Stellingen:

1. The detection of cells immunoreactive with DLT15 (T cell marker) before the thymus of sea bass becomes lymphoid, indicates that these early T cells originate from a different compartment, as also demonstrated for higher vertebrates. *This thesis, chapter 4*

2. Although many authors suppose that body weight correlates better with the development of the immune system than age, in sea bass this appears to be the opposite.
   This thesis, chapter 4

3. After immersion vaccination of sea bass against a *Photobacterium damselae* ssp. *piscicida* bacterin the gill and probably skin but not head kidney or spleen are the major organs for the production of specific antibodies. This is a clear indication for the existence of a local immune system in fish.
   This thesis, chapter 7

4. Intramolecular-induced suppression plays an important role in the regulation of the antibody response of sea bass, which was demonstrated by the extremely low variability observed in the Ig L chain sequences after an DNP-KLH immunisation.
   This thesis, chapter 3

5. Dependent on the public acceptance of genetically modified plants, fish farmers can in principle produce their vaccine producing plants in their own backyard not far from now.

6. Many South European countries, including Portugal, can learn a lot about the lower level of hierarchy and bureaucracy in the Netherlands, resulting in better working conditions and faster decisions.

7. What really makes science grow is new ideas, including the false ones.

8. When the Zodiac canteen reflects the feeding habits of the Dutch people it's astonishing: "soep", "broodjes" and during the week for 5-10 years the same special daily offer ("croquet", "gehaktbal", "uitsmijter", hamburger or loempia from Monday to Friday, respectively).

9. The Dutch way of working is making appointments for everything, which can be highly desirable and efficient for a good organisation. However, when applied in private social life and relations it is all too predictable and does not give much fun.

Stellingen behorende bij het proefschrift
"Development of immunity in sea bass:
a study towards vaccination against pseudotuberculosis"
van N.M.S. dos Santos, Wageningen, 25 oktober 2000

DEVELOPMENT OF IMMUNITY IN SEA BASS: A STUDY TOWARDS VACCINATION AGAINST PSEUDOTUBERCULOSIS

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DEVELOPMENT OF IMMUNITY IN SEA BASS: A STUDY TOWARDS VACCINATION AGAINST PSEUDOTUBERCULOSIS

Nuno Miguel Simões dos Santos

Proefschrift

ter verkrijging van de graad van doctor, op gezag van de rector magnificus van Wageningen Universiteit, dr. ir. L. Speelman, in het openbaar te verdedigen op woensdag 25 oktober 2000 des namiddags te vier uur in de Aula.

Abstract

Nuno M. S. dos Santos (2000). Development of immunity in sea bass: a study towards vaccination against pseudotuberculosis. PhD thesis, Cell Biology and Immunology Group, Wageningen Institute of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

Pseudotuberculosis, caused by the halophilic gram-negative bacterium *Photobacterium damselae* ssp. *piscicida*, has been causing high mortality losses in sea bass (*Dicentrarchus labrax* L.) farming. In the present thesis, basic and applied studies were performed concerning the humoral immune system of sea bass, as part of the development of an effective vaccine for this pathogen and with the aim to determine the earliest age/weight at which sea bass can be vaccinated against that disease. Therefore, monoclonal antibodies (mAb) against the heavy (±78 kDa; WDI 1) and light (±28 kDa; WDI 2 and WDI 3) chains were successfully produced and used. The Ig characterisation has shown that non-uniformly disulphide cross-linking of monomeric (M_r ±210 kDa) and dimeric (M_r ±420 kDa) subunits occurs in the polymerisation of the tetrameric form (M_r ±840 kDa). The sequence of the Ig L chain allowed all characteristic segments (V, J and C) to be deduced, and could be considered as a L1/G isotype. In addition, it was shown to have a cluster-like genomic organisation. Due to its importance for vaccination with T dependent antigens, the ontogenetic development of sea bass T cell was studied using a mAb (DLT 15) specific for that lymphocyte subpopulation. It was shown that early T cells may originate in a different compartment, further differentiate in the thymus and subsequently migrate to peripheral lymphoid organs. Adult proportions were reached around 137 days post-hatch (dph). Studies with different combinations of the mAb against sea bass Ig did strongly suggest the existence of B cell heterogeneity, expressing at least two heavy and two light chains. The percentage of B cells in the different immunological organs was studied using all the mAb. The combination of WDI 1 and WDI 3 appeared to label all or almost all B cells. As in other teleost species, the sea bass head kidney appears to be the primary organ for B-lymphocyte differentiation. The proportion of sIg^+ (B) cells in the different organs increased during the ontogenetic development being higher in lymphocyte populations of the head kidney followed by spleen, PBL, gut and thymus. This sequential order changed during ontogeny until the adult distribution is reached (about 137-145 dph). Additionally, sea bass cytoplasmic Ig^+ (cIg^+) cells (preB cells) were detected earlier in ontogeny than sIg^+ cells. Cytoplasmic Ig^+ plasma cells appear later in ontogeny (87 dph) and might be indicative of immunological maturation, regarding the onset of adaptive humoral immune responses. In the present thesis it was clearly demonstrated that sea bass is able to mount a specific antibody response to different antigens: *Vibrio anguillarum*, *P. damselae* ssp. *piscicida* and DNP. The kinetics of primary and secondary responses were studied in both intraperitoneally (ip) and direct immersion (di) vaccinated fish. While the head kidney was the main ASC-producing organ after ip immunisation, in the di immunised fish it was shown that the gills (and probably the skin) are likely to play a major role in the protection against natural challenges. In addition, a clear age effect was observed in the ASC responses of immersion vaccinated fish, with significantly higher numbers of ASC and faster ASC responses in the oldest groups. More importantly, there was no evidence that direct immersion exposure to *P. damselae* ssp. *piscicida* at the earliest stages used in the present study (0.1g) is tolerogenic. The kinetics and specificity of the invasion ability of *P. damselae* ssp. *piscicida* was studied and supported the results from others suggesting an intracellular phase to the life cycle of the bacterium. The fact that heat-inactivated antiserum obtained from sea bass immunised with a *P. damselae* ssp. *piscicida* bacterin reduced the invasiveness of the bacteria, strongly suggests that the antiserum contained antibodies against factors responsible for the adhesion and invasion of the pathogen. Intramolecular-induced suppression appears to play an important role in the regulation of the antibody response in sea bass, as suggested by the extremely low variability observed in the Ig L chain sequences obtained from DNP_{494}-KLH immunised sea bass. Since intermolecular-induced suppression has been shown in other teleosts, it raises the question about how these mechanisms might influence the specificity of immune responses against vaccine antigens and induction of protection. In conclusion, it may be stated that the bacterin used in this thesis contains immunogenic determinants, which may be important for conferring protection, and that sea bass are immunocompetent enough for vaccination against *P. damselae* ssp. *piscicida* from at least 137-145 dph (~1g), regardless of the route of administration, or from at least 80-100 dph (~0.1g) if immersion vaccination is preferred.

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Aos meus pais
(To my parents)

"Work you have slaved over, work you have tried to put your character and feelings into, can give pleasure and sell." - Vincent van Gogh

In I. F. Walther and R. Metzeger (1997). Vincent van Gogh. The complete paintings. Taschen, Köln, pp. 92.

DEVELOPMENT OF IMMUNITY IN SEA BASS: A STUDY TOWARDS VACCINATION AGAINST PSEUDOTUBERCULOSIS

CONTENTS

CHAPTER 1

General introduction

"You know, this applied science is just as interesting as pure science, and whats more its a damn sight more difficult." - William Bate Hardy

In Mackay, A.L. (1991). A dictionary of scientific quotations. Adam Hilger, Bristol, 197 pp.

1. THE ADAPTIVE HUMORAL IMMUNE SYSTEM

1.1. OVERVIEW ON THE HOST DEFENCE SYSTEM

Water environments contain an enormous variety of infectious organisms (viruses, bacteria, fungi and parasites), that might cause fatal diseases. It is also an extremely easy environment for disease transmission, especially in intensive aquaculture conditions. To overcome this situation, fish, like other vertebrates, possess wide range defensive mechanisms. Physical (scales, skin, mucous) and biochemical (lysozyme) barriers prevent most of the infectious agents from entering the host. However, when the host homeostasis is unbalanced by a variety of stress factors, infection might occur. Immune defence mechanisms are then triggered, shortening the infectious period and avoiding the death of fish. These defence mechanisms involve the recognition of the foreign infectious agents and their elimination. Generally, immune responses fall into two major systems: innate (or non-combinatorial) and adaptive (or combinatorial) immune systems. The adaptive immune response is characterised by higher specificity and memory formation. Both innate and adaptive immune systems comprise cells (leukocytes) and humoral factors responsible for a successful immune response. Thus, while phagocytic cells (macrophages/monocytes), granulocytes (neutrophils, eosinophils and basophils) and non-specific cytotoxic cells (NCC) are characteristic of the innate immune system, lymphocytes (T lymphocytes and B lymphocytes) are essential to all adaptive immune responses. Lysozyme, complement, interferons (α, β and γ), C-reactive protein, transferrin, lectins, hemolysin, proteinase, α2-macroglobulin, chitinase and α-precipitin are characteristic molecules of the humoral innate immune responses, while immunoglobulin (antibody molecule) can be considered as the ultimate effector molecule of an adaptive humoral immune response. In practice, both systems interact considerably, mostly by the release of soluble factors (cytokines and inflammatory mediators) involved in signalling between cells. Antigen presentation by phagocytes and easier recognition of antibody opsonised antigens are other examples of interaction between both systems. Most immune responses comprise a variety of components from both systems with predomination of the innate immune system in the earliest stages of infection and emphasis of the adaptive immune system later.

Because of its importance for vaccination the adaptive immune system will be further described in the following sections: T, B and antigen presenting cells, Ig molecule, humoral immunity and vaccination, with special emphasis for the humoral immune components.

1.2. CELL TYPES INVOLVED IN THE ADAPTIVE IMMUNE RESPONSE

All vertebrate immune responses are mediated by leukocytes (Miller, 1998). As in mammals, fish leukocytes can be classified as T and B lymphocytes, monocytes/macrophages and granulocytes (Miller, 1998; Miller *et al.*, 1998; Partula, 1999). Lymphocytes are the cell type essential to the adaptive immune response because they express the heterodimeric immunoglobulin (Ig) or T-cell receptor (TCR) molecules as antigen-specific-recognition units. It is apparent that the adaptive immune response is present in all extant jawed vertebrates, but is lacking in cyclostomes, lower chordates and protostomes (Marchalonis *et al.*, 1998a,b). Two types of adaptive immune responses can be distinguished: the cell-mediated immune response, mediated by thymus-derived cells and the humoral immune response, involving the production of antibodies by plasma cells (Partula, 1999).

1.2.1. T lymphocytes

T lymphocytes are characterised by the presence of a specific antigen receptor called TCR, which forms the ligand-binding subunit of the TCR-CD3 complex (Partula, 1999). In mammals, two types (γδ and αβ) of TCR are present. Although, TCR-α and –β chain gene sequences have recently been described in teleosts (Partula *et al.*, 1995, 1996a; Hordvik *et al.*, 1996; Zhou *et al.*, 1997; Wilson *et al.*, 1998; Wermenstam *et al.*, 1998; Scapigliati *et al.*, 2000) their expression on the cell surface has not yet been demonstrated. Additionally, the teleost equivalents to the mammalian CD3 (δ, ε, γ) and zeta (ξ) chains (signal-transducing subunit) as well as the major histocompatibility complex (MHC)-binding CD4 (T helper cells) and CD8 (T cytotoxic cells) molecules remain to be investigated. However, the isolation of TCRγδ-like chains in cartilaginous fish (Rast & Litman, 1994; Rast *et al.*, 1995, 1997) as well as CD3 ε-like polypeptide in a chondrostean fish (Alabyev *et al.*, 1998) suggests the existence of TCRγδ-like receptors and CD3-like molecules in all gnathostomes. Unfortunately, only a few monoclonal antibodies (mAb) specific to fish T cells have been obtained yet (Scapigliati *et al.*, 1995; Passer *et al.*, 1996; Rombout *et al.*, 1997, 1998). Apparently, their production is difficult and in most cases mAb against thymocytes cross-react with other cell types. Therefore, division of T cells by their effector function, into T cytotoxic (Tc) and T helper (Th) subpopulations, has only been suggested in functional studies such as mixed leukocyte reactions (MLR; Cuchens & Clem, 1977; Etlinger *et al.*, 1977; Ellis, 1977; Caspi & Avtalion, 1984; Miller *et al.*, 1986; Kaastrup *et al.*, 1988; Luft *et al.*, 1994) and graft rejection (Botham *et al.*, 1980, Botham & Manning 1981) studies and by hapten-carrier assays (Stolen &

Makela, 1976; Miller *et al.*, 1985) and macrophage activating factor (MAF; Graham & Secombes, 1988) production, respectively. More recently, the co-injection of DNAs expressing luciferase and the viral haemorrhagic septicaemia virus glycoprotein was reported as suggesting the presence of cytotoxic T-lymphocyte mediated immune responses in DNA-vaccinated trout (Heppell *et al.*, 1998). In addition, functional studies with catfish T-cell cultures indicated an apparent dichotomy in cytokine production (Wilson *et al.*, 1998), which was suggested by Miller *et al.* (1998) to infer that catfish might have T cell differentiation similar to mammalian Th1 and Th2. In this context, it is suggested that fish T cells have an amplifying or regulatory role similar to that in higher vertebrates (Manning & Nakanishi, 1996), playing a central role in immune responses to protein (T dependent) antigens by elaborating requisite interleukins (Yang *et al.*, 1989) for B cell differentiation (Caspi & Avtalion, 1984; Grondel & Harmsen, 1984), after antigen processing and presentation by accessory cells (Vallejo *et al.*, 1992a,b, 1993; Secombes & Fletcher, 1992) that bear self-MHC molecules (Vallejo *et al.*, 1991a,b). In sea bass, a mAb (DLT 15) to sea bass thymocytes and peripheral T-cells (Scapigliati *et al.*, 1995) has been used in studies that strongly suggested its specificity (Abelli *et al.*, 1996; Picchietti *et al.*, 1997; Romano *et al.*, 1997a; Scapigliati *et al.*, 2000). Flow cytometric analysis of leukocytes fractions using DLT 15 were performed by Romano *et al.* (1997a) and revealed a large number of positive cells in the thymus (~80%) and intestine (~55%) and fewer cells in the spleen (~7%), head kidney (~6%) and PBL (~3%). Moreover, these authors could differentiate two main morphologies of DLT 15^{+}-cells, named type a (with a large round heterochromatic nucleus and light sparse cytoplasm) and type b (more differentiated lymphocytes with an irregular and heterochromatic nucleus and cytoplasm rich in polysomes and mitochondria). In carp, Rombout et al. (1998) described the presence of a special population of putative T cells in mucosal tissues (gut, gills and skin) using mAb WCL38, and because a subpopulation of these cells can be considered as large granular lymphocytes, they are supposed to be analogues of mammalian γδ T cells.

Due to the present lack of specific fish T cell markers (see above) only a limited knowledge is available on the ontogeny of T cells (Abelli *et al.*, 1996; Picchietti *et al.*, 1997; Romano *et al.*, 1999). Thus, studies with early T cell markers have shown that the thymus is the major site for T cell differentiation (Romano *et al.*, 1997b; Hansen & Zapata, 1998; Ellis, 1998). After thymic selection and differentiation, T lymphocytes appear to migrate to peripheral lymphoid organs (Secombes *et al.*, 1983; Tatner, 1985; Jósefsson & Tatner, 1993; Abelli *et al.*, 1996; Ellis, 1998).

1.2.2. B lymphocytes

As in "higher" vertebrates, fish B cells express Ig on their surface, where it acts as specific antigen receptor (Vallejo *et al.*, 1992a; Pilstrom & Bengtén, 1996; Miller, 1998; Ross *et al.*, 1998). Ultrastructural studies showed that surface Ig occurs mainly in clusters on the B cell membrane of fish (van Diepen *et al.*, 1991; Navarro *et al.*, 1993; van der Heijden *et al.*, 1995). Furthermore, it is presently shown that fish B cells have Ig heavy (H)-chain rearrangement and allelic exclusion like those of mammals (Miller *et al.*, 1994). Most B cells express MHC class II molecules (Rodrigues *et al.*, 1995), which are important for cooperative interactions (antigen presentation) with T cells. Some of the studies using mAb against Ig of different fish species (Miller, 1998) suggested B cell heterogeneity. Moreover, it was shown that the adult proportion of surface Ig positive (sIg^+) cells is higher in lymphocyte populations of the peripheral blood leukocytes (PBL) followed by spleen, head kidney and gut, being almost insignificant in the thymus. B cells can be induced by polysaccharides (T independent antigens as defined in mammals; DeLuca *et al.*, 1983; Sizemore *et al.*, 1984; Miller *et al.*, 1985; Miller, 1998) to differentiate into antibody-secreting cells (ASC) with the auxiliary assistance of an interleukin-1 (IL-1) producing accessory cell such as the monocyte or macrophage (Miller *et al.*, 1985, 1987; Clem *et al.*, 1985; Vallejo *et al.*, 1990, 1992a; Kaattari, 1992).

During ontogeny, the head kidney appears to be the primary organ for fish B lymphocyte development (Ellis, 1977; Botham & Manning, 1981; Koumans-van Diepen *et al.*, 1994; Razquin *et al.*, 1990; Chantanachookhin *et al.*, 1991; Jósefsson & Tatner, 1993; Abeli *et al.*, 1996; Romano *et al.*, 1997b; Breuil *et al.*, 1997). The proportion of sIg^+ (B) cells in the different organs increases during ontogenetic development being higher in lymphocyte populations of the head kidney followed by spleen, PBL, gut and thymus, changing this sequential order during ontogeny until the adult distribution is reached (Razquin *et al.*, 1990; Koumans-van Diepen *et al.*, 1994). At least in trout (Castillo *et al.*, 1993), cytoplasmic Ig^+ (cIg^+) cells (preB cells) were detected earlier in ontogeny than sIg^+ cells, which is in agreement with the results reported for amphibians (Zettergren, 1982; Hadji-Azimi *et al.*, 1982) and mammals (Landreth & Kinkade, 1984; van Rees *et al.*, 1990; Francés *et al.*, 1994). Cytoplasmic Ig^+ plasma cells appear later in ontogeny (Koumans-van Diepen *et al.*, 1994) and might be indicative for the onset of adaptive humoral immune responses.

1.2.3. Antigen-presenting cells (APC)

As in mammals, fish monocytes/macrophages and B cells appear as the main APC (Vallejo

et al., 1992a), which also play an important role in adaptive immune responses. These APC are rich in class II MHC molecules (Vallejo *et al.*, 1992a; Rodrigues *et al.*, 1995), which are important for presenting antigenic peptides derived from exogenously acquired proteins to Th cells and thus initiate adaptive immune responses (Lanzavecchia, 1990; Vallejo *et al.*, 1992a; Secombes & Fletcher, 1992). Recently, gene sequences from MHC class I and II have been achieved in several fish species (Dixon *et al.*, 1995; Stet *et al.*, 1996). Their structure and polymorphism suggests that fish MHC molecules function in a way similar to those in mammals (Manning & Nakanishi, 1996). In the case of MHC II internalised antigen, it is initially localised in the endosomes/lysosomes of the APC followed by degradation and redistribution to the plasma membrane (Vallejo *et al.*, 1993) for T cell presentation.

1.3. TELEOST IMMUNOGLOBULINS (Ig)

The antibody molecule is responsible for many effector mechanisms in the specific defence against pathogens. It appears unequivocally in all jawed/gnathostome vertebrates and belongs to a type of proteins biochemically defined as immunoglobulins due to their characteristic domains in three-dimensional structure (Marchalonis *et al.*, 1998b; Pilström *et al.*, 1998). A general tendency of the Ig superfamily loci is that they duplicate themselves, as can be seen in the manifold variable segments of Ig and TCR as well as in the different isotypes (constant segments) of Ig (Pilström *et al.*, 1998).

Serum Ig concentration in fish ranges from very low amounts in Salmonids (around 1mg/ml, which corresponds to 2-6% of the total serum protein) to relatively high amounts in holostean fishes (14-17 mg/ml, which corresponds to 40-50% of the total serum protein). Lower concentrations are found in mucus, bile and in the eggs (Pilström, 1998).

1.3.1. Molecular and Biochemical Characterisation

In fish, there are few classes of Ig with the main type corresponding to the mammalian IgM, which is the first to appear in phylogeny, ontogeny and as Ab in an immune response (Pilström & Bengtén, 1996; Pilström, 1998). The main structure of the antibody (Ab) molecule appears in teleosts as a tetramer (Fig. 1) with a relative molecular mass (M_r) of 600 to 900 kDa (Wilson & Warr, 1992; Pilström, 1998), with up to 16 % of carbohydrate content (Acton *et al.*, 1972; Pilström, 1998) and with higher L-fructose content and galactose:mannose ratio than in mammals (Pilström, 1998). It is composed of four covalently or noncovalently associated Y shaped monomeric units leading to eight binding sites. The tetrameric structure of teleost IgM appears to be caused by the number of amino acids

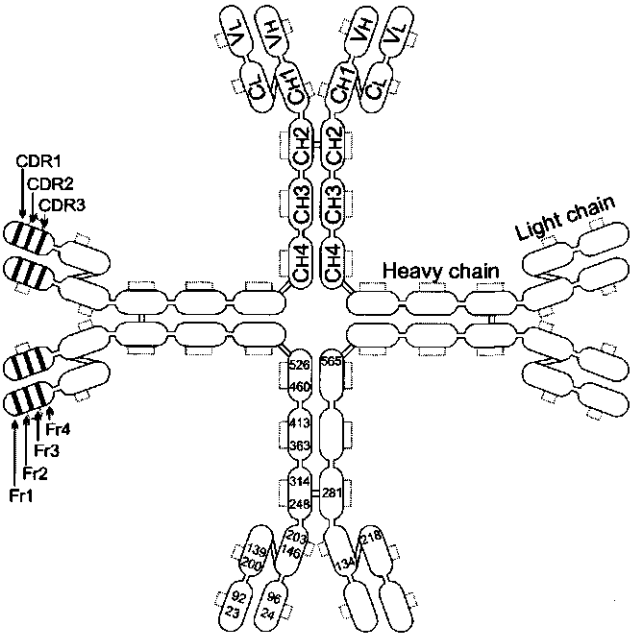

Fig. 1- Diagrammatic representation of the secreted form of the tetrameric IgM of teleosts, showing the potential intradomain (dashed brackets) and interchain (solid lines) disulphide bonds. The numbers represent the positions of available cysteines based on data for salmonids. The illustrated disulphide bonding pattern covalently linking is only one of the probable patterns (see text). Adapted from Wilson & Warr (1992) and Kaattari *et al.* (1998).

downstream from the cysteine residue that forms the disulphide bridges of the polymer, because there may not be room enough for more than one amino acid if a pentameric molecule has to be formed (Pilström & Bengtén, 1996). Thus, a non-planar tetrahedron structure (Shelton & Smith, 1970), with the carboxy amino acids protruding out from the disulphide ring structure and binding sites at all possible directions would be efficient in antigen binding (Pilström & Bengtén, 1996). In additon, the flexibility of the arms is ensured by the conserved proline residues at position 621 (Pilström & Bengtén, 1996). In contrast with mammals where intersubunit disulphide cross-linking appears to be employed in a uniform manner (Davis *et al.*, 1989; Wiersma & Shulman, 1995), teleosts generate considerable structural diversity, by non-uniformly cross-linking subunits ("halfmers", monomers, dimers and trimers) within the tetrameric Ab (Lobb & Clem, 1981a, 1983; Warr, 1983; Sánchez & Dominguez, 1991; Rombout *et al.*, 1993a; Navarro *et al.*, 1993; van der Heijden *et al.*, 1995; Kaattari *et al.*, 1998). Rather than employing distinctly different constant heavy genes to encode for structurally different isotypic forms, the teleost simply relies on post-translational processes which permit great latitude in the formation of intersubunit disulphide bonds (Kaattari *et al.*, 1998).

Although low molecular weight Igs, related or not with the tetrameric IgM form, have been reported in teleosts (Acton *et al.*, 1971; Clem, 1971; Clem & McClean, 1975; Lobb & Clem,

1981a,b; Warr, 1983; Elcombe *et al.*, 1985), any structural or functional equivalency to mammalian IgG has not yet been proven (Wilson & Warr, 1992). Nevertheless, under physiological conditions, a dimeric form of Ig distinct from the plasma Ig, was described in the mucus and bile of sheepshead (Lobb & Clem, 1981b,c,d) . Recently, a novel chimeric Ig heavy chain sharing similarities to IgD was reported in channel catfish (Wilson *et al.*, 1997) and Atlantic salmon (Hordvik *et al.*, 1999).

Each monomeric unit contains two H-light (L) polypeptide chain pairs linked together by covalent and non-covalent forces and can be functionally divided in two (amino terminus) antigen-binding regions (Fab) from each H-L chain pair and one (carboxy-terminal) effector region (Fc). However, the H (M_r ~72 kDa) and L (M_r ~27 kDa) chain in each Ab molecule are respectively identical to another, yielding antigen binding sites with identical specificity and affinity in the same Ab molecule (Wilson & Warr, 1992; Kaattari & Piganelli, 1996). The L chain is bound to the H chain by a cysteine bridge between its constant domain and the first constant domain of the H chain (Pilström *et al.*, 1998).

Both H and L chains can be divided into structural subunits (domains) by their conformational similarities and sequence homology (Kaattari & Piganelli, 1996). Thus, the H chain is characterised by three, four or five domains (one N-terminal variable-heavy: V_H and two to four constant –heavy: C_H) and a carboxy-terminal stretch of amino acids, whereas the L chain is characterised by two domains (one N-terminal variable-light: V_L and one constant – light: C_L). Within the V_H and V_L domains resides the antigen-binding site. The difference between the membrane and secreted form of the teleost Ig (Fig. 2) is given by the fact that the transmembrane-domain coding exons are spliced directly into the C_H3 donor splice site, eliminating the C_H4 domain and the C-terminal stretch of amino acids from the H chain of the secreted form (Wilson *et al.*, 1990; Bengetén *et al.*, 1991; Hordvik *et al.*, 1992; Wilson & Warr, 1992; Lee *et al.*, 1993; Kaattari & Piganelli, 1996; Pilström & Bengtén, 1996; Ross *et al.*, 1998). The two forms are encoded by the same gene and processing of the pre-mRNA determines which form should be synthesised (Pilström & Bengtén, 1996; Pilström, 1998; Ross *et al.*, 1998). The functional significance of the smaller membrane form of the teleost Ig is unknown but probably has little or no consequence (Wilson & Warr, 1992), since the truncated B cell receptor appears to function normally as an antigen receptor and to mediate allelic exclusion (Pilström & Bengtén, 1996; Pilström, 1998; Ross *et al.*, 1998). As in mammals, the cytoplasmic tail of teleost trans-membrane region is too short (three-amino acid sequence of Lys-Val-Lys) to have signaling capability (Wilson *et al.*, 1990), and therefore, there must be a molecule (or molecules) equivalent to the mammalian complex Igα (CD79a)

and Igβ (CD79b) associated with the membrane H chain to transduce the intracellular signal (Ross *et al.*, 1998). Although cDNAs for teleost homologs of these signal transduction accessory molecules have not been cloned, evidence suggests that a functionally homologous B cell receptor complex exists in catfish B cells, although seemingly composed of four peptides (Rycyzyn *et al.*, 1996; Ross *et al.*, 1998).

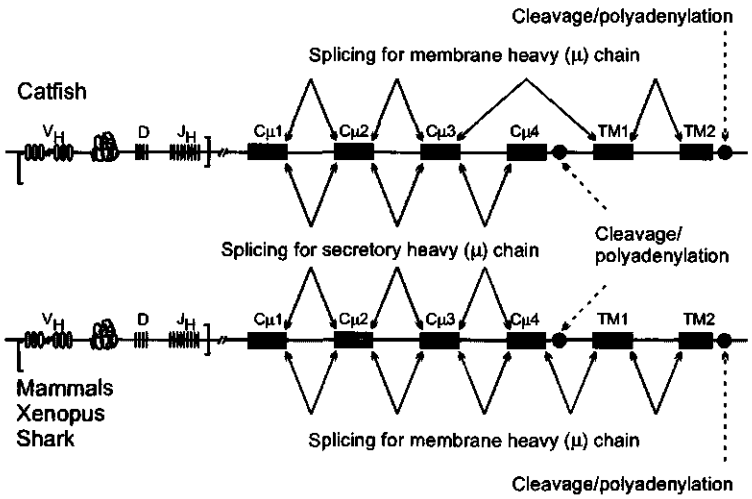

Fig. 2- Diagrammatic representation of mRNA splicing pathways for the alternate production of secreted (μS) and membrane (μM) forms of the heavy (μ) polypeptide in vertebrate species. Adapted from Wilson & Warr (1992) and Ross *et al.* (1998).

1.3.2. Gene organisation and structure

Figure 3 shows the organisation of the loci for the H and L chains in the main vertebrate groups. In teleosts, the H chain locus has a similar type of organisation as presented in amphibia and mammals, called "translocon" arrangement (Marchalonis *et al.*, 1993, 1998b), with multiple V segments located upstream of the diversity (D) segments, followed by multiple joining (J) segments and at most 3' ends the segment encoding a single C region (Ghaffari & Lobb, 1989a,b, 1991, 1992; Amemiya & Litman, 1990; Bengtén, *et al.*, 1991; Hordvik *et al.*, 1992). In addition, it has been shown that teleosts utilise rearrangements similar to that used in the mammalian IgH locus to generate a functional gene (Wilson & Warr, 1992). However, the J_H to C_H1 intron is too short to contain a class-switch

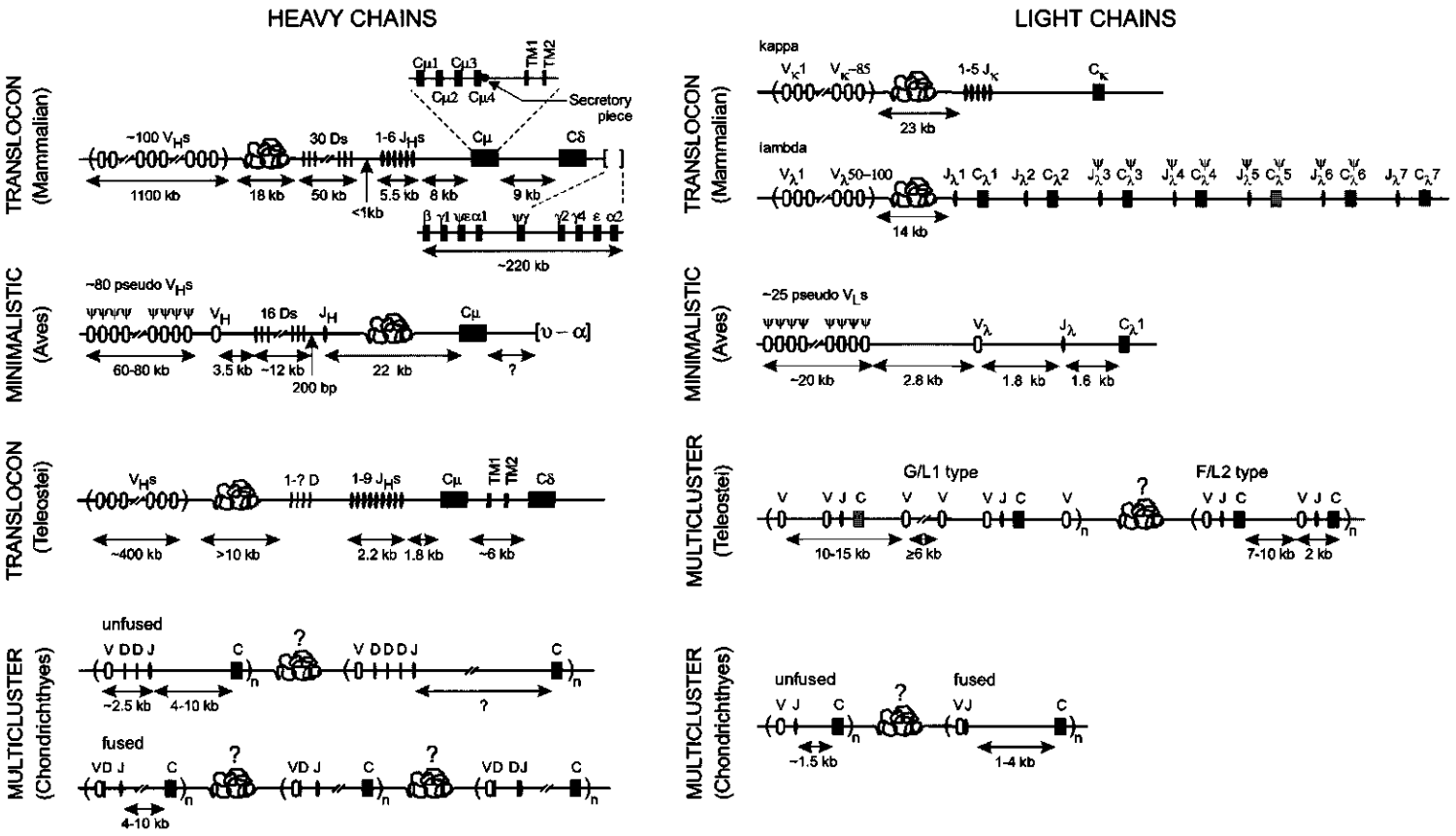

Fig. 3- Genomic organisation of the heavy and light chain genes in the main vertebrate groups. TM, transmembrane exon; ψ, pseudogene. Adapted from Marchalonis *et al.* (1998).

recombination sequence analogous to that known in other vertebrates, and thus, excluding the expression of Ig classes involving the typical class-switch rearrangement demonstrated in the mammals (Wilson & Warr, 1992; Pilström & Bengtén, 1996). As in other vertebrates, fish possess the two extra transmembrane-domain encoding exons, but while the exchange of the C-terminal secretory tail is consistent with what is observed in mammals the exclusion of the C_H4 domain in teleosts (see 1.3.1) appears to be a unique feature of this class of fish (Wilson & Warr, 1992; Kaattari & Piganelli, 1996; Kaattari *et al.*, 1998).

The L chain loci of teleosts, are organised in "multiclusters" of V-J-C or V-V-J-C segments (Ghaffari & Lobb, 1993,1997; Daggfeldt, *et al.*, 1993; Tomana, *et al.*, 1999), as is observed for the H (V-D-J-C, V-J-C, V-D-D-J-C or V-D-D-D-J-C) and L (V-J-C) chain organisation in Chondrichthyes (Hinds & Litman, 1986; Kokubu *et al.*, 1988a,b; Shamblott & Litman, 1989; Hohman *et al.*, 1992; Rast *et al.*, 1994).

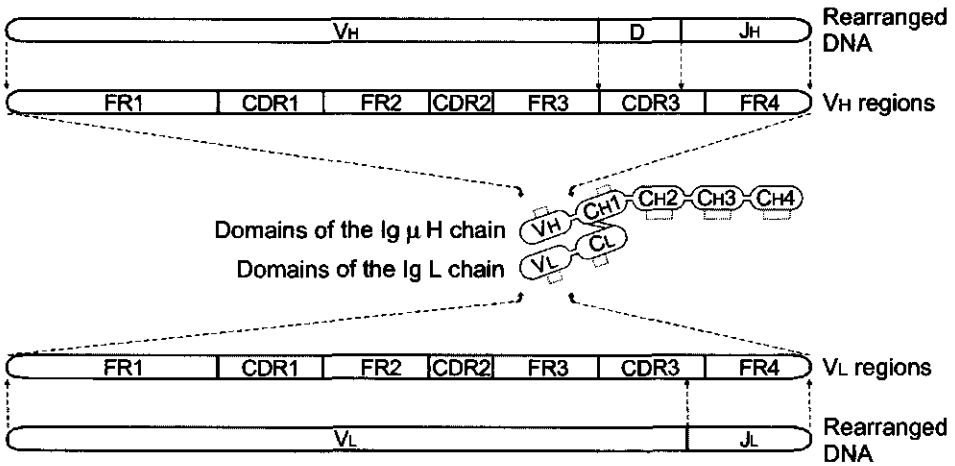

Fig. 4- Diagrammatic representation of the different domains of the immunoglobulin chain and the different DNA-segments encoding the different parts of the variable domains. Adapted from Pilström & Bengtén (1996).

The V domains of both H and L chains can be divided into seven regions (Fig. 4): three regions with high variability, called "complementarity determining regions" (CDRs; approximately at positions 30-38, 48-64 and 94-103 for the V_H and 25-40, 50-60 and 90-99 for the V_L) and four less variable framework regions (FRs) on either side of each CDR. While the CDRs create the antigen-binding site, the FRs are thought to hold the CDRs in an appropriate position for binding the incoming antigen. Only a very limited variability exists within the C regions although responsible for the isotypic heterogeneity of the Ab molecule (Kaattari & Piganelli, 1996; Pilström & Bengtén, 1996; Pilström, 1998).

1.3.3. Antibody heterogeneity

A great diversity of the Ab molecule is necessary to face the myriad number of possible foreign antigenic structures associated with pathogens (Kaattari & Piganelli, 1996).

Variability is particularly great within the CDRs of the V_H and V_L domains. Nevertheless, fish Ig and, thus, their Ab response, have been described of lower affinity and diversity than those of mammals (Mäkelä & Litman 1980; Du Pasquier, 1982). This could be due to deficiencies in the genetic mechanisms for generation of diversity, but seems to be unlikely for teleost fish (Pilström, 1998). In fact, several mechanisms might contribute for the heterogeneity of the Ab molecule:

1. *Sequence diversity*- the great variability within the V domains of both H and L chains is first achieved by the sequence diversity within the V, (D) and J DNA segments (Pilström & Bengtén, 1996) and it is especially confined to the high variability of the CDRs. While the first two CDRs are encoded entirely by the V segments, the third CDR is encoded by the D and J_H segments in the H chain and by the V_L and J_L segments in the L chain (Fig. 4). Thus, the number of different V, (D) and J segments in a species will contribute to its possibilities to diversify the Abs (Pilström & Bengtén, 1996). A similar number of V_H segments (more than 100) and V_H gene families (sequences sharing at least 80 % nucleotide identity) as those of the mammals have been reported in fish, indicating that the number and variability of the V_H segments is similar in those groups (Pilström & Bengtén, 1996; Pilström, 1998). However, only a few V_L segments and families have been described in teleosts (Ghaffari & Lobb, 1993; Daggfeld *et al.*, 1993; Bengtén, 1994). In teleosts, the number of D and J_H segments in the H chain and the V_L and J_L segments in the L chain loci are important for generating variability of the CDR3. The D segments of fish have not been sequenced at the genomic level but at least the number of J_H sements have been in accordance to those found in mammals (Pilström & Bengtén, 1996; Pilström, 1998).

2. *Somatic recombination* - Ig variability can be achieved through somatic recombination where, during B cell differentiation, the generation of an enormous amount of possible functional Ig genes can be generated by random recombination of the different V, (D) and J segments. This is achieved through a process involving recombination-activating genes (RAG), which encode for recombinases that act in very conserved recombination signal sequences (RSS). The RSS consist of a heptamer (consensus sequence CACTGTG) and a nonamer (consensus sequence ACAAAAACC) with a 12 bp spacer downstream of each V segment and the reversed sequences with a 23 bp spacer upstream of each J segment; the D segments have a RSS at each of their ends (Pilström & Bengtén, 1996). In teleosts, a D

segment recombines first with a J_H segment and is followed by the recombination of a V_H segment to the joint D-J_H, while in elasmobranchs half of the clusters show joining of the V_H-D-J_H segments in the germ line and seem to be functional, which will limit the possibility to diversify the Abs (Pilström & Bengtén, 1996; Pilström, 1998). In addition, the rearrangements of D segments take place only within a cluster, although either or both of the two D segments can be involved in the rearrangement (Pilström, 1998). In the L chain gene of the teleosts the clusters are closely linked (1.8-5 kb) and the V_L segments have an opposite transcriptional orientation to the J_L and C_L segments (Pilström & Bengtén, 1996; Pilström, 1998; Pilström *et al.*, 1998) so it is reasonable to suspect that rearrangements between clusters could take place by inversion (Pilström & Bengtén, 1996).

3. *Junctional diversity*- variability can be generated by an imprecise rearrangement of the V-(D)-J segments, where some nucleotides can be removed or added before religation of the DNA, which increases the variability of the CDR3 and also operates in teleost fish (Pilström & Bengtén, 1996; Pilström, 1998).

4. *Gene conversion*- after rearrangement, the functional V is diversified by the transfer of sequences from gene segments present in an upstream pool of pseudogenes (chicken) or functional genes (rabbit). It might be possible that teleosts also use this mechanism, not only in the H chain but also between clusters to generate variability of the L chain (Pilström & Bengtén, 1996).

5. *Somatic mutation*- during the maturation of the immune response, the binding site might be altered through the process of somatic mutation (Berek & Milstein, 1987) where one or more base pairs can be added or deleted. Additionally, there is some evidence that the region of DNA encoding the variable region may be particularly susceptible to mutation with up to 90 % of B cells expressing V_H genes which have undergone somatic mutation (Hay, 1993). Although there is evidence that somatic mutation might occur in fish, the fact that they do not present affinity maturation, at least as observed in mammals, is probably related to the absence of suitable sites for clonal selection (Pilström & Bengtén, 1996).

6. *Isotypes*- following the mammalian paradigm, isotypes are structural differences among Ig originated by specific sequences encoded by distinct C genes that are shared by all members of a species and which lead to different functional capabilities (e.g., transfer to mucosal surfaces: IgAs) of a certain Ab isotype (Winkelhake, 1979; Kaattari & Piganelli, 1996). Isotypic diversity in fish Ig were primarily suggested by the use of mAb (Lobb & Olson, 1988; Killie *et al.*, 1991; Koumans-van Diepen *et al.*, 1995). However, unlike isotypes, these serological forms were induced at all stages of an immune response and not subject of

isotype switching events (Lobb & Olson, 1988; Kaattari *et al.*, 1998). Currently, isotypic differences in fish Ig were supported by sequencing different genes encoding the C region, although a surprisingly low amount of diversity among those sequences was reported (Ghaffari & Lobb, 1989b; Hordvik *et al.*, 1992; Nakao *et al.*, 1998). However, as mentioned before, teleosts can generate considerable structural diversity by non-uniformly cross-linking subunits (see 1.3.1), leading to a varied redox forms (Kaattari *et al.*, 1998). The precise reason for this structural diversity is presently unknown, however, it is likely that this might represent a post-translational mechanism whereby functional diversity might be introduced into immunoglobulin molecules without requiring the dedication of specific structural genes to accomplish the same goals (Kaattari & Pianelli, 1996; Kaattari *et al.*, 1998). In addition, a peculiar form of IgD has been found in the channel catfish (Wilson *et al.*, 1997) and Atlantic salmon (Hordvik *et al.*, 1999). Light chain isotypes have also been described (Partula *et al.*, 1996b; Ghaffari and Lobb, 1997;Wermenstam, N. E. and Pilström, L., unpublished results) but it is difficult to use the traditional classification of κ and λ, though they form a branch close to the κ isotype (with exception of F/L2 isotype from catfish, rainbow trout and cod), indicating that these are more like κ than λ (Pilström *et al.*, 1998).

7. *H and L chain association*- random selection and association of H and L chains in Ab formation during B cell differentiation also increases the possible heterogeneity of the Ig molecule (Kaattari & Pianelli, 1996; Pilström & Bengtén, 1996).

1.3.4. Gene expression

Transcription regulation in the strict sense refers to the repressing or activating the action of proteins (transcription factors) that recognise and bind to specific gene control sequence motifs (Staudt & Lenardo, 1991; Opstelten, 1996). General transcription factors are those required for RNA polymerases to start transcription. The specific control sequences are localised in two types of regions generally referred to as promoters and enhancers (Pilström & Bengtén, 1996; Pilström *et al.*, 1998). Ig promoters, in which transcription initiates, are located upstream of each V segment and consist on several conserved DNA elements (among them a TATA-box and an octamer, of consensus sequence ATGCAAAT or its reverse complement). The Ig enhancers consist of a number of DNA elements (one type of enhancer motif is the E-box) that can affect the transcription of the linked promoters over long distances independent of their orientation and their location 5' or 3' of the promoter (Pilström & Bengtén, 1996; Pilström *et al.*, 1998). However, some events upstream and downstream of transcription are involved in the mechanism of transcription regulation. Thus, environmental

factors/substances can interact directly or indirectly with intracellular or surface receptors triggering signal transduction pathways represented by a balanced action of protein kinases and phosphatases; somewhere down the line, proteins translocate from the cytoplasm to the nucleus where they may enhance, directly or indirectly, the initiation of transcription of specific genes; while in most cases the initiation of transcription is the most important stage of regulation, post-transcriptional mechanisms may operate to further control protein levels as well as the transport of mRNA from the nucleus; finally, there are many ways of regulating the activity of a protein (Opstelten, 1996).

Although very little is known about the transcriptional regulation of the Ig genes in fish, conserved promoter (octamer and TATA-box in teleosts and nonamer/decamer in elasmobranchs) and enhancer structures have been reported in fish; additionally, strong evolutionary conservation in function of the nuclear transcription factors have been reported for the H chain gene while those for the L chain gene might be less conserved (Pilström & Bengtén, 1996; Pilström, 1998; Pilström *et al.*, 1998).

