish (intraperitoneally and by immersion) showed no detectable IgM antibody, as expected.

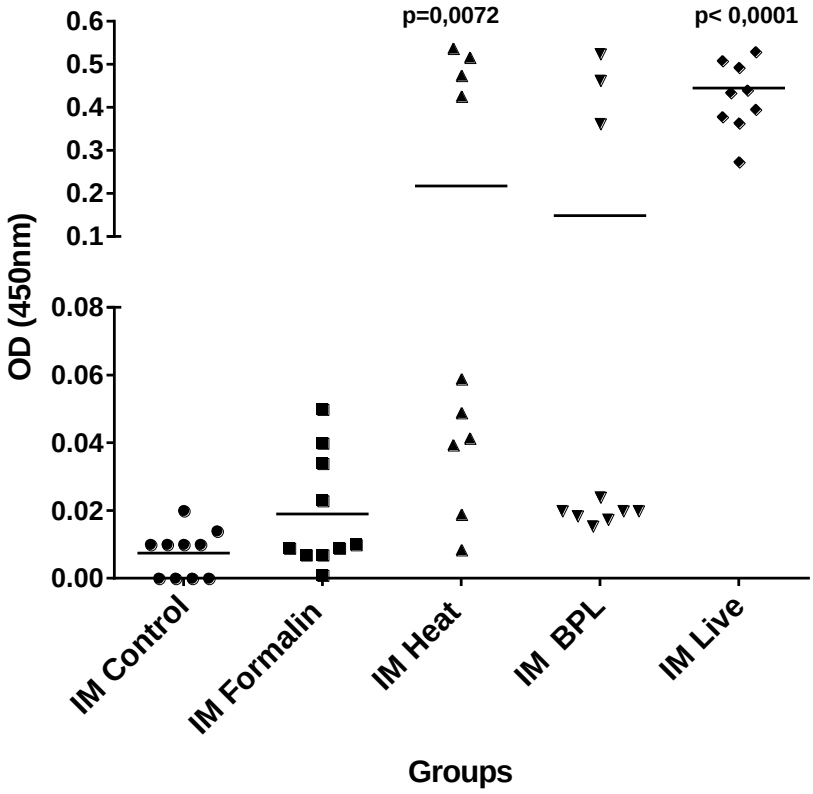

Figure 2. Detection of VERv specific IgM after immersion immunization with different viral preparations. Dilutions 1/60 of sera from different groups were tested by Indirect ELISA using as antigen 10^6 TCID50 per well of viral preparation and Pab IgM (diluted 1:10000). Background values (no viral preparation added) were automatically subtracted from OD 450 nm values of samples.

Serum neutralization test showed neutralizing antibodies against VERv 283 only in IP formalin group (average titre GMT 3.8±2.2), whereas all other groups showed negative results.

Real-time quantitative PCR after intraperitoneal vaccination with formalin-inactivated VERv, was performed to analyse the expression of antiviral immune response genes in

head kidney and gut. Transcripts levels of Mx and ISG12 genes are shown in Fig. 3 as a scatter plot of individual samples, with each dot representing single samples at 24 and 48 hours after injection, and using the average of 3 muscle samples of control fish as calibrator and comparing the expression with control group. Regarding the interferon-inducible Mx gene, a significant upregulation of Mx gene ($p<0.05$) has been observed at 48 hours in gut (Fig. 3a), whereas in head kidney a non significant mean increase tendency has been observed at 24 hours (Fig. 3c). A significant modulation of ISG12 gene ($p<0.05$) has been observed at 48 hours in gut (Fig. 3b) and head kidney (Fig. 3d).