1.4. HUMORAL IMMUNITY

1.4.1. Antibody effector mechanisms

Ab molecules present a number of effector mechanisms that can, directly or indirectly, influence the destruction of the foreign antigen. *Neutralisation* of the antigen by blocking a critical function (important receptor, enzymatic active site or toxigenic determinant) appears as the most direct effect of the Ab on its antigen (Kaattari & Piganelli, 1996). Creation of macromolecular Ab-antigen complexes sufficiently large to come out of solution (*precipitation* when the antigen is molecular and *agglutination* when the antigen is cellular) is another direct effect of the Ab on its antigen. Moreover, this particulate matter is more easily phagocytosed and the close proximity of many Abs within the complex also facilitates the activation of complement or adherence of the Abs to the Fc receptors of phagocytes (Kaattari & Piganelli, 1996). The latter process is an example of *opsonisation*, which is usually referred to the coating of bacteria, fungi and parasites with specific Abs or other immunologically related molecules that often promotes their phagocytosis (Honda *et al.*, 1986; White *et al.*, 1990). *Specific activation of the complement system* by Ab (classical pathway) appears as an indirect but extremely potent effect of the Ab with its antigen. The binding of the Ab to an antigen results in a conformational change of the Ab's Fc region(s) which permits the binding and activation of the first component of the complement system which in turn triggers the complement cascade. At each step of this process proteolytic products are produced that can

opsonise or lyse pathogens as well as act as pharmacological mediators of host vascular, muscular or immune tissue modifications (Kaattari & Piganelli, 1996). Both the alternative (immunoglobulin-independent) pathway and the classical (immunoglobulin-dependent) activation pathway of the complement system have been reported in fish (Sakai, 1992; Yano, 1996).

1.4.2. Humoral immune response and memory

The hallmark of a humoral immune response is the production of specific Abs by plasma cells, triggering a number of effector mechanisms (see 1.4.1) that leads to the ultimate destruction of the invasive antigen. As mentioned before (see 1.2.2) B cells can be induced directly or indirectly to differentiate into antibody secreting cells (ASC), depending whether the antigen is of polysaccharidic (T_{ind}) or proteic nature (T_{dep}), respectively. If the antigen is of a different composition (e.g., glycolipid or nucleic acid), data from mammalian research suggest that it is dealt with as either a T_{ind} or a T_{dep} antigen (Kaattari & Piganelli, 1996). However, in most cases the immune system is challenged by multideterminant antigens, which elicit an Ab response to only some immunodominant determinants at the expense (suppression) of others present in the same (intramolecular-induced suppression) or distinct (intermolecular-induced suppression) molecule (Pross & Eidinger, 1973; Celada & Sercarz, 1988; Killie & Jørgensen, 1994). In fish both intramolecular- (Avtalion & Milgrom, 1976; Killie & Jørgensen, 1994) and intermolecular-induced (Killie & Jørgensen, 1995) suppression to T_{dep} antigens appears to exist.

An important line of defence in fish is the mucosal surface. Whereas mammals present specialised mucosal immune organs and a secretory IgA, fish do not show such apparently organised structures nor a distinct IgA-like isotype. However, teleosts present lymphoid cells scattered through the lamina propria of the gut (Rombout *et al.*, 1993b; Rombout & Joosten, 1998) with specialised function of antigen uptake and processing of antigens in the second segment (Stroband & Kroon, 1981a; Stroband & Van der Veen, 1981b; Iida & Yamamoto, 1985; Georgopoulou *et al.*, 1985; Rombout *et al.*, 1985,1986, 1989; Hart *et al.*, 1988; McLean & Donaldson, 1990; Joosten *et al.*, 1995). In addition, it has been shown that fish possess a differential mucosal immune system as observed in mammals, since Ab (Fletcher & White, 1973; Kawai *et al.*, 1981; St. Louis-Cormier *et al.*, 1984; Lobb, 1987; Rombout *et al.*, 1993a; Loghothetis & Austin, 1994; Lumsden *et al.*, 1993, 1995) and ASC (Davidson *et al.*, 1993, 1997; Joosten *et al.*, 1997) can be specifically induced in mucosal organs.

Immunological memory is related with the capacity of mounting a more effective response

the concentration of antigen is not limited (Tatner, 1987). Although being less stressful than the hyperosmotic immersion it is also limited by the number of fish that can be immunised per unit volume of vaccine, an the method is expensive when relatively large fish are vaccinated. *Bath* and *spray vaccination* are considered modifications of the direct immersion method, where the fish are exposed to dilute suspensions of the antigen for long periods or just sprayed with the vaccine, respectively. Moore *et al.* (1998) found that long exposure periods enhances the antigen uptake and can compensate for use of diluted particle suspensions agreeing with several observations on the enhancement of protection with prolonged diluted vaccine exposure (Kusuda *et al.*, 1980; Tatner & Horne, 1985). *Oral immunisation* would obviously be the ideal way for mass vaccination. It is non-stressful, does not interfere with the normal routine husbandry and, as for immersion vaccination, it can be used for fish of all sizes. Nevertheless, inconsistent results concerning its success have been reported (Evelyn, 1984; Hart *et al.*, 1987; McLean & Donaldson, 1990). This is probably due to degradation of the antigens in the anterior/gastric segment of the gut (Kaattari & Piganelli, 1996), because when antigens are delivered by anal intubation specific responses can be measured (Joosten *et al.*, 1996b). New oral delivery systems protecting the antigen from the gastric pH, allowing their uptake or the release of the antigen in the second segment of the gut (see 1.4.2), have shown promising results (Piganelli *et al.*, 1994; Joosten *et al.*, 1997). Induction of both systemic and mucosal responses have been reported after oral delivery. Stimulation of the mucosal compartment is of major importance since most naturally acquired infections occur through mucosal routes, especially in the aquatic environment where fish live (Kaattari & Piganelli, 1996). However, clear studies concerning the dose and period of administration are essential for protein antigens to avoid the induction of tolerance (Joosten *et al.*, 1996a, 1997). This might be problematic because in routine feeding regimes not all the fish get the same amount of feed.

More recently, vaccines based on recombinant DNA technology appear as a valuable approach to immunisation against viruses and parasites. The potential of the technology is steadily increasing, and it is probably a question of when, rather than whether, recombinant vaccines will appear as an efficient tool for prevention of several important diseases in aquacultured fish (Lorenzen, 1999). *Recombinant protein/antigens* expressed in prokaryotic or eukaryotic cells by fermentation under strictly controlled laboratory conditions (Gilmore *et al.*, 1988; Lecocq-Xhonneux *et al.*, 1994; He *et al.*, 1997), *live attenuated pathogens* (Vaughan *et al.*, 1993) or *nonpathogenic recombinant microorganisms* carrying foreign pathogen genes (Noonan *et al.*, 1995; Zhang & Hanson, 1996), and *genetic or naked DNA*

vaccines (Anderson *et al.*, 1996; Lorenzen *et al.*, 1998) have all been demonstrated to work in fish to a certain extent, under experimental conditions (Lorenzen, 1999). Recombinant protein vaccines have been shown to induce insufficient or inconsistent immunity, probably due to poor antigenicity and/or immunogenicity of the products (Lorenzen & Olesen, 1997; Leong *et al.*, 1997). More advantageous, the live recombinant vaccines can eventually be delivered by immersion (Lorenzen, 1999). However, since practical application of such vaccines inevitably implies release of recombinant organisms into the environment, safety concerns, both in terms of the vaccinated animals and in terms of environmental aspects, is probably the main reason for such vaccines not having received more attention in relation to aquaculture (Lorenzen, 1999). Most direct gene transfer and DNA vaccine experiments have been carried out in mammals, and thus relatively little is known about the expression of pathogen genes in fish (Heppell *et al.*, 1998). Conservation of native structure and thus the antigenicity of proteins is a very important issue in vaccine development, and is one of the clear advantages of DNA over many subunit vaccines such as peptides, recombinant proteins or whole killed pathogens (Heppell *et al.*, 1998). In addition, DNA vaccines offer several other desirable features such as inducing both humoral and cell-mediated immune responses (Lorenzen *et al.*, 1998; Heppell *et al.*, 1998), development of long-lasting immunity without apparent side-effects, are noninfectious, stable, and easy and cheap to produce under reproducible quality control conditions as well as allowing the possibility to mix plasmids to make multivalent vaccines (Heppell *et al.*, 1998, Lorenzen, 1999). The need for intramuscular immunisation, with no apparent scarring or any other permanent damage to the muscle tissue, rather than intraperitoneal injection facilitates the use of fully-automated injection devices (Heppell *et al.*, 1998). However, it is still necessary to develop delivery methods for mass vaccination of small fish under field conditions (Lorenzen *et al.*, 1998), such as the use of an attenuated enteric bacterial pathogen to deliver DNA to the endgut mucosa or the fusion of the gene for the vaccine antigen to the mucosal binding domain of the cholera toxin B or the tetanus toxin C (Leong *et al.*, 1997). Similar to the live recombinant vaccines, DNA vaccines have a number of theoretical human and environmental safety implications related to unintended spread and effects on non-target species, unintended mutation initiated by uncontrolled environmental factors, spontaneous recombination and horizontal tranfer of DNA within and between natural ecosystems (Traavik, 1997; Lorenzen, 1999). Thus, until basic research has been carried out, such aspects need to be addressed not only from scientific but also from ethical and regulatory points of view (Lorenzen, 1999).

2. PSEUDOTUBERCULOSIS

Pseudotuberculosis (Kubota *et al.*, 1970) is the name designed for the bacterial septicaemia caused by the halophilic bacterium *Photobacterium damselae* ssp. *piscicida*, due to the fact that, in the chronic form, the diseased fish shows whitish tubercles in the internal organs which consist of bacterial accumulations (Magariños *et al.*, 1996a). Due to the former designation of its etiological agent as *Pasteurella piscicida* (Janssen & Surgalla, 1968), the disease is still often referred as Pasteurellosis. Since its first diagnosis in 1963, following massive kill of white perch (*Morone americanus*) and striped bass (*M. saxatilis*) at the east coast of Chesapeake Bay in the USA (Sniezsko *et al.*, 1964), the disease has been isolated from many different fish species and locations (Table 1). However, the disease has mainly become of considerable economic importance in yellowtail (*Seriola quinqueradiata*) culture in Japan (Kubota *et al.*, 1970; Kimura & Kitao, 1971; Kusuda & Salati, 1993), and more recently, in gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) farming in Europe and in hybrid striped bass (*M. saxatilis* x *M. chrysops*) in the USA (Table 1). The fact that increasingly resistant strains of the bacterium to antibiotics have been reported (Kusuda & Salati, 1993) emphasise the importance and the need of vaccination for its prevention.

2.1. CLINICAL SIGNS, EPIZOOTIOLOGY AND DIAGNOSIS OF PSEUDOTUBERCULOSIS

In the acute form of the septicaemia only a few gross pathological signs may be observed, such as darkening of the body colour and/or slight haemorrhagic areas in the head and gills (Ohnishi *et al.*, 1982; Yasunaga *et al.*, 1983; Tung *et al.*, 1985; Toranzo *et al.*, 1991). Internally, multifocal necrosis and the presence of bacterial accumulations, free and within phagocytes, in the capillaries and in the interstitial spaces, may be observed histologically (Tung *et al.*, 1985; Hawke *et al.*, 1987; Noya *et al.*, 1995a). In the chronic form of the disease, granulomatous-like deposits (whitish tubercle-like lesions) of about 0.5 to 3.5 mm in diameter may develop in the internal organs, depending on which fish species is infected, whether the infection is natural or experimentally induced and whether or not antibiotic treatment was used (Magariños *et al.*, 1996a). Histologically, the granulomatous deposits showed masses of bacteria, epithelial cells and fibroblasts (Kubota *et al.*, 1970). Moreover, the spleen, kidney and liver showed accumulations of macrophages, many of which were necrotic and contained numerous intact bacteria (Hawke *et al.*, 1987).

Table 1- Epizootics caused by *P. damselae* ssp. *piscicida* (adapted from Magariños *et al.*, 1996a; * Zorrilla *et al.*, 1999)

SPECIES	COUNTRY
WILD FISH	
White perch (*Morone americanus*)	USA
Striped bass (*Morone saxatilis*)	USA
Mullet (*Liza rumada*)	Portugal
CULTURED FISH	
Yellowtail (*Seriola quinqueradiata*)	Japan
Ayu (*Plecoglossus altivelis*)	Japan
Black sea bream (*Mylio macrocephalus*)	Japan
Red sea bream (*Pagrus major*)	Japan
Young black sea bream (*Acanthopagrus schlegeli*)	Japan
Oval file fish (*Navodan modestus*)	Japan
Red grouper (*Epinephelus akaara*)	Japan
Snake-head fish (*Channa maculata*)	Taiwan
Striped bass (*Morone saxatilis*)	USA
Striped jack (*Pseudocaranx dentex*)	Japan, China
Gilthead sea bream (*Sparus aurata*)	Spain, Italy, Israel, Greece, Portugal
Sea bass (*Dicentrarchus labrax*)	Spain, France, Italy, Israel
Mullet (*Mugil* sp.)	Italy
Yatable blenny (*Pictiblennius yatabei*)	Japan
Hybrid striped bass (*Morone saxatilis* x *M. Chrysops*)	USA, Israel
Japanese flounder (*Paralichthys olivaceus*)	Japan
Sole (*Solea senegalensis*)*	Spain

The disease appears mostly related with the summer months when water temperatures rise above 23° C (Magariños *et al.*, 1996a). Although some studies have reported that the water would not be a primary reservoir for the bacterium, which remained in a culturable form for only 4-5 days (Janssen & Surgalla, 1968; Toranzo *et al.*, 1982), it appears that the *P. damselae* ssp. *piscicida* possesses the capacity to enter into a dormant non-culturable but infective state in seawater and sediment (Magariños *et al.*, 1994a). The fact that gilthead seabream (Magariños *et al.*, 1995a) can be infected by the water route supports the view that the aquatic environment constitutes a vehicle of transmission of *P. damselae* ssp. *piscicida*. It has also been suggested that carrier fish provide a reservoir of infection (Janssen & Surgalla, 1968 10; Kitao, 1993), although it has not been possible to isolate the pathogen from surviving challenged fish (Toranzo *et al.*, 1991) probably due to the diagnostic methods employed. Both the gills and skin (Kawahara & Kusuda, 1987; Magariños *et al.*, 1995b), in addition to the gastro-intestinal tract (Wakabayashi *et al.*, 1977; Tung *et al.*, 1985), have been mentioned as possible routes of infection.

The primary isolation of *P. damselae* ssp. *piscicida* can be performed from the kidney, spleen and/or liver of infected fish using either media such as Tryptic Soy Agar (TSA), Brain

Heart Infusion Agar (BHIA) and blood agar, all supplemented with 1 to 2% of NaCl, or Marine Agar 2216E (Magariños *et al.*, 1996a). Typically shiny, grey-yellow, entire, convex colonies with approximately 1-2 mm in diameter (Kusuda & Yamaoka, 1972) develop after 48 to 72h incubation at 22-25° C. Presumptive diagnosis is based on the isolation of Gram-negative bacteria with bipolar staining, non-motile and rod-shaped cells, which are oxidase and catalase positive, fermentative, sensitive to the vibriostatic agent O/129, and with a strict salt requirement (Magariños *et al.*, 1996a). Although the *P. damselae* ssp. *piscicida* is not yet included in the API20E codex index, this system is valuable for a rapid presumptive identification (identification number: 2 005 004), with no false-positive or –negative reactions (Santos *et al.*, 1993). Serological tests using specific antisera (Kimura & Kitao, 1971; Kusuda *et al.*, 1978; Yasunaga *et al.*, 1984, Ueki *et al.*, 1990) or direct and indirect fluorescent antibody techniques (Kitao & Kimura, 1974; Mori *et al.*, 1976) can be used for definitive diagnosis. A commercial diagnostic kit (Bionor AS: Mono-Pp, Mono-Pp^{+} and Aquarapid-Pp, Norway), based on ELISA methods, showed high sensitivity and specificity for a correct and early presumptive diagnosis of pseudotuberculosis within 1 h (Romalde *et al.*, 1995a,b). Moreover, PCR and DNA probe-based detection procedures based on amplification of DNA fragments obtained from a genomic library of *P. damselae* ssp. *piscicida* (Zhao & Aoki, 1989) and on amplification of plasmid sequences of the bacterium (Zhao & Aoki, 1992; Aoki *et al.*, 1997) also appeared as useful and promising techniques for detecting and identifying the bacterium in infected tissues and/or colonies of the pathogen on culture plates. More recently, nested PCR and DNA probes based on 16S rRNA gene sequences of *P. damselae* ssp. *piscicida* have been shown to be at least 100 times more sensitive than the serological approach, which indicates that the method is suitable for fast, precise, and sensitive detection of this fish pathogen not only in diseased fish but also in asymptomatic carriers (Osorio *et al.*, 1999).

2.2. CHARACTERISATION OF P. damselae ssp. piscicida

Photobacterium damselae ssp. *piscicida* is a pleomorphic bacterium changing from coccoidal to long rods under different culture conditions. Although the bacterium was primarily reported as unencapsulated (Koike *et al.*, 1975), Bonet *et al.* (1994) demonstrated that *P. damselae* ssp. *piscicida* was able to produce capsular material with 99.6% of carbohydrate and 0.4% protein. Serologically, the bacterium has been described as a highly homogeneous group, which was supported by the similar patterns of LPS, total cell envelops and outer membrane proteins (Nomura & Aoki, 1985; Magariños *et al.*, 1992a), in addition to

practically identical fingerprint patterns generated by digestion with the restriction endonucleases *Eco*RI and *Bam*HI (Magariños *et al.*, 1992a). However, recently, antigenic differences could be detected by Western blot analysis with monoclonal antibodies against whole cells of the bacterium (Bakopoulous *et al.*, 1997a) and agglutinating and immunodiffusion analysis (Kawahara *et al.*, 1998). Moreover, different plasmid content among the strains have also been reported (Toranzo *et al.*, 1983; Zhao & Aoki, 1992; Magariños *et al.*, 1992a), and ribotype analysis revealed the existence of genetic diversity among *P. damselae* ssp. *piscicida* strains, with three ribotypes identified and proving to be applicable as an epizootiological typing system (Magariños *et al.*, 1996a). The morphophysiological and biochemical characteristics of *P. damseale* ssp. *piscicida* have been widely reported (Kimura & Kitao, 1971; Kusuda & Yamaoka, 1972; Hawke *et al.*, 1987; Toranzo *et al.*, 1991; Baptista *et al.*, 1996; Candan *et al.*, 1996; Zorrilla *et al.*, 1999) and are summarised in table 2.

Table 2- General morphological and biochemical characteristics of *P. damseale* ssp. *piscicida*.

Test	Result	Test	Result
Gram staining	–	Tryptophan deaminase	–
Bipolar staining	+	β-galactosidase (ONPG)	–
Motility	–	Urease	–
Oxidase	+	Gelatinase	–
Catalase	+	Caseinase	–
Methyl red	+	Lipase (Tween 80)	+
Voges-Proskauer	+[1]	Phospholipase	+
Indole production	–	Amylase	–
Nitrate reduction	–	Haemolysis: sheep erythrocytes	–
Citrate utilisation	–	salmon erythrocytes	–
H_2S production	–	Acid production from:	
Glucose oxidation/fermentation	–[1]/+	Glucose	+
Gas from glucose	–	Mannose	+
Growth at: 5° C	–	Galactose	+
15° C	+	Fructose	+
25° C	+	Maltose	–[1]
37° C	–	Sucrose	–
Growth in: 0% NaCl	–	Rhamnose	–
3% NaCl	+	Arabinose	–
5% NaCl	–	Amygdaline	–
Growth on: TSA + 2% NaCl	+	Melibiose	–
BHIA + 2% NaCl	+	Mannitol	–
Marine agar	+	Inositol	–
TCBS	–	Sorbitol	–
McConkey agar	–	Glycerol	–
Arginine dihydrolase	+	Lactose	–
Lysine decarboxylase	–	Salicin	–
Ornithine decarboxylase	–	O/129 sensitivity	+

+, positive reaction; –, negative reaction; 1, differs from Hawke *et al.*, 1987

2.3. *PHATHOGENICITY MECHANISMS OF Photobacterium damseale ssp. piscicida*

There is no doubt that *P. damseale* ssp. *piscicida* is highly pathogenic, causing mass mortalities (fifty percent lethal dose- LD_{50}: ranging from 10^3 to 10^6 cells; Magariños *et al.*, 1996a) in a wide range of fish species (Table 1). The bacterium does not seem to exhibit host specificity and it may be a risk for marine fish species in which the disease has not been described (Magariños *et al.*, 1996a). At least for gilthead sea bream, susceptibility to the disease appears to depend on their age, with fish larger than 50 g becoming resistant (Noya *et al.*, 1995b).

Concerning the adherence and invasive capacities of *P. damselae* ssp. *piscicida*, the bacterium has been shown to have weak or moderately adherent capacity to fish cell lines, but with a great binding capacity to gilthead sea bream, sea bass and turbot intestinal tissues (Magariños *et al.*, 1996b). Adhesive capacities were affected by heat and sugars but not by proteinase K or by treatment with antisera raised against the LPS, indicating components of glycoprotein(s) as ligands in the adhesion process (Magariños *et al.*, 1996b). The pathogen has been described as moderately invasive, and the inhibition of the invasion process by cytochalasin D indicates the active participation of the host cell cytoskeleton in the internalisation of the bacterium, which was shown to be capable of surviving for at least 6 days within the host cells and to spread from cell-to-cell, causing destructive plaques in the cell monolayer (Magariños *et al.*, 1996b). In addition, *P. damselae* ssp. *piscicida* has been reported as being moderately hydrophobic and weakly or non-heamagglutinating (Sakai *et al.*, 1993; Magariños *et al.*, 1996b). The presence of capsule has been shown to play an important role in the pathogenesis of the bacterium by conferring resistance to serum killing and decreasing the LD_{50} values by 2-3 log units (Magariños *et al.*, 1996b), and reducing the index of phagocytosis, though it had no significant effect on the macrophage bactericidal activity (Arijo *et al.*, 1998). It has been described that *P. damselae* ssp. *piscicida* possess an iron acquisition system (siderophore-mediated mechanism), which allows them to survive in the restricted iron conditions within the host (Magariños *et al.*, 1994b). This system consists of a non-phenolate, non-hydroxamate siderophore that may be related to multocidin produced by *Pasteurella multocida* (Magariños *et al.*, 1994b), and iron-regulated outer membrane proteins (IROMPs; Magariños *et al.*, 1994b; Bakopoulous *et al.*, 1997b). All the *P. damselae* ssp *piscicida* strains reported by Magariños *et al.* (1994b) were able to utilise haemin and haemoglobin as sole iron sources by direct binding of these compounds to iron regulated membrane receptors. The virulence of the strains increased when the fish were pre-inoculated

with ferric ammonium citrate, haemin or haemoglobin. Although this pathogen has been considered a non-proteolytic bacterium in normal culture conditions (Magariños *et al.*, 1992b), it was found that under iron-depleted conditions it was able to synthesise some proteolytic enzymes (Magariños *et al.*, 1994b), which may also play a role in the virulent mechanism of the pathogen. The extracellular products (ECP) secreted by a variety of bacterial fish pathogens are important virulence factors since they can contribute to the development of the disease in terms of bacterial nutrition or as aggressins enabling the bacteria to counteract the host's defence mechanisms (Magariños *et al.*, 1996a). The ECP of *P. damselae* ssp. *piscicida* were reported as being strongly toxic for gilthead sea bream, sea bass, turbot and rainbow trout (LD_{50} ranging from 1.0 to 4.6 μg protein per g fish; Magariños *et al.*, 1992b), which clearly demonstrated their importance in the pathogenesis of pseudotuberculosis (Magariños *et al.*, 1992b; Mazzolini *et al.*, 1998). These authors have shown slight variations for caseinase, gelatinase and dermonecrotic activities but strong phospholipase, cytotoxic and haemolytic activities in the ECP of *P. damselae* ssp. *piscicida*.

2.4. CONTROL OF PSEUDOTUBERCULOSIS

When pseudotuberculosis first appeared in yellowtail culture in Japan, it was easily controlled by a wide variety of chemotherapeutic agents such as ampicillin, amoxycillin, novobiocin, thiamphenicol, florfenicol, oxolinic acid, flumequine and sodium nifurstryenate (Kitao, 1993). However, with the wide use of antibiotics the bacterium rapidly developed multiple resistance to several of these compounds (Aoki & Kitao, 1985; Takashima *et al.*, 1985; Kusuda *et al.*, 1988a, 1990). The resistance to some of the antibiotics was reported as being R plasmid encoded (Takashima *et al.*, 1985) while resistance to quinolones was coded for by chromosomal genes (Nakano *et al.*, 1989). Within Europe, the different isolates have shown similar antibiotic sensitivity patterns, with all strains showing resistance to erythromycin, kanamycin and streptomycin and some to potentiated sulphonamides, cephalosporins and penicillins (Toranzo *et al.*, 1991; Balebona *et al.*, 1992; Bakopoulous *et al.*, 1995; Baptista *et al.*, 1996). Resistance of Japanese strains to a wider range of antibiotics than European strains may reflect the effect of the long-term use of these antibiotics for the control of pseudotuberculosis in Japan (Bakopoulous *et al.*, 1995). In general, the European strains are still sensitive to a range of chemotherapeutics and epizootics can be controlled by treatment with chloramphenicol and ampicillin (Magariños *et al.*, 1996a). More recently, bicozamycin, florfenicol and phosphomycin have been shown to be useful for controlling

pseudotuberculosis (Kitao *et al.*, 1992; Kim & Aoki, 1993; Sano *et al.*, 1994), although some resistant strains to florfenicol have already been described (Kim *et al.*, 1993).

As for other bacterial disorders, prophylaxis of pseudotuberculosis by vaccination is undoubtedly the most straightforward way for reduction of the use of chemotherapeutic agents and for better controlling the disease. This is further supported by the fact that the pathogen has a period of intracellular parasitism during the infection, which makes it very difficult to treat with antibiotics (Kusuda & Salati, 1993). Although many attempts have been made to develop *P. damselae* ssp. *piscicida* vaccines, only one small-scale field test has been reported (Kawakami *et al.*, 1997). However, several types of experimental vaccine formulations have been shown as being effective to *P. damselae* ssp. *piscicida* (Kitao, 1993; Kusuda & Salati, 1993; Magariños *et al.*, 1996a; Kawakami *et al.*, 1997), although the results were not always reproducible (Kusuda & Salati, 1993; Magariños *et al.*, 1996a). Antigens from whole-cells (Fukuda & Kusuda, 1982), the LPS (Fukuda & Kusuda, 1985; Kawahara & Kusuda, 1987), ECP (Magariños *et al.*, 1994c) and a mixture of all these antigens (Kawakami *et al.*, 1997) have been suggested to be important for effective protection. In addition, the use of ribosomal fractions of the bacterium (Kusuda *et al.*, 1988b; Ninomiya *et al.*, 1989), live attenuated bacteria (Kusuda & Hamaguchi, 1988) and IROMPs enriched bacterin (Magariños *et al.*, 1994c) have shown promising results in preliminary experiments. However, the ribosomal fraction-based vaccines are difficult to produce on a large scale and the live attenuated vaccines are still problematic for being licensed. Since antigenic differences could recently be detected among *P. damselae* ssp. *piscicida* isolates (see 2.2), it might become important to evaluate monovalent or polyvalent vaccines from *P. damselae* ssp. *piscicida* in protection trials against the different isolates. In addition, studies concerning the understanding of which immune mechanisms are involved in the protection are necessary.

Although, a mixture of β-1,3/1,6-glucans derived from *Saccharomyces cerevisiae* have shown increased phagocytic activity and increased resistance of gilthead sea bream against pseudotuberculosis when delivered intraperitoneally or as a diet supplement, further studies are required in order to clarify the potential effects of these or any other type of immunostimulants for use as a preventive measure against pseudotuberculosis (Magariños *et al.*, 1996a).

3. AIM AND OUTLINE OF THIS THESIS

While food fish production from capture fisheries grew at an average compound growth rate of 1.5% equivalent to the growth of the human population, aquaculture has been the world's fastest growing food production system for the past decade (Tacon, 1997). In Europe, the sea bass/bream industry intends to copy the success of salmon growers (Lem & Shehadeh, 1997). To increase output, the productive process has been improved and intensified in a way that the highest number of individuals in the smallest space can be produced. This growing increase of animal densities has also facilitated the emergence of many diseases as well as their rapid dissemination. This might be the cause of increased mortalities and weak growth and food conversion rates, endangering farm viability. Even when the environmental conditions are good and the fish are healthy certain infectious agents, if introduced into the farm, are so virulent that mass mortalities can and do occur (Ellis, 1988). In fact, pathogens constitute the most important cause of economic loss for fish and shellfish farmers (Meyer, 1991).

Over many years, drugs have been used to treat or prevent disease losses. However, a serious problem is that few approved chemotherapeutic agents are available for use in food because of growing concern for consumer liability and for the accumulation of substances in the environment. There is also concern about the increase of antibiotic-resistant strains of bacteria in the aquatic environment surrounding locations where the drugs are used. In addition, there is no effective chemotherapy for control of viral diseases (Ellis, 1988).

It is against this background that vaccines have been perceived as potentially playing an important benificial role in aquaculture. Supporting this is the increasing success of vaccines in preventing aquatic animal diseases, which has caused a decline in antibiotic use in the salmon farming industry in Norway (Nash, 1995; Torrissen *et al.*, 1995; Aiken & Sinclair, 1995; Markestad & Grave, 1997).

Most of the studies in fish immunology have been performed in salmonid and cyprinid species with a general lack of information in marine species, although recently data are becoming available on the sea bass immune system. In addition, with the intensification of sea bass production, serious problems have arisen concerning the appearance of bacterial and viral epizootics. Of these, the halophilic gram-negative bacteria *P. damselae* ssp. *piscicida* have been causing high mortality losses in sea bass farming (Magariños *et al.*, 1996a; Candan *et al.*, 1996), and thus, studying the host immune response as part of the development of an effective vaccine for this pathogen became an important task to be accomplished. Moreover,

in fish, at the time of hatching, the lymphoid system is still developing, being important to determine the earliest age when fry can be successfully immunised without inducing tolerance.

In the present thesis, basic and applied studies were performed concerning the humoral immune system of sea bass, especially the antibody response and the ontogenetic development, with the aim of finding out the earliest age/weight at which sea bass can be vaccinated to *P. damselae* ssp. *piscicida*. Thus, monoclonal antibodies to sea bass Ig heavy and light chains were produced, characterised and used in studies of B cell populations (**Chapter 2**) as well as in later studies concerning the development of immunity and antibody responses. In addition, the variability of the sea bass Ig light chain after hapten-carrier immunisation was studied in order to evaluate whether intramolecular-induced regulation and suppression also occurs in sea bass (**Chapter 3**) as described for other fish species (see 1.4.2 and 1.5). Basic knowledge of the maturation of the adaptive immune system appears essential concerning the advantageous early vaccination of fish. Therefore, the ontogeny of sea bass B and T cells was studied. Since, from a practical viewpoint, fish are graded and grouped on the basis of their average weight during the production cycle, the influence of weight and age on the ontogeny of sea bass B cells was also studied by comparing groups of fish with different growth rates from the same spawn and by following the offspring from different spawns (**Chapter 4**). Knowledge of the time course of an antibody response is important for evaluating both the protective effect of a vaccine after experimental and/or natural challenge and the sampling time of immune serum for research proposes. Thus, the kinetics of juvenile sea bass systemic and mucosal antibody secreting cell response to a *P. damselae* ssp. *piscicida* bacterin was compared with the ones to *V. anguillarum* and DNP-KLH (**Chapter 5**). The effectiveness of the *P. damselae* ssp. *piscicida* bacterin was then verified *in vitro* by studying the influence of sea bass specific anti-*P. damselae* ssp. *pisicida* serum on the ability of the bacterium to invade epithelial cells (**Chapter 6**). Finally, the development of the antibody secreting cell responses following direct immersion of different aged/sized sea bass in the bacterin was performed in order to find out whether tolerance would be induced at early stages of the development and which organs were involved in the specific response following this route of immunisation (**Chapter 7**), one of the most suitable routes for mass vaccination of young fish. All these points are brought together in **chapter 8** in an attempt to summarise and pinpoint important conclusions for a successful strategy to vaccinate sea bass against the *P. damselae* ssp. *piscicida*.

References

Abelli, L., Picchietti, S., Romano, N., Mastrolia, L. & Scapigliati, G. (1996). Immunocytochemical detection of thymocyte antigenic determinants in developing lymphoid organs of sea bass *Dicentrarchus labrax* (L.). *Fish & Shellfish Immunology* **6**, 493-505.

Acton, R. T., Niedermeier, W., Weinhamer, P. F., Clem, L. W., Leslie, G. A. & Bennett, J. C. (1972). The carbohydrate composition of immunoglobulins from diverse species of vertebrates. *Journal of Immunology* **109**, 371-381.

Acton, R. T., Weinhamer, P. F., Hall, S. J., Niedermeier, W., Shelton, E. & Bennett, J. C. (1971). Tetrameric macroglobulins in three orders of bony fish. *Proceedings of the National Academy of Sciences* **68**, 107-111.

Aiken, D & Sinclair, M. (1995). From capture to culture: exploring the limits of marine productivity. *World Aquaculture* **26**(3), 21-34.

Alabyev, B. Y., Guselnikov, S., Mechetina, L. V., Najakshin, a. M. & Taranin, A. V. (1998). Identification of the CD3 epsilon and TCR alpha chain genes in chondrostean *Acipenser rhutenus*. Poster Bp5 at the Nordic Symposium on Fish immunology, Hirtshals, Denmark.

Amemiya, C. T. & Litman, G. W. (1990). Complete nucleotide sequence of an immunoglobulin heavy-chain gene and analysis of immunoglobulin gene organization in a primitive teleost species. *Proceedings of the National Academy of Sciences USA* **87**, 811-815.

Amend, D. F. & Finder, D. C. (1976). Uptake of bovine serum albumin by rainbow trout from hyperosmotic solutions: a model for vaccinating fish. *Science* **192**, 796-804.

Anderson, E. D., Mourich, D. V., Fahrenkrug, S., LaPatra, S., Shepard, J. & Leong, J. C. (1996). Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. *Molecular Marine Biology and Biotechnology* **5**, 114-122.

Antipa, R. & Amend, D. F. (1977). Immunization of pacific salmon: comparison of intraperitonel injection and hyperosmotic infiltration of *Vibrio anguillarum* and *Aeromonas salmonicida* bacterins. *Journal of Fish Research Board of Canada* **34**, 203-208.

Antipa, R., Gould, R. & Amend, D. F. (1980). Vibrio anguillarum vaccination of sockeye salmon, Oncorhynchus nerka (Walbaum), by direct and hyperosmotic immersion. *Journal of Fish Diseases* **3**, 161-165.

Aoki, T. & Kitao, T. (1985). Detection of transferable R-plasmids in strains of the fish-pathogenic bacterium *Pasteurella piscicida*. *Journal of Fish Diseases* 8, 345-350.

Aoki, T., Ikeda, D., Katagiri, T. & Hirono, I. (1997). Rapid detection of the fish-pathogenic bacterium *Pasteurella piscicida* by polimerase chain reaction targeting nucleotide sequences of the species-specific plasmid pZP1. *Fish Pathology* **32**, 143-151.

Arijo, S., Borrego, J. J., Zorilla, I., Balebona, M. C. & Moriñigo, M. A. (1998). Role of the capsule of *Photobacterium damselae* subsp. *piscicida* in protection against phagocytosis and killing by gilt-head seabream (*Sparus aurata*, L) macrophages. *Fish & Shellfish Immunology* **8**, 63-72.

Avtalion, R. R. & Milgrom, L. (1976). Regulatory effect of temperature and antigen upon immunity in ectothermic vertebrates. I. Influence of hapten density on the immunological and serological properties of penicilloyl-carrier conjugates. *Immunology* **31**, 589-594.

Bakopoulous, V., Adams, A. & Richards, R. H. (1995). Some biochemical properties and antibiotic sensitivities

of *Pasteurella piscicida* isolated in Greece and comparison with strains from Japan, France and Italy *Journal of Fish Diseases* **18**, 1-7.

Bakopoulous, V., Adams, A. & Richards, R. H. (1997a). Serological relationship of *Photobacterium damselae* subsp. *piscicida* isolates (the causative agent of fish pasteurellosis) determined by Western blot analysis using six monoclonal antibodies. *Diseases of Aquatic Organisms* **28**, 69-72.

Bakopoulous, V., Adams, A. & Richards, R. H. (1997b). The effect of iron limitation growth conditions on the cell and extracellular components of the fish pathogen *Pasteurella piscicida*. *Journal of Fish Diseases* **20**, 297-305.

Balebona, M. C., Moriñigo, M. A., Sedano, J., Martinez-Manzanares, E., Vidaurreta, A., Borrego, J. J. & Toranzo, A. E. (1992). Isolation of *Pasteurella piscicida* from sea bass in southwestern Spain. *Bulletin of the European Association of Fish Pathologists* **12**, 168-170.

Baptista, T., Romalde, J. L. & Toranzo, A. E. (1996). First occurrence of pasteurellosis in Portugal affecting cultured gilthead seabream (*Sparus aurata*). *Bulletin of the European Assiciation of Fish Pathologists* **16**, 92-95

Bengtén, E., Leandersson, T. & Pilstrom, L. (1991). Immunoglobulin heavy chain cDNA from the teleost Atlantic cod (*Gadus morhua* L.): nucleotide sequence of secretory and membrane bound forms show an unusual splicing pattern. *European Journal of Immunology* **21**, 3027-3033.

Berek, C. & Milstein, C. (1987). Mutation drift and repertoire shift in the maturation of the immune response. *Immunologiacal Reviews* **21**, 3027-3033.

Bonet, R., Magariños, B., Romalde, J. L., Simón-Pujol, M. D., Toranzo, A. E. & Congregado, F. (1994). Capsular polysaccharide expressed by *Pasteurella piscicida* grown in vitro. *FEMS Microbiology Letters* **124**, 285-290.

Botham, J. W., Grace, M. F. & Manning, M. J. (1980). Ontogeny of first-set and cecond-set alloimmune reactivity in fishes. In *Phylogeny of Immunological Memory* (M. J. Manning, ed.) pp. 83-92. Amsterdam: Elsevier/North Holland.

Botham, J. E. & Manning, M. J. (1981). The histogenesis of the lymphoid organs in carp (*Cyprinus carpio* L.) and the ontogenetic development of allograft reactivity. *Journal of Fish Biology* **19**, 403-414.

Breuil, G., Vassiloglou, B. Pepin, J. F. & Romestand, B. (1997). Ontogeny of IgM-bearing cells and changes in the immunoglobulin M-like protein level (IgM) during larval stages in sea bass (*Dicentrarchus labrax*). *Fish & Shellfish Immunology* **7**, 29-43.

Candan, A., Ang Kucker, M. & Karatas, S. (1996). Pasteurollosis in cultured sea bass (*Dicentrarchus labrax*) in Turkey. *Bulletin of the European Association of Fish Pathologists* **16**, 150-153.

Caspi, R. R. & Avtalion, R. R. (1984). The mixed leucocyte reaction (MLR) in carp: Bidirectional and unidirectional MLR responses. *Developmental and Comparative Immunology* **8**, 631-636.

Castillo, A., Sánchez, C., Dominguez, J., Kaattari, S. L. & Villena, A. J. (1993). Ontogeny of IgM and IgM-bearing cells in rainbow trout. *Development and Comparative Immunology* **17**, 419-424.

Celada, F. & Sercarz, E. E. (1988). Preferential pairing of T-B specificities in the same antigen: the concept of directional help. *Vaccine* **6**, 94-98.

Chantanachookhin, C., Seikai, T. & Tanaka, M. (1991). Comparative study on the ontogeny of the lymphoid organs of three species of marine fish. *Aquaculture* **99**, 143-156.

Clem, L. W. (1971). Phylogeny of immunoglobulin structure and function. IV. Immunoglobulins of the giant grouper, *Epinephelus itaira*. *Journal Biol. Chem.* **246**, 9-15.

Clem, L. W. & McClean, W. E. (1975). Phylogeny of immunoglobulin structure and function. VII. Monomeric and tetrameric immunoglobulins of the margate, a marine teleost. *Immunology* **29**, 791-799.

Clem, L. W., Sizemore, R. C., Ellsaesser, C. F. & Miller, N. W. (1985). Monocytes as accessory cells in fish immune responses. *Developmental and Comparative Immunology* **9**, 803-809.

Cuchens, M. A. & Clem, L. W. (1977). Phylogeny of lymphocyte heterogeneity. II Differential effects of temperature on fish T-like and B-like cells. *Cellular Immunology* **34**, 219-230.

Daggfeldt, A., Bengtén, E. & Pilström, L. (1993). A cluster type organization of the loci of the immunoglobulin light chain in Atlantic cod (*Gadus morhua* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) indicated by nucleotide sequences of cDNA and hybridization analysis. *Immunogenetics* **38**, 199-209.

Davidson, G. A., Ellis, A. E. & Secombes, C. J. (1993). Route of immunisation influences the generation of antibody secreting cells in the gut of rainbow trout (*Oncorhynchus mykiss*). *Developmental and Comparative Immunology* **17**, 373-376.

Davidson, G. A., Lin, S-H., Secombes, C. J. & Ellis, A. E. (1997). Detection of specific and "constitutive" antibody secreting cells in the gills, head kidney and peripheral blood leukocytes of dab (*Limanda limanda*). *Veterinary Immunology and Immunopathology* **58**, 363-374.

Davis, A. C., Roux, K. H. & Shulman, M. J. (1989). On the structure of polymeric IgM. *European Journal of Immunology* **18**, 1001-1008.

DeLuca, D., Wilson, M. & Warr, G.W. (1983). Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. *European journal of Immunology* **13**, 546-551.

Dixon, B., van Erp, S. H. M., Rodrigues, P. N. S., Egberts, E. & Stet, R. J. M. (1995). Fish major histocompatibility genes: an expansion. *Developmental and Comparative Immunology* **19**, 109-133.

Du Pasquier, L. (1982). Antibody diversity in lower vertebrates; why is it so restricted? *Nature* **296**, 311-313.

Elcombe, B. M., Chang, R. J., Taves, C. J. & Winkelhake, J. L. (1985). Evolution of antibody structure and effector functions: comparative hemolytic activities of monomeric and tetrameric IgM from rainbow trout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology* **80B**, 697-706.

Ellis, A. E. (1977). Ontogeny of the immune response in *Salmo salar*. Histogenesis of the lymphoid organs and appearance of membrane immunoglobulin and mixed luecocyte reactivity. In *Developmental Immunobiology* (J. B. Solomon and J. D. Horton, eds) pp. 225-231. Amsterdam: Elsevier/North Holland.

Ellis, A. E. (1988). Current aspects of fish vaccination. *Diseases of Aquatic Organisms* **6**, 201-215.

Ellis, A. E., (1998). Ontogeny of the immune system. In *Handbook of Vertebrate Immunology* (Pastoret, P.-P., Griebel, P., Bazin, H. & Govaerts, A., eds.) pp. 26-30, London: Academic Press.

Ellis, A. E. (1999). Immunity to bacteria in fish. *Fish & Shellfish Immunology* **9**, 291-308.

Etlinger, H. M., Hodgins, H. O. & Chiller, J. M. (1977). Evolution of the lymphoid system. III Evidence for immunoglobulin determinants on rainbow trout lymphocytes and demonstration of mixed leukocyte reaction. *European Journal of Immunology* **7**, 881-886.

Feldmann, M. (1993). The generation of diversity. In *Immunology* (I. M. Roitt, J. Brostoff & D. K. Male, eds.; third edition). pp. 7.1-7.16. London: Mosby-Year Book Europe Ltd.

Francés, V., Paudran-Garcia, D., Guret, C., Ho, S., Wang, Z., Duvert, V., Sacland, S. & Martinez-Valdez, H. (1994). A surrogate 15 kDa JCκ protein is expressed in combination with μ heavy chain by human B cell precursors. *EMBO Journal* **13**, 5937-5943.

Fukuda, Y. & Kusuda, R. (1982). Detection and characterization of precipitating antibody in the serum of immature yellowtail immunised with *Pasteurella piscicida* cells. *Fish Pathology* **17**, 125-127.

Fukuda, Y. & Kusuda, R. (1985). Vaccination of yellowtail against pseudotuberculosis. *Fish Pathology* **15**, 421-425.

Georgopoulou, U., Sire, M. F. & Vernier, J. M. (1985). Immunological demonstration of intestinal absorption of proteins by epithelial cells of the posterior intestinal segment and their intracellular digestion in rainbow trout. Ultrastructural and biochemical study. *Biology of the Cell* **5**, 269-282.

Ghaffari, S. H. & Lobb, C. J. (1989a). Cloning and sequence analysis of channel catfish heavy chain cDNA indicate phylogeny diversity within the IgM immunoglobulin family. *Journal of Immunology* **142**, 1356-1365.

Ghaffari, S. H. & Lobb, C. J. (1989b). Nucleotide sequence of channel catfish heavy-chain cDNA and genomic blot analysis. Implications for the phylogeny of Ig heavy chains. *Journal of Immunology* **143**, 2730-2739.