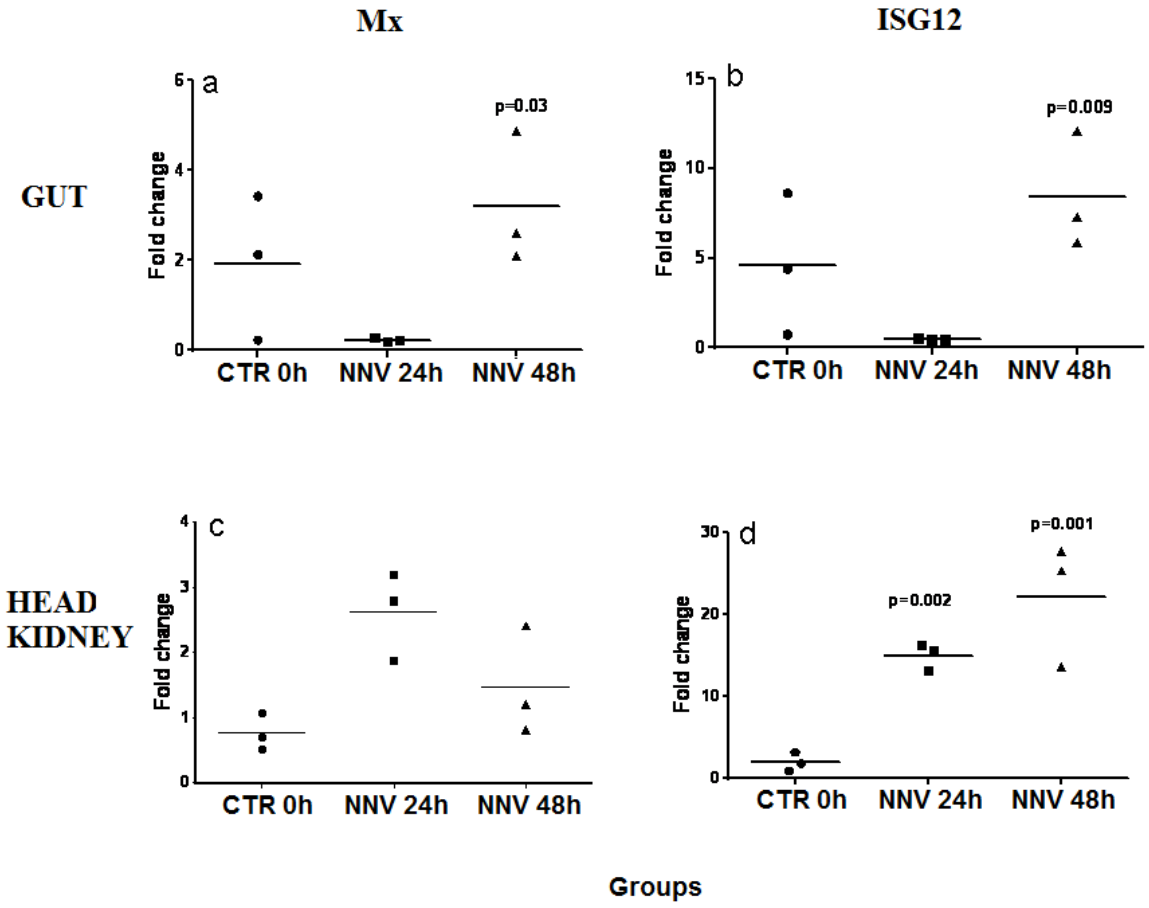

Figure 3. Quantitative PCR in gut and head kidney of intraperitoneally immunized sea bass. The quantitative expression of Mx (3 a-c) and ISG12 (3 b-d) genes (normalized respect to18S rRNA and using the average of 3 muscle samples of control fish as calibrator) are shown in control fish (CTR) and in intraperitoneally immunizated fish (NNV) at differents sampling points (24 and 48 hours post immunization). Results are expressed as the mean ± SD of three single samples. One-way ANOVA followed by Bonferroni's multiple comparison tests was used to determine differences from the

control at each time point. Data analysis was performed using the GraphPad Prism 6.0 software statistical package.

Vaccine potency

Challenge was performed only in the IP formalin-inactivated group as described above. Cumulative mortality in the IP formalin group was 12%, whereas in the positive control group was 64% (Fig. 4), with significant differences between groups ($p<0.001$). The RPS of the IP formalin group was 81.9 %.

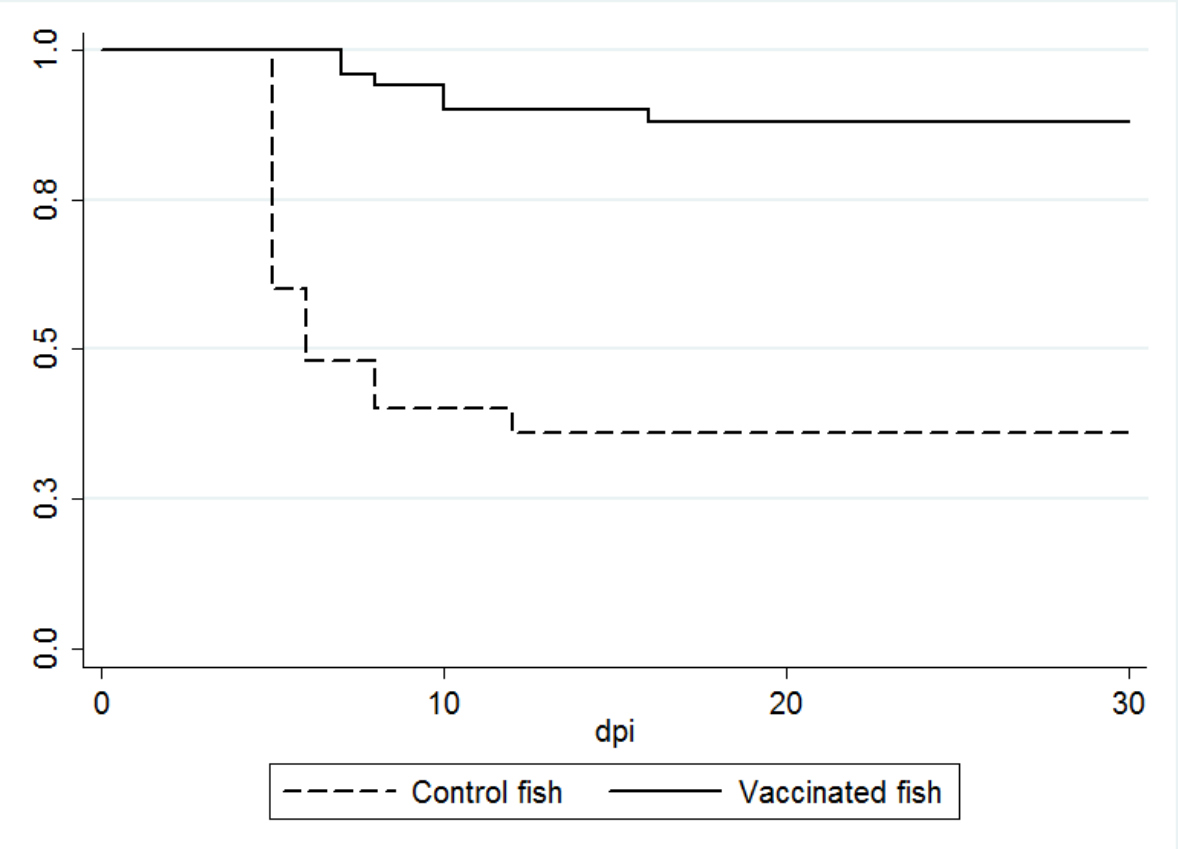

Figure 4. Kaplan-Meier survival stimates. Control fish (CTR); intraperitoneally immunizated fish with formalin-incativated virus (IP Formalin).

6.5 DISCUSSION

Betanodavirus infection is characterized by a high pathogenicity and is associated with extensive mortalities in fish farms (Chen et al., 2014). Different tentative vaccine formulations have been tested by inactivating the virus with formalin (Kai and Chi, 2008; Pakingking et al., 2009; Yamashita et al., 2005), binary ethylenimine (BEI) (Dos Santos et al., 2004; Kai and Chi, 2008), by the use of recombinant capsid proteins (Husgard et al., 2001; Lin et al., 2007; Sommerset et al., 2005), and by DNA-based

vaccines (Sommerset et al., 2005; Vimal et al., 2014a). However, to the present, no vaccines are available to be used in *D. labrax*, and with the aim of producing an effective antigen, in the present study different inactivation ways for Betanodavirus were tested, namely by using formylation, BPL and temperature.

The inactivated antigen was then administered to fish through systemic (intraperitoneal) and mucosal (immersion) routes and serological results showed that formylation was the best inactivation way for the production of specific and neutralizing serum IgM after IP immunization. On the other hand, fish vaccinated IP with BPL and temperature-inactivated virus also showed the presence of specific serum IgM, but the high variability between individuals resulted in a poor statistical significance of data. This latter observation could be explained by assuming a denaturation of antigenic sites during the inactivation process, and remembering the resistence of Betanodavirus to chemical and physical inactivation (Munday et al., 2002; Liltved et al., 2006; Kai and Chi, 2008). Indeed, several attempts to decrease the concentration or the duration of treatment with formalin, BPL and temperature were performed without success (data not shown). However, the proposed inactivation protocols and reported in this thesis were able to completely inactivate VERv.

The effectiveness of immunization with formalin-inactivated Betanodavirus administered intraperitoneally was previously tested in other fish species. A significant specific antibody titer was measured in groupers (*E. septemfasciatus*) together with protection after challenge with live virus (Yamashita et al., 2005; Kai and Chi, 2008). Also, a single injection with formalin-inactivated Betanodavirus (RGNNV genotype) induced a humoral response and protection in Asian sea bass (Pakingking et al., 2009). These data suggest that formylation can be a useful protocol for antigen preparation.

After having confirmed the presence of a humoral response induced by IP administration in sea bass, the effects on the transcription of genes coding for antiviral responses were examined through quantitative analysis of transcripts related to Mx and ISG-12 genes. After vaccination their transcripts were significantly upregulated in head kidney and gut, thus indicating an immunological effect of the formylated antigenic preparation. Based on promising serological and molecular results, the efficacy of the vaccination with formylated antigen given IP was tested by challenging fish with live virus (RGNNV 283.2009). The results showed a high protection rate (81.9 %) and, therefore, a single dose of IP-injected, formalin-inactivated RGNNV can be considered

sufficient to elicit an *in vivo* protection of sea bass. The potential of the formalin-inactivated viral preparation as a vaccine was confirmed by: i) a measurable humoral response; ii) a modulation in the expression of antiviral genes; iii) a seroconversion assay positivity; iv) a high protection after viral challenge.

The viral preparations were investigated immunizing smaller sea bass through mucosal routes, namely by immersion. The efficacy of immersion route in eliciting humoral and cell-mediated immune responses has been already described is other fish species, i.e. groupers. (Angelidis et al., 2006; Villumsen and Raida, 2013). Other interesting approaches have been explored by using inactivated VERv in immersion vaccination, as for example employing nano-encapsulated formalin-inactivated or BEI-inactivated Betanodavirus preparations for the protection of grouper larvae (*E. coioides*) from NNV disease (Kai and Chi, 2008).

By analyzing ELISA results of antigens administered by immersion low, but detectable, specific serum IgM in fish treated with formylated virus was observed, suggesting that a single immersion induced a weak humoral response in sea bass. Also for bath vaccinated groups with antigen treated with BPL- and temperature- inactivated virus a scattered presence of specific serum IgM was observed, resulting in a high individual variability and poor statistical significance of data, again attributable to a possible denaturation of the antigen.

It should be noted that infection with sub-optimal doses of live virus by immersion has an effect measured by the presence of significant antibody titers after 30 days (Fig. 1), and this could raise the possibility of vaccinating fish with a live/attenuated virus, as already shown in a grouper species (*E. Septemfasciatus*) (Oh et al., 2013; Nishizawa et al., 2012). However, there is a disagreement on the use of live pathogens for fish vaccination for problems of their containment, for their long term safety, and for the possibility of vertical/horizontal transmission (Thiéry et al., 2004; Chen et al., 2014). To confim these concerns the presence of VERv was observed by RT-PCR in all brain samples of fish infected by sub-optimal doses of live virus (data not shown). For these reason, live vaccines are not totally safe and therefore their use is not advisable.

In conclusion, it is possible to affirm that the formylated antigen preparation employed in this study is able to modulate serum and transcriptomic antiviral responses, and confer a protection against live pathogen. This, in turn, suggests that acquired and

memory responses play a role in the vaccination protocol developed, and these acquired responses to viral stimulation will be investigated in the future.

CHAPTER 7: General Discussion and Conclusions

7.1 Mucosal immunity

Gills are a mucosal immune tissue that contains a gill associated lymphoid tissue (GIALT) armed with a diverse repertoire of innate and adaptive immune cells and molecules that is involved in the protection of fish against pathogens. A subpopulation of 10% to 20% of GIALT cells are T cells as determined by counting with a monoclonal antibody against pan-T cell (mAb DLT15) (Scapigliati et al., 2000), but despite their obvious importance in mucosal defences, the knowledge on mucosal T cells from fish is limited. Hence, studies on immune cells from gills are of importance for a greater understanding of mucosal immune responses which, in turn, can be very important for the designing of effective mucosal vaccines.