Ghaffari, S. H. & Lobb, C. J. (1991). Heavy chain variable region gene families evolved early in phylogeny. Ig complexity in fish. *Journal of Immunology* **146**, 1037-1046.

Ghaffari, S. H. & Lobb, C. J. (1992). Organization of immunoglobulin heavy chain constant and joining region genes in the channel catfish. *Molecular Immunology* **29**, 151-159.

Ghaffari, S. H. & Lobb, C. J. (1993). Structure and genomic organization of immunoglobulin light chain in the channel catfish. *Journal of Immunology* **151**, 6900-6912.

Ghaffari, S. H. & Lobb, C. J. (1997). Structure and genomic organization of a second class of immunoglobulin light chain genes in the channel catfish. *Journal of Immunology* **159**, 250-258.

Gilmore, R. D. Jr., Engelking, H. M., Manning, D. S. & Leong, J. C. (1988). Expression in *Escherichia coli* of an epitope of the glycoprotein of infectious haematopoietic necrosis virus protects against challenge. *Bio/Technology* **6**, 295-300.

Graham, S. & Secombes, C. J. (1988). The production of a macrophage-activating factor from rainbow trout *Salmo gairdneri* leucocytes. *Immunology* **65**, 293-297.

Grondel, J. L. & Harmsen, E. G. M. (1984). Phylogeny of interleukins: growth factors produced by leucocytes of the cyprinid fish, *Cyprinus carpio* L. *Immunology* **52**, 477-482.

Gudmundsdottir, B. & Magnadottir, B. (1997). Protection of Atlantic salmon (*Salmo salar* L.) against and experimental infection of *Aeromonas salmonicida* ssp. *achromogenes*. *Fish & Shellfish Immunology* **7**, 55-69.

Hadji-Azimi, I., Schwager, J. & Thiebaud, C. (1982). B-lymphocyte differentiation in *Xenopus laevis* larvae. *Developmental Biology* **90**, 253-258.

Hansen, J. D. & Zapata, A. G. (1998). Lymphocyte development in fish and amphibians. *Immunological Reviews* **166**, 199-220.

Hart, S., Wrathmell, A. B., Doggett, T. A. & Harris, J. E. (1987). An investigation of the biliary and intestinal immunoglobulin and the plasma cell distribution in the gall bladder and liver of the common dogfish,

Scyliorhinus canicula L. *Aquaculture* **67**, 147-155.

Hart, S., Wrathmell, A. B., Harris, J. E. & Grayson, T. H. (1988). Gut immunology in fish: a review. *Developmental and Comparative Immunology* **12**, 453-480.

Hawke, J. P., Plakas, S. M., Minton, R. V., McPhearson, R. M., Snider, T. G. & Guarino, A. M. (1987). Fish pasteurollosis of cultured striped bass (*Morone saxatilis*) in coastal Alabama. *Aquaculture* **65**, 193-204.

Hay, F. (1993). The generation of diversity. In *Immunology* (I. M. Roitt, J. Brostoff & D. K. Male, eds.; third edition). pp. 5.1-5.14. London: Mosby-Year Book Europe Ltd.

He, J., Yin, Z., Xu, G., Gong, Z., Lam, T. J. & Sin, Y. M. (1997). Protection of goldfish against *Ichthyophthirius multifiliis* by immunisation with recombinant vaccine. *Aquaculture* **158**, 1-10.

Heppell, J., Lorenzen, N., Armstrong, N. K., Wu, T., Lorenzen, E., Einer-Jensen, K., Schorr, J. & Davis, H. L. (1998). Development of DNA vaccines for fish: vector design, intramuscular injection and antigen expression using viral haemorrhagic septicaemia virus genes as model. *Fish & Shellfish Immunology* **8**, 271-286.

Hinds-Frey, K. R., Nishikata, H., Litman, R. R. & Litman, G. W. (1993). Somatic variation precedes extensive diversification of germline sequences and combinatorial joining in the evolution of immunoglobulin heavy chain diversity. *Journal of Experimental Medicine* **178**, 815-824.

Hinds, K. R. & Litman, G. W. (1986). Major reorganization of immunoglobulin V_H-segmental elements during vertebrate evolution. *Nature* **320**, 546-549.

Hohman, V. S., Schluter, S. F. & Marchalonis, J. J. (1992). Complete sequence of cDNA clone specifying sandbar shark immunoglobulin light chain: gene organisation and implications for the evolution of light chains. *Proceedings of the National Academy of Sciences, USA* **89**, 276-280.

Honda, A., Kodama, H., Moustafa, M., Yamada, F. Mikami, T. & Izawa, H. (1986). Phagocytic activity of macrophages of rainbow trout against *Vibrio anguillarum* and the opsonizing effect of antibody and complement. *Res. Vet. Sci.* **40**, 238-332.

Hordvik, I., Jacob, A. L. J., Charlemagne, J. & Endresen, C. (1996). Cloning of T-cell receptor β chain cDNAs from Atlantic salmon (*Salmo salar*). *Immunogenetics* **45**, 9-14.

Hordvik, I., Thevarajan, J. Samdal, I., Bastani, N. & Crossoy, B. (1999). Molecular cloning and phylogenetic analysis of the Atlantic salmon immunoglobulin D gene. *Scandinavian Journal of Immunology* **50**, 202-210.

Hordvik, I., Voie, A. M., Glette, J., Male, R. & Endresen, C. (1992). Cloning and sequence analysis of two isotypic IgM heavy chain genes from Atlantic salmon, *Salmo salar* L. *European Journal of Immunology* **27**, 2957-2967.

Iida, H. & Yamamoto, T. (1985). Intracellular transport of horseradish peroxidase in the absorptive cells of goldfish hindgut *in vitro*, with special reference to the cytoplasmic tubules. *Cell & Tissue Research* **240**, 553-560.

Janssen, W. A. & Surgalla, M. J. (1968). Morphology, physiology, and serology of *Pasteurella* species pathogenic for white perch. *Journal of Bacteriology* **96**, 1606-1610.

Joosten, P. H. M., Avilés-Trigueros, M., Sorgeloos, P. & Rombout, J. H. W. M. (1995). Oral vaccination of juvenile carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*) with bioencapsulated *Vibrio*

anguillarum bacterin. *Fish & Shellfish Immunology* **5**, 289-299.

Joosten, P. H. M., Engelsma, M. Y., van der Zee, M. D. & Rombout, J. H. W. M. (1996a). Induction of oral tolerance in carp (*Cyprinus carpio* L.) after feeding protein antigens. *Veterinary Immunology and Immunopathology* **60**, 187-196.

Joosten, P. H. M., Kruijer, W. J. & Rombout, J. H. W. M. (1996b). Anal immunisation of carp and rainbow trout with different fractions of a *Vibrio anguillarum* bacterin. *Fish & Shellfish Immunology* **6**, 541-551.

Joosten, P. H. M., Tiemersma, E., Threels, A., Caumartin-Dhieux, C. & Rombout, J. H. W. M. (1997). Oral vaccination of fish against Vibrio anguillarum using alginate microparticles. *Fish & Shellfish Immunology* **7**, 471-485.

Jósefsson, S. & Tatner, M. F. (1993). Histogenesis of the lymphoid organs in sea bream (*Sparus aurata* L.). *Fish & Shellfish immunology* **3**, 35-49.

Kaastrup, P., Nielson, B., Horlyck, V. & Simonsen, M. (1988). Mixed lymphocyte reactions (MLR) in rainbow trout (*Salmo gairdneri*) sibling. *Developmental and Comparative Immunology* **12**, 801-808.

Kaattari, S. L. (1992). Fish B lymphocytes: Defining their form and function. *Annual Review of Fish Diseases* **2**, 161-180.

Kaattari, S. L., Evans, D. & Klemer, J. (1998). Varied redox forms of teleost IgM: an alternative to isotypic diversity? *Immunological Reviews* **166**, 133-142.

Kaattari, S. L. & Piganelli, J. D. (1996). The specific immune system: humoral defense. In *The Fish Immune System: Organism, Pathogen and Environment* (G. Iwama & T. Nakanishi, eds.) pp. 207-254. San Diego: Academic Press.

Kawahara, E., Fukuda, Y. & Kusuda, R. (1998). Serological differences among *Photobacterium damselae* subsp. *piscicida* isolates. *Fish Pathology* **33**, 281-285.

Kawahara, E. & Kusuda, R. (1987). Location of *Pasteurella piscicida* antigens in tissues of yellowtail *Seriola quinqueradiata* vaccinated by immersion. *Nippon Suisan Gakkaishi* **54**, 1101-1105.

Kawakami, H., Shinohara, N., Fukuda, Y., Yamashita, H., Kihara, H. & Sakai, M. (1997). The efficacy of lopopolysaccharide mixed chloroform-killed cell (LPS-CKC) bacterin of *Pasteurella piscicida* on yellowtail, *Seriola quinqueradiata*. *Aquaculture* **154**, 95-105.

Killie, J.-E., Espelid, S. & Jørgensen, T. Ø. (1991). The humoral immune response in Atlantic salmon (*Salmo salar* L.) against the hapten carrier antigen NIP-LPH: the effect of determinant (NIP) density and the isotype profile of anti-NIP antibodies. *Fish & Shellfish Immunology* **1**, 33-46.

Killie, J.-E. & Jørgensen, T. Ø. (1994). Immunoregulation in fish I: intramolecular-induced suppression of antibody responses to haptenated protein antigens studied in Atlantic salmon (*Salmo salar* L). *Developmental and Comparative Immunology* **18**, 123-136.

Killie, J.-E. A. & Jørgensen, T. Ø. (1995). Immunoregulation in fish II: intermolecular-induced suppression of antiody responses studied by haptenated antigens in Atlantic salmon (*Salmo salar* L). *Developmental and Comparative Immunology* **19**, 389-404.

Kim, E.-H. & Aoki, T. (1993). Drug resistance and broad geographical distribution of identical R plasmids of *Pasteurella piscicida* isolated from cultured yellowtail in Japan. *Microbiology and Immunology* **37**, 103-109.

Kim, E.-H., Yoshida, T. & Aoki, T. (1993). Detection of R plasmids encoded with resistance to florfinicol in *Pasteurella piscicida*. *Gyobyo Kenkyu* **28**, 165-170.

Kimura, T. & Kitao, T. (1971). On the causative agent of tuberculosis of yellowtail. *Fish Pathology* **6**, 8-14.

Kitao, T. (1993). Pasteurellosis. In *Bacterial diseases of fish* (V. Inglis, R. J. Roberts and N. R. Bromage, eds.) pp. 159-165. Blackwell Scientific Publications.

Kitao, T. & Kimura, M. (1974). Rapid diagnosis of pseudotuberculosis in yellowtail by means of the fluorescent antibody technique. *Bulletin of the Japanese Society of Scientific Fisheries* **40**, 889-893.

Kitao, T., Yoshida, T., Kusuda, R., Matsuoka, T., Nakano, S., Okada, M. & Ooshima, T. (1992). In vitro antibacterial activity of bicozamycin against *Pasteurella piscicida*. *Gyobyo Kenkyu* **27**, 109-113.

Koike, Y., Kuwahara, A., Fujiwara, H. (1975). Characterisation of *Pasteurella piscicida* isolated from white perch and cultivated yellowtail. *Japanese Journal of Microbiology* **19**, 241-247.

Kokubu, F., Hinds, K. Litman, R., Shamblott, M. J. & Litman, G. W. (1988a). Complete structure and organisation of immunoglobulin heavy chain constant region genes in a phylogenetically primitive vertebrate. *The EMBO Journal* **7**, 1979-1988.

Kokubu, F., Hinds, K. Litman, R., Shamblott, M. J. & Litman, G. W. (1988b). Diverse organisation of immunoglobulin V_H gene loci in a primitive vertebrate. *The EMBO Journal* **7**, 3413-3422.

Koumans-van Diepen, J. C. E., Egberts, E., Peixoto, B.R., Taverne, N. & Rombout, J. H. W. M. (1995) B cell and immunoglobulin heterogeneity in carp (*Cyprinus carpio* L.); an immuno(cyto)chemical study. *Developmental & Comparative Immunology* 19, 97-108.

Koumans-van Diepen, J. C. E., Taverne-Thiele, J. J., van Rens, B. T. T. M. & Rombout, J. H. W. M. (1994). Immunocytochemical and flow cytometric analysis of B cells and plasma cells in carp (*Cyprinus carpio* L.); an ontogenetic study. *Fish & Shellfish Immunology* **4**, 19-28.

Kubota, S. S, Kimura, M., Egusa, S. (1970). Studies of a bacterial tuberculosis of the yellowtail. I. Symptomatology and histopathology. *Fish Pathology* **4**, 111-118.

Kusuda, R. & Hamaguchi, M. (1988). The efficacy of attenuated live bacterin of *Pasteurella piscicida* against pseudotuberculosis in yellowtail. *Bulletin of the European Association of Fish Pathologists* **8**, 50-52.

Kusuda, R., Itaoka, M. & Kawai, K. (1988a). Drug sensitivity of *Pasteurella piscicida* isolated from cultured yellowtail from 1984 to 1985. *Nippon Suisan Gakkaishi* **54**, 1521-1526.

Kusuda, R., Kawai, K. & Masui, T. (1978). Etiological studies on bacterial pseudotuberculosis in cultured yellowtail with *Pasteurella piscicida* as causative agent. II. On the serological properties. *Fish Pathology* **23**, 191-196.

Kusuda, R., Kawai, K. & Itami, T. (1980). Efficacy of bath immunization against vibriosis in cultured ayu. *Bulletin of the Japanese Society of Scientific Fisheries* **46**, 1053.

Kusuda, R., Ninomiya, M., Hamaguchi, M. & Muraoka, A. (1988b). The efficacy of ribosomal vaccine prepared from *Pasteurella piscicida* against pseudotuberculosis in cultured yellowtail. *Fish Pathology* **23**, 191-196.

Kusuda, R. & Salati, F. (1993). Major bacterial diseases affecting mariculture in Japan. *Annual Review of Fish Diseases* **3**, 69-85.

Kusuda, R., Sugiura, H. & Kawai, K. (1990). Drug sensitivity of *Pasteurella piscicida* isolated from cultured yellowtail from 1986 to 1988. *Nippon Suisan Gakkaishi* **56**, 239-242.

Kusuda, R. & Yamaoka, M. (1972). Etiological studies on bacterial pseudotuberculosis in cultured yellowtail with *Pasteurella piscicida* as causative agent. I. On the morphology and biochemical properties. *Bulletin of the Japanese Society of Scientific Fisheries* **38**, 1325-1332.

Lamers, C. H. J., de Haas, M. H. J. & van Muiswinkel, W. B. (1985). Humoral response and memory formation in carp after injection of Aeromonas hydrophila bacterin. *Development and Comparative Immunology* **9**, 65-75.

Landreth, K. S. & Kincade, P. W. (1984). Mammalian B lymphocyte precursors. *Development and Comparative Immunology* **8**, 773-790.

Lanzavecchia, A. (1990). Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annual Review of Immunology* **8**, 773-793.

Lecocq-Xhonneux, F., Thiry, M., Dheur, I., Rossius, M., Vanderheijden, N., Martial, J. & de Kinkelin, P. (1994). A recombinant viral haemorrhagic septicaemia virus glycoprotein expressed in insect cells induces protective immunity in rainbow trout. *Journal of General Virology* **75**, 1579-1587.

Lee, M. A., Bengtén, E., Daggfeldt, A., Rytting, A.-S. & Pilström, L. (1993). Characterization of rainbow trout cDNAs encoding a secreted and membrane-bound Ig heavy chain and the genomic intron upstream of the first constant exon. *Molecular Immunology* **30**, 641-648.

Lem, A. & Shehadeh, Z. H. (1997). International trade in aquaculture products. *FAO Aquaculture Newsletter* **17**, 3-13.

Leong, J. C., Anderson, E., Bootland, L. M. Chiou, P.-W., Johnson, M., Kim, C., Mourich, D. & Trobridge, G. (1997). Fish vaccine antigens produced or delivered by recombinant DNA technologies. In *Fish Vaccinology* (R. Gudding, A. Lillehaug, P. J. Midtlyng & F. Brown, eds.) pp. 267-277. Basel (Switzerland): Karger AG.

Lillehaug, A. (1989). A cost-effectiveness study of three different methods of vaccination against vibriosis in salmonids. *Aquaculture* **83**, 227-236.

Lobb, C. J. (1987). Secretory immunity induced in catfish, Ictalurus punctatus, following bath immunization. *Developmental and Comparative Immunology* **11**, 727-738.

Lobb, C. J. & Clem, L. W. (1981a). Phylogeny of immunoglobulin structure and function. X. Humoral immunoglobulins of the sheepshead, *Archosargus probatocephalus*. *Developmental and Comparative Immunology* **5**, 271-282.

Lobb, C. J. & Clem, L. W. (1981b). Phylogeny of immunoglobulin structure and function. XI. Secretory immunoglobulin in the cutaneous mucus of the sheepshead. *Developmental and Comparative Immunology* **5**, 587-596.

Lobb, C. J. & Clem, L. W. (1981c). Phylogeny of immunoglobulin structure and function. XII. Secretory immunoglobulin in the bile of the marine teleost *Archosargus probatocephalus*. *Molecular Immunology* **18**, 615-619.

Lobb, C. J. & Clem, L. W. (1981d). The metabolic relationships of the immunoglobulins in fish serum, cutaneous mucus and bile. *Journal of Immunology* **127**, 1525-1529.

Lobb, C.J. & Clem, L. W. (1983). Distinctive subpopulations of catfish serum antibody and immunoglobulin. *Molecular Immunology* **20**, 811-818.

Lobb, C.J. & Olson, M. O. J. (1988). Immunoglobulin heavy H chain isotypes in a teleost fish. *Journal of*

Immunology **141**, 1236-1245.

Loghothetis, P. N. & Austin, B. (1994). Immune response of rainbow trout (*Oncorhynchus mykiss*, Walbaum) to *Aeromonas hydrophila*. *Fish & Shellfish Immunology* **4**, 239-254.

Lorenzen, N. (1999). Recombinant vaccines: experimental and applied aspects. *Fish & Shellfish Immunology* **9**, 361-365.

Lorenzen, N., Lorenzen, E., Einer-Jensen, K., Heppel, J., Wu, T. & Davis, H. (1998). Protective immunity to VHS in rainbow trout (Oncorhynchus mykiss, Walbaum) following DNA vaccination. *Fish & Shellfish Immunology* **8**, 261-270.

Lorenzen, N. & Olesen, N. J. (1997). Immunization with viral haemorhagic septicaemia antigens. In *Fish Vaccinology* (R. Gudding, A. Lillehaug, P. J. Midtlyng & F. Brown, eds.) pp. 201-209. Basel (Switzerland): Karger AG.

Luft, J. C., Clem, L. W. & Bly, J. E. (1994). *In vitro* mitogen-induced and MLR-induced responses of leucocytes from the spotted gar, a holostean fish. *Fish & Shellfish Immunology* **4**, 153-156.

Lumsden, J. S., Ostland, V. E., Byrne, P. J. & Ferguson, H. W. (1993). Detection of a distinct gill-surface antibody response following horizontal infection and bath challenge of brook trout *Salvelinus fontinalis* with *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease. *Diseases of Aquatic Organisms* **16**, 21-27.

Lumsden, J. S., Ostland, V. E., MacPhee, D. D. & Ferguson, H. W. (1995). Production of gill-associated and serum antibody by rainbow trout (*Oncorhynchus mykiss*) following immersion immunization with acetone-killed *Flavobacterium branchiophilum* and the relationship to protection from experimental challenges. *Fish & Shellfish Immunology* **5**, 151-165.

Magariños, B., Pazos, F., Santos, Y., Romalde, J. L. & Toranzo, A. E. (1995b). Response of *Pasteurella piscicida* and Flexibacter maritimus to skin mucuc of marine fish. *Diseases of Aquatic Organisms* **21**, 103-108.

Magariños, B., Romalde, J. L., Bandín, I., Fouz, B. & Toranzo, A. E. (1992a). Phenotypic, antigenic, and molecular characterisation of *Pasteurella piscicida* strains isolated from fish. *Applied and Environmental Microbiology* **58**, 3316-3322.

Magariños, B., Romalde, J. L., Barja, J. L. & Toranzo, A. E. (1994a). Evidence of a dormant but infective state of the fish pathogen *Pasteurella piscicida* in seawater and sediment. *Applied and Environmental Microbiology* **60**, 180-186.

Magariños, B., Romalde, J. L., Lemos, M. L., Barja, J. L. & Toranzo, A. E. (1994b). Iron uptake by Pasteurella piscicida and its role in pathogenicity for fish. *Applied and Environmental Microbiology* **60**, 2990-2998.

Magariños, B., Romalde, J. L., Noya, M., Barja, J. L. & Toranzo, A. E. (1996b). Adherence and invasive capacities of the fish pathogen *Pasteurella piscicida*. *FEMS Microbiological Letters* **138**, 29-34.

Magariños, B., Romalde, J. L., Santos, Y., Casal, J. F., Barja, J. L. & Toranzo, A. E. (1994c). Vaccination trials on gilthead seabream (*Sparus aurata*) against *Pasteurella piscicida*. *Aquaculture* **120**, 201-208.

Magariños, B., Santos, Y., Romalde, J. L., Rivas, C., Barja, J. L. & Toranzo, A. E. (1992b). Pathogenic activities of live cells and extracellular products of the fish pathogen *Pasteurella piscicida*. *Journal of General Microbiology* **138**, 2491-2498.

Magariños, B., Toranzo, A. E. & Romalde, J. L. (1995a). Different susceptibility of gilthead seabream and turbot to *Pasteurella piscicida* infection by the water route. *Bulletin of the European Association of Fish Pathologists* **15**, 88-90.

Magariños, B., Toranzo, A. E. & Romalde, J. L. (1996a). Phenotypic and pathobiological characteristics of *Pasteurella piscicida*. *Annual Review of Fish Diseases* **6**, 41-64.

Mäkelä, O. & Litman, G. W. (1980). Lack of heterogeneity in anti-hapten antibodies of a phylogenetically primitive vertebrate shark. *Nature* **287**, 639-640.

Manning, M. J., Grace, M. F. & Secombes, C. J. (1982a). Developmental aspects of immunity and tolerance in fish. In *Microbial Diseases of Fish* (R. J. Roberts, ed.) pp. 31-46. Academic Press, London.

Manning, M. J., Grace, M. F. & Secombes, C. J. (1982b). Ontogenetic aspects of tolerance and immunity in carp and rainbow trout: studies on the role of thymus. *Developmental and Comparative Immunology* **supplement 2**, 75-82.

Manning, M. J. & Mughal, M. S. (1985). Factors affecting the immune responses of immature fish. In *Fish and Shellfish Pathology* (A. E. Ellis, ed.) pp. 27-40. Academic Press, London.

Manning, M. J. & Nakanishi, T. (1996). The specific immune system: cellular defenses. In *"The Fish Immune System; Organism, Pathogen and Environment"* (Iwama, G. & Nakanishi, T., eds.) pp. 159-205. San Diego: Academic Press.

Marchalonis, J. J., Hohman, V. S. & Schluter, S. F. (1993). Antibodies of sharks. Novel methods of generation diversity. *The Immunologist* **1**, 115-120.

Marchalonis, J. J., Schluter, S. F., Bernstein, R. M. & Hohman, V. S. (1998a). Antibodies of sharks: revolution and evolution. *Immunological Reviews* **166**, 103-122.

Marchalonis, J. J., Schluter, S. F., Bernstein, R. M., Shen, S. & Edmundson, A. B. (1998b). Phylogenetic emergence and molecular evolution of the immunoglobulin family. *Advances in Immunology* **70**, 417-506.

Markestad, A. & Grave, K. (1997). Reduction of antibacterial drug use in Norwegian fish farming due to vaccination. In *Fish Vaccinology* (R. Gudding, A. Lillehaug, P. J. Midtlyng & F. Brown, eds.) pp. 365-369. Basel (Switzerland): Karger AG.

Mazzolini, E., Fabris, A., Ceschia G., Vismara, D., Magni, A., Amadei, A., Passera, A., Danielis, L. & Giorgetti, G. (1998). Pathogenic variability of *Pasteurella piscicida* during *in vitro* cultivation as a preliminary study for vaccine production. *Journal of Applied Ichthyology* **14**, 265-268.

McLean, E. & Donaldson, E. (1990). Absorption of bioactive peptides by the gastrointestinal tract of fish: a review. *Journal of Aquatic Animal Health* 2, 1-11.

Meyer, F. P. (1991). Aquaculture disease and health management. *Journal of Animal Sciences* **69**, 4201-4208.

Miller, N. W., (1998). Leukocytes and their markers. In *Handbook of Vertebrate Immunology* (Pastoret, P.-P., Griebel, P., Bazin, H. & Govaerts, A., eds.) pp. 6-9, London: Academic Press.

Miller, N. W., Bly, J. E., van Ginkel, F., Ellsaesser, C. F. & Clem, L. W. (1987). Phylogeny of lymphocyte heterogeneity: identification and separation of functionally distinct subpopulations of channel catfish lymphocytes with monoclonal antibodies. *Developmental and Comparative Immunology* **11**, 739-747.

Miller, N. W., Deuter, A. & Clem, L. W. (1986). Phylogeny of lymphocyte heterogeneity: the cellular requirement for the mixed leucocyte reaction in channel catfish. *Immunology* **59**, 123-128.

Miller, N. W., Rycyzyn, M., Wilson, M., Warr, G. W., Naftel, J. P. & Clem, L. W. (1994). Development and characterization of channel catfish long term B cell lines. *Journal of Immunology* **152**, 2180-2189.

Miller, N. W., Sizemore, R. C. & Clem, L. W. (1985). Phylogeny of lymphocyte heterogeneity: the cellular requirement for *in vitro* antibody responses of channel catfish leucocytes. *Journal of Immunology* **134**, 2884-2888.

Miller, N., Wilson, M., Bengtén, E., Stuge, T., Warr, G. & Clem, W. (1998). Functional and molecular characterization of teleost leukocytes. *Immunological Reviews* **166**, 187-197.

Moore, J. D., Ototake, M. & Nakanishi, T. (1998). Particulate antigen uptake during immersion immunisation of fish: the effectiveness of prolonged exposure and the roles of skin and gill. *Fish & Shellfish Immunology* **8**, 393-407.

Mori, S., Kitao, T. & Kimura, M. (1976). A field survey by means of the direct fluorescent antibody technique for diagnosis of pseudotuberculosis in yellowtail. *Fish Pathology* **11**, 11-16.

Nakanishi, T. & Ototake, M. (1997). Antigen uptake and immune responses after immersion vaccination. In *Fish Vaccinology* (R. Gudding, A. Lillehaug, P. J. Midtlyng and F. Brown, Eds.) pp. 59-68. Developments in Biological Standardization. Basel, Karger.

Nakano, S., Aoki, T. & Kitao, T. (1989). In vitro antimicrobial activity of pyridonecarboxilic acid against fish pathogens. *Journal of Aquatic Animal Health* **1**, 43-50.

Nakao, M., Moritomo, T., Tomana, M., Fujiki, K. & Yano, T. (1998). Isolation of cDNA encoding the constant region of the immunoglobulin heavy-chain from common carp (*Cyprinus carpio* L.). *Fish & Shellfish Immunology* **8**, 425-434.

Nash, C. E. (1995). Salmon farming then and now. *World Aquaculture* **26**(2), 4-10.

Navarro, V., Quesada, J. A., Abad, M. E., Taverne, N. & Rombout, J. H. W. M. (1993). Immuno(cyto)chemical characterisation of monoclonal antibodies to gilthead seabream *Sparus aurata* immunoglobulin. *Fish & Shellfish Immunology* **3**, 167-177.

Ninomiya, M., Muraoka, A. & Kusuda, R. (1989). Effect of immersion vaccination of cultured yellowtail with a ribosomal vaccine prepared from *Pasteurella piscicida*. *Nippon Suisan Gakkaishi* **55**, 1773-1776.

Nomura, J. & Aoki, T. (1985). Morphological analysis of lipopolysaccharide from Gram negative fish pathogenic bacteria. *Fish Pathology* **20**, 193-197.

Noonan, B., Enzmann, P. J. & Trust, T. J. (1995). Recombinant infectious hematopoietic necrosis virus and viral hemorrhagic septicemia virus glycoprotein epitopes expresses in *Aeromonas salmonicida* induce protective immunity in rainbow trout (*Oncorhynchus mykiss*). *Applied and Environmental Microbiology* **61**, 3586-3591.

Noya, M., Magariños, B. & Lamas, J. (1995b). Interactions between peritoneal exudadte cells (PECs) of gilthead seabream (*Sparus aurata*) and *Pasteurella piscicida*. A morphological study. *Aquaculture* **131**, 11-21.

Noya, M., Magariños, B., Toranzo, A. E. & Lamas, J. (1995a). Sequential pathology of experimental pasteurollosis in gilthead seabream, *Sparus aurata*. A light and electron microscopic study. *Disease of Aquatic Organisms* **21**, 177-186.

Ohnishi, K., Watanabe, K. & Jo, Y. (1982). *Pasteurella* infection in young black seabream. *Fish Pathology* **16**, 207-210.

Opstelten, D. (1996). B lymphocyte development and transcription regulation *in vivo*. *Advances in Immunology*

63, 197-268.

Osorio, C. R., Collins, M. D., Toranzo, A. E., Barja, J. L. & Romalde, J. L. (1999). 16S rRNA gene sequence analysis of *Photobacterium damseale* and nested PCR method for rapid detection of the causative agent of fish pasteurollosis. *Applied and Environmental Microbiology* **65**, 2942-2946.

Partula, S. (1999). Surface markers of fish T-cells. *Fish & Shellfish Immunology* **9**, 241-257.

Partula, S., de Guerra, A., Fellah, J. S. & Charlemagne, J. (1995). Structure and diversity of T-cell antigen receptor β-chain in a teleost fish. *Journal of Immunology* **155**, 699-706.

Partula, S., de Guerra, A., Fellah, J. S. & Charlemagne, J. (1996a). Structure and diversity of T-cell antigen receptor α–chain in a teleost fish. *Journal of Immunology* **157**, 207-212.

Partula, S., Schwager, J., Timmusk, S., Piltrom, L. & Charlemagne, J. (1996b). A second immunoglobulin light chain isotype in the rainbow trout. *Immunogenetics* **45**, 44-51.

Passer, B. J., Chen, C-L., Miller, N. W. & Cooper, M. D. (1996). Identification of a T cell lineage antigen in catfish. *Developmental and Comparative Immunology* **20**, 441-450.

Picchietti, S., Terribili, F. R., Mastrolia, L., Scapigliati, G. & Abelli, L. (1997). Expression of lymphocyte antigenic determinants in developing gut-associated lymphoid tissue of the sea bass *Dicentrarchus labrax* (L.). *Anatomy and Embryology* **196**, 457-463.

Piganelli, J. D., Zhang, J. A., Christensen, J. M. & Kaattari, S. L. (1994). Enteric coated microspheres as an oral method for antigen delivery to salmonids. *Fish & Shellfish Immunology* **4**, 179-188.

Pilström, L. (1998). Immunoglobulins. In *Handbook of Vertebrate Immunology* (Pastoret, P.-P., Griebel, P., Bazin, H. & Govaerts, A., eds.) pp. 15-23, London: Academic Press.

Pilström, L. & Bengtén, E. (1996). Immunoglobulin in fish- genes, expression and structure. *Fish & Shellfish Immunology* **6**, 243-262.

Pilström, L., Lundqvist, M. L. & Wermenstam, N. E. (1998). The immunoglobulin light chain in poikilothermic vertebrates. *Immunological Reviews* **166**, 123-132.

Pross, H. & Eidinger, D. (1973). In vitro studies of "antigenic competition" II. Reconstitution of the immune defect and the relationship between antigen-induced suppression and non-specific enhancement. *Immunology* **25**, 269-281.

Rast, J. P., Andersson, M. K., Ota, T., Litman, R. T., Margittai, M., Shamblott, M. J. & Litman, G. W. (1994). Immunoglobulin light chain class multiplicity and alternative organizational form in early vertebrate phylogeny. *Immunogenetics* **40**, 83-99.

Rast, J. P., Andersson, M. K., Strong, S. J., Luer, C., Litman, R. T. & Litman, G. W. (1997). α, β, γ and δ T cell antigen receptor genes arose early in vertebrate phylogeny. *Immunity* **6**, 1-11.

Rast, J. P. & Litman, G. W. (1994). T-cell receptor gene homologs are present in the most primitive jawed vertebrates. *Proceeding of the National Academy of Sciences, USA* **91**, 9248-9252.

Rast, J. P., Haire, R. N., Litman, R. T., Pross, S. & Litman, G. W. (1995). Identification and characterization of T-cell antigen receptor-related genes in phylogenetically diverse vertebrate species. *Immunogenetics* **42**, 204-212.

Razquin, B. E., Castillo, A., López-Fierro, P., Alvarez, F., Zapata, A. & Villena, A. J. (1990). Ontogeny of IgM-producing cells in the lymphoid organs of rainbow trout, *Salmo gairdneri* Richardson: an immuno- and enzyme-histochemical study. *Journal of Fish Biology* **36**, 159-173.

Rodrigues, P. N. S., Hermsen, T. T., Rombout, J. H. M. W., Egberts, E. & Stet, R. J. M. (1995). Detection of MHC class II transcripts in lymphoid tissues of the common carp (Cyprinus carpio L.). *Developmental and Comparative Immunology* **19**, 483-495.

Romalde, J. L., Le Breton, A., Magariños, B. & Toranzo, A. E. (1995a). Use of BIONOR Aquarapid-Pp kit for the diagnosis of *Pasteurella piscicida* infections. *Bulletin of the European Association of Fish Pathologists* **15**, 64-66.

Romalde, J. L., Magariños, B., Fouz, B., Bandín, I., Núnez, S., & Toranzo, A. E. (1995b). Evaluation of BIONOR mono-kits for rapid detection of bacterial fish pathogens. *Disease of Aquatic Organisms* **21**, 25-34.

Romano, N., Taverne-Thiele, J. J., Fanelli, M., Baldassini, M. R., Abelli, L., Mastrolia, L., van Muiswinkel, W. B. & Rombout, J. H. M. W. (1999). Ontogeny of the thymus in a teleost fish, *Cyprinus carpio* L.: developing thymocytes in the epithelial microenvironment. *Developmental and Comparative Immunology* **23**, 123-137.

Romano, N., Taverne-Thiele, J. J., van Maanen, J. C. & Rombout, J. H. M. W. (1997b). Leucocyte subpopulations in developing carp (*Cyprinus carpio* L.): immunocytochemical studies. *Fish & Shellfish Immunology* **7**, 439-453.

Rombout, J. H. W. M., Blok, L. J., Lamers, C. H. J. & Egberts, E. (1986). Immunisation of carp (*Cyprinus carpio*) with a Vibrio anguillarum bacterin: indications for a common mucosal immune system. *Developmental and Comparative Immunology* **10**, 341-351.

Rombout, J. H. W. M. & Joosten, P. H. M. (1998). Mucosal immunity. In *Handbook of Vertebrate Immunology* (Pastoret, P.-P., Griebel, P., Bazin, H. & Govaerts, A., eds.) pp. 39-40, London: Academic Press.

Rombout, J. H. W. M., Joosten, P. H. M., Engelsma, M. Y., Vos, A. P., Taverne, N. & Taverne-Thiele, J. J. (1998). Indications for a distinct putative T cell population in mucosal tissue of carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology* **22**, 63-77.

Rombout, J.H.W.M., Lamers, C. H. J., Hellfrich, M. H., Dekkee, A. & Taverne-Thiele, J. J. (1985). Uptake and transport of intact macromolecules in the intestinal epithelium of the carp (*Cyprinus carpio*) and the possible immunological implications. *Cell & Tissue Research* **239**, 519-530.

Rombout, J.H.W.M., Taverne, N., van de Kamp, M. & Taverne-Thiele, A. J. (1993a). Differences in mucus and serum immunoglobulin of carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology* **17**, 309-317.

Rombout, J.H.W.M., Taverne-Thiele, A.J &. Villena, M. I. (1993b). The gut-associated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): an immunohistochemical analysis. *Developmental and Comparative Immunology* **17**, 55-66.

Rombout, J. H. W. M., van den Berg, A. A. (1989). Immunological importance of the second gut segment of carp. I. Uptake and processing of antigens by epithelial cells and macrophages. *Journal of Fish Biology* **31**, 13-18.

Rombout, J. H. W. M., van de Wal, J. W., Companjen, A., Taverne, N. & Taverne-Thiele, J. J. (1997). Characterization of a T cell lineage marker in carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology* **21**, 35-46.

Ross, D. A., Wilson, M. R., Miller, N. W., Clem, L. W. & Warr, G. W. (1998). Evolutionary variation of

immunoglobulin μ heavy chain RNA processing pathways: origins, effects, and implications. *Immunological Reviews* **166**, 143-151.

Russell, W. J., Voss, E. W. & Sigel, M. M. (1970). Some characteristics of anti-dinitrophenyl antibody of the gray snapper. *Journal of Immunology* **105**, 262-264.

Sakai, D. K. (1992). Repertoire of complement in immunological defense mechanisms of fish. *Annual Review of Fish Diseases* **2**, 223-247.

Sakai, M., Atsuta, S., Kobayashi, M., Kawakami, H. & Yoshida, T. (1993). The cell surface hydrophobicity and hemaglutinating properties of the bacterial fish pathogen, *Pasteurella piscicida*. *Bulletin of the European Association of Fish Pathologists* **13**, 168-170.

Sánchez, C. & Dominguez, J. (1991). Trout immunoglobulin populations differing in light chains revealed by monoclonal antibodies. *Molecular Immunology* **11**, 1271-1277.

Sano, M., Nakano, H., Kimura, T. & Kusuda, R. (1994). Therapeutic effect of fosfomicin on experimentally induced pseudotuberculosis in yellowtail. *Fish Pahtology* **29**, 187-192.

Santos, Y., Romalde, J. L., Bandín, I., Magariños, B., Núnez, S., Barja, J. L. & Toranzo, A. E. (1993). Usefulness of the API-20E system for the identification of bacterial fish pathogens. *Aquaculture* **116**, 111-120.

Scapigliati, G., Mazzini, M., Mastrolia, L., Romano, N. & Abelli, L. (1995). Production and characterisation of a monoclonal antibody against the thymocytes of the sea bass *Dicentrarchus labrax* (L.) (Teleostea, Percicthydae). *Fish & Shellfish Immunology* **5**, 393-405.

Scapigliati, G., Romano, N., Abelli, L., Meloni, S., Ficca, A. G., Buonocore, F., Bird, S. & Secombes, C. J. (2000). Immunopurification of T-cells from sea bass *Dicentrarchus labrax* (L.). *Fish & Shellfish Immunology* **10**, ???-???.

Secombes, C. J. & Fletcher, T. C. (1992). The role of phagocytes in the protective mechanism of fish. *Annual Review of Fish Diseases* **2**, 53-71.

Secombes, C. J., van Groningen, J. M., van Muiswinkel, W. B. & Egberts, E. (1983). Ontogeny of the immune system in carp (*Cyprinus carpio* L.). The appearance of antigenic determinants on lymphoid cells detected by mouse anti-carp thymocyte monoclonal antibodies. *Development and Comparative Immunology* **7**, 455-464.

Shamblott, M. J. & Litman, G. W. (1989). Genomic organization and sequences of immunoglobulin light chain genes in a primitive vertebrate suggest coevolution of immunoglobulin gene organization. *The EMBO Journal* **8**, 3733-3739.

Shelton, E. & Smith, M. (1970). The ultrastructure of carp (Cyprinus carpio) immunoglobulin: a tetrameric macroglobulin. *Journal of Molecular Biology* **54**, 615-617.

Sizemore, R. C., Miller, N. W., Cuchens, M. A., Lobb, C. J. & Clem, L. W. (1984). Phylogeny of lymphocyte heterogeneity: the cellular requirements for *in vitro* mitogenic response of channel catfish leukocytes. *Journal of Immunology* **133**, 2920-2924.

Sniezsko, S. F., Bullock, G. L., Hollis, E. & Boone, J. G. (1964). *Pasteurella* sp. from an epizootic of white perch (*Roccus americanus*) in Chesapeak Bay tidewater areas. *Journal of Bacteriology* **88**, 1814-1815.

Stet, R. J. M., Dixon, B., van Erp, S. H. M., van Lierop, M-J. C., Rodrigues, P. N. S. & Egberts, E. (1996). Interference of structure and function of fish Major Histocompatibility Complex (MHC) molecules

from expressed genes. *Fish & Shellfish Immunology* **6**, 305-318.

St. Louis-Cormier, E. A., Osterland, C. K: & Anderson, P. D. (1984). Evidence for a cutaneous secretory immune system in rainbow trout (Salmo gairdneri). *Developmental and Comparative Immunology* **8**, 71-80.

Stolen, J. S. & Makela, O. (1976). Cellcollaboration in marine fish: the effect of carrier preimmunization on the anti-hapten response to NIP and NNP. In *Phylogeny of thymus and bone marrow-bursa cells* (R. K. Wright and E. L. Cooper, eds.), pp 93-97. Elsevier/North Holland, Amsterdam.

Stroband, H. W. J. & Kroon, A. G. (1981a). The development of the stomach in *Carias lazera* and the intestinal absorption of protein macromolecules. *Cell & Tissue Research* **215**, 397-425.

Stroband, H. W. J. & Van der Veen, F. H. (1981b). The localization of protein absorption during the trasport of food along the intestine of the grass carp, *Ctenopharyngodon idella* (val.). *Histochemistry* **215**, 397-425.

Tacon, A. G. J. (1997). Importance of aquaculture to global food fish supplies. *FAO Aquaculture Newsletter* **17**, 2.

Takashima, N., Aoki, T. & Kitao, T. (1985). Epidemiological surveillance of drug-resistant strains of *Pasteurella piscicida*. *Fish Pathology* **20**, 209-217.

Tatner, M. F. (1985). The migration of labelled thymocytes to the peripheral lymphoid organs in the rainbow trout, *Salmo gairdneri* Richardson. *Developmental and Comparative Immunology* **9**, 85-91.

Tatner, M. F. (1987). The quantitative relationship between vaccine dilution, length of immersion time, and antigen uptake, using radiolabelled *Aeromonas salmonicida* bath in direct immersion experiments with rainbow trout, *Salmo gairdneri*. *Aquaculture* **62**, 173-185.

Tatner, M. F. (1996). Natural changes in the immune system of fish. In "*The Fish Immune System; Organism, Pathogen and Environment*" (Iwama, G. & Nakanishi, T., eds.) pp. 255-287. San Diego: Academic Press.

Tatner, M. F. & Horne, M. T. (1984). The effects of early exposure to *Vibrio anguillarum* vaccine on the immune response of the fry of rainbow trout, *Salmo gairdneri* Richardson. *Aquaculture* **41**, 193-202.

Tatner, M. F. & Horne, M. T. (1985). The effects of vaccine dilution, length of immersion time, and booster vaccinations on the protection levels induced by direct immersion vaccination of brown trout, *Salmo trutta*, with *Yersinia ruckeri* (ERM) vaccine. *Aquaculture* **46**, 11-18.

Tomana, M., Nakao, M., Moritomo, T., Fujiki, K. & Yano, T. (1999). Isolation of cDNA encoding immunoglobulin light chain from common carp (*Cyprinus carpio* L.). *Fish & Shell fish Immunology* **9**, 71-80.

Toranzo, A. E., Barja, J. L., Colwell, R. R. & Hetrick, F. M. (1983). Characterization of plasmids in bacterial fish pathogens. *Infection and Immunity* **39**, 184-192.

Toranzo, A. E., Barja, J. L. & Hetrick, F. M. (1982). Survival of *Vibrio anguillarum* and *Pasteurella piscicida* in estuarine and fresh waters. *Bulletin of the European Association of Fish Pathologists* **3**, 43-45.

Toranzo, A. E., Barreiro, S., Casal, J. F., Figueras, A., Magariños, B. & Barja, J. L. (1991). Pasteurollosis in cultured gilthead seabream (*Sparus aurata*): first report in Spain. *Aquaculture* **99**, 1-15.

Traavik, T. (1997). Environmental issues of recombinant vaccines. In *Fish Vaccinology* (R. Gudding, A. Lillehaug, P. J. Midtlyng & F. Brown, eds.) p. 381. Basel (Switzerland): Karger AG.

Tung, M.-C., Tsai, S.-S., Ho, L.-F., Huang, S. T. & Chen, S. C. (1985). An acute septicemic infection of *Pasteurella* organism in pond-cultured Formosa snake-head fish (*Channa maculata* Lacepeda) in Taiwan. *Fish Pathology* **20**, 143-148.

Ueki, N., Kayano, Y. & Muroga, K. (1990). *Pasteurella piscicida* infection in juvenile red grouper (*Epinephelus akaara*). *Fish Pathology* **25**, 43-44.

Vallejo, A. N., Miller, N. W. & Clem, L. W. (1991a). Phylogeny of immune recognition: Processing and presentation of structurally-defined proteins in channel catfish immune responses. *Developmental Immunology* **1**, 137-148.

Vallejo, A. N., Miller, N. W. & Clem, L. W. (1991b). Phylogeny of immune recognition: Role of alloantigens in antigen presentation in channel catfish immune responses. *Immunology* **74**, 165-168.

Vallejo, A. N., Miller, N. W. & Clem, L. W. (1992a). Antigen processing and presentation in teleost immune responses. *Annual Review of Fish Diseases* **2**, 73-89.

Vallejo, A. N., Miller, N. W. & Clem, L. W. (1992b). Cellular pathway(s) of antigen processing and presentation in fish APC: Effect of varying *in vitro* temperatures on antigen catabolism. *Developmental and Comparative Immunology* **16**, 367-381.

Vallejo, A. N., Miller, N. W., Harvey, N. E., Cuchens, M. A., Warr, G. W., & Clem, L. W. (1993). Cellular pathways of antigen processing and presentation in fish APC: endosomal involvement and cell-free antigen presentation. *Developmental Immunology* **3**, 51-65.

Vallejo, A. N., Miller, N. W., Jorgensen, T. & Clem, L. W. (1990). Phylogeny of immune recognition: Antigen processing/presentation in channel catfish immune responses to hemocyanins. *Cellular Immunology* **130**, 362-377.

van der Heijden, M. H. T., Rooijakkers, J. B. M. A., Booms, G. H. R., Rombout, J. H. W. M. & Boon, J. H. (1995). Production, characterisation and applicability of monoclonal antibodies to european eel (Anguilla anguilla L.) immunoglobulin. *Veterinary Immunology and Immunopathology* **45**, 151-164.

van Diepen, J. C. E., Wagenaar, G. T. M. & Rombout, J. H. W. M. (1991). Immunocytochemical detection of membrane antigens of carp leucocytes using light and electron microscopy. *Fish & Shellfish Immunology* **1**, 47-57.

van Muiswinkel, W. B. & Wiegertjes, G. F. (1997). Immune responses after injection vaccination of fish. In *Fish Vaccinology* (R. Gudding, A. Lillehaug, P. J. Midtlyng & F. Brown, eds.) pp. 55-57. Basel (Switzerland): Karger AG.

van Rees, E. P., Dijkstra, C. D. & Sminia, T. (1990). Ontogeny of the rat immune system: an immunocytochemical approach. *Developmental and Comparative Immunology* **14**, 9-18.

Vaughan, L. M., Smith, P. R. & Foster, T. J. (1993). An aromatic-dependent mutant of the fish pathogen *Aeromonas salmonicida* is attenuated in fish and is effective as a live vaccine against the salmonid disease furuncolosis. *Infection and Immunity* **61**, 2172-2181.

Voss, E. W., Groberg, W. J. & Freyer, J. L. (1978). Binding affinity of tetrameric coho salmon Ig anti-hapten antibodies. *Immunochemistry* **15**, 459-464.

Wakabayashi, H., Toyota, H. & Egusa, S. (1977). Artificial infection of yellowtail with gastral administration of cultured *Pasteurella piscicida* cells. *Fish Pathology* **11**, 207-211.

Warr, G. W. (1983). Immunoglobulin on the toadfish, *Spheroids glaber*. *Comparative Biochemistry and*

Physiology **76B**, 507-514.

Wermenstam, N. & Pilstrom, L. (1998). Characterization of the T-cell antigen receptor genes in Atlantic cod (*Gadus morhua* L.). Poster Bp9 at the Nordic Symposium on Fish Immunology, Hirtshals, Denmark.

White, S. K., Chappell, L. H. & Secombes, C. J. (1990). Protection of rainbow trout, *Oncorhynchus mykiss* (Richardson), against *Diplostomum spathaceum* (Dignea): the role of specific antibody and activated macrophages. *Journal of Fish Diseases* **13**, 281-291.

Wiersma, E. J. & Shulman, M. J. (1995). Assembly of IgM. Role of disulfide bonding and noncovalent interaction. *Journal of Immunology* **154**, 5265-5272.

Wilson, M. R., Bengtén, E, Miller, N. W., Clem, L. W., Du Pasquier, L. & Warr, G. W. (1997). A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. Proceedings of the National Academy of Sciences USA **94**, 4593-4597.

Wilson, M. R., Marcus, A., van Ginkel, F., Miller, N. W., Clem, L. W., Middleton, D. & Warr, G. W. (1990). The immunoglobulin M heavy chain constant region of the channel catfish, *Ictalurus punctatus*: an unusual mRNA splice pattern produces the membrane form of the molecule. *Nucleics Acids Research* **18**, 5227-5233.

Wilson, M.R. & Warr, G.W. (1992). Fish immunoglobulins and the genes that encode them. *Annual Review of Fish Diseases* **2**, 201-221.

Wilson, M. R., Zhou, H., Bengtén, E., Clem, L. W., Stuge, T. B., Warr, G. W. & Miller, N. W. (1998). T-cell receptors in channel catfish. Structure and expression of TCR α and β genes. *Molecular Immunology* **35**, 545-557.

Yang, M.-C. W., Miller, N. W., Clem, L. W. & Buttke, T. M. (1989). Unsaturated fatty acids inhibit IL-2 production in thymus-dependent antibody responses *in vivo*. *Immunology* **68**, 181-184.

Yano, T. (1996). The nonspecific immune system: humoral defense. In *The Fish Immune System: Organism, Pathogen and Environment* (G. Iwama & T. Nakanishi, eds.) pp. 105-157. San Diego: Academic Press.

Yasunaga, N., Hatai, K. & Tsukahara, J. (1983). *Pasteurella piscicida* from an epizootic of cultured red sea bream. *Fish Pathology* **18**, 107-110.

Yasunaga, N., Yasumoto, S., Hirakawa, E. & Tsukahara, J. (1984). On a massive mortality of oval fish (*Novodan modestus*) caused by *Pasteurella piscicida*. *Fish Pathology* **19**, 51-55.

Zapata, A. G., Torroba, M., Varas, A. & Jiménez, E. (1997). Immunity in fish larvae. In *Fish Vaccinology* (R. Gudding, A. Lillehaug, P. J. Midtlyng and F. Brown, eds.) pp. 23-32. Developments in Biological Standardization. Basel, Karger.

Zettergren, L. D. (1982). Ontogeny and distribution of cells in B lineage in the American frog, *Rana pipiens*. *Developmental and Comparative Immunology* **6**, 311-320.

Zhang, H. G. & Hanson, L. A. (1996). Recombinant channel catfish virus (Ichtalurid herpsvirus 1) can express foreign genes and induce atibody production against the gene product. *Journal of Fish Diseases* **19**, 121-128.

Zhao, J. & Aoki, T. (1989). A specific DNA hybridization probe for detection of *Pasteurella piscicida*. *Disease of Aquatic Organisms* **7**, 203-210.

Zhao, J. & Aoki, T. (1992). Plasmid profile analysis of Pasteurella piscicida and use of plasmid DNA probe to

identify the species. *Journal of Aquatic Animal Health* **4**, 198-202.

Zhou, H., Bengtén, E., Miller, N. W., Warr, G. W., Clem, L. W. & Wilson, M. R. (1997). T cell receptor sequences in the channel catfish. *Developmental and Comparative Immunology* **21**, 238-

Zorrilla, I., Balebona, M. C., Moriñigo, M. A., Sarasquete, C. & Borrego, J. J. (1999). Isolation and characterization of the causative agent of pasteurellosis, *Photobacterium damselae* ssp. *piscicida*, from sole, *Solea senegalensis* (Kaup). *Journal of Fish Diseases* **22**, 167-172.

CHAPTER 2

Characterisation of monoclonal antibodies specific for sea bass (*Dicentrarchus labrax* L.) IgM indicates the existence of B cell subpopulations

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"Only when you perfectly dominate the technique, you will quit of being its slave and might be able to paint as you wish." - Katsushika Hokusai (1760-1849)

Japanese painter cited by J. M. Parramón (1999) *in* Materiais e Técnicas. Curso Práctico de Pintura a Aguarela. Lema SL, Barcelona, pp. 43.

Abstract

Three monoclonal antibodies (mAb) against sea bass IgM (*Dicentrarchus labrax* L.) were selected (WDI 1-3) based on ELISA, Western blot and FACS analysis. Further characterisation was done by immunofluorescence on cytocentrifuge slides and immuno-electron microscopy. All mAb were found to be of the IgG1/κ isotype and were effective in detecting *Vibrio anguillarum-ordalii* antibody titres with an ELISA. Under denaturing conditions in Western blots, all mAb reacted with a tetrameric, dimeric and monomeric form of serum IgM. Under reducing conditions WDI 1 recognises the heavy (H) chain (±78 kDa) and both WDI 2 (slightly) and WDI 3 (strongly) recognise the light (L) chain (±28 kDa) in Western blots of whole serum and protein A isolated sea bass IgM. The average percentages (n=6) of surface IgM^+ (B) cells in peripheral blood leukocytes (PBL), head kidney, trunk kidney, spleen, thymus and gut for the different MAb were estimated by FACS. The highest was in PBL (33 %) stained with WDI 3 and the lowest in thymus (1%). All mAb showed specific immunogold labelling of a subpopulation of lymphoid cells in both PBL and spleen and of plasma cells and some macrophages in spleen. Additional percentage staining in FACS analysis using different combinations of the three mAb, as well as double immunofluorescence staining of cytocentrifuge preparations and Coomassie blue staining of SDS-PAGE of protein A purified IgM under reduced conditions, strongly suggest the existence of at least two H and L chains expressed in different combinations in at least two subpopulations of B cells in sea bass. From these results it is also concluded that the combination of WDI 1 with WDI 3 stains all or almost all B cells.

Key words: Sea bass, *Dicentrarchus labrax*, monoclonal antibodies, IgM, B cells, plasma cells, *Vibrio anguillarum*.

1. INTRODUCTION

Monoclonal antibodies (mAb) have been extremely useful in studies of fish immunology and, although several mAb to fish IgM (channel catfish: Lobb & Clem, 1982; rainbow trout: DeLuca *et al.*, 1983, Thuvander *et al.*, 1990, Sánchez *et al.*, 1993; carp: Secombes *et al.*, 1983; sea bream: Navarro e*t al.*, 1993; turbot: Estévez *et al.*, 1994; eel: van der Heijden, 1995) are available, it was only recently that mAb against sea bass IgM have been produced (Romestand *et al.*, 1995). However, these authors did not demonstrate the applicability of the mAb to specific immune responses or studies with living cells. The main goal of this work was to produce mAb against sea bass IgM to be used to monitor specific immune responses and characterise leucocyte populations.

2. MATERIALS AND METHODS

2.1. FISH

Sea bass (*Dicentrarchus labrax* L.) of about 220 g were kindly supplied by Eng. José da Costa Canas (Figueira da Foz, Portugal) and kept in two circular tanks in FOZAQUA Sociedade Aquicola, Lda (Figueira da Foz, Portugal). Fish were fed with expanded and pelleted sea bass feed (SORGAL SA, Ovar, Portugal). One group (N=11) was immunised intramuscularly (100 µl at each side of the back) with a commercial *Vibrio anguillarum-ordalii* bacterin (Biovax 1300, Biomed, WA, USA), containing 10^{10} formalin-killed bacteria ml^{-1}, emulsified with Freund's complete adjuvant 1:1 (FCA; Difco, Detroit, MI, USA). Fish received a second immunisation, 37 days after the first, with bacterin emulsified in Freund's incomplete adjuvant (FIA; Difco, Detroit, MI, USA). Twenty days after the first immunisation and 14 days after the second immunisation, blood from anaesthetised (tricaine methanesulfonate, Crescent Research Chemicals) immunised and non-immunised fish was taken from the caudal vein using 2.5 ml heparinized syringes. Blood samples were centrifuged for 15 min at 1100 *g* and 4° C and subsequently plasma was stored at –80° C.

For selection and characterisation of mAb, sea bass weighing 100 g were obtained from a local farm (Seafarm, Goes, The Netherlands) and kept in aquaria at 19 ± 1° C in recirculating, filtered and UV sterilised artificial salt water (35‰) at the Wageningen Agricultural University. Fish were fed with Kordon's golden gate brine shrimp (California, USA).

2.2. PURIFICATION OF SEA BASS PLASMA Ig

To a pool of immunised and non-immunised sea bass plasma, 2% (v/v) of a 10% (w/v) dextran sulphate 500 solution in distilled water (Sigma, St Louis, MO, USA) and 10% (v/v) of 1 M $CaCl_2.2H_2O$, was added. After stirring for 30 min on ice and centrifugation for 15 min at 12000 *g* and 4° C, the supernatant was dialysed (Spectra/Por, MWCO: 6-8000; Spectrum Medical Industries Inc., Houston, TX, USA) overnight against Tris buffered saline (TBS; 20 mM TRIS, 50 mM NaCl; pH 7.6) in a cold room (4° C). The remaining solution was then precipitated with saturated ammonium sulphate (40% v/v) by stirring in the cold room for 4 h. After centrifugation at 12000 *g* for 30 min and 4° C, the supernatant was again precipitated (50% v/v) overnight. The suspension was then centrifuged at 12000 *g* for 30 min at 4° C, the

pellet resuspended in TBS and dialysed overnight as above. The dialysed solution was stored at -80° C before immunisation of mice.

Further purification was done using 6 ml of the above sample, diluted 1:2 in phosphate buffered saline (PBS; 140 mM NaCl, 9 mM $Na_2HPO_4.2H_20$, 1.9 mM $NaH_2PO_4.H_2O$; pH 8.0), in a Protein A affinity chromatography column. Briefly, the sample was filtered in a disposable filter holder (0.2 µm) and run through the Protein A cartridge using a peristaltic pump with a flow rate of 1 ml min^{-1} (Pharmacia, Sweden). The column was then rinsed with PBS and the Ig eluted with 0.1 M glycine-HCl (pH 2.5) with the same flow rate. After collection, the pH of the eluted Ig was neutralised with 1.5 M Tris (pH 10) and the amount measured from the absorbance at 280 nm.

The purity of the samples was checked in a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), using a Mini-Protean II unit (BioRad). The non-purified and saturated ammonium sulphate purified sera were first diluted 1:30 and 1:20 in distilled water, respectively. The samples were then diluted 4:1 in non-reducing and reducing buffer (containing dithiothreitol, DTT, or 2β-mercaptoethanol), boiled for 5 min at 100° C and loaded onto wells of 4% stacking gels and run (100 V) in electrode buffer (24.76 mM Tris, 186.49 mM glycine, 3.47 mM SDS, 0.77 mM NaN_3) in a 5% and 10% SDS-PAGE, respectively. The gels were fixed and stained with Coomassie blue and destained with a solution of 7.1% (v/v) acetic acid and 21.4% (v/v) ethanol in distilled water. High and low molecular weight prestained SDS-PAGE standards (BioRad) were used in the non-reduced and reduced gels, respectively.

2.3. PRODUCTION OF mAb

Balb/C mice were immunised by subcutaneous (sc) injection with 200 µl of saturated ammonium sulphate purified sea bass Ig (SAS-SBIg) emulsified 1:1 (v/v) with FIA. After 3 weeks, serum samples were taken to check antibody titres with a dot blot assay. Five weeks after sc immunisation, mice received an intraperitoneal (ip) injection of 200 µl of SAS-SBIg, without FIA. Three days later splenic lymphocytes were fused with cells of the mouse myeloma cell line SP2/0-Ag-14 using 50% polyethylene glycol 1500. Hybridomas were cultured (10^5 cells per well in 100 µl) in 96-well cell culture cluster dishes (Costar, Cambridge, MA, USA) using AH-medium (RPMI supplemented with 10% v/v fetal calf serum, 1% w/v glutamine, 1% w/v pyruvate, 1% v/v of a penicillin-streptomycin solution, 2%

w/v hypoxanthine and 1% w/v azaserine). The culture medium was changed every 3-5 days and after 14 days the supernatant from the growing hybridomas was screened. For the final antibody producing clones, cell culture flasks (Costar) of 25, 75 and 162 cm^2 were used. The cells were cultured at 37° C using 5% CO_2.

The IgG isotypes of the final selected mAb were verified using a mouse monoclonal antibody isotyping kit (Iso*Strip*; Boehringer Mannheim) following the instructions of the supplier.

2.4. SELECTION AND CHARACTERISATION OF mAb

Screening of hybridomas was first performed by an enzyme-linked immunosorbent assay (ELISA) for production of antibodies of the IgG class, since IgM antibodies have some disadvantages (Schots *et al.*, 1992), followed by flow cytometric (FACS) analysis of peripheral blood leukocytes (PBL) and Western blotting. The chosen clones were then subcloned and stabilised in H-medium (the same as AH-medium but without azaserine) by limiting dilution and screened again using FACS analysis and Western blot in reducing and non-reducing conditions. Additional characterisation was done with immuno-electron microscopy, immunofluorescence on cytocentrifuge slides and ELISA for *V. anguillarum-ordalii*-immunised sea bass plasma.

In all experiments the same batch of a 12 ml culture supernatant from each mAb was used, taken into account the optimal dilution of the culture supernatant in each technique as established in preliminary experiments.

2.5. ELISA

For the selection of mAb of IgG class, flat bottom microtitre plates (Greiner Labortechnik) were coated with 50 µl of goat-anti-mouse IgG (Aurion, Wageningen, The Netherlands), diluted 1:1000 in 50 mM carbonate buffer (15 mM Na_2CO_3, 34.87 mM $NaHCO_3$ and 7.69 mM NaN_3). After 2 h of incubation at 37° C, plates were washed twice for 15 s in tap water containing 0.05% (v/v) Tween 20 (water Tween) and blocked with 200 µl per well of PBS (pH 7.4) containing 0.1% (v/v) Tween 20, 10% (w/v) milk powder and 0.1% (w/v) bovine serum albumin (BSA; Boehringer Mannheim, Indianapolis, USA). After washing twice, the plates were incubated for 2 h at room temperature (RT) with hybridoma culture supernatant (100 µl of each supernatant per well), then washed 3 times and incubated for 2 h at 37° C with

100 µl per well of goat-anti-mouse horseradish peroxidase (GAM-HRP; BioRad, Richmond, CA, USA) diluted 1:2000 in 50 mM carbonate buffer. After several washes, 100 µl of 0.04% (w/v) O-phenylenediamine dihydrochloride (OPD; Sigma, St Louis, MO, USA) and 0.124% (v/v) H_2O_2 in substrate solution (0.1 M citric acid monohydrate and 0.2 M $Na_2HPO_4.2H_2O$; pH 5.0) was added to each well of the plates and incubated for 30 min at RT. The reaction was stopped with 50 µl per well of 2.5 M H_2SO_4 and the absorbance measured with an ELISA reader 2001 (Anthos Labtec Instruments, Salzburg, Austria) at 492 nm.

To measure the specific antibody titres of immunised sea bass, showing the specificity of the selected mAb for the sea bass Ig, flat bottom microtitre plates (Greiner) were coated with 100 µl per well of a *V. anguillarum-ordalii* suspension (10^9 bacterial cells ml^{-1} in distilled water) and dried overnight at 37° C in a circulating-air stove. The plates were washed twice in water Tween and blocked (90 min, RT) with 200 µl per well of PBS (pH 7.4) containing 1% (w/v) milk powder. After washing twice the plates were incubated for 90 min at 37° C with 100 µl of $\log_2$ serial dilutions of the test plasma in PBS. Then, following washing and incubation (90 min, 37° C) with 100 µl per well of different dilutions (1:50, 1:200 and 1:800) of culture supernatant of the mAb, the plates were washed again and incubated for 90 min at 37° C with 100 µl per well of GAM-HRP diluted 1:2000 in PBS. As a control, incubations with only PBS instead of the mAb or GAM-HRP were carried out. Plates were again washed twice before colour development. All steps were carried out in a moist chamber. Using data regression, antigen-specific antibody titres were estimated by calculating dilution at an optical absorbance (OD) of 0.4. An unpaired Student's *t*-test was used to compare the means of immunised and non-immunised fish. Cross-reaction with carp IgM was checked using plasma from *V. anguillarum-ordalli* immunised carp.

2.6. WESTERN BLOT

After SDS-PAGE, the samples (sea bass whole serum, SAS-SBIg and protein A isolated IgM), were electrophoretically transferred (300 mA, 1h 30 min) onto a 0.45 µm pore size nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using 20.8 mM Tris, 160 mM glycine and 16.67% (v/v) methanol in distilled water as running buffer. The nitrocellulose was blocked (overnight; 4° C) with TBS containing 0.05% (v/v) Tween 20 (TTBS) and 1% (w/v) BSA and incubated (RT, 1h) with the mAb undiluted and diluted (1:2 to 1:4 dilutions of culture supernatant) in antibody buffer (TBS containing 0.1% w/v BSA). The strips were then

washed three times for 5 min in TTBS, incubated for 1 h at RT with goat anti-mouse (Ig)-alkaline phosphatase (GAM-AP; BioRad; 1:2000 in antibody buffer), washed three times for 5 min in TTBS and once for 10 min in substrate buffer (100 mM Tris, 100 mM NaCl and 5 mM $MgCl_2$; pH 9.5) and stained for 30 min with substrate solution (substrate buffer containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate). The colour reaction was stopped by washing with distilled water. High and low molecular weight prestained SDS-PAGE standards (BioRad) were used in the non-reduced and reduced gels, respectively. Serum from carp was used as positive control, as well as to give an estimation of the molecular weight (when stained with WCI 12, specific for carp IgM H chain; Rombout *et al.*, 1993), and negative control (when stained with the presented mAb). Strips incubated with TBS containing 0.1% w/v BSA without the mAb or GAM-AP were also used as controls.

2.7. FLOW CYTOMETRY

Blood was collected with a heparinised syringe, diluted 1:4 in sea bass-RPMI (SB-RPMI; RPMI + 42 mM NaCl + 1% w/v BSA + 0.01% v/v of a 1.5 M NaN_3 stock solution) and centrifuged for 15 min at 200 *g* and 4° C to separate the red blood cells. The supernatant was again centrifuged for 10 min at 550 *g* and 4° C, the pellet resuspended in 2-3 ml of SB-RPMI, added carefully onto Lymphoprep (Nycomed Pharma AS, Oslo, Norway; density 1.077 ± 0.001 mgL^{-1}) and centrifuged for 30 min at 1100 *g* and 4° C. Cells at the interface were harvested, washed twice in SB-RPMI (10 min, 550 *g*, 4° C) and counted and diluted to 3×10^6 cells ml^{-1}. The lymphoid cells (7×10^5 cells) were incubated with the optimal dilution (in SB-RPMI) of a mAb culture supernatant for 45 min at 4° C, washed twice with SB-RPMI (10 min, 550 *g*, 4° C) and incubated for 45 min at 4° C with fluorescein isothiocyanate- or R-Phycoerythrin-labelled goat-anti-mouse Ig (GAM-FITC or GAM-RPE; Dako, 1:100 in SB-RPMI). For the controls the same procedure was used but the cells were incubated with SB-RPMI instead of a mAb. After washing as described above, cell suspensions were analysed with a FACStar flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 5 W argon laser, tuned at 488 nm.

Leukocytes from head kidney (HK), trunk kidney (TK), spleen (SP), thymus (THY) and gut were also used. Cell suspensions were made by pressing the organ through a 100 µm mesh gauze using SB-RPMI. The cells were then washed twice for 10 min at 550 *g* and 4° C, the pellet resuspended in 2-3 ml of SB-RPMI and added onto Lymphoprep as described for PBL.

Additional staining, combining different mAb (equal volumes of optimal dilution of the mAb culture supernatant), in PBL, HK and SP was also performed. The same controls as mentioned above were applied.

Carp PBL were also used as control.

2.8. IMMUNOFLUORESCENCE ON CYTROCENTRIFUGE PREPARATIONS

Cell suspensions were obtained from blood, HK and SP as explained for FACS analysis. Cells were centrifuged (5 min, 50 *g*, 4° C) onto 0.01% w/v poly-L-lysine (PLL) coated slides and, after removing the supernatant, centrifuged again (2 min, 50 *g*, 4° C). The slides were dried for 15 min at RT and fixed for 5 min in cold acetone. After rinsing in PBS (pH 7.4) and incubation for 30 min at RT with optimal dilution (in PBS) of each mAb, the slides were washed (2×) and incubated for 30 min with the rabbit anti-mouse (RAM)-FITC (1:50 in PBS). The slides were washed again (2×), incubated for 45 min with non-immune mouse serum, and after rinsing in PBS, incubated again for 30 min with optimal dilution (in PBS) of other mAb in different combinations. After washing (2×), slides were incubated for 30 min with RAM-TRITC (tetramethyl rhodamine isothiocyanate; Dakopatts, Glostrup, Denmark; 1:50 in PBS). Finally, the slides were washed twice again in PBS, embedded in 90% (v/v) PBS and 10% (v/v) glycerine containing 0.1% (w/v) p-phenylenediamine and observed under a fluorescence microscope equipped with phase-contrast (Nikon, Microphot FXA). As controls, slides incubated with PBS instead of one of the mAb were used, as well as slides incubated twice with the same mAb.

2.9. IMMUNO-ELECTRON MICROSCOPY

The same procedure as described above for FACS analysis was carried out on sea bass SP cells and PBL, but with dilutions of the mAb culture supernatants twice as much concentrated. However, in this case the cells were incubated for 1 h on ice with 100 µl undiluted goat anti-mouse Ig coupled to 25 nm gold particles (Aurion, Wageningen, The Netherlands). After washing once and resuspension in SB-RPMI, a pellet was produced by centrifuging the cells in an Eppendorff tube. The supernatant was removed and the cells fixed for 1 h on ice with 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). The cells were washed in cacodylate buffer followed by distilled water, post-fixed for 1 h on ice with 1% OsO_4 and, after washing twice with distilled water, contrasted with 0.5% uranyl acetate (1 h, RT, dark).

The cells were washed twice again, dehydrated in ethanol and propylene oxide, embedded in Epon 812 and ultrathin sections made on a Reichert Ultracut S. Finally the sections were contrasted with lead citrate (1 min) and examined in a Philips 208 electron microscope. The same controls as for flow cytometry were performed.

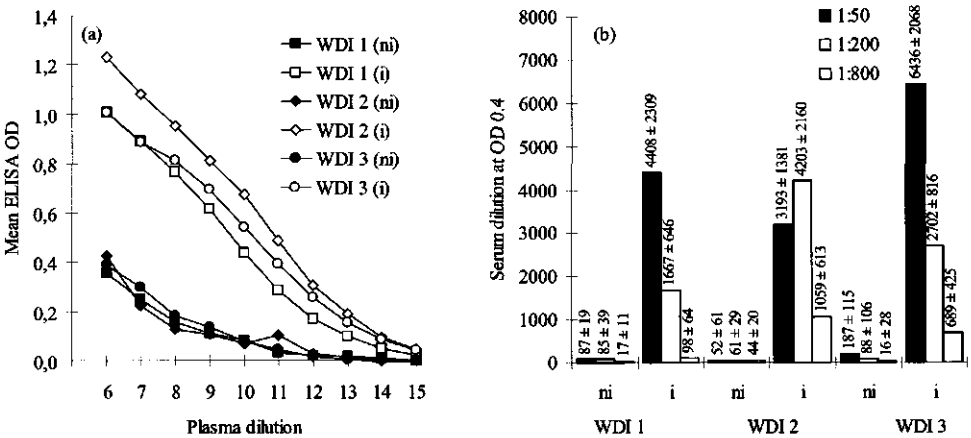

Fig. 1- (a) ELISA titration curve with different mAb when diluted 1:200; ni: non-immunised; i: immunised. (b) Specific antibody titres of V. anguillarum-ordalii immunised and non-immunised sea bass at OD 0.4 with different mAb dilutions (1:50, 1:200 and 1:800); top numbers represent the average of eight fish ± SD.

3. RESULTS

3.1. PRODUCTION AND SELECTION OF mAb

More than 500 clones (> 30% of the total wells) produced antibodies of the IgG class. From these, 380 clones were tested in a FACS analysis and, based on the results, 37 were used for Western blotting under reduced conditions. Finally, after subcloning, the culture supernatant of 3 mAb (WDI 1-3) producing hybridomas were further characterised and found to be of the IgG1/κ isotype.

3.2. *MONOCLONAL CHARACTERISATION*

3.2.1. *ELISA*

All mAb showed positive reaction in ELISA when used at 1:50 to 1:200 dilution of the culture supernatant (Fig. 1). Figure 1 shows the mean specific antibody titres (± SD) for *V. anguillarum-ordalii* immunised (N= 8; secondary response) and non-immunised sea bass (N= 8) at OD of 0.4 and with different mAb dilutions (1:50, 1:200 and 1:800 of the culture supernatant). Antibody titres of non-immunised and immunised sea bass were significantly different (P<0.001 and P<0.0003 for WDI 1, P<0.0004 and P<0.001 for WDI 2 and P<0.00006 and P<0.00005 for WDI 3) when the mAb were used diluted 1:50 and 1:200, respectively. Even when the mAb were diluted 1:800 the two groups showed significant differences (P<0.01 for WDI 1, P<0.05 for WDI 2 and P<0.002 for WDI 3). All controls were negative.

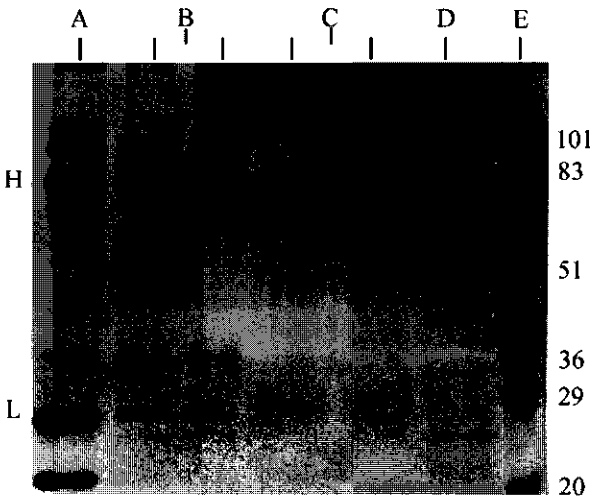

Fig. 2- Coomassie blue stained reduced SDS-PAGE of sea bass whole and purified serum samples. Lane A, sea bass whole serum; lanes B, saturated ammonium sulphate fraction of sea bass serum; lanes C, Protein A purified sea bass serum; lane D, carp serum; lane E, low molecular weight prestained BioRad standards with their respective molecularweights (kDa). Note the existence of two H cahins of about 78 kDa. The L chain is esimated at 28 kDa.

3.2.2. *SDS-PAGE / Western blot*

Protein A-affinity chromatography showed that this might be an effective mean of isolating IgM from sea bass serum.

On a Coomassie blue-stained reduced SDS-PAGE, the Protein A isolated protein appeared to have two H chains of approximately 78 kDa and at least one light (L) chain of about 28 kDa (Fig. 2).

All mAb selected were reactive with a tetrameric, dimeric and monomeric form of the IgM, when used to stain immunoblots of non-reduced sea bass whole serum, SAS-SBIg and Protein A purified sea bass IgM [Fig. 3(a)]. The monomeric form appeared to have a relative molecular weight (M_r) of about 210 kDa and consequently the M_r of dimeric and tetrameric forms might be estimated to be about 420 and 840 kDa, respectively. This was supported by immunoblots of non-reduced carp whole serum stained with WCI 12, which showed similar pattern but with slightly lighter representatives (see also Rombout *et al.*, 1993).

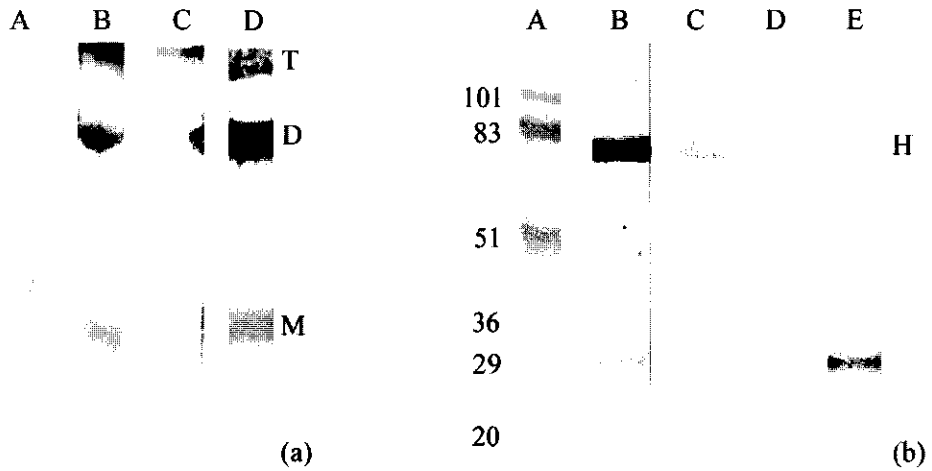

Fig. 3- Western blot of non-reduced (a) and reduced (b) Protein A-purified sea bass serum. (a) Lane A, high molecular prestained Bio Rad (210 kDa); lane B, immunoreaction with WDI 1; lane C, Immunoreaction with WDI 2; lane D, immunoreaction with WDI 3. Note the immunostained tetrameric IgM (T; ±840 kDa), dimeric IgM (D; ±420 kDa) and monomeric IgM (M; ±210 kDa). (b) Lane A, low prestained BioRad standard with their respective molecular weights (kDa); lane B, blotted Coomassie blue staining; lane C, immunoreaction with WDI 1; lane D, immunoreaction with WDI 2; lane E, immunoreaction with WDI 3. Note the immunostaining of the IgM heavy chain (H; ±78 kDa) with WDI 1 and light chain (L; ±28 kDa) with WDI 3.

Figure 3(b) shows the immunoreactions of the mAb in Western blots of reduced (2β-mercaptoethanol) Protein A purified IgM. WDI 1 showed a clear reaction with the H chain of sea bass IgM. Nevertheless, this mAb reacted most strongly with the lower band of the H chain. WDI 2 showed a slight reaction with the L chain in the Protein A purified IgM, although not strongly enough to be photographed. WDI 3 showed a strong reaction with the L chain. Using DTT as reducing agent the immunoreaction of WDI 1 was strongly decreased

and that of WDI 2 completely absent. The same results were obtained when whole serum or SAS-SBIg were used.

When reduced and non-reduced carp serum was stained with WDI 1-3 no reaction was observed. No staining occurred in the other controls.

3.2.3. Immuno-electron microscopy

All mAb showed a specific immunogold labelling of surface Ig-positive ($sIgM^+$) lymphocytes both in PBL and SP. The gold particles were mainly found in small clusters at the external membrane but also inside vacuoles (Figs 4 & 5). Plasma cells showed less surface labelling but a remarkably higher amount of endocytosed gold particles (Fig. 5). A limited number of labelled macrophages (Fig. 6) were detected. Erythrocytes, thrombocytes, granulocytes and the majority of lymphoid cells, were completely negative. There was no staining when mAb were omitted. The same negative result was observed when carp leucocytes were used.

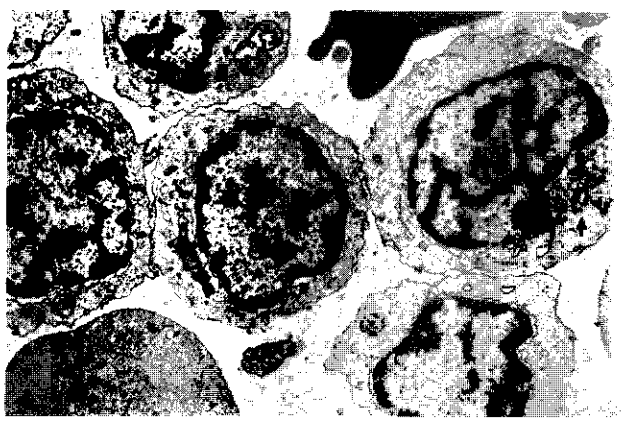

Fig. 4- Electronmicrograph (12500×) of WDI 1-immunoreactive lymphocyte (top and middle) and an early plasma cell (bottom), from sea bass spleen labelled with 25 nm gold particles. Note the accumulated vesicular structures (arrow) present in the plasma cell and some non-reactive lymphocytes and erythrocytes.

3.2.4. Flow cytometry

The optimal dilution of the culture supernatant was determined with PBL for each mAb (WDI 1, 1:50; WDI 2 and WDI 3, 1:100).

The average percentages and standard deviation (SD) of $sIgM^+$ cells in PBL, HK, TK, SP, THY and gut for the different mAb are summarised in Table 1 (average of six fish). The gated lymphoid cells in forward/side (90°) scatter (FSC/SSC) dot plot and corresponding fluorescence histograms of a representative fish are shown in Fig. 7 for PBL. Additional

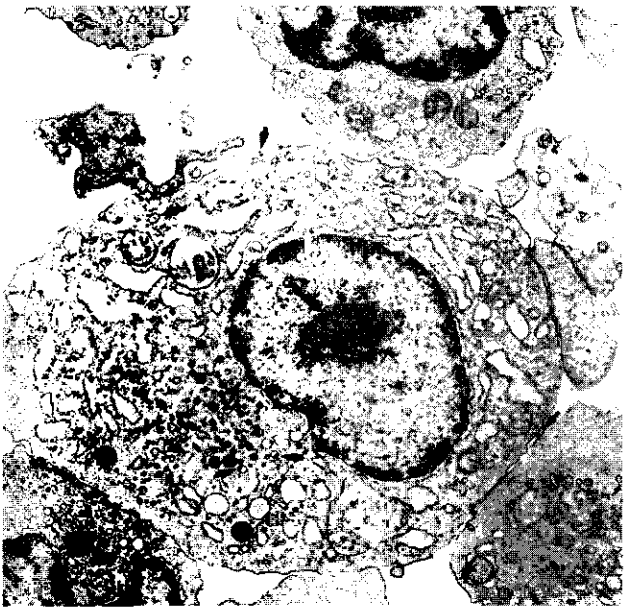

Fig. 5- Electronmicrograph of a WDI 3-immunoreactive plasma cell (16200×). Note the presence of 25 nm gold particules (arrows) inside vesicular structures of the plasma cell.

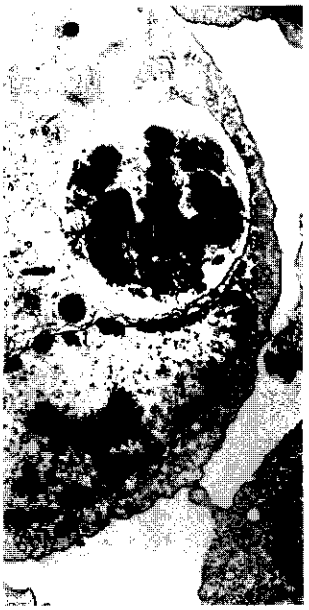

Fig. 6- Electronmicrograph of a WDI 3-immunoreactive macrophage (19500×).

staining with different combinations in equal volumes of optimal diluted mAb, incubated with PBL, HK and SP suspensions, is given in Fig. 8, for a fish showing the highest percentage of B cells. However, the same staining pattern was found for other specimens. When WDI 1 and WDI 2 were combined, a greater percentage of positive-staining cells was found in all cell suspensions, when compared with the percentage obtained for each mAb when used alone. The combination of WDI 1 and WDI 3 gave additional staining with PBL but not with HK or SP, when compared with the WDI 3 single staining. No additional staining was found when WDI 2 and WDI 3 were combined, compared with WDI 3 single staining. All controls were negative.

3.2.5. Immunofluorescence on cytocentrifuge preparations

All mAb showed specific immunoreaction in cytocentrifuge slides of PBL, HK and SP cells. Both $sIgM^+$ lymphocytes and cells with strongly stained cytoplasm (plasma cells) were observed. Double staining of SP cells with one mAb followed by another mAb is shown in Fig. 9. When WDI 1 was followed by WDI 2 or WDI 3 (or vice versa) additional stained cells (with only red fluorescence) were found in all combinations, as well as double stained cells

(orange-yellowish fluorescence), although when WDI 2 was used as the second mAb less additional reaction was observed. When WDI 2 was followed with WDI 3 the same result as described above was observed, but not when WDI 3 was followed by WDI 2.

Preparations stained twice with the same mAb did not stain additional cells, i.e., following initial staining with a mAb/RAM-FITC there was no further staining when cells were incubated again with the same mAb followed by RAM-TRITC. When only one of the mAb was used followed by the two RAM-conjugates in succession, only the fluorescence from the first RAM-conjugated was observed, indicating that the mAb was blocked by the first RAM-conjugate. Hence, the additonal staining with the second RAM-conjugate when using two different mAb, as described above, indicates the mAb recognise different epitopes.

Table 1. Mean percentages ± S.D. of $sIgM^+$ cells labelled by indirect immunofluorescence with WDI 1, WDI 2 and WDI 3 in peripheral blood leukocytes (PBL), head kidney (HK), trunk kidney (TK), spleen (SP), thymus (THY) and gut cell suspensions determinated using gated lymphoid cells as shown in Fig. 7.

mAb	$N = 6$					
	PBL	HK	TK	SP	THY	GUT
WDI 1	28.5 ± 6.5	17.5 ± 9.3	13.1 ± 3.3	17.2 ± 4.9	1.4 ± 0.7	10.1 ± 2.7
WDI 2	22.1 ± 4.0	14.9 ± 7.7	10.9 ± 2.5	15.1 ± 4.8	1.1 ± 0.5	10.4 ± 4.1
WDI 3	33.1 ± 6.6	18.8 ± 10.0	15.2 ± 3.1	19.9 ± 5.4	1.4 ± 0.9	11.9 ± 4.2

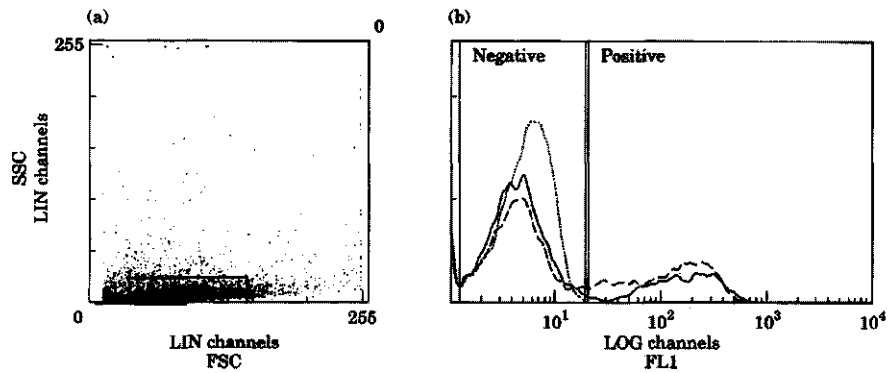

Fig. 7- Flow cytometry analysis of lymphoid cells from PBL. (a) FSC/SSC dot plot of PBL showing the lymphoid cell gate used. (b) Combined (smoothed) RPE fluorescence histogram of gated cells showing the control (. . .), WDI 1 (- - -) and WDI 2 (—) reactions. WDI 3 shows similar pattern as WDI 1.

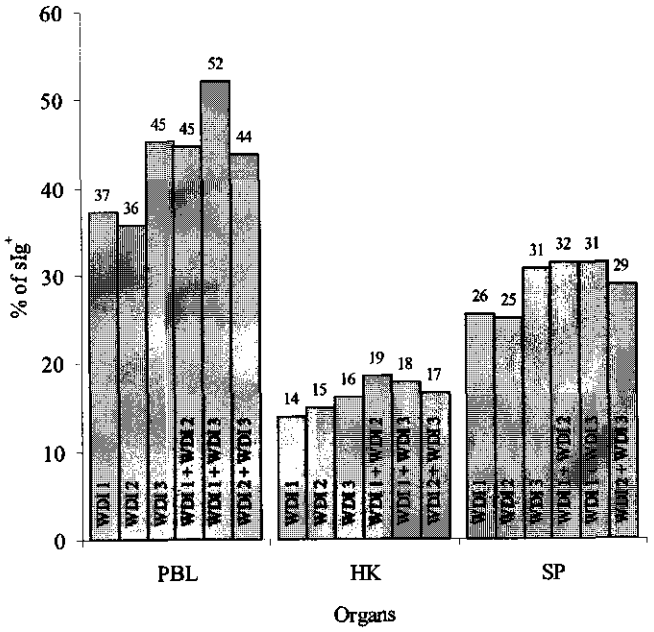

Fig. 8- Percentage of fluorescently stained cells using different combinations (equal volumes of optimal dilution) of the mAb incubated with PBL, HK and Sp lymphoid cell suspensions. Top numbers represent the percentage of $sIgM^+$ stained cells for individual and/or combined mAb.

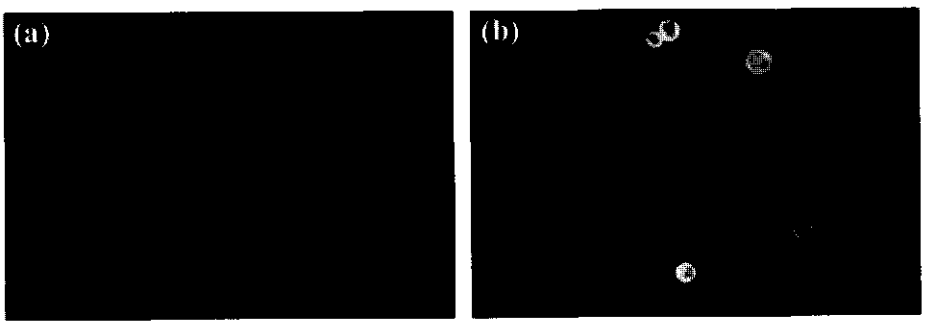

Fig. 9- Immunoreaction in cytocentrifuge preparations of sea bass spleen cells when stained with WDI 2 and RAM-FITC followed by WDI 3 and RAM-TRITC. Panel (a) photographed for green fluorescence and panel (b) for red fluorescence.