By using updated technology and in order to improve molecular knowledge of T cells, a first strategy was the obtainment of a whole RNA transcriptome from gills by deep sequencing of RNA from an healthy sea bass. The transcriptome appears to be quite complete when compared to the *Gasterosteus aculeatus* (stickleback) genome. In fact, the 90.65% of all the proteins predicted from the stickleback genome found a significant match (tBLASTn e-value lower than 1×10^{-10}) within the sea bass transcriptome. The transcriptome has been analyzed to group of expressed genes in a family of immune-related and named "immunome", a term originated in the lab where my PhD work has been done (Randelli et al., 2009). The sea bass gills immunome describes some features of gill-associated T cells (Nuñez Ortiz et al., 2014), among which, for instance, are that the most abundant PFAM domains found were leucine-rich repeats (LRR), a motif present in Toll-like receptors (TLR), and immunoglobulin (Ig) domains that should very likely correspond to T cell receptors, co-receptors, and immunoglobulins, all of which have been already described to be present in gills (Purcell et al., 2006; Ribeiro et al., 2010; Sundaram et al., 2012; Salinas et al., 2011). Moreover, main identified putative expressed sequences in the gills immunome were related to main functions related to T cells including Th1/Th2/Th17/Treg cell subpopulations. The identified sequences were T cell receptors (TCRα, TCRβ, and TCRγ), CD coreceptors (CD3, CD4, CD6, CD8, CD28), associated protein-kinases Lck and Zap-70, key cytokines of T cell subpopulations of the adaptive immune response (IFN-γ, IL-2, IL-4, IL-10, IL-17, IL-21, IL-22) and master regulators for T regulatory (Treg) cells (Foxp3, T-bet and

GATA3). On the base of this observationits can be hypothesized that a whole and putatively active subset of T-cells is present within the gills, although further characterization of these cell types is required to determine their functional role and similarities with existing mammalian subsets.

Of importance, RAG gene sequences were not found in the gills and therefore it can be speculated that either the expression of genes of somatic recombination of Ig-domains is below the detection threshold in the gills or somatic recombination of T cell receptors does not occur in the gills.

Basic functional features of T cells include proliferation induced by lectins, and GIALT leukocytes gills were tested by proliferation with two lectins, ConA and PHA. The results showed that GIALT leukocytes were able to proliferate in response to both lectins, as measured by CFSE incorporation, with PHA resulting a better inducer of proliferation with respect to ConA after 48 hours. Importantly, it was demonstrated that proliferating gills leukocytes contained sea bass T cells using two antibodies, the mAb DLT15 likely specific for pan-T cells (Scapigliati et al., 2000) and mAb DLT22 specific for CD45-T cells (Marozzi et al., 2012). The mean percentages of cells recognized by both antibodies doubled after 48 h of *in vitro* stimulation by lectins. Another confirmation of proliferating T cell came from qPCR experiments of proliferated leukocytes, where significant increases of some marker genes for T cell-related activities (TRβ, TRγ, CD4, CD8, CD45, and IL-10 genes) were measured. Transcription was particularly high for TRβ, TRγ and IL-10 followed by CD8, CD45 and CD4, compared to control cells. To our knowledge, this is the first report on leukocyte and T cell proliferation from fish gills.

The mAb DLT15 was also able to recognize by IHC numerous T cells in the gills. T cells were located in the epithelium of the filaments but rare T lymphocytes were found in the epithelium of the branchial lamellae and a T cell-containing interbranchial lymphoid tissue, observed in Atlantic salmon (*Salmo salar*), was not found in sea bass.

Another feature associated with vertebrate mucosal immunity is the presence of secretory immunoglobulins, IgA (mammals, birds, and reptiles), IgX (amphibians), and IgT in teleost fish. Thus, investigations on functional immunology of fish mucosal tissues require reagents to detect and quantitate IgT-producing B cells. At present, antibodies recognizing the immunoglobulin IgT have only been developed to recognize trout IgT (Zhang et al., 2010; Olsen et al., 2011; von Gersdorff Jorgensen et al., 2011).

A new polyclonal rabbit antibody against sea bass IgT, called RAIgT, has been produced by immunizing with synthetic peptides (Picchietti et al., Abstract 2nd ISFSI Congress, Portland (USA), 2016), and following this in this thesis I reported on the characterization of IgT-expressing cells in tissues of sea bass. IgT+ B cells were detected in leukocytes preparations from different mucosal and non mucosal tissues by indirect immunofluorescence and flow cytometry. The highest number of immunoreactive cells were detected in leukocytes from head kidney, followed by spleen and GIALT leukocytes, their size (ca. 5-8 μm) and morphology confirmed by fluorescence microscopy was consistent with that of lymphocytes. The large proportion of positive cells in head kidney and spleen can be explained by considering that head kidney is a major hematopoietic organ and a site of production of B cell precursors in fish (Rombout and Joosten, 1998; Press and Evensen, 1999), whereas the spleen is a site of functional maturation of B cells playing roles in systemic antibody responses, as previously shown for IgT in rainbow trout spleen (Castro et al., 2013). Having obtained a putative good antiserum against sea bass IgT, a necessary step has been to demonstrate that RAIgT is specific for IgT class, and thus the following step was to immunopurify RAIgT-expressing cells. We took advantage from magneting sorting using magnetic beads (Dynabeads technology), from head kidney leukocytes were obtained two cell fractions: a RAIgT-positive and a RAIgT-negative fractions. The analysis of fractions by RT-PCR showed higher expression of IgT in RAIgT-positive cells and of IgM in RAIgT-negative, thus confirming the antibody specificity. Regarding secreted IgT, antibody titers were detected by Indirect ELISA in sera and intestinal mucus. Intestinal mucus samples presented higher amounts of secretd IgT as previously described in trout (Zhang et al., 2010), and was confirmed by Immunohistochemistry (Picchietti et al., Abstract 2nd ISFSI Congress, Portland (USA), 2016).

Two works have recently associated by gene expression analysis the presence of IgT with antiviral (IHNV) responses in rainbow trout (Ballesteros et al., 2013; Castro et al., 2013). Future studies will investigate in sea bass the involvement of either IgT-producing cells and presence of secreted IgT during VERv infection of sea bass by employing the RAIgT.

7.2 Immune responses of European sea bass against Betanodavirus

Viral encephalopathy and retinopathy disease caused by Betanodavirus (VERv) affects fish species worldwide. Betanodavirus is a highly pathogenic virus that infects during youth and adulthood and presents a high mortality rate (Munday et al., 2002; Chérif et al., 2009). There are differents genotypes of VERv, but of particular interest is the RGNNV genotype because it is the most widespread in the Mediterranean Sea and the most infectious for sea bass.

VERv can be detected by RT-PCR analysis of brain tissue employing amplification primers specific to RNA1 or RNA2 (Baud et al., 2015) and by immunohistochemistry. Also different ELISA assays have been developed to detect VERv in some fish species (Arimoto et al., 1992; Romestand and Bonami, 2003; Fenner et al., 2006; Shieh and Chi, 2005; Breuil et al., 2001). But, at present, ELISAs to detect and quantitate Betanodavirus in sea bass are not available. Then a capture-based ELISA system was developed to facilitate the diagnosis and detection of VERv in biological samples of adult or juveniles sea bass, and the system can be used as a tool when RT- PCR cannot be employed. Principal antibodies permitting the assay are the capture antibody pAb 283 against the isolate 283.2009 RGNNV (VERv 283), and the detection antibody mAb 4C3 (IgG2a) that recognizes peptides corresponding to residues 141–162 and 181–202 in the VERv capsid protein (Costa et al., 2007). A working dilution of PAb 283 (20 µg mL^{-1}) and sensitivity (2 µg mL^{-1}) were determined by Indirect ELISA and was probed that this antibody is able to detect by Western blot both viral proteins, protein A (polymerase) and capsid protein of a concentrated (by ultracentrifuge) viral preparation. The quality of the preparation was monitored by electron microscopy and resulted excellent.

The developed capture-based ELISA presented a sensitivity for the antigen in solution of 17 µg mL^{-1} (35 x 10^5 TCID50 per mL) and resulted reliable and useful to reveal the presence of VERv in brain homogenates of *in vivo* infected sea bass. The standard curve of the capture-based ELISA, obtained using different concentrations of inactivated VERv 283 preparation, allowed to quantify virus concentration in brain homogenates.

The capture-based ELISA system may detects other RGNNV strains, but viruses not belonging to the family Nodaviridae can not be detected. It can be hypothesized, even if further work is necessary to verify, that the ELISA system might be also reactive with other genotypes, because of the conservation of capsid protein residues 193–212

(Nishizawa et al., 1995) and for the similarities between SJNNV/TPNNV and BFNNV/RGNNV, sharing 76% and 81% homology in nucleotide and amino acid sequences, respectively (Munday et al., 2002). Thus, from this part of work it is possible to conclude that a good working system to detect VERv during sea bass infection, and associate the virus presence with immune responses has been obtained.

By considering the above reported high infectivity of VERv and the induced mortality in sea bass there is a great demand for effective vaccines to fight the pathology, especially during young stages where fish are prone to infection, and a part of experimental work has been devoted to investigate immune responses during immunization of sea bass against VERv.

The majority of experimental vaccines against VERv disease have been developed to be used on grouper (Epinephelus spp.) (Yuasa et al., 2002; Thiéry et al., 2006; Kai et al., 2014; Pakingking et al., 2009). Very few vaccines have been performed to be tested in sea bass, which were based on recombinant proteins or synthetic peptides injected intramuscularly (Coeurdacier et al. 2003; Thiéry et al. 2006) and so far no vaccines are available to be used in this species.

Betanodaviruses are extremely resistant to chemical and physical inactivation (Munday et al. 2002; Liltved et al. 2006; Kai and Chi, 2008). Promising results have been obtained inactivating Betanodavirus with formalin (Kai and Chi, 2008; Pakingking et al., 2009; Yamashita et al., 2005) and binary ethyleneimine (BEI) (Patent PCT/EP2004/001543, 2005; Kai and Chi, 2008). For example nano-encapsulated formalin-inactivated or BEI-inactivated betanodavirus preparations given by immersion immunization have been shown in protecting grouper larvae (*E. coioides*) from VNN disease (Kai and Chi, 2008). Immersion vaccination is an interesting and an useful method to immunize fry sea bass because is stress-free and it is known that Betanodavirus preferentially infects during young life stages (Munday et al., 2002, Chérif et al., 2009). Moreover, immersion immunization increases specific antibodies and antibody-secreting cells in the mucosal tissues, pointing to the potential to induce local or mucosal immunity (Esteban, 2012; dos Santos et al., 2001; Rombout et al., 2014). On the other hand, intraperitoneal immunization is in general very effective inducing systemic humoral responses and resulting in an optimal protection (Yamashita et al., 2005; Kai and Chi, 2008).

With the aim of producing an effective antigen and to find an effective route of administration, different protocols to inactivate Betanodavirus were tested, namely by formylation, BPL and temperature and at the same time, molecular and cellular immune responses of sea bass immunized through systemic (intraperitoneal) and mucosal (immersion) routes with these preparations were investigated.

The first approach consisted of immersion immunization of fry sea bass with formalin-inactivated Betanodavirus. This round of immunization without boosting resulted in a weak humoral response in sea bass, no or very few specific serum IgM was detected. Furthermore, no proliferation of CFSE labelled leukocytes from gills and spleen was detected by flow cytometry after incubation with VERv preparation and neither protection after challenge with virulent virus. Nevertheless, VERv uptake was detected by immunohistochemistry (IHC) in gills epithelium using two different antibodies that recognize VERv (Mab 4C3 and pAb 283) and also was obtained a modulation in transcriptional activity of antiviral genes Mx and ISG-12 in gills at 24 hours post immersion. These results, however, should be interpreted also by considering the detection limits of the ELISA assay on sera of very young fish, the high individual variability in immune responses, and the antigen formylation that might decrease the antigenicity. Different immersion times have been tested, but no significant differences were found.

Subsequently, all the inactivated preparations were used to immunize fish by immersion and intraperitoneally. Serological analyses of fish immunized by immersion and intraperitoneally with BPL- and temperature- inactivated Betanodavirus presented a scattered presence of specific serum IgM, resulting in a high individual variability and poor statistical significance of data in fish, suggesting a possible denaturation of the antigen. But only the protocols described here were able to completely inactivate VERv. Importantly, sea bass infected by immersion with a sub-optimal dose of virulent VERv showed significant IgM titers. Previously has been demonstrated the potentiality of a live vaccine administered by bath or intraperitoneally for sevenband grouper *Epinephelus septemfasciatus* (Oh et al., 2013; Nishizawa et al., 2012). However, it is not advisable the use of live pathogens because VERv was detected in brain samples from infected-fish (30 dpv) by RT-PCR analysis, Betanodavirus can be transmitted vertically and horizontally and the confinement of the vaccine cannot be guaranteed.