4. DISCUSSION

Evidence that the mAb produced in this study are specific for the sea bass IgM is provided by their reactivity with anti-*V. anguillarum-ordalii* antibodies in immunised sea bass plasma using ELISA. Significant differences were found between non-immunised and immunised sea bass with all three mAb selected. The titration curves for the three selected mAb were very similar and the immunised sea bass had very high anti-*V. anguillarum* antibody titres.

Although the saturated ammonium sulphate purification technique appeared to be effective for the immunisation of mice in order to obtain mAb against the H and L chains of sea bass IgM, the purity of the sea bass IgM from Protein A affinity chromatography isolation was much higher as shown in the SDS-PAGE. It was not the aim of this work to prove whether the protein A-isolated IgM sample was completely pure or contained all IgM, nevertheless, the success of protein A affinity chromatography in the isolation of fish IgM has already been shown by other authors when applied to turbot serum (Estévez *et al.*, 1993, 1994), although it failed when used with carp (unpublished data) and salmon (Ellis, personal communication).

It is generally accepted that the major serum IgM of teleosts has a tetrameric form with a M_r between 600-900 kDa (Wilson & Warr, 1992; Rombout *et al.*, 1993; Navarro *et al.*, 1993; Koumans-van Diepen *et al.*, 1995; van der Heijden *et al.*, 1995). In Western blots produced from non-reducing SDS-PAGE, all selected mAb showed reactions not only with a tetrameric (±840 kDa) form but also with a dimeric (±420 kDa) and monomeric (±210 kDa) form of sea bass IgM, which has also been described for carp (Rombout *et al.*, 1993) and eel (van der Heijden *et al.*, 1995). However, Bourmaud *et al.* (1995) and Romestand *et al.* (1995) only reported the existence of a tetrameric form of sea bass serum IgM of about 880 kDa while Palenzuela *et al.* (1996) reported tetrameric sea bass serum IgM of about 850 kDa. The differences between these weights and the present estimated M_r (840 kDa) may be due to the different methodologies applied. Heterogeneity in teleost IgM by different net charge of the tetrameric form or different dissociation of that form into smaller molecular subunits have also been described for other teleost species (Wilson & Warr, 1992; Rombout *et al.*, 1993; Navarro *et al.*, 1993; Koumans-van Diepen *et al.*, 1995; van der Heijden *et al.*, 1995). Most of these results showed that the high molecular weight IgM from teleosts consists of repeating monomeric units, presented as H_2L_2 and containing equimolar amounts of H and L chains. Additionally, Lobb & Clem (1983) showed that the high molecular weight IgM from catfish could be dissociated, in the presence of denaturing agents, into eight distinct subunits formed by covalently-linked combinations of H-L chain pairs ("halfmers"). Differences in secretory and serum immunoglobulins were also studied in sheepshead (Lobb & Clem,1981), rainbow trout (St. Louis-Cormier *et al.*, 1984), catfish (Lobb, 1987) and carp (Rombout *et al.* 1993). Whether these different molecules represent distinct isotypes of IgM and/or they have a different physiological significance remains to be investigated. Nevertheless, the mAb described react with peptides which, with respect to their M_r resemble the H (WDI 1) and L (WDI 2 and WDI 3) chains of IgM. The M_r of H (±78 kDa) and L (±28 kDa) chains described

here agree with those found by Romestand *et al.* (1995) and Palenzuela *et al.* (1996). In contrast to WDI 3, WDI 2 only slightly stained the light chain, which might be due to the reduced condition of the determinant rather than to lower concentration of antibody, since these two mAb worked optimally with the same dilution of culture supernatant in the FACS experiments as well as in ELISA and were both used in the Western blots undiluted and/or diluted 1:2 to 1:4. It is known that configuration, as well as differences in charge, optical configuration and steric conformation, are important for antibody binding (Roitt *et al.* 1993). In this context an antibody may bind to a certain determinant in the native form but not when its configuration is changed, and vice versa .

In immuno-electron microscopy, the mAb stained the surface of lymphocytes with the gold particles appearing on the cell surface as clusters, as described for carp (van Diepen *et al.*, 1991), sea bream (Navarro *et al.*, 1993) and eel (van der Heijden *et al.*, 1995). Nevertheless, the presence of gold particles inside vacuoles were frequent, suggesting that endocytosis was not blocked completely during the incubation time and hence the phenomenon of patching cannot be excluded. Plasma cells showed less reaction at their surface than found in carp (van Diepen *et al.*, 1991) and more closely resemble mammalian plasma cells which loose their surface Ig completely during differentiation (Hämmerling *et al.*, 1976). The presence of gold particles inside vesicular structures of early and mature plasma cells suggests their lysosomal nature and possibly the involvement in endocytosis. Vesicular granules, referred to as cytoplasmic dense granules, were also reported by Meseguer *et al.* (1991) but only in developing plasma cells. All mAb were reactive with at least some lymphocytes. The number of labelled macrophages was very low in spleen suspensions of sea bass. However, this might indicate the presence of Ig receptors at the surface of a subpopulation of macrophages as suggested for carp by Koumans-van Diepen *et al.* (1994a). More studies have to be done to achieve better conclusions. Specificity of the mAb was shown by the lack of any gold particle in the other controls. No cross-reaction was observed when using a mAb with carp leukocytes.

Most importantly, the results strongly indicate the existence of B cell heterogeneity in sea bass, expressing at least two different H and L chains. When WDI 1 was combined with either WDI 2 or WDI 3 an increase in the percentage of staining cells could be observed in FACS analysis as well as in double staining of cytocentrifuge preparations. The existence of two H chains is also supported by the Coomassie Blue stained SDS-PAGE with reduced protein A-isolated IgM; two bands with the same intensity, appear in the H chain molecular weight region. Additionally, these two bands were both stained by WDI 1 on a Western blot, although

less reactivity was observed with the upper molecular weight H chain, indicating that both H chains might have some similar determinants. Although, Romestand *et al.* (1995) and Palenzuela *et al.* (1996) reported the existence of only one H chain in sea bass, the present work clearly indicates the presence of at least two H chains. Palenzuela *et al.* (1996) also reported the existence of two L chains (a major band with 27.5 kDa and a minor band of 28.5 kDa) in sea bass IgM. The existence of more than one H and L chain is not new, since evidence of diversity in molecular weights, structure and/or antigenicity in IgM of other teleost species are reported (Wilson & Warr, 1992; Rombout *et al.*, 1993; Koumans-van Diepen, 1995; van der Heijden, 1995; Warr, 1995; Palenzuela *et al.*, 1996). In the FACS analysis, when WDI 2 and WDI 3 were combined, additional staining was not or hardly observed, when compared with WDI 3 on its own. However, when used singly, WDI 2 stained a lower number of B cells when compared with WDI 3, indicating that they might react against the same L chain but, probably to different determinants, since the difference cannot be explained by differences in affinity, because both mAb could be used in a similar dilution in FACS and ELISA. This result is also supported by the immunofluorescence in cytocentrifuge preparations, since an additional cell population could be seen when staining with WDI 2 was followed by WDI 3, though not in the opposite order. The highest proportion of staining cells was obtained with the combination of WDI 1 and WDI 3 suggesting that together they stain all or almost all B cells in PBL, HK and SP, because 52% of IgM^+ PBL appears to be high compared with other species (46% in trout: DeLuca *et al.*, 1983, Thuvander *et al.*, 1990, Sánchez *et al.*, 1993; 37% in catfish: Ainsworth *et al.*, 1990; 48% in carp: Koumans-van Diepen *et al.*, 1994b; 26% in eel: van der Heijden *et al.*, 1995). However, this extremely high percentage was only found in one fish and the variation of the individual percentages of the mAb between the six fish was considerable. This individual variation in percentage B cells could be influenced by fluctuating percentages of thrombocytes which are also present in the FSC/SSC gate used, at least in the case of carp (Rombout *et al.*, 1996). Moreover, van der Heijden *et al.* (1995) justified the low percentage of B cells in PBL of eel by the abundance of thrombocytes present in PBL suspensions. So, it may be concluded that the percentage of B cells found is within the range of the other teleost species studied.

In conclusion, all mAb produced here were shown to be specific for sea bass IgM and were useful for the detection of specific IgM in ELISA, as well as strongly suggesting the existence of at least two H and L chains expressed in different combinations in at least two subpopulations of B cells in sea bass.

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References

Ainsworth, A.J., Dexigang, C. & Greenway, T. (1990). Characterization of monoclonal antibodies to channel catfish, *Ictalurus punctatus*, leucocytes. *Veterinary Immunology and Immunopathology* **26**, 81-92.

Bourmaud, C.A-F., Romestand, B. & Bouix, G. (1995). Isolation and partial characterization of IgM-like sea bass (*Dicentrarchus labrax* L. 1758) immunoglobulins. *Aquaculture* **132**, 53-58.

DeLuca, D., Wilson, M. & Warr, G.W. (1983). Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. *European Journal of Immunology* **13**, 546-551.

Estévez, J., Leiro, J., Sanmartin, M.L. & Ubeira, F.M. (1993). Isolation and characterization of turbot (*Scophthalmus maximus*) immunoglobulins. *Comparative Biochemistry and Physiology* **105A**, 275-281.

Estévez, J., Leiro, J., Santamariana, M.T., Domínguez & Ubeira, F.M. (1994). Monoclonal antibodies to turbot (*Scophthalmus maximus*) immunoglobulins: characterization and applicability in immunoassays. *Veterinary Immunology and Immunopathology* **41**, 353-366.

Hämmerling, U., Chin, A.F. & Abbott, J. (1976). Ontogeny of murine B lymphocytes: sequence of B-cell differentiation from surface-immunoglobulin-negative precursors to plasma cells. *Proceedings of National Academy of Science U.S.A.* **73**, 2008-2012.

Koumans-van Diepen, J.C.E., Egberts, E., Peixoto, B.R., Taverne, N., & Rombout, J.H.W.M. (1995). B cell and immunoglobulin heterogeneity in carp (*Cyprinus carpio* L.); an immuno(cyto)chemical study. *Developmental and Comparative Immunology* **19** (1), 97-108.

Koumans-van Diepen, J.C.E.,Taverne-Thiele, J.J., van Rens, B.T.T.M. & Rombout, J.H.W.M. (1994a). Immunocytochemical and flow cytometric analysis of B cells and plasma cells in carp (*Cyprinus carpio* L.); an ontogenetic study. *Fish and Shellfish Immunology* **4**, 19-28.

Koumans-van Diepen, J.C.E., van de Lisdonk, M.H.M., Taverne-Thiele, A.J., Verburg-van Kemenade, B.M.L. & Rombout, J.H.W.M. (1994b). Characterisation of immunoglobulin-binding leucocytes in carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology* **18**, 45-56.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriopage T4. *Nature* **227**, 680-685.

Lobb, C.J. (1987). Secretory immunity in catfish, *Ictalurus punctatus*, following bath immunisation. *Developmental and Comparative Immunology* **11**, 727-738.

Lobb, C.J. & Clem, L.W. (1981). Phylogeny of immunoglobulin stucture and function-X. Humoral immunoglobulins of the sheepshead, *Archosargus probatocephalus*. *Developmental and Comparative Immunology* **5**, 271-282.

Lobb, C.J. & Clem, L.W. (1982). Fish lymphocytes differ in the expression of surface immunoglobulin. *Developmental and Comparative Immunology* **6**, 473-479.

Lobb, C.J. & Clem, L.W. (1983). Distinctive subpopulations of catfish serum antibody and immunoglobulin. *Molecular Immunology* **20** (8), 811-818.

Meseguer, J., Esteban, M.A. and Agulleiro, B. (1991). Stromal cells, macrophages and lymphoid cells in the head-kidney of sea bass (*Dicentrarchus labrax* L.). An ultrastructural study. *Archivum of Histology and Cytology*, **54** (3), 299-309.

Navarro, V., Quesada, J.A., Abad, M.E., Taverne, N. & Rombout, J.H.W.M. (1993). Immuno(cyto)chemical characterisation of monoclonal antibodies to gilthead seabream (*Sparus aurata*) immunoglobulin. *Fish And Shellfish Immunology* **3**, 167-177.

Palenzuela, O., Sitjà-Bobadilla, A., & Álvarez-Pellitero, P. (1996). Isolation and partial characterization of serum immunoglobulins from sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.). *Fish and Shellfish Immunology* **6**, 81-94.

Roitt, I., Brostoff, J. & Male, D. (1993). *Immunology* (third edition) Chapter 6, pp. 6.4-6.5.

Rombout, J.H.W.M., Koumans-van Diepen, J.C.E., Emmer, P.M., Taverne-Thiele, J.J. & Taverne, N. (1996). Characterisation of carp (*Cyprinus carpio* L.) thrombocytes with specific monoclonal antibodies. *Journal of Fish Biology* in press.

Rombout, J.H.W.M., Taverne, N., van de Kamp, M. & Taverne-Thiele, A.J. (1993). Differences in mucus and serum immunoglobulin of carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology* **17**, 309-317.

Romestand, B., Breuil, G., Bourmaud, C.A-F., Coeurdacier, J.L. & Bouix, G. (1995). Development and characterisation of monoclonal antibodies against sea bass immunoglobulins *Dicentrarchus labrax* Linnaeus, 1758. *Fish and Shellfish Immunology* **5**, 347-357.

Sánchez, C., López-Fierro, P., Zapata, A., & Dominguez, J. (1993). Characterisation of monoclonal antibodies against heavy and light chains of trout immunoglobulin. *Fish and Shellfish Immunology* **3**, 237-251.

Schots, A., Pomp, R. & van Muiswinkel, W.B. (1992). *Techniques in Fish Immunology* (first edition). Stolen, J.S., Fletcher, T.C., Anderson, D.P., Kaatari, S.L. and Rowley, A.F. (eds). SOS Publications, 43 DeNormandie Ave., Fair haven, NJ. 07704-3303 USA. Chapter 1, pp. 1-18.

Secombes, C.J., van Groningen, J.J.M. & Egberts, E. (1983). Separation of lymphocyte subpopulations in carp *Cyprinus carpio* L. by monoclonal antibodies: immunohistochemical studies. *Immunology* **48**, 165-175.

St. Louis-Cormier, E.A., Osterland, C.K. & Anderson, P.D. (1984). Evidence for a cutaneous secretory immune system in rainbow trout (*Salmo gairdneri*). *Developmental and Comparative Immunology* **8**, 71-80.

Thuvander, A., Fossum, C. & Lorenzen, N. (1990). Monoclonal antibodies to salmonid immunoglobulin: characterization and applicability in immunoassays. *Developmental and Comparative Immunology* **14**, 415-423.

van der Heijden, M.H.T., Rooijakkers, J.B.M.A., Booms, G.H.R., Rombout, J.H.W.M. & Boon, J.H. (1995). Production, characterisation and applicability of monoclonal antibodies to European eel (*Anguilla anguilla* L., 1758) immunoglobulin. *Veterinary Immunology and Immunopathology* **45**, 151-164.

van Diepen, J.C.E., Wagenaar, G.T.M. & Rombout, J.H.W.M. (1991). Immunocytochemical detection of membrane antigens of carp leucocytes using light and electron microscopy. *Fish and Shellfish immunology* **1**, 47-57.

Warr, G.W. (1995). The immunoglobulin genes of fish. *Developmental and Comparative Immunology* **19**, 1-12.

Wilson, M.R. & Warr, G.W. (1992). Fish immunoglobulins and the genes that encode them. *Annual Review of Fish Diseases* **2**, 201-221.

CHAPTER 3

Ig light chain variability in DNP_{494}-KLH immunised sea bass (*Dicentrarchus labrax* L.): evidence for intramolecular-induced suppression

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"Life is just a word of three letters" - Carl Sagan and Ann Druyan (1992)

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Abstract

The complete coding sequence of the sea bass light chain was obtained by sequential anchored PCR on a head kidney cDNA library of a DNP_{494}-KLH immunised sea bass. The cDNA sequence obtained codes for a leader peptide of 21 aa and a mature IgL chain of 223 aa. Both the amino acid sequence comparisons and neighbor-joining trees showed that the IgL chain of sea bass obtained is of the L1/G type and can not be readily classified as a mammalian κ or λ isotype. To study the variability of the light chain additional PCRs on the cDNA library and cDNA from pooled head kidneys were performed. Multiple alignment of unique sequences (19) could be performed without introducing gaps, and showed extremely low variability in CDR1, and no variability in CDR2 or CDR3. A possible explanation for this low variability might be the enhanced expression of monospecific anti-DNP antibodies.

Key words: *Dicentrarchus labrax*, immunoglobulin light chain, expression, variability, DNP_{494}-KLH

1. INTRODUCTION

Knowledge of Ig gene structure and function will allow the understanding of fish immunity and its evolution as well as the potential genetic manipulation of fish immunity (Warr, 1995). To date, IgL chain sequences have been obtained from a limited number of teleost species from different orders (Ghaffari & Lobb, 1993, 1997; Daggfeldt *et al.*, 1993; Partula *et al.*, 1996; Tomana *et al.*, 1999) but none from the perciformes. Those studies have shown that the IgL *loci* of teleost species have a cluster type of organisation with repeated clusters of either (V-V-J-C) or (V-J-C). However, the V-segments of the Ig L1/G isotypes described in teleosts, unlike those of elasmobranch fish (Shamblott & Litman, 1989; Marchalonis *et al.*, 1998), have an opposite transcriptional orientation to the J and C segments which might allow rearrangement between clusters and, thereby, increase the potential L-chain variability (Ghaffari & Lobb, 1993; Pilström & Lundqvist, 1998). It has been shown that variability is mostly confined to the CDRs, although a lesser degree of diversity can also be found in the FRs (Tomana *et al.*, 1999; Wildholm *et al.*, 1999). Additional diversity can be also created at the junctions of the V and J segments by exonuclease activity leading to different lengths of the gene segments. In addition, the length variability of the CDR1 and CDR3 regions are considered important for the variability and capacity to bind antigen (Hsu & Steiner, 1999).

It has been suggested that fish antibodies are of lower affinity and diversity than those of mammals and birds (Mäkelä & Litman, 1980; Du Pasquier, 1982). However, many reports have shown that fish respond in a specific manner to different antigens, including either

combination with λgt10 forward and reverse primers (see 2.3.1).

1.1.3. PCR to obtain specific probes for the southern blot

Probes specific for the VL and CL regions were generated by PCR using the cloned and characterised cDNA fragments as templates. Specific primers in the FR1 (see 2.3.2) and in the FR4 (DilaIgVL/Rv: 5'- CAC CCA AGC ACT GTT TAT TTC ATA AT- 3') of the V region were deduced from the complete sequence and used in a PCR mixture to obtain the specific VL-probe (nt 75-375 in Fig. 1). Specific primers in the 5'-end (DilaIgCL/Fw: 5'- GCG TCC CAC CCT GAC CGT CCT- 3') and in the 3'-end (see 2.3.2) of the C region were deduced from the complete sequence and used in a PCR mixture to obtain the specific CL-probe (nt 423-718 in Fig. 1). The probes were labelled by random priming according to Feinberg & Vogelstein (1983).

1.4. CLONING AND DNA SEQUENCING

Ligation and transformation of the PCR products were performed using the pGEM®-T (Promega Corporation, Madison, USA) easy vector system. White colonies were selected and used for overnight growth in LB/ampicilin medium. Plasmid purification was performed using the QIAprep® (Qiagen) spin miniprep kit. DNA sequences were determined using the Bigdye (Perkin-Elmer, Branchbury, New Jersey, USA) terminator kit using both plasmid sequencing primers T7 and SP6 and analysed on a automated sequencer (ABI 377; Applied Biosystems, Foster City, CA, USA).

1.5. SEQUENCE ANALYSIS

The deduced amino acid sequences were analysed using the DNA Strider™ software and the multiple alignments were made using CLUSTAL W program (Thompson *et al.*, 1994). Sequence analysis and sequence retrievals were performed on the EMBL server at WWW.ebi.ac.uk. The neighbor-joining tree was constructed using the program MEGA (Kumar *et al.*, 1993). The phylogenetic tree of the nucleotide sequences was tested for reliability using 1000 bootstrap replications.

1.6. SOUTHERN BLOT

Genomic DNA was isolated from sea bass erythrocytes as described by (Stet *et al.*, 1993) and digested with the restriction enzymes *Eco*RI, *Hin*DIII and *Bam*HI. The digested DNA was

separated on a 0.8% agarose gel and transferred to a nylon membrane Hybond-N^+ (Amersham, Amserham, UK) by vacuum blotting with 0.4M NaOH. The filters were fixed by UV cross-linking (150 mJ), prehybridised and hybridised with ^{32}P-labelled random-primed probes according to Feinberg & Vogelstein (1983) in a buffer (modified from Church & Gilbert, 1984) containing 0.5 M $NaH_2PO_4/NaHPO_4$ (pH 7.2), 1 mM ethylenediaminetetraacetate (EDTA) and 7% (w/v) sodium dodecylsulphate (SDS) at 65° C. Following hybridisation, the filters were washed twice at different stringencies (VLprobe: 1x SSC at 50° C; CLprobe: 0.1x SSC at 65° C) and exposed to a Biomax-MR film (Eastman Kodak Co., Rochester, NY, USA) for 72 h at –70° C.

3. RESULTS

3.1. COMPLETE IgL SEQUENCE FROM THE LIBRARY

The complete cDNA sequence was obtained by sequential anchored PCR on the amplified library prepared from a single immunised sea bass. The 5'-end of the sequence was obtained using anchored PCR with the λgt10 and DegIgCL/Rv primers. Products of about the expected size (600bp) were cloned, sequenced and analysed. Three clones showed to encode the 5' UT, the LP, the V and part of the C IgL region (nt 624 in Fig. 1). From the second anchored PCR using the specific primer DilaIgL/Fw, 3 clones were obtained and were shown to encode the remaining of the sequence, denoted Dila. The sequences of the 3'-end of the clones obtained from the first anchored PCR and the 5'-end of one of the clones of the second anchored PCR matched in the overlap (153 nt), allowing the compilation of the complete sequence (Fig. 1).

The complete cDNA sequence obtained, is characterised by a 5'-untranslated segment (87 nt), the coding region for a leader peptide of 21 amino acids and a mature IgL chain of 223 amino acids (V: 104, J: 13 and C: 106) followed by an 3'-untranslated segment (234 nt) including the polyadenylation signal and the poly-A tail. The V and C regions contain the characteristic cysteine residues that likely to form the intradomain disulphide interactions (C-43, C-114, C-164 and C-223). The C region contains the characteristic cystein residue expected to form the interchain disulphide linkage (C-242) between the heavy and light chains.

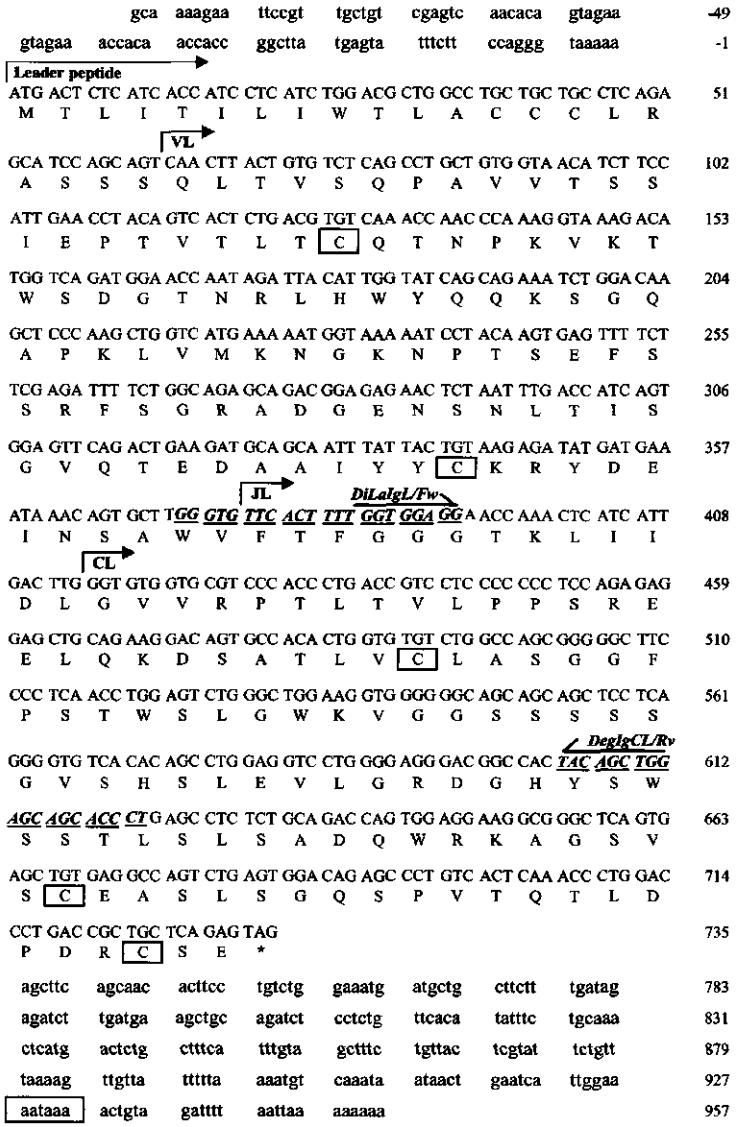


Fig. 1- Complete nucleotide and inferred amino acid sequence of sea bass IgL chain. Boxed are the cysteines residues likely to form the intradomain (C-43, C-114, C-164 and C-223) and interchain (C-242) disulphide interactions, and the polyadenylation signal. The stop codon is indicated by *. Primers used to obtain the sequence are indicated by underlined bold/italic cases.

3.2. PHYLOGENETIC ANALYSIS

Comparison of the IgL amino acid sequence of the sea bass (Dila) with those of other teleost fish (Fig. 2), showed high similarity with the Atlantic cod IgL1 isotype (C: 75.7%; V: 45.5%), channel catfish IgLG (C: 56.2%; V: 49%) and rainbow trout IgL1 (C: 54.7%; V: 40.2%) isotypes. A lower percentage similarity was obtained from comparisons with the

Ig Light Variable Regions

```
                    FR1          ><        CDR1       ><       FR2       ><   CDR2   ><                    FR3                      ><  CDR3
Sea bass   ***QLTVSQP AVV*TSSIEP TVTLTCQT*N PKVKTWSDGT NRLHWYQQK* SGQAPKLVMK NGKN****PT SE******** FSSRFSGRA* *DGENSNLTI SGVQTEDAAI YYCKRYDEIN S
Cod/L1     ***-V--T-T P--*SVTPGT ------K-*- SA-YD****G DE-------* P-E----I-- LVNR****LV -P******** TPA----SG* *RRTDFS--- T-A-R----V -H-QSFHYPG -
Cod/L2     ***A-VLT-E KTL*SVNRGD N-KIS-TE*T DSSSN***** YV-T-----* --SP--YLLT TTGS****RA -G******** IP---T-SGT KNDKTEY-L- N-I-D--E-T ---ACAVGCG A(+)
Trout/L1   ***-YV-T-T P--KAVLP-Q --S-N-K-*S SD-HS****G YY-A-----* P-G--Q-LIY RATT****LQ -G******** TP-----SG* *S-SDFT--- ---KV---GD ---QSAHYS- -
Trout/L2   ***-KA-T-T PS-L-V-TKG -A-FH-DI*T KGEGN***** *VVI--K-V* P-G--QY-LR YYHTWSSFTS -SPDQYGSGP S-D--TSK-T *SDKDYQFI- -N-EET-S-V ---QTW-SS* *
Catfish/F  SFGEIVLT-S PGSQSV-PGQ S-----RA*S QG-YD***** *D-Q--L--* P-E----LIY -DVT****RF TG******** VPD----SG* *R-TEFS--- ----S---GD ---QQSET** *
Catfish/G  ***-V--T-T PA-KSALPGE ---IN-R-*- -A-YYY-SYG HY-------* P-E----LI- FANQ****LH -G******** IPA----SG* *S-SDFT--- --------GD ---QS-HS** *
Sturgeon   ***-Y--T-T PAEKSVLPGD ----S-KV*S SA-HN****N DN-C-----* P-E--T-LIY WANT****LQ -G******** IPT----SG* *S-TDFT--- ----A---GD ---QS-H-PS *
H-Shark    **DTIIMT-S PPALSVTLGQ -A-I--TA*S QS-ND***** *YVV----R* D--K-TPLLY R-TV****RH TR******** T-D--T-SQ* *QNLKFT--- -D--N--V-D ---QQTYS** *
S-Shark    ***DPVLT-- GSI*S--PGK ---I--TM*S GGTISS***Y WAS*--W--* PDS--VF-WS ESDR****MA -G******** IPN--A-SVD SSSNKMH--- TN--S---TD ---AAAASRS *
Skate      ***IPVLN-T PTSGPV-AAQ -AR-E-R-Q- GN-AS***** YTIF--R-R* P-ES--WLVT YETDNDIHRG -G******** ITD--QPSRD TSANSYI--- GT-EPG---V ---AVWETD* *
Chicken    SLV-AALT-- SS-*SANPGE --KI--SG*D RSYYG***** ****-----A P-S--VT-IY ANT-****RP -D******** IP-----SK* *S-STAT--- T---AD-E-V ---GSA-SSS *
Duck       SLV-AALT-- -SK*SVNPGD --QI--SG*S SSDYG***** ****-P---T P-S--VT-IY QNNK****RP -G******** IP-----SK* *S-STAT--- T---A--E-V ---GS--SS* *
Human/κ    ***DIQMT-S PSSLSA-VGD R--I---A*S QDINN***** *Y-N-----* P-KT---LIY GAS-****LE TG******** VP-----SG* *S-TDFIP-- -SL-P--I-T ---QQ--N** *
Human/λ    ***-SALT-- -S-*SG-PGQ SI-IS-TG*T NNDVGS***Y -LVS----H* P-K---IMIY EVSK****RP -G******** V-N----SK* *S-NTAS--- --L-A--E-D ---CS-AGS* *
(+ DTGFGGGTEVILAR)
```

Ig Light Constant Regions

```
              FR4      ><
Sea bass   ****GVVRPT LTVLPPSREE LQ*KDSATLV CLASGGFPST WSLGWKVGGS SSSSG*VSHS LEVLGR*DGH YSWSSTLSLS ADQWRKAGSV SCEASLSGQ* SPVTQTLDPD RCSE***
Cod/L1     ****---Q-- -S------V- -E*QG----- -T-I-----D -K-------- -W-V-*--D- -G-P-K*--- ------MT-P ---------- -----KN--T Q------N-G E---***
Cod/L2     ****SPSP-S -ELMVATQPP VPGLGGP--- ---K-FH-AG AA-S-SED-A -VRGEE-QAG VAQRQP*--S --LN-V-L-P STR--SGHTP T-HV-H-ALS --LSKSVSSQ Q--L***
Trout/L1   ****SNSA-- -------S-- -S*STT---M ---NK----D -T-S---D-T -QKQ***EA- PT--EK*--L -------T-T GQE-T---E- T---QQKS-* T---K--RRA D--G***
Trout/L2   ***STLPP-V --I----SD- -K*SSKV--- ----QMAMGY ADVS-TA--T PVTG-*IAT- GP-PQA*-KT FQL--C-TVD TSE-NQDKVP --KVTVGSK* *FAEKDIKKS E--TE--
Catfish/F  ***GPT-K-S VSL----SLQ -S*EG--S-L --L-AYS-QG ALVS-T-D-- EVKD-*-LT- A-ERKK**DG -TH----T-- KAL-E-GEEP V-KV-HDNVD H---**FRKS Q-EV***
Catfish/G  **G*RLTQ-S V------SV- ---*QEKV--- -V-YK----D -R-S---D-- -W---*E-R- TA--QA*--L ---------H PE---NK*V- T----KDN-* P--VS-VNTE Q-*****
Sturgeon   **GSPTAPSS VSL----KL- -DS-GK---- --VNNFY-DV VDIK-T-D-V AQ---*-LT- TMKQK**--K --A--S-T-T KAV-NSKETV T-TVKHEAVS T-RSESIKRS E-TLLDA
H-Shark    LSREKSQP*- --L----E-- VKA-GT---- ---DHFY-DE VGVE--KD-A AI-A-*-QT- NYLRAS*-ST --C--L-T-- GSD-ESNARF --ALIHETLS --LSKSVSRS E-V****
S-Shark    **GSPRSP*- VS-----SDQ ITA-NM---- --V--FV-GA ABIE-T-D-- VRGN-*-ET- RIQQEA*-NT F-V--Y-T-- -SD-NSHELY --VVKHET-A N-LQTSISRS S-M****
Skate      *KSSESRK-S -LL----P-- TG*TG----S --V--FK-GL VA-R-A-D-V ETE--*-TTG AVSPDA*EQT -RL--Y-RVP -AA-G-GT-Y --SVAH-SLG --LRH-VSSS S-AN***
Chicken    **GQPK-A-- I-LF---K-- -NEATK---- --INDFY--P VTVD-VID-- TR-G***ETT APQRQS*NSQ -MA--Y---- -SD-SSHETY T-RVTHN-T* *SI-K--KRS E-*****
Duck       **GQPK-S-- VH-F---D-- ISSQNK---- --M-DFY--P VTVT---N-- TR---*-ET- ASQRQS*NSK -MA--Y-T-- -SE-KG-N-- V-QVTHD-T* *-IEK--NKS E-*****
Human/κ    ***TVAAP*S VFIF---D-Q -KS*GT-SV- --LNNFY-RE AKVQ---DNA LQ-GN*SQE- VTEQDSK-ST --L-N--T-- KADYE-HKVY A--VTHQ-LS ----KSFNRG E-*****
Human/λ    **GQPKAA-S V-LF---S-- --*ANK---- --I-DFY-GA VTVA--ADS- PVKA-*-ETT TPSKQS*NNK -AA--Y---T PE--KSHR-Y --QVTHE-S* *T-EK-VA-T E--****
```

Fig. 2- Alignment of the inferred amino acid sequences of the sea bass VL and CL segments with those of different vertebrate species (access numbers from EMBL databank): sea bass (*Dicentrarchus labrax*; acc n°: AJ400216), Atlantic cod (*Gadus morhua*; L1 acc n°: X76517, L2: Wermenstam, N. E., personal communication), rainbow trout (*Oncorhynchus mykiss*; L1 acc n°: X68517, L2 acc n°: U69987), channel catfish (*Ictalurus punctatus*; F acc n°: U25705, G acc n°: L25533), Siberian sturgeon (*Acipenser baerii*; acc n°: X90557), horn shark (*Heterodontus francisci*; acc n°: L25561), sandbar shark (*Carcharhinus plumbeus*; acc n°: M81314), skate (*Raja erinacea*; acc n°: L25568), chicken (*Gallus gallus*; acc n°: M24403), duck (*Anas platyrhynchos*; acc n°: X82069) and human (*Homo sapiens*; acc n°: κ acc n°: AF113887, λ acc n°: X57823). Residues identical to the sea bass sequences are denoted by *dashes* and gaps introduced to optimise similarity between sequences are indicated by *asterisks*. Putative frameworks (FR) and complementary determining segments (CDR) are indicated above sequences for the Dila sequence.

channel catfish IgLF (C: 35.5%; V: 30.7%), Atlantic cod IgL2 (C: 28.4%; V:24.1%) and rainbow trout IgL2 (C: 28.0%; V: 27.8%) isotypes. Comparison of the sea bass IgL V and C sequences with different light chain sequences from other vertebrates, ranged from 25.9-42.2% and 31.9-43% for the V and C segments, respectively. These results were further supported by distance trees from vertebrate V and C chain amino acid sequences (Fig. 3). The sea bass IgL chain C region clustered together with the cod IgL1, catfish IgLG and trout IgL1, which was supported by a high bootstrap value. The V region of the sea bass Ig was found clustered with the same sequences as seen in the C region tree, with the inclusion of the sturgeon. However, this cluster is not supported by a high bootstrap value.

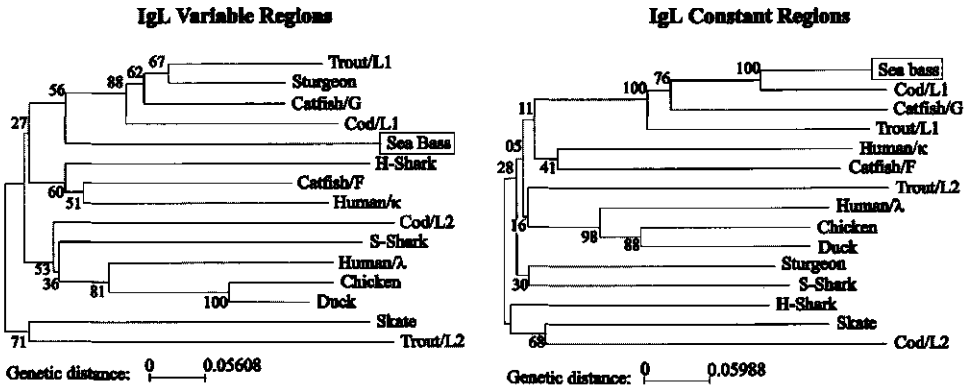


Fig. 3- Neighbor-joining tree of amino acid sequences of the VL and CL regions from different vertebrates species (see Fig. 4), using p-distance parameter and complete deletion of gaps. Bootstrap replications are indicated as a percentage at each node.

3.3. GENE ORGANISATION (SOUTHERN BLOT)

Southern blot analysis of genomic DNA using VL- and CL-specific probes showed multiple scorable hybridising bands, with at least 10 (single enzyme digests) to 19 (multiple enzyme digests) V and 12 (single enzyme) to 15 (multiple enzymes) C fragments (Fig. 4). These results, suggest a cluster type of organisation. Many of the bands appear longer than 2 kb, some of which probably contain several copies, as judged from the intensity of the signal.

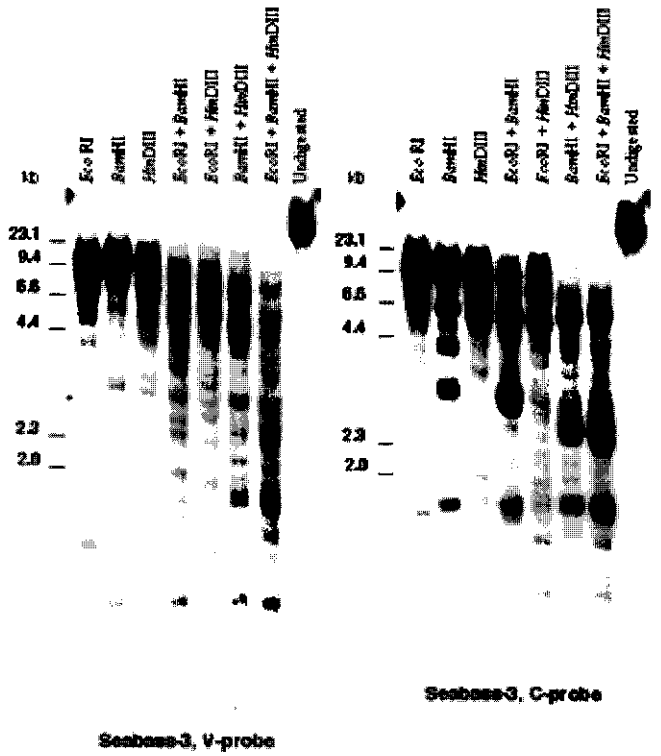
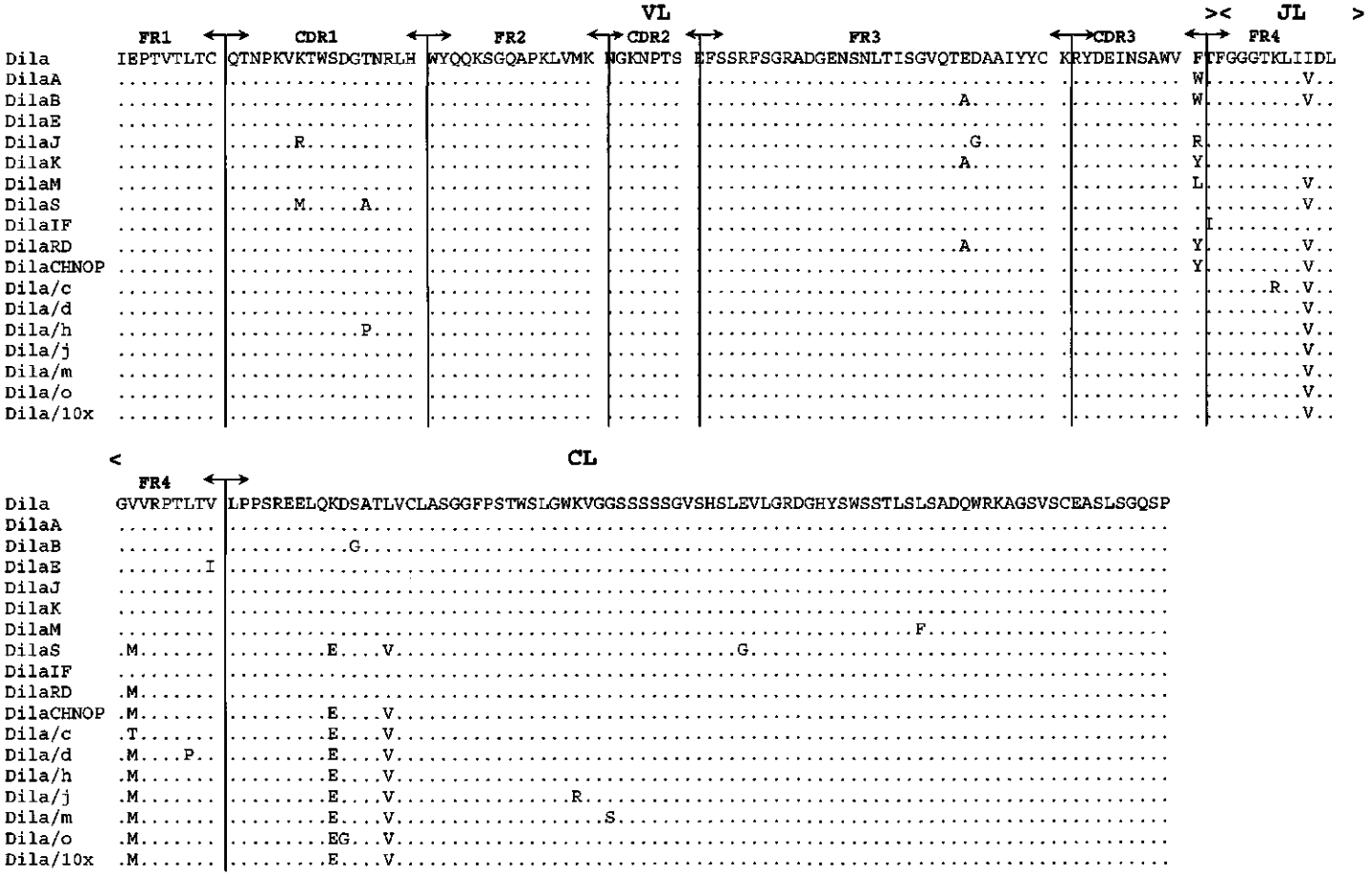


Fig. 4- Southern blots of sea bass DNA (individual n° 3), digested with *Eco*RI, *Bam*HI and *Hin*III separately and in combinations. The filters were hybridised with a VL- or CL-specific probes. The DNA size markers (kb) are indicated on the left.

1.4. IgL CHAIN V AND C REGION DIVERSITY

An anchored PCR using the λgt10 forward and reverse primers in combination with the DilaIgCL/Rv specific primer was performed on the amplified library. Surprisingly, only 3 clones were obtained encoding the IgL chain, denoted Dila/10x (see figure 5 legend), and showed to be identical, and differed from Dila (see 3.1) only in the J and C region (Fig. 5). To obtain additional sequences, a PCR was performed on the amplified library using the DilaIgVL/Fw and DilaIgCL/Rv specific primers. Seventeen clones, denoted Dila# (# as an uppercase letter for each clone), were obtained, coding for 10 additional unique IgL sequences. An extremely reduced variability was observed among these sequences and the variability was confined to the CDR1 (1-2 aa), FR3 (1 aa), and J (1-2 aa) and C (1-4 aa) regions. No variability was observed in CDR2 and CDR3. Finally, a similar PCR (same specific primers) was performed on the cDNA of pooled head kidneys, resulting in an additional 12 clones, denoted Dila/# (# as a lowercase letter for each clone). The clones represented 6 additional unique sequences compared with the previous obtained sequences.