Fish intraperitoneally immunized with formalin-inactivated virus presented a high significant titer of specific serum IgM and also seroconversion was positive in this group. Supported by these encouraging results, the quantitative expression of Mx and ISG-12 genes was controlled and a significant upregulation of both antiviral genes was detected in head kidney and gut. In addition, the formylated antigen administered intraperitoneally provided protection after challenging with virulent VERv (RPS=81.9%). These results suggest that a single dose of IP injected formalin-inactivated VERv is sufficient to protect juvenile sea bass against VERv, inducing a high serological and molecular immune response. Further studies will be necessary to evaluate the length of the protection provided by IP injected formalin-inactivated VERv in sea bass, as well as the level of cross protection against different Betanodavirus genotypes, but at present, this is the first study evaluating the efficacy of different vaccines and dissecting immune response of sea bass immunized against VERv.

Overall, the work of this thesis produced important results to better understand molecular and cellular immune responses of a fish species, both in healthy conditions and during stimulation with a natural pathogen. The obtained results, published in journals and presented in meetings, will have importance for the knowledge in comparative immunology of Vertebrates, and may find possible application in veterinary fish immunology.

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Curriculum vitae

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EDUCATION

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Period of Research abroad: "Nasal vaccination in sea bass against *Vibrio* anguillarum and viral (Betanodavirus) infections".

University of Murcia, Spain. Advisor: Victor Mulero (vmulero@um.es).

M.S. **Master of Advanced Biotechnology** (2011-2012).

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Occupation: Lab Technician.

Main responsibilities and activities: Production of antimicrobial peptides, using insects as biofactories. In this case, I used the baculovirus expression vector technology for the accumulation of the protein of interest in lepidopteran larvae (*Trichoplusia ni*) and Sf21 cells. Antimicrobial activity assays *in vitro.* Antimicrobial activity assays *in vivo* using Zebrafish as model.

B.S. **Biology**, 2010.

University of Seville, Spain.

EXPERIENCE

- **NATURALIA XXII, American Garden of Seville, 2010.**

Type of Business of sector: Environmental Company

Occupation: Monitor environmental education, Scholarship.

Main responsibilities and activities: to help social groups and individuals gain a basic understanding of the environment and acquire awareness and sensitivity to the total environment and its allied problems.

- **AGUAS DEL HUESNA S.L, Utrera, Sevilla, March-June 2009**.

Type of Business of sector: Sewage Company

Occupation: Lab technician.

Main responsibilities and activities: Physicochemical analysis to sewage maintenance as free chlorine residual, nitrate, fluoride, pH, turbidity…Testing microbiological quality (by the measurement of indicator bacteria).

- **LABAQUA S.A., Seville, December-March 2009**

Type of Business of sector: Sewage Company

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Main responsibilities and activities: Physicochemical analysis water analysis: fluoride, nitrate, arsenic, lead and Aesthetic parameters.

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Professional training

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- Cell culture training, University of Malaga, Spain, 2012.

-Biomedical laboratory instrumental techniques, University of Malaga, Spain, 2012.

- Chemical and Biological safety in laboratories, University of Seville, Spain, 2008.

Linguistic skills

-Mother tongue: **Spanish**
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English: Advanced Listener, Intermediate Speaker, Advanced Reading and Writing. PET certificate University of Cambridge (Council of Europe Level B1) and B2 level course at Tuscia University.
Italian: Advanced Listener, Advanced Speaker, Advanced Reading and Writing.

Computing Skills

-Applications: Microsoft Office Suite, Internet Explorer and several e-mail packages.
-Operating Systems: Windows.
-Confident in use of various software packages: Graph pad Prism, Adobe photoshop, Win MDI2.9, FlowJo Vx 10.0.4, EXPO32 ADC Software.
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Technical skills

-Molecular biology: PCR, primer design, cloning, RNA/DNA extraction, purification of genetic material, Western blotting, Bradford and Coomasie blue.
-Cell culture/cell biology, leukocytes preparation and proliferation.
-Flow cytometry: Beckman Coulter Epics XL MCL and BD FACSCalibur.
-Animal handling.
-Monoclonal antibody production.
-Microbiological Techniques: Preparation and sterilization of culture medium, Isolation and culture of microorganisms…
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-Writing and presenting reports.
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-Made presentations to both academic and non-specialist audiences.

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PUBLICATIONS

- **ABSTRACTS**

G Scapigliati, E Randelli, S Picchietti, N Nuñez, V Stocchi, C Bernini, F Buonocore. "On the origin of lymphocytes: a review". Invertebrate Survival Journal 11: 54-65 (2014).

N. Nuñez Ortiz, V. Stocchi, E. Randelli, F. Buonocore, G. Scapigliati. "A polyclonal antiserum for sea bass (*Dicentrarchus labrax*) IgT". Invertebrate Survival Journal 12: 89-102 (2015).

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S. Picchietti, L. Guerra, N. Nuñez Ortiz, F. Buonocore, D. Cervia, G. Scapigliati. "Localization of IgT ex pressing cells in sea bass (*Dicentrarchus labrax*)". Invertebrate Survival Journal 12: 89-102 (2015).

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- **PEER REVIEWED ARTICLE**

Núñez Ortiz N., Gerdol M., Stocchi V., Marozzi C., Randelli E., Bernini C., Buonocore F., Papeschi C., Toffan A., Pallavicini A., Scapigliati G. T CELL TRANSCRIPTS AND T CELL ACTIVITIES IN THE GILLS OF THE TELEOST FISH SEA BASS (*DICENTRARCHUS LABRAX*). Dev Comp Immunol 47(2):309-18 (2014). doi:10.1016/j.dci.2014.07.015

Nuñez-Ortiz N., Stocchi V., Toffan A., Pascoli F., Sood N., Buonocore F., Picchietti S., Papeschi C., Thompson K.D., Scapigliati G. QUANTITATIVE IMMUNOENZIMATIC DETECTION OF VIRAL ENCEPHALOPATHY AND RETINOPATHY VIRUS (BETANODAVIRUS) IN SEA BASS *DICENTRARCHUS LABRAX*. Journal of fish diseases. Journal of fish diseases (2015).

Nuñez-Ortiz N., Stocchi V., Toffan A., Pascoli F., Buonocore F., Picchietti S., Scapigliati G. EFFICACY OF FORMALIN INACTIVATED VACCINE AGAINST VIRAL ENCEPHALOPATHY AND RETINOPATHY (VER) DISEASE IN EXPERIMENTALLY INFECTED EUROPEAN SEA BASS (*D. LABRAX*). (Submitted Vaccine 2016).

- **Work in progress:**

N. Nuñez-Ortiz, F. Buonocore, V. Stocchi, S. Picchietti, E. Randelli, A.Toffan, F. Pascoli, and G. Scapigliati. THE ENCEFALOPATHY AND RETINOPATHY VIRUS OF EUROPEAN SEA BASS *DICENTRARCHUS LABRAX:* STRATEGIES FOR ITS DETECTION AND JUVENILES IMMUNIZATION. European Association of Fish Pathologists (2016).

Nuñez-Ortiz N., Stocchi V., Toffan A., Pascoli F., Sood N., Buonocore F., Picchietti S., Papeschi C., Thompson K.D., Scapigliati G. MUCOSAL IMMUNIZATION OF SEA BASS (*DICENTRARCHUS LABRAX*) AGAINST VIRAL ENCEPHALOPATHY AND RETINOPATHY VIRUS (BETANODAVIRUS). (in preparation 2016).

CONFERENCES ATTENDED AND CONTRIBUTED PRESENTATIONS

2ND INTERNATIONAL CONFERENCE OF FISH & SHELLFISH IMMUNOLOGY, 2016, PORTLAND MAINE. Simona Picchietti*, Francesco Buonocore*, Noelia Nuñez Ortiz*, Valentina Stocchi*, Laura Guerra, Elisa Randelli, Giuseppe Scapigliati§. "IGT AND IGD FROM SEA BASS (*DICENTRARCHUS LABRAX*): LOCALIZATION OF EXPRESSING AND IMMUNOREACTIVE CELLS IN LYMPHOID TISSUES."

XVII Meeting IADCI-Italian Association of Developmental and Comparative Immunobiology (IADCI). Lecce, February 11-13, 2016. Nuñez-Ortiz N., Pascoli F., Toffan A., Buonocore F., Scapigliati G. “Strategies for detection and vaccination of juveniles european sea bass (*Dicentrarchus labrax*) against Betanodavirus”.

76° Congresso Nazionale dell'Unione Zoologica Italiana (UZI), Tuscia University. 15/09/2015 - 15/09/2015.

The 13th International Society of Developmental and Comparative Immunology (ISDCI). Murcia, Spain, 28 June to 3 July 2015. Poster presentation: Núñez Ortiz N., Stocchi V., Buonocore F., Gerdol M., Randelli E., Papeschi C., Toffan A., Pallavicini A., Scapigliati G. “T cell transcripts and T cell activities in the gills of the teleost fish sea bass (*Dicentrarchus labrax*)”.

Fish for research: indicazioni per una corretta scelta e utilizzo delle specie ittiche impiegate a fini sperimentali. Torino, April, 23 2015.
Stocchi V., Nuñez N., Buonocore F., Picchietti S., Toffan* A., Pascoli* F., Scapigliati G. “Utilizzo di Teleostei per studi immunologici legati alle biotecnologie delle produzioni e per la salute umana”.

XVI Meeting IADCI-Italian Association of Developmental and Comparative Immunobiology (IADCI). Trieste, February 18-20, 2015.

1. N. Nuñez Ortiz, V. Stocchi, E. Randelli, F. Buonocore, G. Scapigliati. "A polyclonal antiserum for sea bass (*Dicentrarchus labrax*) IgT". Oral presentation

2. V. Stocchi, N. Nuñez Ortiz, M. Gerdol, A. Pallavacini, E. Randelli, F. Buonocore. "Identification of teleost fish (IgD) and T (IgT) immunoglobulins in sea bass (*Dicentrarchus labrax*) from a gills trascriptome". Oral presentation

3. S. Picchietti, L. Guerra, N. Nuñez Ortiz, F. Buonocore, D. Cervia, G. Scapigliati. "Localization of IgT ex pressing cells in sea bass (*Dicentrarchus labrax*)".Oral presentation

4. F. Buonocore, N. Nuñez Ortiz, E. Randelli, V. Stocchi, A. Toffan, F. Pascoli, S. Picchietti, G. Scapigliati. "Immunization of sea bass (*Dicentrarchus labrax*) against Nodavirus". Oral presentation

9th International Symposium on Viruses of Lower Vertebrates- Malaga, October 1-4, 2014.

F. Buonocore, N. Nuñez, V. Stocchi, S. Picchietti, E. Randelli, C. Papeschi, A.Toffan°, F. Pascoli°, Niels Lorenzen*, K.D Thompson", and G. Scapigliati.

"The encefalopathy and retinopathy virus of european sea bass (*Dicentrarchus labrax*): strategies for its detection and juveniles immunization".

XV Meeting IADCI-Italian Association of Developmental and Comparative Immunobiology (IADCI). Ferrara, February 12-14, 2014.

G Scapigliati, E Randelli, S Picchietti, N Nuñez, V Stocchi, C Bernini, F Buonocore

"On the origin of lymphocytes: a review".

COLLABORATION IN CONFERENCE ORGANIZATION

76° Congresso Nazionale dell'Unione Zoologica Italiana, Tuscia University. 15/09/2015 - 15/09/2015.

XI Congreso Luso – Español de Herpetología / XV Congreso Español de Herpetología, University of Seville. 06/10/2010 - 09/10/2010.