VL
>< JL >
FR1 CDR1 FR2 CDR2 FR3 CDR3 FR4
Dila IEPTVTLTC QTNPKVKTWSDGTNRLH WYQQKSGQAPKLVMK NGKNPTS EFSSRFSGRADGENSNLTISGVQTEDAAIYYC KRYDEINSAWV FTFGGGTKLIIDL
DilaA .... W.....V..
DilaB ....A..... ..... W.....V..
DilaE ....
DilaJ .....R..... ....G..... ..... R.....
DilaK ....A..... ..... Y.....
DilaM .... L.....V..
DilaS .....M.....A.... ....V..
DilaIF .... .I.....
DilaRD ....A..... ..... Y.....V..
DilaCHNOP .... Y.....V..
Dila/c ....R..V..
Dila/d ....V..
Dila/h .....P.... ....V..
Dila/j ....V..
Dila/m ....V..
Dila/o ....V..
Dila/10x ....V..
< CL
FR4
Dila GVVRPTLTV LPPSREELQKDSATLVCLASGGFPSTWSLGWKVGGSSSSSGVSHSLEVLGRDGHYSWSSTLSLSADQWRKAGSVSCEASLSGQSP
DilaA ....
DilaB .....G..... .....
DilaE .....I ....
DilaJ ....
DilaK ....
DilaM ....F.....
DilaS .M..... .....E....V.....G..... .....
DilaIF ....
DilaRD .M..... .....
DilaCHNOP .M..... .....E....V..... .....
Dila/c .T..... .....E....V..... .....
Dila/d .M....P.. .....E....V..... .....
Dila/h .M..... .....E....V..... .....
Dila/j .M..... .....E....V.....R..... .....
Dila/m .M..... .....E....V.....S..... .....
Dila/o .M..... .....EG...V..... .....
Dila/10x .M..... .....E....V..... .....

Again, an extremely low variability was observed among the sequences as well as compared to the former ones. The additional variability was manly found in the J and C regions. Thus, a total of 18 unique sequences were obtained showing an extremely low variability in CDR1 and no variability in CDR2 or CDR3 (Fig. 5). Only 5 different V genes were found. All sequences could be aligned without introducing gaps in both V and J segments, and thus, no differences in the length of the CDRs were observed. The deduced CDR1, CDR2 and CDR3 were composed of 17, 7 and 11 amino acids, respectively. Analysis of the constant regions revealed 12 different C genes, which differed by up to 4 amino acids. An analysis of the isoelectric point (pI) showed that the CDR2 and CDR3 have a pI of 10.1 and 6.94, respectively, while in the CDR1 the pI ranged from 10.1 to 11.3.

2. DISCUSSION

This is the first IgL chain sequence from a representative species of the perciformes. To date IgL chain sequences have been obtained from a limited number of fish species from different orders.

From the first anchored PCR, using λgt10 primers in combination with a degenerated primer corresponding to conserved sequences in the middle of the C region of known vertebrate IgL genes (Tomana *et al.*, 1999), a partial sequence could be obtained encoding the 5' UT, the LP, the V and part of the C IgL region. Based on the sequence, a specific primer was designed in the VJ segments (Dila IgL/Fw). Since it has been shown in other teleost species that high variability occurs in those segments (Ghaffari & Lobb, 1993; Daggfeldt *et*

Figure 5 (former page)- Multiple alignment of the deduced amino acid sequences of the VL and CL regions obtained by PCR on the amplified library or cDNA from pooled sea bass head kidneys. Amino acid identities are indicated as dots. Putative frameworks (FR) and complementary determining segments (CDR) are indicated above sequences. On top, VL, JL and CL regions are indicated. Dila# (# as an uppercase letter for each clone obtained by PCR with the VL and CL specific primers on the amplified library) and Dila/# (# as a lowercase letter for each clone obtained by PCR with the VL and CL specific primers on the cDNA of pooled head kidneys). Dila/10x represents 10 identical sequences obtained by anchored PCR on the amplified library (3) and by PCR with the VL and CL specific primers on the cDNA of pooled head kidneys (6 clones) and on the amplified library (1 clone). Acc n°: AJ400216-33

al., 1993; Tomana *et al.*, 1999; Wildholm *et al.*, 1999) it was anticipated that the primer would be specific allowing the amplification of the remaining 3'-end of the sequence. Thus, the complete sequence presented in this study was obtained by the contig of the two sequences that completely matched in the overlap (153 nt). However, it should be noted that in subsequent experiments using the DilaIgVL/Fw and DilaIgVC/Rv specific primers, sequences were found identical in the C segment but differed by a few amino acid substitutions in the V and J segment (clones DilaJK in Fig. 5). Thus, the 5'-end of the clones could also be amplified by the primer used and the annealing temperature (50°) would allow for mismatches in the VJ segments where the primer was located.

All characteristic segments (V, J and C) coding for the V and C domains of the IgL chain could be deduced based on sequence comparisons with other teleost and vertebrate species. The typical cysteine residues that likely form the intra- and inter-chain interactions were also conserved in the sea bass.

Both the amino acid sequence comparisons and neighbor-joining trees showed that the IgL chain of sea bass sequenced in the present study can be considered a L1/G isotype (Ghaffari & Lobb, 1993; Daggfeldt *et al.*, 1993). Whether sea bass has additional light chain isotypes remains to be investigated. However, the fact that different isotypes have recently been characterised in the rainbow trout (Partula *et al.*, 1996), channel catfish (Ghaffari & Lobb, 1997) and Atlantic cod (Wermenstam, N. E. and Pilström, L., unpublished results) suggests that it is likely that a second light chain isotype may be present in sea bass. This is further supported by the fact that serum immunoglobulins from sea bass, purified using affinity chromatography and analysed by reducing SDS-PAGE, showed two different molecular weight (27.5 and 28.5 kDa) IgL polypeptides (Palenzuela *et al.*, 1996). Moreover, flow cytometry analysis using different combinations of monoclonal antibodies to Ig heavy and light chains, as well as double staining of immunofluorescence on cytospin preparations also seems to suggest the existence of more than one L chain, present on B cells in sea bass (dos Santos *et al.*, 1997). In accordance to what has been reported for other teleost species (Ghaffari & Lobb, 1993; Daggfeldt *et al.*, 1993; Tomana *et al.*, 1999), the sea bass IgL1 isotype sequenced in the present study can not be readily classified as a mammalian κ or λ light chain isotype, thought it seems more related to κ (Fig. 3)(Pilström & Lundqvist, 1998).

Southern blot analysis with specific V and C probes, suggested that the sea bass IgL genes have a cluster-like genomic organisation, reminiscent of the cluster organisation of the cartilaginous fish IgL genes (Shamblott & Litman, 1989; Marchalonis *et al.*, 1998), and as

shown in other teleost species (Ghaffari & Lobb, 1993; Daggfeldt *et al.*, 1993; Tomana *et al.*, 1999).

The sequence diversity within the V and J segments constitutes the first mechanism to create variability (Pilström & Bengtén, 1996). In addition, it has been shown that variability is mostly confined to the CDRs, although a lesser degree of diversity can also be found in the FRs (Ghaffari & Lobb, 1997; Wildholm *et al.*, 1999). To assess the variability of the sea bass IgL chain, PCRs were performed on the cDNA library and cDNA from pooled head kidneys. Seventeen additional unique sequences were obtained and showed an extremely low variability (Fig. 5). The 12 different C genes found, differed only up to 4 aa, and thus, indicate that these sequences represent closely related genes. This is consistent with the results from the Southern blot analysis where the specific C probe strongly hybridised with multiple genomic restriction fragments. Within the V segments variability was only confined to the CDR1 (4 different sequences with only 1 or 2 aa substitutions) and FR3 (3 different sequences with only 1 aa substitution). Thus, all V segments isolated in this study belong to a single family. Variability was greater in the J segment (9 different sequences) but only confined to 1 or 2 aa substitutions and without any gap. Although, the length of the CDR1 and CDR3 is considered important for the variability and capacity to bind antigen (Hsu & Steiner, 1999), this feature was absent in the present clones. However, the length of the CDR1 (17 amino acids) and CDR3 (11 amino acids) in the studied clones showed to match with the most common length of the CDR1 and CDR3 from the Atlantic cod (Wildholm *et al.*, 1999). CDR 2 is constant at 7 amino acids as expected. With the exception of the pI from the CDR2, both CDR1 and CDR3 showed to be close or within the range of pI reported for Atlantic cod (Wildholm *et al.*, 1999). Since only minor differences were observed in the CDR1, variation of the pI values were minor as well.

The low variability observed in the sequences obtained in the present study together with the fact that the head kidney was used as the source of the RNA could suggest that this organ would have mainly an haematopoietic function or that the Ig variability could be generated in another organ, as observed in chickens (Sayegh *et al.*, 1999). However, the head kidney has been shown to produce the highest number of specific antibody secreting cells when sea bass were immunised intraperitoneally with different antigens (*Photobacterium damselae* ssp *damselae*, *Vibrio anguillarum* and DNP_{494}-KLH), which indicates that is indeed a major organ for specific antibody production (dos Santos *et al.*, 2000).

Although, it can not be excluded that either the sea bass IgL chain might have natural low

variability not contributing for the overall variability of the antibody molecule or that another IgL isotype might be preferentially used, a more plausible explanation is that the immunisation has introduced a bias towards the induction of monospecific antibodies to DNP. High numbers of specific anti-DNP antibody secreting cells were produced by sea bass immunised with DNP_{494}-KLH (dos Santos *et al.*, 2000). It has been observed in experiments in carp immunised with the DNP_{494}-KLH (Wiegertjes *et al.*, 1996), which has shown high titre response to the DNP in the absence of an antibody response to the KLH (Wiegertjes, personal communication). The hypothesis proposed based on such observation has been termed intramolecular-induced suppression of anti-carrier response, and has been shown to occur when teleost species are immunised with haptenated-carriers (Avtalion & Milgrom, 1976). Thus, the same might have occurred in the immunised sea bass, although the anti-carrier responses have not been measured (dos Santos *et al.*, 2000). Furthermore, studies in BALB/c mice, immunised either once or twice with hapten(Xmp)-KLH, revealed that the V region gene complex associated with the anti-Xmp antibodies is encoded by a single VH gene, a conserved D segment sequence, one of three different JH genes and a single Vκ and Jκ gene combination, with only few somatic point mutations (Lou *et al.*, 1992). The IgL chain variability reported showed few differences in the CDRs and the J segment reminiscent of the sequence data presented in this study. Additionally, all CDRs and J segments of the L chain expressed in those mice hybridomas showed no length variation. The long CDR1 segments (17 aa) observed in the sea bass and mice hybridomas IgL chain sequence, could be the result of the fact that the canonical structures with long loop form small pocket at the antigen binding sites and are more often used in the recognition of small molecules as haptens (Ota *et al.*, 2000). In conclusion, the above strongly supports that the results obtained in the present study underpin the proposed mechanism of intramolecular-induced suppression.

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References

Warr, G.W. (1995). The immunoglobulin genes of fish. *Developmental and Comparative Immunology* **19**, 1-12.

Ghffari, S.H. & Lobb, C.J. (1993). Structural and genomic organization of immunoglobulin light chain in the channel catfish. An unusual genomic organizational pattern of segmental genes. *Journal of Immunology* **151**, 6900-6912.

Daggfeldt, A., Bengtén, E. & Pilström, L. (1993). A cluster type organization of the loci of the immunoglobulin light chain in Atlantic cod (*Gadus morhua* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) indicated by nucleotide sequences of cDNAs and hybridization analysis. *Immunogenetics* **38**, 199-209.

Partula, S., Schwager, J., Timmusk, S., Pilström, L. & Charlesmagne, J. (1996). A second immunoglobulin light chain isotype in the rainbow trout. *Immunogenetics* **45**, 44-51.

Ghaffari, S.H. & Lobb, C.J. (1997). Structure and genomic organisation of a second class of immunoglobulin light chain genes in the channel catfish. *Journal of Immunology* **159**, 250-258.

Tomana, M., Nakao, M., Moritomo, T., Fujiki, K. & Yano, T. (1999). Isolation of cDNA encoding immunoglobulin light chain from common carp (*Cyprinus carpio* L.). *Fish & Shellfish Immunology* **9**, 71-80.

Shamblott, M.J. & Litman G.W. (1989). Genomic organization and sequences of immunoglobulin light chain genes in a primitive vertebrate suggest coevolution of immunoglobulin gene organization. *EMBO Journal* **8**, 3733-3739.

Marchalonis, J.J., Schluter, S.F., Bernstein, R.M., Hohman, V.S. (1998). Antibodies of sharks: revolution and evolution. *Immunological Reviews* **166**, 103-122.

Pilström, L., Lundqvist, M.L. & Wermenstam, N. (1998). The immunoglobulin light chain in poikilothermic vertebrates. *Immunological Reviews* **166**, 123-132.

Wildholm, H., Lundbäck, A.-S., Daggfeldt, A., Magnadottir, B., Warr, G.W. & Piltröm, L. (1999). Light chain variable region diversity in Atlantic cod (*Gadus morhua* L.). *Developmental and Comparative Immunology* **23**, 231-240.

Hsu, E. & Steiner, L.A. (1992). Primary structure of immunoglobulin through evolution. *Current Opinion on Structural Biology* **2**, 422-431.

Mäkelä, O. & Litman, G.W. (1980). Lack of heterogeneity in anti-hapten antibodies of a phylogenetically primitive shark. *Nature* **287**, 639-640.

Du Pasquier, L. (1982). Antibody diversity in lower vertebrates-why is it so restricted? *Nature* **296**, 311-313.

Leslie, G.A. & Clem, L.W. (1976). Production of anti-hapten antibodies by several classes of lower vertebrates. *Journal of Immunology* **103**, 613-617.

Avtalion, R.R. & Milgrom, L. (1976). Regulatory effect of temperature and antigen upon immunity in ectothermic vertebrates. I. Influence of hapten density on the immunological and serological properties of penicilloyl-carrier conjugates. *Immunology* **31**, 589-594.

Cossarini-Dunier, M., Desvaux, F.-X. & Dorson, M. (1986). Variability in humoral responses to DNP-KLH rainbow trout (Salmo gairdneri). Comparison of antibody kinetics and immunoglobulins spectrotypes between normal trouts and trouts obtained by gynogenesis or self-fertilization. *Developmental and Comparative Immunology* **10**, 207-217.

Killie, J.-E., Jørgensen, T.Ø. (1994). Immunoregulation in fish I: intramolecular-induced suppression of antibody responses to haptenated protein antigens studied in Atlantic salmon (*Salmo salar* L). *Developmental*

and Comparative Immunology **18**, 123-136.

Killie, J.-E.A. & Jørgensen, T.Ø. (1995). Immunoregulation in fish II: intermolecular-induced suppression of antibody responses studied by haptenated antigens in Atlantic salmon (*Salmo salar* L). *Developmental and Comparative Immunology* **19**, 389-404.

Lamers, C.H.J., de Haas, M.H.J. & van Muiswinkel, W.B. (1985). Humoral response and memory formation in carp after injection of *Aeromonas hydrophila* bacterin. *Development and Comparative Immunology* **9**, 65-75.

Espelid, S., Hjelmeland, K. & Jørgensen, T. (1987). The specificity of Atlantic salmon antibodies made against the fish pathogen *Vibrio salmonicida*, establishing the surface protein VS-P1 as the dominanting antigen. *Developmental and Comparative Immunology* **11**, 529-537.

Boesen, H.T., Pedersen, K., Koch, C. & Larsen, J.L. (1997). Immune response of rainbow trout (*Oncorhynchus mykiss*) to antigenic preparations from *Vibrio anguillarum* serogroup O1. *Fish & Shellfish Immunology* **7**, 543-553.

Bricknell, I.R., King, J.A., Bowden, T.J. & Ellis, A.E. (1999). Duration of protective antibodies, and the correlation with protection in Atlantic salmon (*Salmo salar* L.), following vaccination with an *Aeromonas salmonicida* vaccine containing iron-regulated outer membrane proteins and secretory polysaccharides. *Fish & Shellfish Immunology* **9**, 139-151.

Voss, E.W., Groberg, W.J.Jr. & Freyer, J.L. (1978). Binding affinity of tetrameric coho salmon Ig anti-hapten antibodies. *Immunochemistry* **15**, 459-464.

Stolen, J. & Mäkelä, O. (1980). Cell collaboration in a marine teleost. A demonstration of specificity in the carrier effect. *Immunological Letters* **1**, 341-345.

Wetzel, M.C. & Charlemagne, C. (1985). Antibody diversity in fish. Isoelectrofocalisation study of individually-purified specific antibodies in three teleost fish species: tench, carp and goldfish. *Developmental and Comparative Immunology* **9**, 261-270.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extration. *Analytical Biochemistry* **162**, 156-159.

Feinberg, P. & Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6-13.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressives multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**(22), 4673-4680.

Kumar, S., Tamura, K. & Nei, M. (1993). MEGA: Molecular Evolutionary Genetics Analysis, version 1.01. The Pennsylvania State University, University Park, PA 16802.

Stet, R.J.M., van Erp, S.H.M., Hermsen, T., Sültmann & Egberts, E. (1993). Polymorphism and estimation of the number of *MhcCyca* class I and class II genes in laboratory strains of the common carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology* **17**, 141-156.

Church, G.M. & Gilbert, W. (1984). Genomic sequencing. *Proceedings of the National Academy of Sciences USA* **81**, 1991-1995.

Palenzuela, O., Sitjà-Bobadilla, A. & Álvarez-Pellitero, P. (1996). Isolation and partial characterisation of serum

immunoglobulins from sea bass (*Dicentrachus labrax* L.) and gilthead sea bream (*Sparus aurata* L.). *Fish & Shellfish Immunology* **6**, 81-94.

dos Santos, N.M.S., Taverne, N., Taverne-Thiele, A.J., de Sousa, M. & Rombout, J.H.W.M. (1997). Characterisation of monoclonal antibodies specific for sea bass (Dicentrarchus labrax L.) IgM indicates the existence of B cell subpopulations. *Fish & Shellfish Immunology* **7**, 175-191.

Pilström, L. & Bengtén, E. (1996). Immunoglobulin in fish-genes, expression and structure. *Fish & Shellfish Immunology* **6**, 243-262.

Sayegh, C.E., Demaries, S.L., Iacampo, S. & Ratcliffe, M.J.H. (1999). Development of B cells expressing surface immunoglobulin molecules that lack V(D)J-encoded determinants in the avian embryo bursa of Fabricius. *Proceedings of the National Academy of Sciences USA* **96**, 10806-10811.

dos Santos, N.M.S., Taverne-Thiele, A.J., Barnes, A.C., Ellis, A.E. & Rombout, J.H.W.M. (2000). Kinetics of juvenile sea bass (*Dicentrarchus labrax*, L.) systemic and mucosal antibody secreting cell response to different antigens (*Photobacterium damselae* ssp. *piscicida*, *Vibrio anguillarum* and DNP). *Fish & Shellfish Immunology* **submitted**.

Wiegertjes, G.F., Bongers, A.B.J., Voorthuis, P., Doulabi, B.Z., Groeneveld, A., van Muiswinkel, W.B. & Stet, R.J.M. (1996). Characterization of isogenetic carp (*Cyprinus carpio* L.) lines with a genetically determined high or low antibody production. *Animal Genetics* **27**, 313-319.

Lou, S.C., Winter, D., Mayers, G.L. & Bankert, R.B. (1992). Nucleotide sequence of messenger RNA encoding V_HDJ_H and $V_\kappa J_\kappa$ of a highly conserved idiotype-defined primary response anti-hapten antibody. *Journal of Immunology* **149**, 3944-3952.

Ota, T., Sitnikova, T. & Nei, M. (2000). Evolution of the vertebrates immunoglobulin variable gene segments. In *Origin and evolution of the vertebrate immune system.* (L. Du Pasquier L & G.W. Litman, eds.) pp:221-245. Current Topics in Microbiology and Immunology.

CHAPTER 4

Ontogeny of B and T cells in sea bass (*Dicentrarchus labrax* L.)

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"...complex systems can be understood only by meticulous data collection, logical analysis, and repeated practical scale investigation to identify the governing factors in each real situation. Neglect of any part of this combination quickly leads to nonsense." - L. P. Smith (1973)

Life in a changing climate. *In* N. Calder (ed.) Nature in the round. Wedenfeld and Nicolson, London, pp. 37-47.

Abstract

Monoclonal antibodies specific to sea bass Ig heavy (WDI 1) and light (WDI 3) chains and T cells (DLT15) were used in an ontogenetic study of sea bass by flow cytometry and immunocytochemistry. The influence of weight and age, as well as season, on B cell development was studied in the fastest and slowest growing offspring from the same spawn (5 to 305 days post hatch- dph). Additionally, B and T cell development was followed in samples of different offspring (5-137 dph). The results strongly suggest that DLT15 recognises very early (pre-?) T cells as well as mature T cells and that these very early T cells might have their origin in a different compartment and subsequently mature in the thymus. They also appeared much earlier in ontogeny (between 5-12 dph onwards) than pre-B cells having cytoplasmic Ig (from 52 dph onwards). With the monoclonal antibodies used, adult levels of T and B cells were both reached between 137-145 dph, suggesting that sea bass is immunologically mature from at least that age onwards. As in other teleosts, the thymus appears to be the primary organ for T lymphocytes and head kidney the primary organ for B lymphocytes. For sea bass, age seems to be more important in determining B cell maturation than body weight.

Key words: Sea bass, *Dicentrarchus labrax*, ontogeny, B cells, plasma cells, T cells

1. INTRODUCTION

While vaccination of fish is obviously the best way to control diseases, care has to be taken concerning the immunocompetent state of fish, since tolerance, instead of protection, can arise if fish are immunised when too young (van Loon *et al.*, 1981; Joosten *et al.*, 1995). Vaccination at early stages has economic advantages (less mortality at early stages and less vaccine needed) and is easier to perform. From the practical point of view, fish are graded and grouped on the basis of their average weight during the production cycle, allowing for a study on the importance of weight *v.* age in determining immunological maturation for fish vaccination. From the literature, indications are available that weight might correlate better with the development of the immune system than age (Tatner, 1996).

The appearance of lymphoid organs, as well as lymphocytes within them, does not correlate with their active functionality or maturation. Even if morphologically mature lymphoid cells are present at early stages, fish might not be functionally immunocompetent until later (Tatner, 1996). The appearance of surface Ig^+ B cells and cytoplasmic Ig^+ plasma cells might better indicate the functional maturity of the lymphoid system as suggested for salmon, *Salmo salar* (Ellis, 1977), carp, *Cyprinus carpio* (van Loon *et al.*, 1981; Koumans van Diepen *et al.*, 1994), skate, *Bathyraja aleutica* (Kobayashi *et al.*, 1985), rainbow trout,

Oncorhynchus mykiss (Razquin *et al.*, 1990; Castillo *et al.*, 1993), and sea bass, *Dicentrachus labrax* (Breuil *et al.*, 1997). As the responses to most antigens (T dependent antigens) depend on both T and B cell recognition, the ontogeny of these cell types is important. Several studies have indicated differences in the immune response to T cell dependent and T cell independent antigens during ontogeny (Tatner, 1996). Therefore, attention must also be paid to the ontogeny of T cells, because of their central role in the immune responses. As only a few T cell markers are available for fish (Scapigliati *et al.*, 1995; Passer *et al.*, 1996; Rombout *et al.*, 1997; Rombout *et al.*, 1998) only a limited number of studies are available on the ontogeny of T cells (Abelli *et al.*, 1996; Picchietti *et al.*, 1997; Romano *et al.*, 1999).

In this paper, the ontogeny of sea bass B and T cells was studied using specific monoclonal antibodies (mAb; dos Santos *et al.*, 1997; Scapigliati *et al.*, 1995; Scapigliati *et al.*, 2000). The influence of weight and age on the ontogeny of sea bass B cells was studied by comparing groups of fish with different growth rates from the same spawn and by following the offspring from different spawns.

2. MATERIALS AND METHODS

2.1. FISH SAMPLING

Sea bass samples were kindly supplied by a local farm (Rio Alto, A. Coelho & Castro Lda; Povoa do Varzim, Portugal). Two different samplings were made from different spawners and seasons. From the first spawn, samples were collected from 5 until 335 days post-hatch (dph). At 83 dph the larvae (same spawn/age) were graded (different weight) and divided into 3 groups. The groups containing the largest and the smallest fish were used in experiments. From the second spawn (one year later), samples were taken from 5 until 137 dph. Table 1 shows average rearing temperatures and weights for both spawns.

2.2. FLOW CYTOMETRY

Before 80 dph cell suspensions were made by macerating whole larvae/fry (more than 100) through a 100 μm mesh gauze using sea bass-RPMI (SB-RPMI; RPMI + 42 mM NaCl + 1% w/v BSA + 0.01% v/v of a 1.5 M NaN_3 stock solution). The cells were then washed twice using SB-RPMI for 10 min at 550 *g* and 4° C and the pellet resuspended in 2-3 ml of SB-RPMI. The cells were carefully layered onto Percoll (Sigma; 1.07 and 1.02 mg l^{-1} density

Table 1- Average of daily rearing temperatures ± 1° C and fish weights for first and second spawns. Underlined temperatures mean artificial heated water; temperatures not underlined mean natural water temperatures. dph: days post-hatch; g: grams; lf: larger fish; sf: smaller fish.

FIRST SPAWN (1997 → 1998)																	
dph (day/month)	5 (11/03)	12 (18/03)	19 (25/03)	25 (31/03)	32 (07/04)	39 (14/04)	—	53 (28/04)	—	68 (13/05)	83 (18/05)	98 (12/06)	117 (01/07)	—	145 (29/07)	208 (30/09)	335 (04/02)
T° C	16.1	18.3	17.9	17.8	14.9	15.6	—	16.1	—	16.2	16.5	18.6	17.3	—	15.5	16.7	14.3
g/lf	—	—	—	—	—	—	—	—	—	—	0.50	1.24	2.35	—	6.65	11.48	34.49
g/sf	—	—	—	—	—	—	—	—	—	—	0.12	0.18	0.63	—	2.59	4.55	10.95
SECOND SPAWN (1998)																	
dph (day/month)	5 (02/04)	12 (09/04)	—	24 (21/04)	31 (28/04)	38 (05/05)	45 (12/05)	52 (26/05)	59 (26/05)	66 (02/06)	87 (23/06)	101 (07/07)	116 (22/07)	137 (12/08)	—	—	—
T° C	17.6	18.8	—	15.6	14.8	15.2	14.7	13.5	13.7	14.0	16.2	16.3	17.6	16.6	—	—	—
g	—	—	—	—	—	—	0.009	0.015	0.026	0.038	0.078	0.117	0.468	1.006	—	—	—

Table 2- Percentages of sIg^+ cells in different organs (arranged in decreasing order) of young sea bass of different age and growth rate. The cells were stained by indirect immunofluorescence with combination of WDI1 and WDI3 in peripheral blood leucocytes (PBL), spleen (Sp), head kidney (Hk), gut and thymus (Thy) cell suspensions. The percentage of sIg^+ cells was determined using gated lymphoid cells and propidium iodide dead cell exclusion as shown in Fig. 1a,b. Fish samples from same spawn after grading (different weight). dph: days post-hatch; g: weight in grams.

	LARGER ANIMALS						SMALLER ANIMALS					
dph (g)	*(% of sIg^+)* + → −					**dph (g)**	*(% of sIg^+)* + →					
83 (0.493)	Hk *(5.69)*	Sp *(3.90)*	Thy *(0.22)*	Gut *(-0.10)*		**83 (0.116)**	Hk *(5.52)*	Sp *(3.66)*	Gut *(0.41)*	Thy *(0.30)*		
98 (1.237)	Hk *(11.02)*	PBL *(9.62)*	Sp *(7.21)*	Gut *(0.76)*	Thy *(0.30)*	**98 (0.175)**	Sp *(14.10)*	Hk *(11.03)*	PBL *(4.34)*	Gut *(1.58)*	Thy *(0.64)*	
117 (2.351)	Hk *(16.84)*	Sp *(16.06)*	PBL *(8.87)*	Gut *(1.12)*	Thy *(0.47)*	**117 (0.628)**	Sp *(11.66)*	Hk *(9.57)*	PBL *(6.39)*	Gut *(0.44)*	Thy *(0.03)*	
145 (6.649)	PBL *(34.25)*	Sp *(30.60)*	Hk *(13.55)*	Gut *(2.29)*	Thy *(0.41)*	**145 (2.594)**	PBL *(45.30)*	Sp *(34.73)*	Hk *(19.87)*	Gut *(1.99)*	Thy *(0.52)*	
208 (11.480)	Sp *(35.99)*	PBL *(35.25)*	Hk *(15.20)*	Gut *(1.83)*	Thy *(1.23)*	**208 (4.550)**	PBL *(26.67)*	Sp *(24.65)*	Hk *(13.26)*	Gut *(2.87)*	Thy *(1.28)*	
335 (34.490)	PBL *(44.16)*	Sp *(20.62)*	Hk *(8.10)*	Gut *(6.60)*	Thy *(1.19)*	**335 (10.954)**	PBL *(27.65)*	Sp *(20.66)*	Hk *(10.84)*	Gut *(2.34)*	Thy *(1.06)*	

3. RESULTS

3.1. FLOW CYTOMETRY

3.1.1. First spawn

Surface Ig-positive (sIg^+) cells could clearly be detected in macerated larvae at 68 dph. The combination of WDI1 and WDI3 (equal volumes of optimal dilutions) showed the highest percentage (1.84 %). WDI1 and WDI3 single staining showed 0.76 and 1.23 %, respectively.

Percentages of sIg^+ cells for the larger and smaller fish, detected by combination of WDI1 and WDI3 in PBL, spleen, head kidney, gut and thymus are shown in Table 2 in decreasing order of percentage sIg^+ cells contained in each organ. In the larger fish, the percentage of sIg^+ cells in blood and gut continuously increased until 335 dph. Percentages of sIg^+ cells reached its maximum value at 208 dph in spleen while in head kidney it reached a plateau from 117 to 208, followed by a decline in both organs between 208 and 335 dph. In the smaller fish, the highest percentage of sIg^+ cells was reached at 145 dph for blood, spleen and head kidney, followed by a decline between 145 and 208 dph, thereafter remaining constant in all organs observed. Early in ontogeny, head kidney showed the highest percentage of sIg^+ cells followed by spleen, gut and thymus. During ontogeny, a shift in the sequential distribution of the

percentages of sIg^+ cells in the different organs was observed, ending with blood containing the highest percentage of sIg^+ cells, followed by spleen, head kidney, gut and thymus. Similar results were obtained when WDI1 or WDI3 were used alone.

3.1.2. Second spawn

Percentages of sIg^+ cells (WDI1 and/or WDI3) and T cells (DLT15) in macerated whole larvae, PBL, spleen, head kidney, gut and thymus are shown in Fig. 2a-f. T cells could be detected in macerated larvae (Fig. 2a) with DLT15 between 5-12 dph (0.34-1.34 %) with increasing percentages until 66 dph (12.71 %), showing 0.97 correlation with age. Surface Ig^+ cells could clearly be detected in whole macerated larvae (Fig. 2a) at 59 dph (0.7 % with WDI1 and 2.41 % with WDI3).

The percentages of T cells was maximal at 101 dph for blood, spleen and head kidney and at 87 dph for gut and thymus. Subsequently, the percentage of $DLT15^+$ cells declined in all organs investigated (Fig. 2b-f). No clear time effect in the percentage of sIg^+ cells could be observed in the lymphoid organs between 87 and 116 dph. However, in all organs (except thymus) the highest values for percentage of sIg^+ cells were reached at 137 dph. The sequential distribution of the percentages of sIg^+ cells in the different organs and its shift during ontogeny is comparable to that found for the first spawn. Similar results were obtained when WDI1 or WDI3 were used alone. WDI3 single staining showed higher percentages of sIg^+ cells than WDI1 in all organs, but always lower than the combination of the two mAbs (Fig. 2b-f).

3.2. IMMUNOFLUORESCENCE ON CYTOCENTRIFUGE SLIDES

All mAbs showed specific immunoreaction on cytocentrifuge slides. In all samples most of the $DLT15^+$ cells were small surface-stained lymphocytes, although some strong and faint cytoplasmically stained cells could also be observed (Fig. 3a). In thymus, almost all cells appeared to be $DLT15^+$. Scarce cytoplasmic Ig^+ (cIg^+) cells were first detected at 52 dph (Fig. 3b). At 59 dph, cIg^+ cells could be detected much more easily and were shown to have stronger staining. At this age, sIg^+ cells could also be observed (Fig. 3c). From 87 dph onwards, cIg^+ and sIg^+ cells could be detected in all lymphoid organs, with the exception of the thymus (Fig. 3d,e,f). Cytoplasmic Ig^+ cells from excised organs appeared to have stronger staining when compared with the ones from macerated samples. Spleen followed by head kidney appeared to be the organs with the highest percentage of cIg^+ cells.

al., 1996) and gut (Picchietti *et al.*, 1997) of sea bass larvae, at 30 dph, three days after the thymus became lymphoid. The differences in the results might be explained by the higher sensitivity of flow cytometric analysis. This suggests that DLT15 might react with an antigenic determinant already present on very early (pre-?) T cells from a different compartment which further mature in the thymus as happens in mammals and birds (Lydyard & Grossi, 1993), because the thymus is not yet lymphoid (Abelli *et al.*, 1996; Breuil *et al.*, 1997) when $DLT15^+$ cells were first detected. Future studies with probes to the ikaros gene, expressed in early lymphoid cells (Trede & Zon, 1998), might help to clarify the real nature of these early $DLT15^+$ cells. Previous morphological studies have shown that in fish, the thymus is the first lymphoid organ to contain lymphocytes during ontogeny (Ellis, 1977; Grace & Manning, 1980; Josefsson & Tatner, 1993; Abelli *et al.*, 1996; Breuil *et al.*, 1997). In sea bass, the head kidney is the first organ to develop, but becomes lymphoid after the thymus (Abelli *et al.*, 1996; Breuil *et al.*, 1997). Whether the head kidney already contains stem cells is not known yet, but the yolk sac and the liver are also possible candidates as these are sources of stem cells in mammals (Lydyard & Grossi, 1993). Low numbers of large cytoplasmic $DLT15^+$ cells could also be detected in all cytocentrifuge preparations in both macerated larvae and organs. Abelli *et al.* (1996) also stated the presence of immunoreactive molecules within the cytoplasm. Nevertheless, their relatively low number and large size indicate that these cytoplasmic $DLT15^+$ cells represent a cell type different from the large granular lymphocytes (type b) described by Romano *et al.* (1997a). The nature and function of these large cytoplasmic $DLT15^+$ cells remain to be investigated.

Cytoplasmic Ig^+ cells were detected 1-2 weeks earlier in ontogeny (52 dph) than sIg^+ cells. The appearance of early cIg^+ cells (preB cells) during ontogeny is supported by the results on trout by Castillo *et al.* (1993) and are in agreement with the results reported for amphibians (Zettergren, 1982; Hadji-Azimi *et al.*, 1982) and mammals (Landreth & Kinkade, 1984; van Rees *et al.*, 1990). In mammals, it is known that preB cells express μ heavy chains in the cytoplasm and some of them have small amounts of surface μ chains associated with surrogate light chain VpreB and λ5 (Karasuyama *et al.*, 1996), while in chickens, rearrangement of Ig heavy and light chain loci appears to occur simultaneously to produce sIg^+ B cells (Weill & Reynaud, 1995). However, it is important to note that the cIg^+ cells described here for sea bass were detected using a mAb to the L chain of sea bass Ig and at 52 dph they showed a less dense fluorescent staining pattern than the ones from adult control fish and fish of 59 dph onwards which are regarded as plasma cells. These results suggest that cells detected at 52

dph might indeed be preB cells and that in sea bass, the L chain might be transcribed in high amounts from early stages of B cell development. Whether there is co-expression of L and H chain in these cells or whether the expression of the H chain precedes the expression of the L chain remains to be investigated. Both flow cytometry and cytocentrifuge preparations support the appearance of sIg^+ cells between 59-68 dph. Breuil *et al.* (1997) found similar percentages of sIg^+ cells as the ones reported here, although they claimed their appearance earlier in ontogeny using flow cytometric analysis. The differences encountered earlier in ontogeny might have been due to the high contamination of melanin present in the macerated samples (also stated by the mentioned authors) and/or variable cross reactivity of the conjugated antibody, leading to higher fluctuation of the measurements.

Early in ontogeny, percentages of T cells were highest in thymus, followed by gut, PBL, head kidney and spleen. They were also present in higher percentages than B cells in the sampled organs, with the exception of spleen. Additionally, the high percentage found in PBL at this stage might indicate that some migration occurs from thymus to other organs, as has been suggested by others (Secombes *et al.*, 1983; Tatner, 1985; Josefsson & Tatner, 1993; Abelli *et al.*, 1996). Later, the percentage of T cells decreased in PBL, spleen and head kidney but not in thymus and gut. Adult levels of the percentages of $DLT15^+$ cells (Scapigliati *et al.* 1995, Romano *et al.*, 1997a) were present in all organs at the last sampling day (137 dph) with the thymus showing the highest percentage of $DLT15^+$ cells, followed by gut, head kidney and spleen.

It is worth mentioning that the percentage of $DLT15^+$ cells in the thymus and to a large extent in the gut is higher than presented in Fig. 2, because nearly all the cells showed a shift in fluorescence although they did not pass the markers used to gate the cells (figure 1c,d). This was confirmed by the cytocentrifuge preparations in which almost all the thymocytes were $DLT15^+$. Additionally, a tendency for the positive cells to aggregate was observed in the cytocentrifuge preparations of thymus and gut which could also have had a decreasing effect on the flow cytometric percentage of positive cells in these organs.

Both cIg^+ plasma cells and sIg^+ B cells could clearly be observed in spleen, head kidney and gut from 87 dph onwards. The spleen contained the highest percentage of cIg^+ plasma cells followed by head kidney. This is in accordance with the ultrastuctural study of sea bass head kidney (Meseguer *et al.*, 1991) and spleen (Quesada *et al.*, 1990). In fish from both spawns, a similar sequential order for the percentage of sIg^+ cells in different organs was found, with only minor differences between the larger and smaller animals from the first

spawn. As for the T cells, the adult organ distribution of sIg^+ cells (dos Santos *et al.*, 1997) was reached by 137-145 dph. PBL contained the highest percentage sIg^+ cells, followed by spleen, head kidney, gut and thymus. These results suggest that sea bass might be immunologically mature from at least this age. Additionally, the results confirm those of others (Abeli *et al.*, 1996; Breuil *et al.*, 1997), that in sea bass the thymus is the primary organ for T lymphocytes and the head kidney for B lymphocytes, which is similar to other fish species (Ellis, 1977; Botham & Maning, 1981; Koumans-van Diepen *et al.*, 1994; Romano *et al.*, 1997b; Razquin *et al.*, 1990; Chantanachooklin *et al.*, 1991; Josefsson & Tatner, 1993). A surprising feature was the decline in the percentage of sIg^+ cells, observed in both groups of fish of the first spawn, coincident with the Autumn and Winter periods. Since the results are relative percentages, it is not known if this decline was due to the increase of another cell population or a decline in the B cell population. Attention has to be paid to the fact that declining percentages of B cells were observed at 208 dph (29th September), while the temperatures were still high. This suggests that in addition to temperature (Bly & Clem, 1992) other seasonal factors might influence the fish immune system.

No clear tendency for a correlation of the percentages of sIg^+ cells with body weight was found in any of the organs. Direct comparison of the results from the faster and slower growing fish from the first spawn, did not show a clear tendency for different percentages and/or rate of increase of sIg^+ cells in organs of the two groups, with the exception of blood from larger animals. These results were further supported by the data from the second spawn where fish showed similar percentages of sIg^+ cells at equivalent ages, although they experienced a slower growth. Thus, the present results strongly suggest that, at least in sea bass, age is the most important factor relating to the development of immunocompetence, contradicting the suggestions advanced for other species (Tatner, 1996).

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References

Abelli, L., Picchietti, S., Romano, N., Mastrolia, L. & Scapigliati, G. (1996). Immunocytochemical detection of thymocyte antigenic determinants in developing lymphoid organs of sea bass *Dicentrarchus labrax* (L.). *Fish & Shellfish Immunology* **6**, 493-505.

Bly, J. E. & Clem, L. W. (1992). Temperature and teleost immune functions. *Fish & Shellfish Immunology* **2**, 159-171.

Botham, J. E. & Manning, M. J. (1981). The histogenesis of the lymphoid organs in carp (*Cyprinus carpio* L.) and the ontogenetic development of allograft reactivity. *Journal of Fish Biology* **19**, 403-414.

Breuil, G., Vassiloglou, B. Pepin, J. F. & Romestand, B. (1997). Ontogeny of IgM-bearing cells and changes in the immunoglobulin M-like protein level (IgM) during larval stages in sea bass (*Dicentrarchus labrax*). *Fish & Shellfish Immunology* **7**, 29-43.

Castillo, A., Sánchez, C., Dominguez, J., Kaattari, S. L. & Villena, A. J. (1993). Ontogeny of IgM and IgM-bearing cells in rainbow trout. *Development and Comparative Immunology* **17**, 419-424.

Chantanachookhin, C., Seikai, T. & Tanaka, M. (1991). Comparative study on the ontogeny of the lymphoid organs of three species of marine fish. *Aquaculture* **99**, 143-156.

dos Santos, N. M. S., Taverne, N., Taverne-Thiele, A. J., de Sousa, M. & Rombout, J. W. M. (1997). Characterisation of monoclonal antibodies for sea bass (*Dicentrarchus labrax* L.) IgM indicates the existence of B cell subpopulations. *Fish & Shellfish Immunology* **7**, 175-191.

Ellis, A. E. (1977). Ontogeny of the immune response in *Salmo salar*. Histogenesis of the lymphoid organs and appearance of membrane immunoglobulin and mixed leukocyte reactivity. *In* "Developmental immunobiology" (J. B. Solomon & J. D. Horton, eds.), pp. 225-231. Elsevier/North Holland Biomedical press.

Grace, M. F. & Manning, J. J. (1980). Histogenesis of the lymphoid organs in rainbow trout, *Salmo gairdneri* Rich. 1836. *Developmental and Comparative Immunology* **4**, 255-264.

Hadji-Azimi, I., Schwager, J. & Thiebaud, C. (1982). B-lymphocyte differentiation in *Xenopus laevis* larvae. *Developmental Biology* **90**, 253-258.

Jósefsson, S. & Tatner, M. F. (1993). Histogenesis of the lymphoid organs in sea bream (*Sparus aurata* L.). *Fish & Shellfish immunology* **3**, 35-49.

Joosten, P. H. M., Avilés-Trigueros, M., Sorgeloos, P. & Rombout, J. H. W. M. (1995). Oral vaccination of juvenile carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*) with bioencapsulated *Vibrio anguillarum* bacterin. *Fish & Shellfish Immunology* **5**, 289-299.

Karasuyama, H., Rolink, A. & Melchers, F. (1996). Surrogate light chain in B cell development. *Advances in Immunology* **63**, 1-41.

Koumans-van Diepen, J. C. E., Taverne-Thiele, J. J., van Rens, B. T. T. M. & Rombout, J. H. W. M. (1994). Immunocytochemical and flow cytometric analysis of B cells and plasma cells in carp (*Cyprinus carpio* L.); an ontogenetic study. *Fish & Shellfish Immunology* **4**, 19-28.

Kobayashi, K., Tomonaga, S., Teshima, K. & Kajii, T. (1985). Ontogenic studies on the appearance of two classes of immunoglobulin-forming cells in the spleen of the Aleutian skate, *Bathyraja aleutica*, a cartilaginous fish. *European Journal of Immunology* **15**, 952-956.

Landreth, K. S. & Kincade, P. W. (1984). Mammalian B lymphocyte precursors. *Development and Comparative Immunology* **8,** 773-790.

Lydyard, P. & Grossi, C. (1993). Development of the immune system. In *Immunology* (Roitt, I. M., Brostoff, J. & Male, D., Eds.; third edition) pp. 11.1-11.9. Mosby.

Meseguer, J., Esteban, M. A. & Agullero, B. (1991). Stromal cells, macrophages and lymphoid cells in the head-kidney of sea bass (*Dicentrarchus labrax* L.). An ultrastructural study. *Archives of Histology and Cytology* **54,** 299-309.

Passer, B. J., Chen, C-L., Miller, N. W. & Cooper, M. D. (1996). Identification of a T cell lineage antigen in catfish. *Developmental and Comparative Immunology* **20,** 441-450.

Picchietti, S., Terribili, F. R., Mastrolia, L., Scapigliati, G. & Abelli, L. (1997). Expression of lymphocyte antigenic determinants in developing gut-associated lymphoid tissue of the sea bass *Dicentrarchus labrax* (L.). *Anatomy and Embryology* **196,** 457-463.

Queasada, J., Villena, M. I. & Agulleiro, B. (1990). Structure of the spleen of the sea bass (*Dicentrarchus labrax*): a light and electron microscopy study. *Journal of Morphology* **206,** 273-281.

Razquin, B. E., Castillo, A., López-Fierro, P., Alvarez, F., Zapata, A. & Villena, A. J. (1990). Ontogeny of IgM-producing cells in the lymphoid organs of rainbow trout, *Salmo gairdneri* Richardson: an immuno- and enzyme-histochemical study. *Journal of Fish Biology* **36,** 159-173.

Romano, N. Abelli, L., Mastrolia, L. Scapigliati, G. (1997a). Immunocytochemical detection and cytomorphology of lymphocyte subpopulations in a teleost fish *Dicentrarchus labrax*. *Cell Tissue Research* **289,** 163-171.

Romano, N., Taverne-Thiele, J. J., Fanelli, M., Baldassini, M. R., Abelli, L., Mastrolia, L., van Muiswinkel, W. B. & Rombout, J. H. M. W. (1999). Ontogeny of the thymus in a teleost fish, *Cyprinus carpio* L.: developing thymocytes in the epithelial microenvironment. *Developmental and Comparative Immunology* **23,** 123-137.

Romano, N., Taverne-Thiele, J. J., van Maanen, J. C. & Rombout, J. H. M. W. (1997b). Leucocyte subpopulations in developing carp (*Cyprinus carpio* L.): immunocytochemical studies. *Fish & Shellfish immunology* **7,** 439-453.

Rombout, J. H. W. M., Joosten, P. H. M., Engelsma, M. Y., Vos, A. P., Taverne, N. & Taverne-Thiele, J. J. (1998). Indications for a distinct putative T cell population in mucosal tissue of carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology* **22,** No. 1, 63-77.

Rombout, J. H. W. M., van de Wal, J. W., Companjen, A., Taverne, N. & Taverne-Thiele, J. J. (1997). Characterization of a T cell lineage marker in carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology* **21,** No. 1, 35-46.

Scapigliati, G., Mazzini, M., Mastrolia, L., Romano, N. & Abelli, L. (1995). Production and characterisation of a monoclonal antibody against the thymocytes of the sea bass *Dicentrarchus labrax* (L.) (Teleostea, Percicthydae). *Fish & Shellfish Immunology* **5,** 393-405.

Scapigliati, G., Romano, N., Abelli, L., Meloni, S., Ficca, A. G., Buonocore, F., Bird, S., and Secombes, C. J. (2000). Immunopurification of T-cells from sea bass *Dicentrarchus labrax* (L.). *Fish & Shellfish Immunology* **10,** xxx-xxx.

Secombes, C. J., van Groningen, J. M., van Muiswinkel, W. B. & Egberts, E. (1983). Ontogeny of the immune system in carp (*Cyprinus carpio* L.). The appearance of antigenic determinants on lymphoid cells detected by mouse anti-carp thymocyte monoclonal antibodies. *Development and Comparative Immunology* **7,** 455-464.

Tatner, M. F. (1985). The migration of labelled thymocytes to the peripheral lymphoid organs in the rainbow trout, *Salmo gairdneri* Richardson. *Developmental and Comparative Immunology* **9,** 85-91.

Tatner, M. F. (1996). Natural changes in the immune system of fish. In *The Fish Immune System; Organism, Pathogen and Environment* (Iwama, G. & Nakanishi, T., Eds.) pp. 255-287. Academic Press.

Trede, N. S. & Zon, L. I. (1998). Development of T-cells during fish embryogenesis. *Developmental and Comparative Immunology* **22,** 253-263.

van Loon, J. J. A., van Oosterom, R. & van Muiswinkel, W. B. (1981). Development of the immune system in carp (*Cyprinus carpio*). *In* "Aspects of Develpmental and Comparative Immunology" (J. B. Solomon, ed.), Vol. 1, pp. 469-470. Pergamon Press, Oxford.

van Rees, E. P., Dijkstra, C. D. & Sminia, T. (1990). Ontogeny of the rat immune system: an immunocytochemical approach. *Developmental and Comparative Immunology* **14,** 9-18.

Weil, J. C. & Reynaud, C. A. (1995). Generation of diversity by post-rearrangement diversification mechanisms: the chicken and the sheep antibody repertoires. In *Immunoglobulin Genes* (Honjo, T., and Alt, F. W., Eds.), pp. 267-288, Academic Press, London.

Zettergren, L. D. (1982). Ontogeny and distribution of cells in B lineage in the American frog, *Rana pipiens*. *Developmental and Comparative Immunology* **6,** 311-320.

antigen as respective controls for each immunised group. Duplicates were done in different plates to reduce plate variability. Secretion of antibodies was allowed to occur over 6-7 h at 25° C in a vibration-free place. After washing and immersion, as described above, 100 µl of a mixture of the monoclonal antibodies (mAb) to sea bass heavy (WDI1) and light (WDI3) Ig chains (dos Santos *et al.*, 1997) diluted 1:25 and 1:50, respectively, in PBS containing 0.1% BSA was added to each well and incubated overnight at 4° C. Subsequently, the nitrocellulose was removed from the plates and washed (3x) and immersed (5 min) in PBS-Tween (PBS + 0.05% of polyoxyethylene-sorbitan monolaurate) on a tray with shaking. The sheets were then incubated (shaking) for 1 h with goat anti-mouse Ig horseradish peroxidase (GAM-HRP, BIORAD) diluted 1:3000 in PBS-Tween containing 0.1% BSA. After washing in PBS-Tween (2x) and PBS (3x) the spots were developed for 10-15 min at room temperature by adding the chromogen solution containing 258 µg 3-amino-9-ethyl carbazole (Sigma) ml^{-1} and 0.04% H_2O_2 (30%) in 0.05M sodium acetate buffer pH 5.0 (Czerkinsky *et al.*, 1988). The reaction was stopped by washing with running tap water and the nitrocellulose allowed to dry before counting.

2.5. SPOT COUNTING AND STATISTICS

To allow accurate counting of the maximum number of spots (up to 800), a grid was drawn in the nitrocellulose using a scalpel. This also allowed the use of high magnification (40x) for counting real spots. All wells of the dilution steps with more than 5 spots were considered for spot enumeration and the average number of spots was calculated for each treatment taking into account the corresponding number of cells per well. This allowed the reduction of any variation (dilution variability) between wells. The results were expressed as the average number of ASC from duplicated plates per 10^6 leukocytes.

Numbers of ASC in the spleen, head kidney and gut were converted to non-zero format (+1) and transformed by conversion to $\log_e$ before comparison using two-way analysis of variance with a nested design of time (days post-immunisation) in the type of response (primary or secondary). All sampling days were nested in the respective type of response in order to use the maximum degrees of freedom for reducing the error variance. Thus, it is worth mentioning that comparisons were based on means from all sampling days in each type of response. Post-hoc comparisons were carried out using the Scheffé test in order to determine differences between groups. P levels lower or equal to 0.05 were considered

significant. The software STATISTICA 5.0 (STATSOFT INC., 1995) was used for all the statistical tests.

Relative frequencies of ASC were calculated based on the organs of the fish sampled over the periods only where the higher levels of the responses took place. Thus, fish from sampling days 16 and 18 were not considered in the primary response of the gut and the head kidney to *P. damselae* ssp. *piscicida*, while fish from sampling day 22 were not considered for spleen. Sampling days 87 and 90 were not considered for the secondary response to *P. damselae* ssp. *piscicida* of all the organs. In the response to DNP, only the fish from sampling day 80 were not considered in all the sampled organs.

3. RESULTS

ASC were detected in all sampled organs of both primary and secondary responses (Figure 1) and immune leukocytes never gave higher numbers of ASC on heterologous antigen-coated wells than control leukocytes, indicating the responses measured were antigen specific. However, a high variability among the responses of the individuals was observed at each sampling day. Additionally, some individual control fish (PBS/FIA-injected) showed high numbers of ASC for each of the coating antigens or even when no coating was used (blank wells). These were mostly higher in the gut and especially when DNP-BSA was used as coating.

Table 1 shows the p values for comparisons between ASC from organs of immunised and control fish for each of the antigen groups, taken over the whole of the sampling periods of the primary and secondary responses.

3.1. ASC TO Photobacterium damselae ssp. piscicida

A slight and similar elevation of ASC was observed in the head kidney and the gut from immunised fish in the primary response with peaks being reached 22-24 days post-immunisation, although in the gut this was not significantly different from the control group (Fig. 1 and Table 1). In the secondary response, a high elevation of the ASC was observed in the head kidney followed by the spleen (peaking at day 7 post-boost) but not in the gut, which was again not significantly different from the control group (Fig. 1 and Table 1). Despite the higher numbers of ASC in some fish in the secondary responses, no statistical differences were detected between the primary and secondary responses. Additionally, no significant

differences were observed between the organs, although a low p value was observed between the secondary responses of head kidney and gut (0.060).

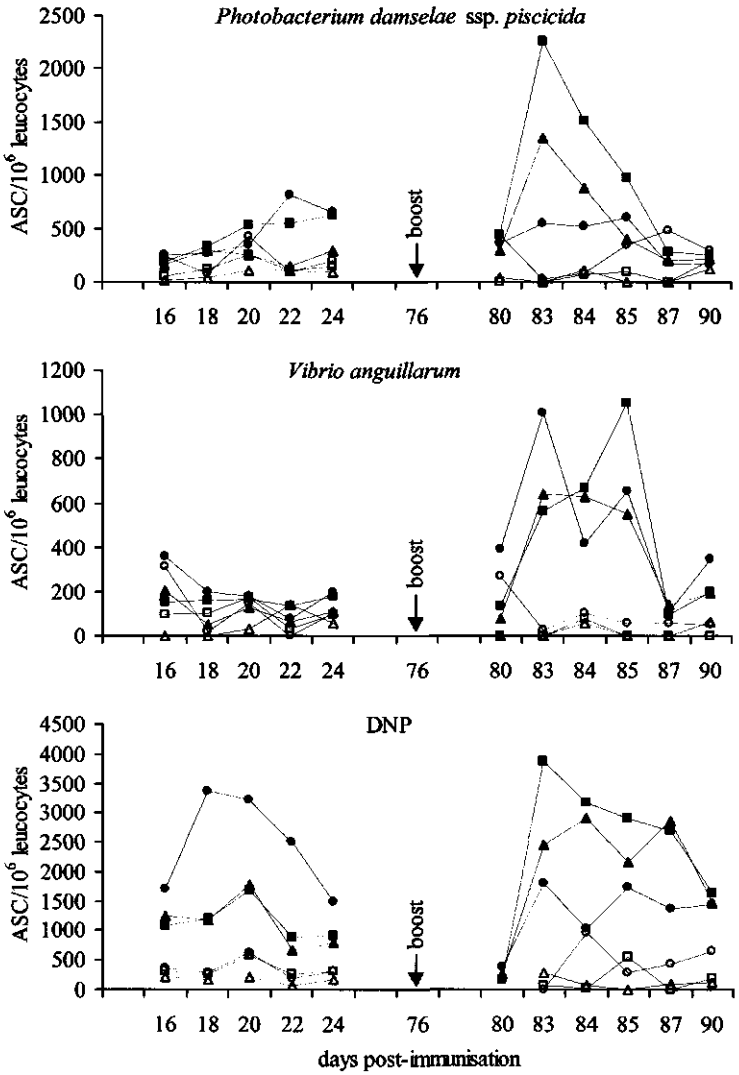


Fig. 1- Number of specific antibody secreting cells (ASC) in the head kidney, spleen and gut after primary and secondary immunisation of sea bass with *Photobacterium damselae* ssp. *piscicida*, *Vibrio anguillarum* or DNP-KLH, determined with the ELISPOT assay. Each point represents the mean number/10^6 leukocytes of N= 6 or N= 3 immunised and control fish, respectively, determined using duplicate counts of all wells with more than 5 spots. Standard error bars are omitted for clarity. Figure legend: head kidney (immunised), ■; head kidney (control), □; spleen (immunised), ▲; spleen (control), △; gut (immunised), ●; gut (control), ○.

Table 1- p values for pst-hoc comparisons between treatments (Immunised vs. controls) for each experimental group (*P. damselae* ssp. *piscicida*, *V. anguillarum* and DNP). SP- spleen; HK- head kidney.

			EXPERIMENTAL GROUP		
			I	II	III
			P. damselae	*V. anguillarum*	DNP
PRIMARY RESPONSE					
SP/immunised	*vs.*	SP/control	0.252441	**0.003162**	**0.039889**
HK/immunised	*vs.*	HK/control	**0.043770**	0.156303	0.225833
Gut/immunised	*vs.*	Gut/control	0.066743	**0.006974**	0.080770
SECONDARY RESPONSE					
SP/immunised	*vs.*	SP/control	**0.000000**	**0.000000**	**0.000000**
HK/immunised	*vs.*	HK/control	**0.000000**	**0.000000**	**0.000000**
Gut/immunised	*vs.*	Gut/control	0.518145	**0.000075**	0.076982

3.2. ASC TO Vibrio anguillarum

No clear elevation of ASC could be observed in the primary response, although significant differences were observed between the spleen and the gut compared with their respective controls (Fig. 1 and Table 1). In the secondary response, a significant increase of ASC was observed in all organs from immunised fish compared with their respective controls, peaking at 7-9 days post-boost (Fig. 1 and Table 1). The head kidney reached the highest number of ASC followed by the gut. Once again, no significant differences were detected between primary and secondary responses nor between the organs.

3.3. ASC TO DNP

A clear increase in number of ASC was observed in the primary response in all organs from immunised fish, especially for the gut, with peaks being reached at 18-20 days post-immunisation (Fig. 1). However, only the spleen showed a significant difference from the respective control (Table 1). A significant increase of ASC was observed in the head kidney and spleen in the secondary response, compared with the respective controls, peaking at day 7-8 post-boost. Although a clear elevation of ASC was observed in the gut, it was not significant compared with the respective control (Fig. 1 and Table 1). No significant differences were detected between primary and secondary responses, although a low p value was obtained between gut primary and secondary responses (0.064). Differences between organs were only

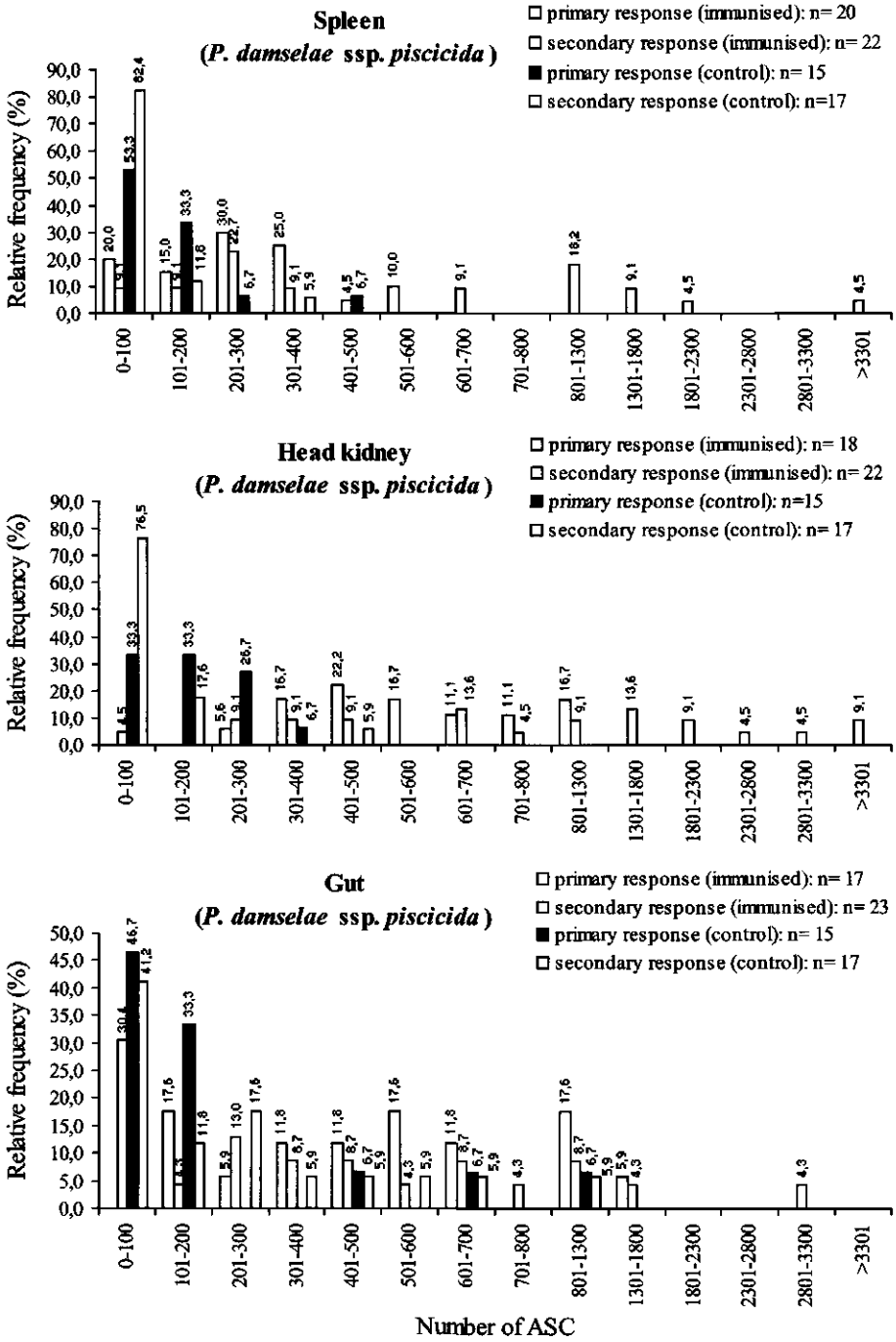
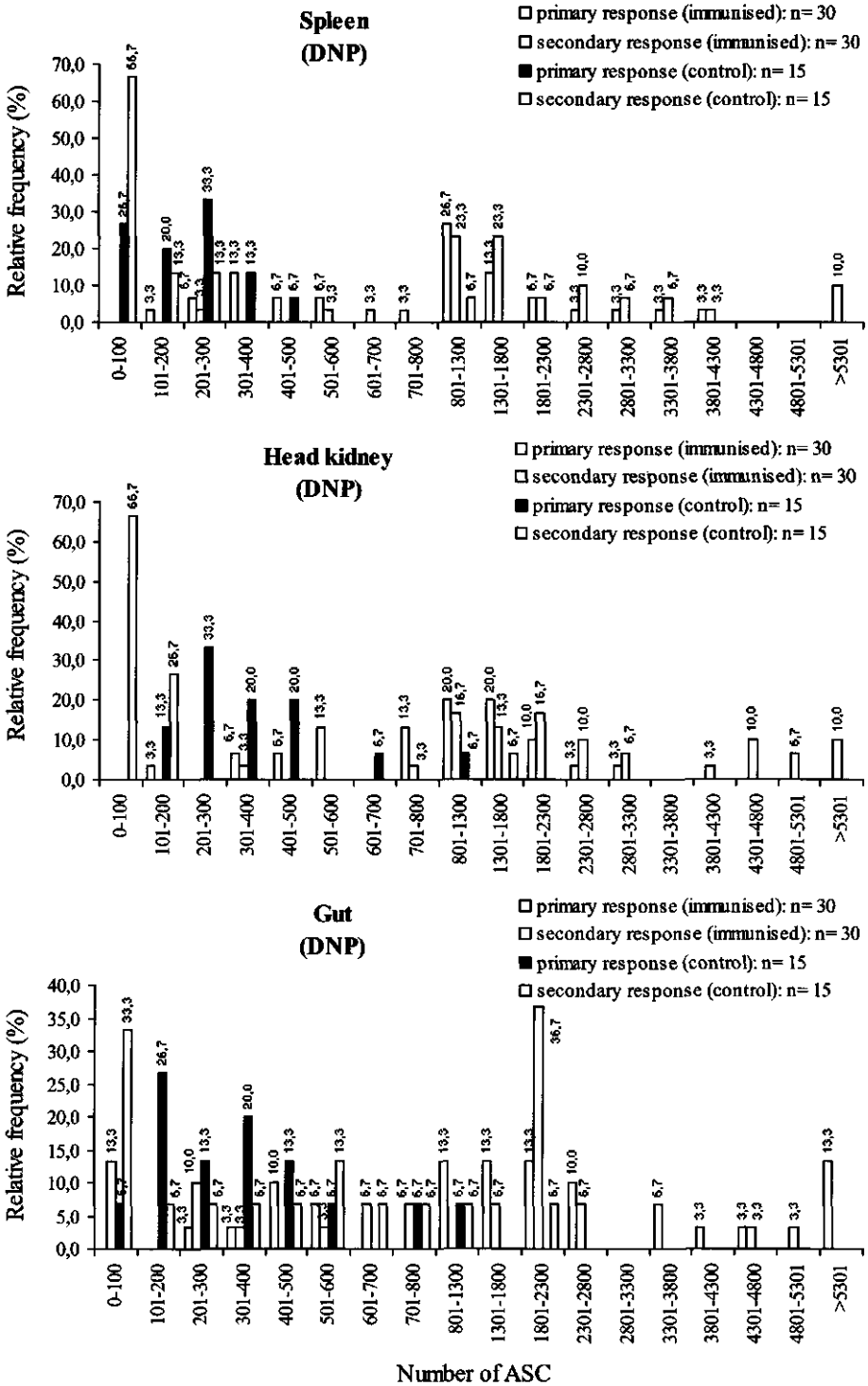


Fig. 2 (this and next page)- Relative frequency distribution of ASC in the spleen, head kidney and gut of the fish immunised with *P. damselae* ssp. *piscicida* and with DNP with respect to the sampling days where a clear response took place and in their respective controls.


Spleen
(DNP)
primary response (immunised): n= 30
secondary response (immunised): n= 30
primary response (control): n= 15
secondary response (control): n= 15
Relative frequency (%)
Head kidney
(DNP)
Gut
(DNP)
0-100
101-200
201-300
301-400
401-500
501-600
601-700
701-800
801-1300
1301-1800
1801-2300
2301-2800
2801-3300
3301-3800
3801-4300
4301-4800
4801-5301
>5301
Number of ASC

by others for a similar number of sample points (Secombes *et al.*, 1991; Davidson *et al.*, 1992, 1997; Siwicki & Dunier, 1993; Bandin *et al.*, 1997). Additionally, the presence in some fish of non-specific ASC for the antigens used also increased the variability. The development of low affinity natural cross-reactive antibodies in PBS-injected fish (Michel *et al.*, 1990; O'Dowd *et al.*, 1999) as well as high natural Ab activities against several haptens (Leslie & Clem, 1970; Rudikoff *et al.*, 1970; Killie *et al.*, 1994, 1995) has been described and it is likely that that natural anti-hapten antibodies were being enhanced upon non-specific adjuvant stimulation in the present study. Thus, the low sample number and high individual variation often made it difficult to draw certain conclusion. In this context, analysis of the frequencies of the responding and non-responding fish might contribute to support the real tendencies of the responses (Bricknell *et al.*, 1999) that might be masked by the high error variance in the statistical analysis. This approach of the data analysis was, therefore, also used.

The fact that fish had been previously bath vaccinated (at 2 g of average weight) with a *Vibrio* bacterin on the farm from where they were obtained was only known at the end of the experiments. The absence of a clear elevation in the number of ASC in the primary response to *V. anguillarum* may therefore have been due to an earlier increase of ASC from a secondary response would have been missed by the sampling regime employed. This is suggested by the slightly higher numbers of ASC on the first day of sampling (day 16 post-immunisation). Indeed, the secondary response to *P. damselae* ssp. *piscicida* and DNP peaked at about day 7 and reached baseline by about day 14. Secondary responses of intraperitoneally injected fish after priming immersions have already been reported (Mughal *et al.*, 1986; Bridges & Manning, 1991). For this reason, no clear conclusions can be drawn from the "primary" response to *V. anguillarum* in comparison with the other antigens. Thus, only the responses to *P. damselae* ssp. *piscicida* and DNP will be subjected to further comparison.

Only head kidney and spleen showed significant primary responses to *P. damselae* ssp. *piscicida* and DNP, respectively, compared with their controls. However, a similar number of ASC and respective kinetics was observed in the head kidney and the spleen of fish immunised with DNP, suggesting that significant differences were probably masked by a higher error variance in the head kidney. This is further supported by the fact that 93.3 % of the control fish produced less than 700 ASC/10^6 kidney leukocytes while 70 % of the immunised fish produced more. The same might be true for the spleen of fish immunised to *P. damselae* ssp. *piscicida*, because 81.8 % of the immunised fish produced more than 200 ASC/10^6 splenic leukocytes while 94.1 % of the control fish produced less. The low p level

observed in the responses of the gut also suggests that a significant effect might be masked by the high error variance and background ASC in control fish, as mentioned before. Again, analysis of the frequencies of the immunised and control fish to those antigens showed that the great majority of the immunised fish produced higher numbers of ASC than their respective controls.

The peak days were observed 4 days earlier in the primary responses to DNP (18 to 20 dpi) than to *P. damselae* ssp. *piscicida* (22-24 dpi). Similar times for primary peak days have been reported for different species when immunised at their physiological optimum temperatures (Davidson *et al.*, 1992; Siwicki & Dunier, 1993; Aaltonen *et al.*, 1994). Bandin *et al.* (1997) reported a peak day at 17 dpi for the primary response of outbred and inbred carp to DNP and *Aeromonas salmonicida* reared at 23° C. A significantly higher number of ASC were observed in the response to DNP compared with the response to *P. damselae* ssp. *piscicida*, mainly in the secondary responses of head kidney and spleen. Higher titres of anti-hapten antibodies when compared with anti-protein and -LPS antibody titres were reported by Killie *et al.* (1994, 1995) in immunised Atlantic salmon. Thus, it appears that haptens are potent immunogens in fish. Comparison of the kinetics of the ASC responses in these two organs in response to these two antigens (time-group interaction) also suggests a longer lasting response in fish immunised with DNP. However, no differences could be detected between the "tertiary" response to *V. anguillarum* and the secondary response to *P. damselae* ssp. *piscicida* nor between the kinetics of those groups.

Induction of antibody production in the gut by parenteral immunisation has also been reported by Davidson *et al.* (1993). Nevertheless, such extremely high numbers of ASC in the primary response of the gut to DNP observed in the present work, when compared with the head kidney and spleen, is surprising. The possibility that higher numbers of natural anti-hapten ASC were non-specifically induced by adjuvant in the gut does not appear to be a convincing explanation because even when the number of ASC in the respective control fish are subtracted from the numbers in the immunised fish, the number of anti-DNP ASC in the gut remains relatively higher. Thus, further investigation might be necessary to explain such differences.

Memory formation is undoubtedly the hallmark of any vaccination strategy. In fish, memory formation is mainly characterised by a heightened and faster secondary antibody production (Kaattari & Piganelli, 1996; van Muiswinkel & Wiegertjes, 1997). In that sense, clear secondary responses can be considered in the present study. Higher responses were

observed in the head kidney (3.6 and 2.3 times higher for *P. damselae* ssp. *piscicida* and DNP, respectively) and the spleen (4.5 and 1.6 times higher for *P. damselae* ssp. *piscicida* and DNP, respectively), although significant differences could not be detected from their primary responses. Nevertheless, a clear trend for higher production of ASC in the secondary responses to these antigens was observed in the frequencies of ASC in the head kidney and spleen compared to the respective primary responses. Additionally, the peaks of the secondary responses (7 dpi) were observed much earlier than the primary ones (20 dpi) being in agreement with those found for carp reared at similar temperatures (Rijkers *et al.*, 1980; Bandin *et al.*, 1997) and where a higher magnitude was shown for the anamnestic response to SRBC and DNP compared with the respective primary responses.

Contrary to the head kidney and spleen, a decrease in the numbers of ASC was seen in the secondary responses of the gut in the groups immunised with *P. damselae* ssp. *piscicida* (26% of primary response) and DNP (47% of primary response) compared with their respective primary response although this was not statistically significant. Taking into account the high individual variability observed mostly in the gut, it might be argued that the low p values observed between the gut primary and secondary response to DNP ($p= 0.064$) as well as comparison between the gut primary response to *P. damselae* ssp. *piscicida* and its control ($p= 0.067$), the gut primary response to DNP and its control ($p= 0.081$) and the gut secondary response to DNP and its control ($p= 0.077$), suggest some suppression of the number of ASC response in the gut. This is further supported by the analysis of the relative frequencies of the production of ASC in this organ. Thus, a higher percentage (36.7%) of primed fish produced a higher number (more than 2300) of ASC to DNP compared with the boosted fish (13.3%). Additionally, a higher percentage of boosted fish (26.7%) produced less than 400 ASC compared with the primed fish (6.7%). A more suggestive result was observed in the response to *P. damselae* ssp. *piscicida*, where 64.7 % of the primed fish produced more than 400 ASC while only 48.4 % of the boosted fish produced more than this. In addition, more boosted fish (30.4 %) produced the lowest number of ASC (0-100) compared with the primed fish (0 %). On the other hand, such a tendency for suppression in the gut ASC response was not observed in the secondary ("tertiary") response to *V. anguillarum*. The fact that the responses may be directed to different types of antigens (LPS and/or protein) and that the fish were immersion vaccinated (different priming route) in the farm might also have had some influence. If the suppression of the numbers of ASC appears to be supported, it is worth highlighting that conclusions about suppression of the immune response might be misleading, taking into

account the assay used. A higher rate of antibody production by plasma cells in secondary responses and/or some degree of affinity maturation (Kaattari & Piganelli, 1996; Pilström & Bengtén, 1996) can not be neglected. In fact, bigger and much sharper spots were observed in secondary responses to all antigens and thus greater amounts of antibody might be produced by a smaller number of ASC in the secondary response.

Although no significant differences could be detected between the organs and between their kinetics (time-organ interaction) within each group, the head kidney appears to contain the highest density of specific ASC, especially in the secondary responses. The highest concentrations of ASC were also observed in the head kidney of dab (Secombes *et al.*, 1991; Davidson *et al.*, 1997), catfish (Waterstrat *et al.*, 1991), roach (Aaltonen *et al.*, 1994) and carp (Bandin *et al.*, 1997).

Specific serum antibodies have been reported to peak about one week later than the corresponding ASC peak (Lamers & Pilarczyk, 1982), therefore, under the conditions used in the present work they would be expected to peak at about 25-27 and 14-16 days after immunisation in primed and boosted fish, respectively. In contrast, Coeurdacier *et al.* (1997) reported a much later peak time for sea bass primary serum antibodies (day 86). Although the rearing temperature is not stated by the authors, the experiment was conducted in an open sea water system (starting on 11th January) and so it was probably much lower than the temperature used in the present study, which might explain such a delay in the antibody response. On the contrary, Bakopoulos *et al.* (1997) reported a much sooner peaking (15 days) of serum antibodies in sea bass kept at 18-19° C and immunised with heat-killed *P. damselae* ssp. *piscicida*. Such a fast primary response has never been reported in fish before and comparison with the present data suggests that this was probably a secondary response and that the fish used by these authors had probably previously contacted the pathogen. Thus, the sea bass immune response can be classified as a fast antibody response but with short duration when compared with cold water species such as salmon, dab or rainbow trout and is more comparable to what has been described for carp (Bandin *et al.*, 1997), another warm water species.

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References

Aaltonen, T. M., Jokinen, E. I. & Valtonen, E. T. (1994). Antibody synthesis in roach (*Rutilus rutilus*); analysis of antibody secreting cells in lymphoid organs with ELISPOT-assay. *Fish & Shellfish Immunology* **4**, 129-140.

Anderson, D. P., Robertson, B. S. & Dixon, O. W. (1979). Cellular immune response in rainbow trout *Salmo gairdneri* Richardson to *Yersinia ruckeri* O-antigen monitored by the passive haemolytic plaque assay test. *Journal of Fish Diseases* **2**, 169-178.

Bakopoulos, V., Volpatti, D., Adams, A. Galeotti, M & Richards, R. (1997). Qualitative differences in the immune response of rabbit, mouse and sea bass, *Dicentrarchus labrax*, L. to *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish Pasteurellosis. *Fish & Shellfish Immunology* **7**, 161-174.

Bandin, I., Dopazo, C. P., van Muiswinkel, W. B. & Wiegertjes, G. F. (1997). Quantitation of antibody secreting cells in high and low antibody responder inbred carp (*Cyprinus carpio* L.) strains. *Fish & Shellfish Immunology* **7**, 487-501.

Boesen, H. T., Pedersen, K., Koch, C. & Larsen, J. L. (1997). Immune response of rainbow trout (*Oncorhynchus mykiss*) to antigenic preparations from *Vibrio anguillarum* serogroup O1. *Fish & Shellfish Immunology* **7**, 543-553.

Bricknell, I. R., King, J. A., Bowden, T. J. & Ellis, A. E. (1999). Duration of protective antibodies, and the correlation with protection in Atlantic salmon (*Salmo salar* L.), following vaccination with an *Aeromonas salmonicida* vaccine containing iron-regulated outer membrane proteins and secretory polysaccharides. *Fish & Shellfish Immunology* **9**, 139-151.

Bridges, A. F., Manning, M. J. (1991). The effects of priming immersions in various human gamma globulin (HGG) vaccines on humoral and cell-mediated immune responses after intraperitoneal HGG challenge in the carp, *Cyprinus carpio*. *Fish & Shellfish Immunology* **1**, 119-129.

Candan, A., Ang Kucker, M. & Karatas, S. (1996). Pasteurollosis in cultured sea bass (*Dicentrarchus labrax*) in Turkey. *Bulletin of the European Association of Fish Pathologists* **16**, 150-153.

Coeurdacier, J. L., Pepin, J. F., Fauvel, C., Legall, P., Bourmaud, A. F. & Romestand, B. (1997). Alterations in total protein, IgM and specific antibody activity of male and female sea bass (*Dicentrarchus labrax* L., 1758) sera following injection with killed *Vibrio anguillarum*. *Fish & Shellfish Immunology* **7**, 151-160.

Cossarini- Dunier, M., Desvaux, F.-X. & Dorson, M. (1986). Variability in humoral responses to DNP-KLH rainbow trout (Salmo gairdneri). Comparison of antibody kinetics and immunoglobulins spectrotypes between normal trouts and trouts obtained by gynogenesis or self-fertilization. *Developmental and Comparative Immunology* **10**, 207-217.

Czerkinsky, C. C., Moldoveanu, Z., Mestecky, J., Nilsson, L-Å. & Ouchterlony, Ö. (1988). A novel two colour ELISPOT assay; I. Simultaneous detection of distinct types of antibody-secreting cells. *Journal of Immunological Methods* **115**, 31-37.

Czerkinsky, C. C., Nilsson, L-Å., Nygren, H., Ouchterlony, Ö. & Tarkowski, A. (1983). A Solid-Phase Enzyme-Linked Immunospot (ELISPOT) Assay for Enumeration of Specific Antibody-Secreting Cells. *Journal of Immunological Methods* **65**, 109-121.

Davidson, G. A., Ellis, A. E. & Secombes, C. J. (1992). An ELISPOT assay for the quantification of specific antibody-secreting cells to *Aeromonas salmonicida* in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **15**, 85-89.

Davidson, G. A., Ellis, A. E. & Secombes, C. J. (1993). Route of immunization influences the generation of antibody secreting cells in the gut of rainbow trout, *Oncorhynchus mykiss*. *Developmental and Comparative Immunology* **17**, 373-376.

Davidson, G. A., Lin, S-H., Secombes, C. J. & Ellis, A. E. (1997). Detection of specific and "constitutive" antibody secreting cells in the gills, head kidney and peripheral blood leukocytes of dab (*Limanda limanda*). *Veterinary Immunology and Immunopathology* **58**, 363-374.

dos Santos, N. M. S., Taverne, N., Taverne-Thiele, A. J., de Sousa, M. & Rombout, J. W. M. (1997). Characterisation of monoclonal antibodies for sea bass (*Dicentrarchus labrax* L.) IgM indicates the existence of B cell subpopulations. *Fish & Shellfish Immunology* **7**, 175-191.

dos Santos, N. M. S., Romano, N., de Sousa, M., Ellis, A. E. & Rombout, J. W. M. (2000). Ontogeny of B and T cells in sea bass (*Dicentrarchus labrax*, L.). *Fish & Shellfish Immunology* **in press**.

Ellis, A. E. (1999). Immunity to bacteria in fish. *Fish & Shellfish Immunology* **9**, 291-308.

Kaattari, S. L. & Piganelli, J. D. (1996). The Specific Immune System: Humoral Defence. In *The Fish Immune System; Organism, Pathogen and Environment* (George Iwama & Teruyuki Nakanishi, Eds.) pp. 207-254. Academic Press, San Diego.

Killie, J.-E. A. & Jørgensen, T. Ø. (1994). Immunoregulation in fish I: intramolecular-induced suppression of antibody responses to haptenated protein antigens studied in Atlantic salmon (*Salmo salar* L). *Developmental and Comparative Immunology* **18**, 123-136.

Killie, J.-E. A. & Jørgensen, T. Ø. (1995). Immunoregulation in fish II: intermolecular-induced suppression of antiody responses studied by haptenated antigens in Atlantic salmon (*Salmo salar* L). *Developmental and Comparative Immunology* **19**, 389-404.

Lamers, C. H. J., de Haas, M. H. J. & van Muiswinkel, W. B. (1985). Humoral response and memory formation in carp after injection of *Aeromonas hydrophila* bacterin. *Development and Comparative Immunology* **9**, 65-75.

Lamers, C. H. J., & Pilarczyk, A. (1982). Immune response and antigen localization in carp (*Cyprinus carpio*) after administration of *Yersinia ruckeri* O-antigen. *Development and Comparative Immunology* **supplement 2**, 107-114.

Leslie, G. A., & Clem, L. W. (1970). Reactivity of normal shark immunoglobulins with nitrophenyl ligands. *Journal of Immunology* **105**, 1547-1552.

Magariños, B., Toranzo, A. E. & Romalde, J. L. (1996). Phenotypic and pathobiological characteristics of *Pasteurella piscicida*. *Annual Review of Fish Diseases* **6**, 41-64.

Michel, C., Gonzalez, R., Bonjour, E. & Avrameas, S. (1990). A concurrent increasing of natural antibodies and enhancement of resistance to furunculosis in rainbow trout. *Annales de Recherches Veterinaires* **21**, 311-218.

Mughal, M. S., Farley-Ewens, E. K. & Manning, M. J. (1986). Effects of direct immersion in antigen on immunological memory in young carp, *Cyprinus carpio*. *Veterinary Immunology and Immunopathology* **12**, 181-192.

O'Dowd, A. M., Bricknell, I. R., Secombes, C. J. & Ellis, A. E. (1999). The primary and secondary antibody responses to IROMP antigens in Atlantic salmon (*Salmo salar*) immunised with A^+ and A^- *Aeromonas salmonicida* bacterins. *Fish & Shellfish Immunology* **9**, 125-138.

Pilström, L. & Bengtén, E. (1996). Immunoglobulin in fish- genes, expression and structure. *Fish & Shellfish Immunology* **6**, 243-262.

Rijkers, G. T., Frederix-Wolters, E. M. & van Muiswinkel, W. B. (1980). The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). *Immunology* **41**, 91-97.

Rudikoff, S., Voss, E. W. & Sigel, M. M. (1970). Biological and chemical properties of natural antibodies in the nurse shark. *Journal of Immunology* **105**, 1344-1352.

Secombes, C. J., White, A., Fletcher, T. C., and Houlihan, D. F. (1991). The development of an ELISPOT assay to quantify total and specific antibody-secreting cells in dab *Limanda limanda* (L.). *Fish & Shellfish Immunology* **1**, 87-97.

Siwicki, A. K. & Dunier, M. (1993). Quantification of antibody secreting cells to *Yersinia ruckeri* by ELISPOT assay after *in vivo* and *in vitro* immunization of rainbow trout (*Oncorhynchus mykiss*). *Veterinary Immunology and Immunopathology* **37**, 73-80.

Toranzo, A. E. & Barja, J. L. (1990). A review of the taxonomy and seroepizootiology of *Vibrio anguillarum*, with special reference to aquaculture in the Northwest of Spain. *Diseases of Aquatic Organisms* **9**, 73-82.

van Muiswinkel, W. B. & Wiegertjes, G. F. (1997). Immune responses after injection vaccination of fish. In *Fish Vaccinology* (R. Gudding, A. Lillehaug, P. J. Midtlyng and F. Brown, Eds.) pp. 55-57. Karger.

Waterstrat, P. R., Brazil, J. & Ainsworth, A. J. (1991). Use of an ELISA-based assay for the detection of antibody-secreting cells in channel catfish, *Ictalurus punctatus* (Rafinesque). *Journal of Fish Diseases* **14**, 669-675.

CHAPTER 6

Invasion of fish epithelial cells by *Photobacterium damselae* subsp. *piscicida*: evidence for receptor specificity, and effect of capsule and serum

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"I have not seen any problem, however complicated, which, when looked at in the right way, did not become still more complicated." - Poul Anderson

In Mackay, A. L. (1991). A dictionary of scientific quotations. Adam Hilger, Bristol, 197 pp.

Abstract

Photobacterium damselae ssp. *piscicida* is a fish pathogen which causes a serious disease in commercial warmwater-fish species. Because information on the initial stages of the infection is scarce, an investigation of the invasion ability of this pathogen was undertaken utilising a fish epithelial cell line (epithelioma papilosum carpio, EPC), a virulent capsulated strain of *P. damselae* (MT1415), an avirulent non-capsulated strain of *P. damselae* (EPOY-8803-II) and *Escherichia coli* HB101 as a non-invasive control. *Photobacterium damselae* was found to be able to adhere to and invade fish epithelial cells and remain inside them for 6-9 h. There were no significant differences in invasiveness between the capsulated and a non-capsulated strain. A kinetics study demonstrated that *P. damselae* invasiveness was more efficient at low multiplicities of infection (MOI), reaching saturation at higher MOI, suggesting internalisation may be receptor mediated. Invasion efficiency (IE) was significantly higher than in the control *E. coli* HB101. Engulfment of bacteria was possibly by an endocytic process and was unaffected by killing the bacteria with UV-light. However, heat-killed bacteria had significantly reduced invasion capability. Ultrastructural studies showed that inside the epithelial cells, the bacteria remained within large vacuoles for a few hours and no evidence of intracellular replication was found, either by fluorescence or electron microscope studies. Normal sea-bass serum slightly reduced the invasion capability of the MT1415 strain, but heat-inactivated normal serum had no effect. On the other hand, heat-inactivated fish antiserum raised against the same strain reduced the percentage of invaded epithelial cells by 50 %. As for other pathogens, an intracellular phase of *P. damsela* may be a mechanism to delay or avoid phagocytosis and host immune responses, favouring the spread of infection.

Keywords: *Photobacterium damselae* subsp. *piscicida*, fish epithelial cells, invasion efficiency

1. INTRODUCTION

Pasteurellosis is a serious bacterial disease caused by *Photobacterium damselae* ssp. *piscicida* (previously *Pasteurella piscicida*) which affects commercially important warmwater-fish species, such as sea-bass, white bass, yellowtail, striped bass, gilthead seabream, etc. (Thune *et al.*, 1993). The pathology of pasteurellosis has been widely reported (Kubota *et al.*, 1970; Wolke, 1975; Hawke *et al.*, 1987; Toranzo *et al.*, 1991; Noya *et al.*, 1995b) and acute and chronic infections have been described.

The pathogenesis of *P. damselae* is poorly understood. It has been demonstrated that the polysaccharide capsular layer has an important role in the virulence of the pathogen (Magariños *et al.*, 1996b), as well as the extracellular products and iron availability (Magariños *et al.*, 1992, 1994). Regarding the interaction of *P. damselae* with phagocytes, the results have been contradictory. While morphologically intact bacteria within macrophages have been found *in vivo* (Kubota *et al.*, 1970; Nelson *et al.*, 1981; Kusuda & Salati, 1993; Noya *et al.*, 1995a, b),

suggesting that *P. damselae* can survive inside macrophages, *in vitro* studies (Skarmeta *et al.*, 1995; Arijo *et al.*, 1998) have indicated that macrophages from three different fish species were able to kill the bacteria. More recently, Barnes *et al.* (1999) have confirmed that this species is unable to respond to oxidative attack such as that experienced during the macrophage respiratory burst. On the other hand, it has been suggested that *P. damselae* could avoid host defence mechanisms and antimicrobial agents by intracellular survival in non-phagocytic cells (Magariños *et al.*, 1996a) since it has been demonstrated that this bacterium is capable of invading different fish cell lines (Magariños *et al.*, 1996a; Yoshida *et al.*, 1997).

The ability to invade epithelial cells is a key determinant of virulence for several human pathogenic bacteria such as *Escherichia coli*, *Yersinia*, *Salmonella* and *Shigella* species (Galán, 1994; Zierler & Galán, 1995). Amongst fish pathogens, this capacity has been demonstrated for *Aeromonas hydrophila* (Leung *et al.*, 1996; Tan *et al.*, 1998) and *Vibrio anguillarum* (Wang *et al.*, 1998). *Photobacterium damselae* is considered weakly or moderately adherent and invasive to various fish cell lines (Romalde & Magariños, 1997). Moreover, it showed a high binding capacity to fish intestines (Magariños *et al.*, 1996*a*). The adherence seemed to be mediated by a protein or glycoprotein receptor in the bacterial cell surface, and the internalisation of the bacteria was an actin microfilament-dependent mechanism (Magariños *et al.*, 1996*a*). Although these authors have indicated that *P. damselae* is able to remain viable inside the cells for at least two days, and to spread from cell to cell, it has not been demonstrated that internalisation in fish cell lines was a specific process.

This study was carried out to analyse and obtain a better understanding of *P. damselae* internalisation, by studying invasion efficiency, the kinetics of invasion, the role of the capsule, and the effects of heating and exposure to UV-light, serum and antiserum on the invasiveness of this species.

2. MATERIAL AND METHODS

2.1. BACTERIA

A virulent capsulated strain (MT1415) and an avirulent non-capsulated strain (EPOY-8803-II) (Magariños *et al.*, 1996*b*) of *Photobacterium damselae* ssp. *piscicida* were used in the present study. The capsulated strain was originally isolated from an outbreak of pasteurellosis in sea bass

(*Dicentrarchus labrax*, L.) in Italy and was obtained from the Marine Laboratory Collection, Aberdeen; the non-capsulated strain was isolated from red grouper (*Epinephelly akaara*) in Japan and was obtained from the Microbiology Department, Málaga University, Spain (Arijo *et al.*, 1998). Strains were cultured on tryptic soy agar supplemented with 2 % NaCl (TSA2) at 22 °C for 48 h, or in tryptone soya broth supplemented with 2 % NaCl (TSB2) overnight at 22 °C with shaking.