PROFESSIONAL MEMBERSHIPS

SIICS- Società Italiana di Immunobiologia Comparata e dello Sviluppo.
ISDCI-International Society of Developmental and Comparative Immunology.

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T cell transcripts and T cell activities in the gills of the teleost fish sea bass (*Dicentrarchus labrax*)

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ABSTRACT

The gills of fish are a mucosal tissue that contains T cells involved in the recognition of non-self and pathogens, and in this work we describe some features of gill-associated T cells of European sea bass, a marine model species. A whole transcriptome was obtained by deep sequencing of RNA from unstimulated gills that has been analyzed for the presence of T cell-related transcripts. Of the putative expressed sequences identified in the transcriptome, around 30 were related to main functions related to T cells including Th1/Th2/Th17/Treg cell subpopulations, thus suggesting their possible presence in the branchial epithelium. The number of T cells in the gills of sea bass, measured with the specific T cell mAb DLT15 range from 10% to 20%, and IHC analysis shows their abundance and distribution in the epithelium. Leukocytes from gills are able to proliferate in the presence of lectins ConA and PHA, as measured by flow cytometry using CFSE fluorescence incorporation, and during proliferation the number of T cells counted by immunofluorescence increased. In lectin-proliferating cells the expression of T cell-related genes TRβ, TRγ, CD4, CD8α, CD45 and IL-10 increased dramatically. Our data represent a first analysis on T cell genes and on basic T cell activities of fish gills, and suggest the presence of functionally active subpopulations of T lymphocytes in this tissue.

1. Introduction

The gills of fish are, in terms of exposed surface, the biggest tissue of most Teleost species (e.g. 1 m²/kg in carp; Oikawa and Itazawa, 1985), they serve to maintain fish omeostasis by the uptake of nutrients and substances, and by forming an active barrier against the entry of pathogens. The thin gill epithelium is a mucosal tissue at direct contact with the water environment, and contains a gill-associated lymphoid tissue (GIALT) with macrophages/granulocytes (Barnett et al., 1996; Lin et al., 1998; Mulero et al., 2008), B cells (Davidson et al., 1997; Dos Santos et al., 2001; Salinas et al., 2011), T cells (Scapigliati et al., 1999), and with high expression levels of T cell-related genes (Boschi et al., 2011). The GIALT is a first-line of defense against the entry of pathogens from the microbe-rich water environment and thus must be armed with either a fast non-self recognition and elimination system, together with an antigen recognition/antigen memory asset.

For these features of innate and acquired immune defenses, the GIALT is regarded as a target tissue for mucosal vaccination and, effectively, vaccination of some fish species is achieved by brief immersion of fish in antigenic mixtures diluted in water, from which the antigen enters the animal through the gills and other mucosal tissues (Salinas et al., 2011).

Of particular importance are lymphocytes of mucosal tissues, where T cells may be present in percentages up to 60% in gut-associated lymphoid tissue (GALT) and of 25% in GIALT (Randelli et al., 2008) and, as in mammals, are considered to be involved in a first line of defense against pathogen entry. However, despite the obvious importance of GIALT in maintaining fish health, the knowledge on its cellular and molecular components is still meagre.

In order to extend our previous observations on T cells and T cell transcripts (Boschi et al., 2011; Pallavicini et al., 2010; Randelli et al., 2009), and to achieve a more comprehensive knowledge of GIALT asset and function in fish we employed the European sea bass (*Dicentrarchus labrax*) as a model to produce a whole gill RNA transcriptome and analyze it for the presence of genes coding for T lymphocyte-related peptides. Moreover, we investigated some basical cellular aspects typical of T cells like *in vitro* induction of

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Quantitative immunoenzymatic detection of viral encephalopathy and retinopathy virus (betanodavirus) in sea bass *Dicentrarchus labrax*

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Abstract

Viral encephalopathy and retinopathy disease caused by betanodavirus, genus of the family *Nodaviridae*, affects marine, wild and farmed species including sea bass, one of the most important farmed species in Europe. This work describes a reliable and sensitive indirect ELISA assay to detect betanodavirus in biological samples using a polyclonal antiserum (pAb 283) against the 283/I09 virus strain, the most common red-spotted grouper nervous necrosis virus (RGNNV) genotype in the Mediterranean area, and a capture-based ELISA using a monoclonal antibody (mAb 4C3) specific to a common epitope present on the capsid protein. Using adsorbed, purified VERv preparation, the detection limit of indirect ELISA was 2 μg mL^{-1} (3×10^5 TCID50 per mL), whereas for capture-based ELISA, the sensitivity for the antigen in solution was 17 μg mL^{-1} (35×10^5 TCID50 per mL). The capture-based ELISA was employed to detect VERv in brain homogenates of *in vivo* infected sea bass and resulted positive in 22 of 32 samples, some of these with a high viral load estimates (about 1.1×10^8 TCID50 per mL). The ELISA system we propose may be helpful in investigations where coupling of viral content in fish tissues with the presence of circulating VERv-specific IgM is required, or for use in samples where PCR is difficult to perform.

Keywords: betanodavirus, capture-based ELISA, *Dicentrarchus labrax*, indirect ELISA.

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Introduction

Betanodavirus is the aetiological agent of viral nervous necrosis (VNN) or viral encephalopathy and retinopathy disease (VER). The VER virus (VERv) belongs to the family *Nodaviridae* and its genome consists of two molecules of single-stranded RNA: RNA1 (3.1 kb) a single open reading frame translated into RNA-dependent RNA polymerase or protein A of about 982 amino acids (Mori *et al.* 1992), and RNA2 (1.4 kb) that encodes the viral coat protein of 338–340 amino acids. Moreover, a subgenomic RNA3 transcript (0.4 kb) of RNA1 is synthesized during RNA replication and encodes a molecule antagonizing host cell RNA interference mechanisms (Fenner *et al.* 2006; Chen, Su & Hong 2009).

Four different genotypes of betanodavirus have been identified according to the variability of the T4 region of RNA2: striped jack nervous necrosis

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