2.2. TISSUE CULTURE

Epithelioma papulosum carpio (EPC) cells were grown in 75 cm^2 flasks containing Glasgow modification of Eagle's minimal essential medium (G-MEM) (ICN) with 10 % foetal bovine serum (FBS, Sigma). In all the experiments, EPC cells were grown to a confluence of 80-90 % (between $2x10^6$ and $1x10^7$ cells/25 cm^2).

2.3. BACTERIAL HYDROPHOBICITY

The cell surface hydrophobicity of the *P. damselae* strains used was studied using the Salt Aggregation Test (SAT). The assay was performed essentially as described by Lindhal *et al.* (1981). Briefly, serial doubling dilutions of ammonium sulphate were made ranging between 4 M and 0.003 M in 0.002 M sodium phosphate buffer (pH 6.8). To 50 µl of each dilution, 50 µl of bacterial suspension (≈ 10^{10} cfu/ml) were added. The lowest concentration at which agglutination occurred for each strain was recorded.

2.4. ADHERENCE ASSAY

Bacteria were grown as indicated above and 25 µl added to each well of a 24-well tissue culture plate containing a sterile glass coverslip seeded with EPC cells. The plates were centrifuged (150 ***g***, 5 min) to achieve contact between bacteria and cells. The infected cultures were incubated at 22 °C from 15 min to 24 h. After incubation, coverslips were washed with PBS to remove non-adherent bacteria, fixed with methanol, stained with Giemsa and mounted with 90 % glycerol in PBS.

required. Normal serum was collected from non-immunised fish. The agglutinating titres were determined as described by Roberson (1990). The titre of the antiserum against strain MT1415 was 1:16. No agglutination was detected after incubation of MT1415 with normal serum.

2.9. TREATMENT WITH NORMAL SERUM AND ANTISERUM

Heat-inactivation of the sera was carried out at 45° C for 15 min in order to inactivate complement (Sakai, 1981).

Invasion assays were conducted as described above. After fluorescein-labelling and washing, the bacteria were suspended in PBS to about 3 x 10^8 ml^{-1}. Aliquots (100 µl) were placed into five microcentrifuge tubes (in triplicate) and incubated with 500 µl of PBS as control, sea bass normal serum, heat-inactivated normal serum, or heat-inactivated antiserum for 10 min at room temperature. After centrifugation (1500 *g*, 5 min), bacteria were washed and resuspended in PBS. Bacteria were added to EPC (MOI≈15-19) and incubated for 2 h. Visualisation of intracellular bacteria were carried out as above with the fluorescence method.

2.10. TRANSMISSION ELECTRON MICROSCOPY

The techniques described by Watanabe *et al.* (1988) for rabbit platelets and by Gutenberger *et al.* (1997) for trout leucocytes were used. EPC cells obtained as described above were infected with bacteria. After centrifugation (150 *g* for 5 min) and gentle resuspension, the infected EPC cells were incubated for 1-9 h at 22° C in a rotary mixer (20 rpm), washed three times with PBS (900 *g* for 5 min) to remove non-attached bacteria and pelleted. The pellet was resuspended in 2 % glutaraldehyde buffered with 0.05M PIPES (piperazine-N,N'-bis [2-ethanesulfonic acid]) (Hayat, 1986) at pH 7.2, and fixed for 1 h at room temperature. After washing twice with 0.025M PIPES and centrifugation at 230 *g* for 10 min, two drops of 2 % agarose (maintained at 37° C) (Agarose type VII, low gelling temperature <30° C) were added. The cells were centrifuged at 230 *g* for 5 min at room temperature and then left on ice until the agarose gelled. The gel was cut into small pieces (1 mm^2), postfixed with 1 % aqueous osmium tetroxide for 1 h, and left overnight at room temperature in 2 % aqueous uranyl acetate. Dehydration in acetone series was followed by embedding in araldite (Durcupam ACM) using propylene oxide as an intermediate solvent. Ultrathin sections (70-80 nm) were obtained with an LKB Ultratome III, stained on the grid with Fahmy's lead citrate (Lewis & Knight, 1977) and examined with a Hitachi H-300 electron microscope.

2.11. STASTISTICAL ANALYSIS

All the results were analysed by applying a unifactorial ANOVA test and probabilities $p<0.05$ were considered significant. The data are presented as mean±standard deviation.

3. RESULTS

3.1. ADHERENCE

Light microscope examination of infected EPC cells demonstrated extracellular *P. damselae* MT1415 bacteria in close association with the plasma membrane of the epithelial cells within 15 min of infection (data not shown).

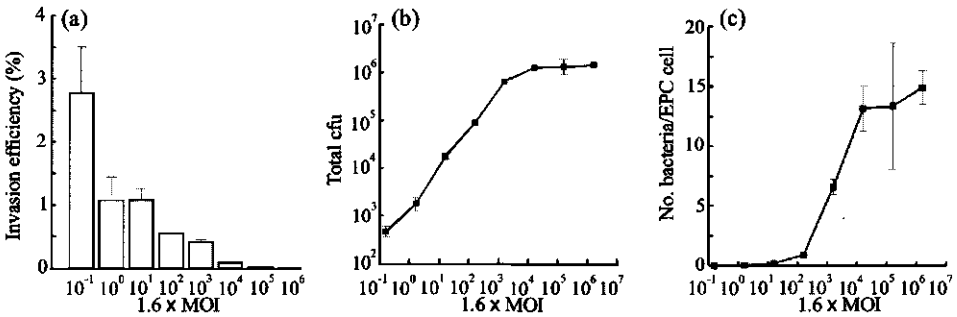

Fig. 1- Characteristics of invasion of EPC cells by *Photobacterium damselae* ssp. *piscicida* MT1415 after 2 h of incubation and 1 h gentamicin treatment. All assays were conducted in duplicate. Results are presented as mean ± SD. (a) Invasion efficiency (IE) of the bacteria at different MOIs. (b) Total number of bacteria internalised at different MOIs. The number of internalised bacteria increased progressively and significantly ($p<0.05$), and reached saturation at MOI=$1.6x10^4$. Thereafter, there were no significant ($p>0.05$) differences when the MOI was increased. (c) Mean number of bacteria internalised per EPC cell at different MOIs. As in (b), the number of bacteria per cell increased up to MOI=$1.6x10^4$ and thereafter no significant increase was recorded.

3.2. KINETICS OF BACTERIAL INVASION

The bactericidal test confirmed that gentamycin concentration and incubation time used for the invasion assay were sufficient to kill the extracellular bacteria. Bacterial invasion efficiency (IE) was calculated as: [number internalised colony forming units (cfu) at the end of the assay/starting inoculum] x 100. Invasion efficiency of *P. damselae* MT1415 was tested at different MOIs, from 0.16 to $1.6x10^6$ bacteria per EPC cell (Fig. 1a). Maximum invasion

efficiency (2.77 %) was observed at an MOI of 0.162 and was reduced to about 1 % at MOIs of 1.6 and 16.0. At higher MOIs, IE values decreased gradually. The non-invasive control *E. coli* HB101 was tested for a similar range of MOIs, and the maximum invasion efficiency was 0.0038 % (data not shown).

When bacterial invasion ability is expressed as the total number of intracellular cfu recovered from the infected EPC cells (Fig. 1b), a progressive increase was found from the lowest MOI, reaching a value of $\approx 1.3 \times 10^6$ at MOI=1.6×10^4. Thereafter, the number increased slightly but not significantly, suggesting a limitation on entry at MOIs$\geq 1.6 \times 10^4$.

The number of internalised cfu per EPC cell resulting from varying the MOI (Fig. 1c) increased slowly for the first four MOIs and then there was a sharp increase up to 6.5 bacteria/EPC cell at MOI=1620. This value doubled at MOI 16000 and thereafter remained constant at MOIs$\geq 1.6 \times 10^4$.

3.3. QUNTIFICATION OF P. damselae INTERNALISATION BY THE FLUORESCENCE METHOD

The fluorescence staining assays confirmed the presence of intracellular micro-organisms (Fig. 2). By using this method, it was possible to quantify the percentage of EPC cells infected over time, as well as the intensity of infection (ie. number of bacteria per infected EPC cell) over time.

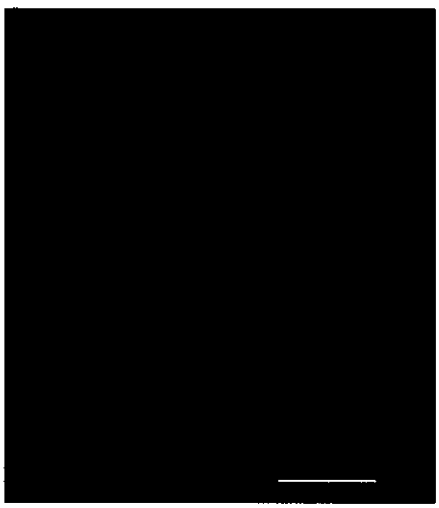

Fig. 2- EPC cell infected by *P. damselae* MT1415 as observed by fluorescence staining. Green bacteria, intracellular; orange bacteria, extracellular. Bar, 100

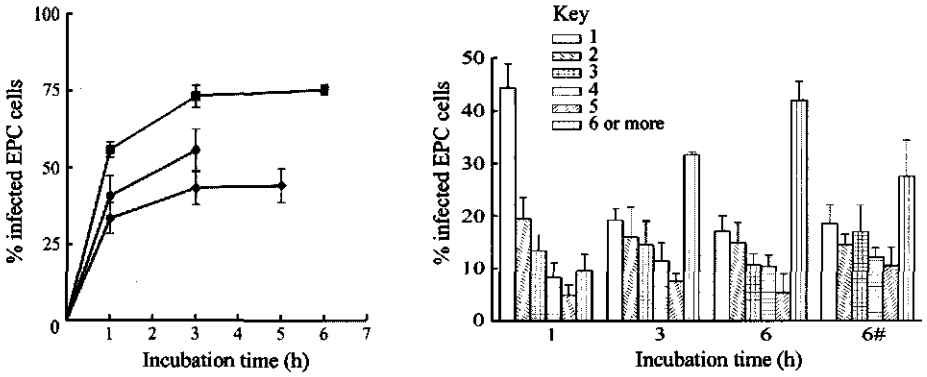

Fig. 3- Percentage of epithelial cells infected by *P. damselae* (MT1415) over time. (a) Cells were infected with 2.28 $\times 10^8$ (◆), 4.64 $\times 10^8$ (●), or 9.92 $\times 10^8$ (■) bacteria (MOI ≈ 5, 30 and 60, respectively). Results are expressed as the means ± SD from three replicates for each experiment. As the bacterial concentration increased, so did the percentage of EPC cells infected. There was a significant ($p<0.05$) increase between 1 and 3 h of incubation, but not between 3 h and longer incubation times. (b) Bars show number of intracellular bacteria per epithelial cell. Time 6# represents a 6 h incubation period in which the bacteria were eliminated from the medium after 3 h. Results are expressed as the means ± SD from a representative experiment carried out in quadruplicate. Bacterial concentration was 9.92 $\times 10^8$.

Uptake of *P. damselae* strain MT1415 by EPC cells was concentration and time dependent (Fig. 3a). Depending on bacterial concentration, between 33-63 % of EPC cells had one or more internalised bacteria within 1 h of *P. damselae* inoculation. The percentage of infected EPC cells reached a plateau by 3 h. Significant differences were always found between 1 and 3 h of incubation, but not between 3 h and longer incubation times (5 or 6 h). The number of intracellular bacteria per epithelial cell was studied over time. In this case, EPC cells were infected as above and incubated for 1, 3, and 6 h. Two 6 h-incubations were carried out (6 and 6#). In 6#, the cells were washed and new medium was added after 3 h of infection in order to remove extracellular bacteria from the medium. After incubation, two hundred EPC cells were randomly examined and the number of bacterial cells (1, 2, 3, 4, 5, 6 or more) in each of the infected cells was counted (Fig. 3b). The number of intracellular bacteria per EPC cell was time dependent. After 1 h infection, 44 % of infected EPC cells had only 1 intracellular bacterium and 9 % had six or more. After 6 h infection, 17 % had one intracellular bacterium and 41 % had six or more. The number of EPC cells with one bacterium was significantly ($p<0.05$) reduced from 1 to 3 and 6 h, whereas the number of epithelial cells with 6 or more bacteria was significantly ($p<0.05$) increased from 1 to 3 and 6 h. However, when the bacteria were removed from the medium after 3 h and the cells incubated for a further 3 h (6#), there was no significant difference

between the values at 3 and 6# h of incubation suggesting that the increase in the number of intracellular bacteria per EPC cell between 3 and 6 h resulted from uptake of further bacteria from the medium, rather than division of bacteria within the cells.

3.4. HYDROPHOBICITY AND INVASION OF THE VIRULENT AND AVIRULENT STRAINS

Non-capsulated strain EPOY-8803-II aggregated with a lower (0.25 M) concentration of ammonium sulphate than the capsulated strain MT1415 (> 4 M). For invasion assays, EPC cells were infected with equivalent bacterial concentrations ($\approx 10^8$ bacteria/ml, MOI≈15) of the virulent MT1415 and non-virulent EPOY-8803-II strains, and incubated for 1 and 3 h and observed by fluorescence microscopy. There were no significant differences between the percentage of EPC cells invaded by both strains, either after 1 h (MT1415: 37.83 ± 1.76 %; EPOY-8803-II: 38.83 ± 1.04 %) or 3 h (MT1415: 49.33 ± 5.13 %; EPOY-8803-II: 49.00 ± 0.87 %) of incubation.

3.5. EFFECT OF HEAT, UV-LIGHT, SERUM, AND ANTISERUM ON BACTERIAL INVASIVENESS

After heat and UV-light treatments no viable bacteria could be detected on TSA2 plates, confirming complete inactivation of the bacteria. Invasiveness of heat-inactivated bacteria was greatly reduced (9.33 ± 1.53 % EPC cells invaded) compared with the non-inactivated bacteria (52.17 ± 4.04 % EPC cells invaded). However, bacteria inactivated by UV-light were still able to invade up to 44.83 ± 1.53 % of EPC cells, which was not significantly ($p>0.05$) different from the non-treated viable bacteria.

Capsulated bacteria incubated with heat-inactivated normal serum invaded a similar percentage of EPC cells (47.5 ± 4.36 %) as the control (bacteria incubated with PBS) (49.17 ± 2.02 %). However, when bacteria were incubated with fresh normal serum the percentage of EPC cells infected was significantly ($p<0.05$), but not greatly, reduced (36 ± 1.50 %). Incubation of the bacteria with heat-inactivated sea-bass antiserum provoked a significant ($p<0.05$) decrease in the number of epithelial cells with intracellular bacteria (24.5 ± 1.00 %).

3.6. ULTRASTRUCTURE OF P. damselae INFECTION

Ultrastructural studies of EPC cells infected with *P. damselae* provided information on the alterations which occurred both in host cells and bacteria. Thus, EPC cells infected with the bacteria were incubated for 1, 3, 5, 7, and 9 h; non-infected EPC cells were used as control. No

changes in cell morphology occurred in the control cell cultures over the experimental period (data not shown).

After 1 h infection, both extra- and intracellular bacteria could be seen. Extracellular bacteria were in close association with the plasma membrane (Fig. 4a). EPC cells showed cytoplasmic extensions around the bacteria which were interpreted as the first steps in the engulfment process (Fig. 4b). The intracellular bacteria were always situated within membrane-bound vacuoles (Fig. 4b, c) and no evidence of membrane disintegration was found. Vacuoles with multiple bacteria were observed (Fig. 4c) which may be a result of several bacteria being engulfed at the same time

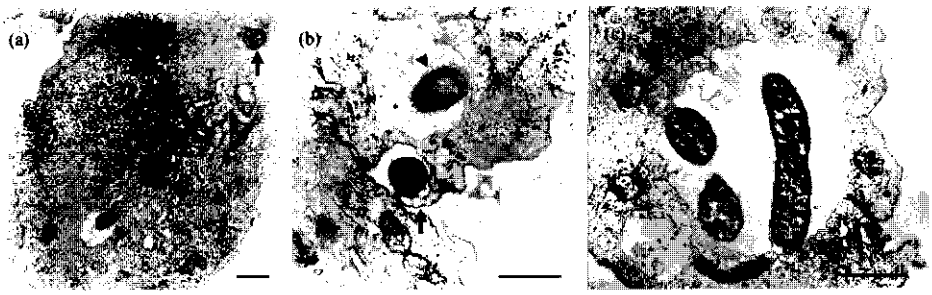

Fig. 4- Transmission electron micrographs of infected EPC cells with *P. damselae* MT1415 after 1-3 h incubation. (a) After 1 h incubation. Both extracellular (arrow) and intracellular (arrow-head) bacteria can be seen. N, nucleus of epithelial cell. (b) After 1 h incubation. A cytoplasmic extension (arrow) is visible around one extracellular bacterium close to a vacuole that already contained an internalised bacterium (arrow-head). (c) After 3 h incubation. Once internalised, bacteria are always located inside membrane-bound vacuoles. Large vacuoles may contain two or more bacteria. Bars, 1 μm.

or by vacuole fusion. After 5 h post-infection most bacteria were located within large membrane-bound vacuoles and no evidence of bacteria free in the cytoplasm was seen.

By 7 h post-infection, EPC cells showed degenerative changes (Fig. 5a, b). There was a decrease in the number of intracellular bacteria and the bacteria-containing vacuoles were now located closer to the EPC cell surface, separated from the medium by a thin edge of cytoplasm. Furthermore, although many extracellular bacteria were found, most of them being associated with cellular membranes, no evidence of rupture of the plasma membrane of EPC cells was observed. At 9 h post-infection, epithelial cells appeared to be lysed, and most bacteria were extracellular. Some instances of extracellular bacterial division were observed (Fig. 5c). Despite many observations, at no time was intracellular replication or bacterial destruction observed.

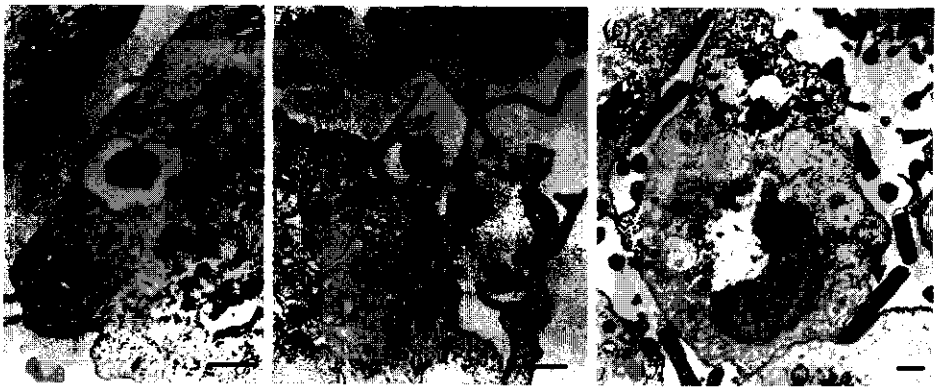

Fig. 5- Transmission electron micrographs of EPC cells infected with *P. damselae* MT1415 after 7-9 h incubation. (a) After 7 h incubation. Bacteria-containing vacuole close to the EPC cell surface. The cytoplasm of the epithelial cell shows degenerative characteristics (asterisk). (b) After 7 h incubation. Exit of *P. damselae* from an EPC cell. The bacteria are still associated with the cellular membrane (arrows). (c) After 9 h incubation. Epithelial cells are lysed and bacteria are extracellular. Some instances of extracellular division can be see (arrow). N, nucleus of epithelial cell. Bars, 1 μm.

4. DISCUSSION

Photobacterium damselae ssp. *piscicida* has the ability to adhere to and invade fish epithelial cells. This invasion ability has been previously demonstrated by Magariños *et al.* (1996a) and Yoshida *et al.* (1997). However, this is the first study of the kinetics and specificity of this process. Visual evidence of the intracellular phase of *P. damselae* inside fish epithelial cells has been provided by electron microscopy observations and the light microscopic fluorescence technique. Moreover, the invasion of fish epithelial cells by *P. damselae* was confirmed by the gentamicin assays, since this antibiotic selectively kills extracellular bacteria.

Magariños *et al.* (1996a) indicated this species was weakly or moderately invasive. They tested various fish cell lines at an MOI of 100 and found that up to 10^3 bacteria were recovered from EPC cells after the antibiotic assay. Unfortunately, the concentration of neither the bacteria or the fish cells was stated in this report so it is not possible to directly compare with the present data. The invasive properties of a species depend greatly on the bacterial strain and cell lines used, as has been demonstrated for *Salmonella*, where the invasion efficiency varied between ≈6 and 38 % depending on the cell lines and the bacterial strain tested (Mills & Finlay, 1994). On the other hand, we have demonstrated that, for this species, MOI has a clear effect on the number of

bacteria that invade the cells. Invasion efficiency of *P. damselae* was maximum at the lowest MOI (0.16), whereas the number of internalised bacteria and the number of bacteria per epithelial cell reached saturation at higher MOIs ($\geq 1.6 \times 10^4$). This relation between MOI and invasion has to be born in mind for future studies on *P. damselae* invasiveness, since the value of MOI chosen represents a compromise between number of internalised bacteria and invasion efficiency.

The maximum invasive efficiency observed for *P. damselae* in the present study represents 2 % of the initial inoculum, a percentage significantly lower than those indicated for some human invasive bacterial pathogens, such as *Salmonella typhimurium* (Huang *et al.*, 1998) or *Yersinia enterocolitica* (Small *et al.*, 1987), that showed invasion efficiency of 48.9 % and 21 %, respectively. However, the level reported for *P. damselae* is higher than those reported for some other invasive species, such as *Campylobacter jejuni* (0.1-0.2 %) (Konkel *et al.*, 1993) and similar to the enteroinvasive *Escherichia coli* 11(2.9 %) (Small *et al.*, 1987). The present study also showed that the induced uptake by the EPC cells of *P. damselae* was not a non-specific property of the cell line, since the cells were not invaded by the non-invasive *E. coli* HB101.

More intracellular organisms and higher number of bacteria per epithelial cell were recorded as the MOI increased, until reaching a saturation level at high MOIs ($\geq 1.6 \times 10^4$). These results suggest that internalisation of *P. damselae* by EPC cells is a process mediated by receptor-ligand interactions, since this type of process is characterized by saturability (García-Peñarrubia *et al.*, 1992). Both epithelial cells and bacteria seem to be involved in the internalisation process. The host cell plays an active role since the internalisation of the bacteria is inhibited by cytochalasin D (Magariños *et al.*, 1996*a*), indicating that it is dependent upon host actin microfilaments. Moreover, the ultrastructural analysis by TEM provides evidence that *P. damselae* is ingested into vacuoles, possibly by an endocytic process, in a similar way to pathogens such as *Salmonella*, *Shigella* spp. and *Yersinia* (Finlay & Falkow, 1988). On the other hand, heat-killed bacteria were unable to invade the epithelial cells, while the UV-light inactivated bacteria kept their invasive capabilities. This suggests that engulfment does not require *P. damselae* to be viable, but integrity of its surface components is necessary to permit interactions between the bacteria and the surface of the host cells. These results agree with Magariños *et al.* (1996*a*) who found that adhesive capacities are affected by heat and sugars, and suggested that adherence may be mediated by a glycoprotein of the bacterial or host cell surface.

Once internalised by non-phagocytic cells, microorganisms have different strategies to survive within the host cells. Some bacteria, such as *Shigella* spp. (Sansonetti *et al.*, 1986) and *Rickettsia* (Heinzen *et al.*, 1993), escape from the vacuole to multiply in the cytoplasm and later infect

adjacent cells. However, there are numerous bacterial species that remain inside the vacuoles of the epithelial cells throughout their infection. Species such as *Salmonella* and *Yersinia* (Finlay & Falkow, 1988; Janda *et al.*, 1991), *Haemophilus influenzae* (Virji *et al.*, 1991) or group B streptococci (Rubens *et al.*, 1992) are included in this group. *Photobacterium damselae* did not seem to escape from the vacuoles. At no time after infection were bacteria observed free in the cytoplasm. Instead, they were found within large cytoplasmic vacuoles where they remained viable for several hours. The intracellular time period seems to be dependent on the cell line used and assay conditions, since Magariños *et al.* (1996*a*) found that *P. damselae* remained inside CHSE-214 cells for at least two days, while in the present study the bacteria were liberated from the EPC cells after 7-9 h following infection.

Some invasive bacteria replicate inside the host cells (Finlay & Falkow, 1988). However, during the present study, examination of a large number of electron micrographs failed to show evidence of intracellular multiplication of *P. damselae*. Data from the fluorescence assay indicated an increasing number of intracellular bacteria per cell at longer incubation times, which could be due either to the continuous invasion or to replication of the organisms inside the EPC cells. However, when the extracellular bacteria were eliminated from the medium there was no further increase in the number of intracellular bacteria, which supports the EM observations that *P. damselae* does not seem to replicate inside the EPC cells. Bacterial replication seemed to occur once the bacterium had left the host cell and was observed extracellularly, after 9 h of infection.

The virulence factors that contribute to invasion of the epithelial cells by *P. damselae* are unknown. Significant differences in invasion between non-capsulated and capsulated *P. damselae* strains were not observed, although their surface hydrophobicity is , according to the criteria proposed by Santos *et al.* (1990), strong for the avirulent non-capsulated EPOY-8803-II strain, while the virulent capsulated strain MT1415 was not hydrophobic. Similar results were found by Magariños *et al.* (1996*a*) who studied the adherence to tissues of capsulated (virulent) and non-capsulated (avirulent) strains of *P. damselae*. The lack of effect of bacterial capsule on adherence or invasion has been reported in other Gram-negative bacteria, such as *H. influenzae* (Lipuma & Gilsdorf, 1987; Roberts *et al.*, 1984) and enterotoxigenic *E. coli* (Guerina *et al.*, 1983). However, studies on the role of bacterial capsules in adherence or invasion to host cells are contradictory. It has been postulated that a capsule may reduce adhesiveness in several species, such as *E. coli* (Runnels & Moon, 1984), *H. influenzae* (St Geme III & Falkow, 1991; Virji *et al.*, 1991), *Actinobacillus pleuropneumoniae* (Jacques *et al.*, 1991) and *Pasteurella multocida* (Jacques *et al.*, 1993). On the other hand, it has been found that *E. coli* capsule may promote adherence

(Davis *et al.*, 1981; Chan *et al.*, 1982). Recent studies with *H. influenzae* (St Geme III & Cutter, 1996) have suggested that the level of encapsulation, and not the presence of capsule by itself, influences the adhesive interactions of the organism with its host cells. In *P. damselae*, the level of encapsulation seems also to be important, since although the constitutive capsule of virulent strains does not interfere with adhesion, a thicker capsular material decreases the adherence to host tissues (Magariños *et al.*, 1996*b*). Since both capsulated and non-capsulated strains of *P. damselae* appear to be able to invade fish epithelial cells, the virulence of the capsulated bacteria cannot be explained on the basis of their ability to invade host cells.

Incubation of the bacteria prior to invasion with fresh normal sea bass serum slightly, but not significantly, reduced the percentage of EPC cells invaded by the encapsulated strain, while incubation with heat-inactivated normal serum had no effect on invasiveness. Although this could suggest a certain degree of inhibition by the complement of fish serum on capsulated bacteria, the percentage of infected epithelial cells was still high. It has been demonstrated that bacterial capsules confer resistance to bactericidal activity of serum (Amaro *et al.*, 1994; Daly *et al.*, 1996), including *P. damselae* (Arijo *et al.*, 1998; Magariños *et al.*, 1996*b*). This slight reduction in invasiveness with fresh normal fish serum may be a consequence of some bacteria with a thinner capsule being killed by the action of complement. Incubation with heat-inactivated antiserum against MT1415 strain had a significant effect on invasion, indicating that antibodies reduce the invasiveness of the bacteria, presumably by blocking the surface receptors involved in adhesion and/or invasion. The effect of antiserum is certainly specific as heat-inactivated normal serum did not decrease invasiveness and did not agglutinate the bacteria as did the antiserum. Thus, nonspecific interactions with fish serum proteins have no effect on the bacterial invasiveness.

The presence of intracellular bacteria in host cells has been interpreted as a mechanism to avoid phagocytosis and host immune responses, reducing their effectiveness and favouring the spread of infection (Virji *et al.*, 1991; Magariños *et al.*, 1996*a*; Galán, 1994; Amaro *et al.*, 1994; Daly *et al.*, 1996). As Virji *et al.* (1991) pointed out, bacteria do not need intracellular replication, only the ability to enter and survive inside the host cells and to exit. The present data show that *P. damselae* can invade fish epithelial cells, remain intracellular for a few hours, leave the host cells by lysing them, and replicate extracellularly. As the capsule confers resistance to complement-dependent killing (Margariños *et al.*, 1996*b*; Arijo *et al.*, 1998), the intracellular environment may be more important to escape contact with phagocytes. Although some studies have indicated that *P. damselae* survive inside macrophages (Kubota *et al.*, 1970; Nelson *et al.*, 1981; Kusuda & Salati, 1993; Noya *et al.*, 1995a, b), Skarmeta *et al.* (1995) and Arijo *et al.*

Jacques, M., Kobisch, M., Bélanger, M. & Dugal, F. (1993). Virulence of capsulated and noncapsulated isolates of *Pasteurella multocida* and their adherence to porcine respiratory tract cells and mucus. *Infect Immun* **61,** 4785-4792.

Konkel, M. E., Mead, D. J. & Cieplak, W., Jr (1993). Kinetic and antigenic characterization of altered protein synthesis by *Campylobacter jejuni* during cultivation with human epithelial cells. *J Infect Dis* **168,** 948-954.

Kubota, S., Kimura, M. & Egusa, S. (1970). Studies on bacterial tuberculoidosis of the yellowtail-I. Symptomathology and histopathology. *Fish Pathol* **4,** 111-118.

Kusuda, R. & Salati, F. (1993). Major bacterial diseases affecting mariculture in Japan. *Ann Rev Fish Dis* **3,** 69-85.

Leung, K. Y., Lim, T. M., Lam, T. J. & Sin, Y. M. (1996). Morphological changes in carp epithelial cells infected with *Aeromonas hydrophila*. *J Fish Dis* **19,** 167-174.

Lewis, P. R. & Knight, D. P. (1977). Staining methods for sectioned material, vol. 5. In *Practical Methods in Electron Microscopy*, pp. 1-311. Edited by A. M. Glauert. Amsterdam: Elsevier North-Holland.

Lindhal, M., Faris, A., Wadstrom, T. & Hjerten, S. (1981). A new test based on "salting out" to measure relative surface hydrophobicity of bacterial cells. *Biochim Biophys Acta* **677,** 471-476.

Lipuma, J. L. & Gilsdorf, J. R. (1987). Role of capsule in adherence of *Haemophilus influenzae* type b to human buccal epithelial cells. *Infect Immun* **55,** 2308-2310.

Magariños, B., Romalde, J. L. (1997). Immunization with bacterial antigens: Pasteurellosis. In *Fish Vaccinology*, pp. 167-177. Edited by R. Gudding, A. Lillehaug, P. J. Midtlying and F. Brown. Basel: Karger.

Magariños, B., Santos, Y., Romalde, J. L., Rivas, C., Barja, J. L. & Toranzo, A. E. (1992). Pathogenic activities of live cells and extracellular products of the fish pathogen *Pasteurella piscicida*. *J Gen Microbiol* **138,** 2491-2498.

Magariños, B., Romalde, J. L., Lemos, M. L., Barja, J. L. & Toranzo, A. E. (1994). Iron uptake by *Pasteurella piscicida* and its role in pathogenicity for fish. *Appl Environ Microbiol* **60,** 2990-2998.

Magariños, B., Romalde, J. L., Noya, M., Barja, J. L. & Toranzo, A. E. (1996*a*). Adherence and invasive capacities of the fish pathogen *Pasteurella piscicida*. *FEMS Microbiol Lett* **138,** 29-34.

Magariños, B., Bonet, R., Romalde, J. L. Martínez, M. J., Congregado, F. & Toranzo, A. E. (1996*b*). Influence of the capsular layer on the virulence of *Pasteurella piscicida* for fish. *Microb Pathog* **21,** 289-297.

Mills, S. D. & Finlay, B. B. (1994). Comparison of *Salmonella typhi* and *Salmonella typhimurium* invasion, intracellular growth and localization in cultured human epithelial cells. *Microb Pathogen* **17,** 409-423.

Nelson, J. S., Kawahara, E., Kawai, K. & Kusuda, R. (1981). Macrophage infiltration in pseudotuberculosis of yellowtail, *Seriola quinqueradiata*. *Bull Mar Sci Fish Kochi Uni* **11,** 17-22.

Noya, M., Magariños, B. & Lamas, J. (1995*a*). Interaction between peritoneal exudate cells (PECs) of gilthead seabream (*Sparus auratus*) and *Pasteurella piscicida*. A morphological study. *Aquaculture* **131,** 11-21.

Noya, M., Magariños, B., Toranzo, A. E. & Lamas, J. (1995*b*). Sequential pathology of experimental pasteurellosis in gilthead seabream *Sparus aurata*. A light- and electron-microscopic study. *Dis Aquat Org* **21,** 177-186.

Roberson, B. S. (1990). Bacterial agglutination. In *Techniques in Fish Immunology*, chapter 9, pp.81-86. Edited by J. S. Stolen, T. C. Fletcher, D. P. Anderson, B. S. Roberson and W. B. van Muiswinkel. Fair Aven, USA: SOS Publications.

Roberts, M., Jacobs, R. F., Haas, J. E. & Smith, A. L. (1984). Adherence of Haemophilus influenzae to monkey respiratory tissue in organ cultures. J Gen Microbiol 130, 1437-1447.

Romalde, J. L. and Magariños, B. (1997). Immunisation with bacterial antigens: pasteurellosis. Dev Biol Standardization 90, 167-177.

Rubens, C. E., Smith, S., Hulse, M., Chi, E. Y. & van Belle, G. (1992). Respiratory epithelial cell invasion by group B streptococci. Infect Immun 60, 5157-5163.

Runnels, P. L. & Moon, H. W. (1984). Capsule reduces adherence of enterotoxigenic Escherichia coli to isolated intestinal epithelial cells of pigs. Infect Immun 45, 737-740.

Sakai, D. K. (1981). Heat inactivation of complements and immune hemolysis reaction in rainbow trout, masu salmon, coho salmon, goldfish and tilapia. Bull Japan Soc Sci Fish 47, 565-571.

Sansonetti, P. J., Ryter, A., Clerc, P., Maurelli, A. & Mounier, J. (1986). Multiplication of Shigella flexneri within Hela cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect Immun 51, 461-469.

Santos, Y., Bandín, I., Nieto, T. P., Bruno, D. W., Ellis, A. E. & Toranzo, A. E. (1990). Comparison of the cell surface hydrophobicity of bacterial fish pathogens by different procedures. In Pathology in Marine Sicence, pp. 101-115. Edited by T. C. Cheng and F. O. Perkins. New York: Academic Press.

Skarmeta, A. M., Bandín, I., Santos, Y. & Toranzo, A. E. (1995). In vitro killing of Pasteurella piscicida by fish macrophages. Dis Aquat Org 23, 51-57.

Small, P. L. C., Isberg, R. R. & Falkow, S. (1987). Comparison of the ability of enteroinvasive Escherichia coli, Salmonella typhimurium, Yersinia pseudotuberculosis, and Yersinia enterocolitica to enter and replicate within HEp-2 cells. Infect Immun 55, 1674-1679.

Snieszko, S. F., Bullock, G. L., Hollis, E. & Boone, J. G. (1964). Pasteurella sp. from an epizootic of white perch (Roccus americanus) in Chesapeake Bay tide-water areas. J Bacteriol 88, 1814-1815.

St. Geme, III, J. W. & Cutter, D. (1996). Influence of pili, fibrils, and capsule on in vitro adherence by Haemophilus influenzae type b. Mol Microbiol 21, 21-31.

St. Geme, III, J. W. & Falkow, S. (1991). Loss of capsule expression by Haemophilus influenzae type b results in enhanced adherence to and invasion of human cells. Infect Immun 59, 1325-1333.

Tan, E., Low, K. W., Wong, W. S. F. & Leung, K. Y. (1998). Internalization of Aeromonas hydrophila by fish epithelial cells can be inhibited with a tyrosine kinase inhibitor. Microbiol 144, 299-307.

Thune, R. L., Stanley, L. A. & Cooper, R. K. (1993). Pathogenesis of Gram-negative bacterial infections in warmwater fish. Ann Rev Fish Dis 3, 37-68.

Toranzo, A. E., Barreiro, S., Casal, J. F., Figueras, A., Magariños, B. & Barja, J. L. (1991). Pasteurellosis in cultured gilthead seabream, Sparus auratus: first report in Spain. Aquaculture 99, 1-15.

Virji, M., Kayhty, H., Ferguson, D. J. P., Alexandrescu, C. & Moxon, E. R. (1991). Interactions of Haemophilus influenzae with cultured human endothelial cells. Microb Pathog 10, 231-245.

Wang, X. H., Oon, H. L., Ho, G. W. P., Wong, W. S. F., Lim, T. M. & Leung, K. Y. (1998). Internalization and cytotoxicity are important virulence mechanisms in vibrio-fish epithelial cell interactions. Microbiol 144, 2987-3002.

Watanabe, S., Sasaki, J., Wanda, T., Tanaka, Y. & Otsuka, N. (1988). Low gel temperature agarose encapsulation of small specimens for electron microscopy. J Electron Microsc 37, 89-91.

"... although this is projected for several years in the future, the picture of a fish farmer preparing vaccine-tomatoes in his own backyard may one day turn out to be more than a fantasy." - Niels Lorenzen (1999)

Recombinant vaccines: experimental and applied aspects. *Fish & Shellfish Immunology* **9,** 361-365.

Abstract

Extremely high numbers of antibody secreting cells (ASC) were observed in the gills of sea bass fry immunised at three different age/sizes (initial weight of 0.1, 2 and 5 g) by direct immersion in a *Photobacterium damselae* ssp. *piscicida* bacterin. The relatively low ASC production in the head kidney and spleen suggests that the systemic compartment was only slightly stimulated upon immersion vaccination. There was no response of corresponding magnitude in the gut as the one observed in the gills. A clear age effect was observed in the ASC response of the different groups, especially visible in the gills. Significantly higher numbers of specific ASC were observed in the gills of the two oldest groups (initial weight of 2 and 5 g) compared with the youngest fish (initial weight of 0.1 g), but the oldest groups were not significantly different from each other. Additionally, a more rapid response was observed with the ageing of the fish, with peak responses in all the organs at day 18, 16 and 8 post-immunisation in the smallest to largest fish, respectively. There was no evidence that direct immersion exposure to *P. damselae* ssp. *piscicida* at the earliest stages used in the present study (0.1 g) was tolerogenic. In the context of present knowledge, this study strongly supports the importance of the route of immunisation to locally stimulate ASC and the importance that the gills might have in specific responses.

Key words: Sea bass, *Dicentrarchus labrax*, gills, ontogeny, ELISPOT, antibody secreting cells, *Photobacterium damselae* ssp. *piscicida*

1. INTRODUCTION

Pseudotuberculosis, caused by the halophilic Gram negative bacterium *Photobacterium damselae* ssp. *piscicida* (formerly *Pasteurella piscicida*), has been one of the most important diseases in sea bass (*Dicentrarchus labrax* L.) farming (Magariños *et al.*, 1996; Candan *et al.*, 1996). Additionally, increasing resistance to antibiotics has been reported (Kim *et al.*, 1993), emphasising the importance of vaccination for its prevention. Due to their small size, vaccination of sea bass fry by immersion rather than injection is required.

The development of the capacity to produce circulating and/or local antibodies by mature plasma cells is an accurate way to determine the age at which fish can be vaccinated without inducing tolerance as well as the efficiency of a delivery method. In the present study, the systemic (spleen and head kidney) and mucosal (gut and gills) antibody secreting cell (ASC) responses to a *P. damselae* ssp. *piscicida* bacterin, delivered by direct immersion to sea bass of different ages, was monitored in an ELISPOT assay using a mixture of monoclonal antibodies (mAb) to sea bass Ig heavy (WDI1) and light (WDI3) chains (dos Santos *et al.*, 1997), to maximise the chance of detecting any possible isotypic-specific responses in different organs.

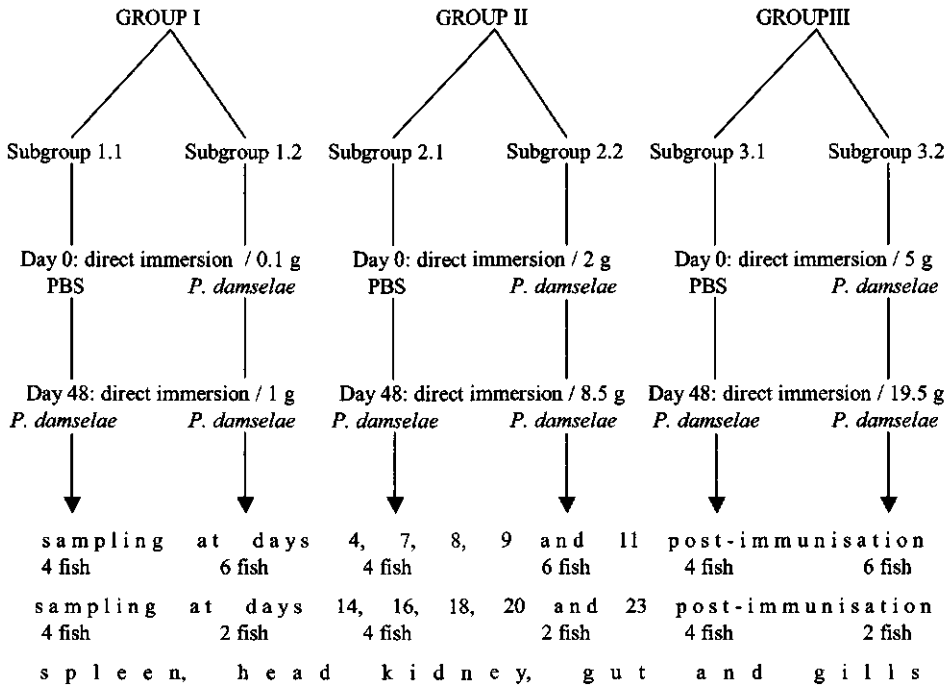

Fig. 1- Experimental design.

2. MATERIALS AND METHODS

2.1. ANTIGEN

The *P. damselae* ssp. *piscicida* bacterin (Photogen®; 11mg bacterial wet weight pellet ml^{-1}) was kindly supplied by Aqua Health Europe, Ltd (Stirling, Scotland, U.K.).

2.2. IMMUNISATION OF FISH

Figure 1 summarises the experimental protocol. Sea bass juveniles were divided into 3 groups according to their initial average weight (group1: 0.1g, group2: 2g and group3: 5g). Each group was divided into 2 subgroups. Fish in one of each subgroup were primed (day 0) and boosted (day 48) by direct immersion in a 10-fold dilution of the Photogen® bacterin in the rearing water for 60 s. After washing with clean water, they were replaced into their respective tanks. Fish in the other subgroups had the same treatment but were immersed on day 0 in 10-fold dilution of Phosphate Buffered Saline (PBS; 140 mM NaCl, 9 mM

$Na_2HPO_4.2H_20$, 1.9 mM $NaH_2PO_4.H_2O$; pH 7.4) in the rearing water instead of the bacterin. Spleen, head kidney, gut and gills were dissected from each fish at days 7, 8, 9, 11, 14, 16, 18, 20 and 23 after immunisation. Sampling days were firstly determined in preliminary experiments where organs from 2 fish were sampled every second day from day 4 to 30 after primary and from day 4 to 14 after secondary intraperitoneal immunisation, respectively.

Fish were kept in a recirculation UV -treated artificial salt-water (22 ‰) system at 22±1° C and fed commercial sea bass feed at a ratio of 3.5% of the body weight.

2.3. LEUKOCYTE ISOLATION

Fish were killed by overdose of tricaine methane sulphonate (5mg/10L; Crescent Research Chemicals, Phoenix, U.S.A) and bled from the caudal vein. The gills were cut and allowed to bleed for a while before being removed and then, placed in a small Petri dish with sea bass-RPMI (SB-RPMI; RPMI + 42 mM NaCl + 0.5 % w/v BSA) until they became whitish. Cell suspensions of spleen, head kidney, gut and gills were made by macerating the organs through a 100 µm mesh gauze using SB-RPMI. The cells were washed for 10 min at 550 *g* and 4° C, resuspended in 1 ml of SB-RPMI and layered onto Lymphoprep (Nycomed Pharma AS, Oslo, Norway; density 1.077 ± 0.001 mg l^{-1}). After centrifuging (30 min, 1100 *g*, 4° C), cells at the interface were harvested, washed in SB-RPMI (10 min, 550 *g*, 4°C), counted and diluted to the appropriate concentration (see below).

1.4. DETECTION OF ANTIBODY SECRETING CELLS (ASC)

Different types of dot blot plates, coating antigen concentrations, cell incubation periods and temperatures were optimised during preliminary experiments. The optimal conditions were then used as described. Wells of a 96-well dot blot apparatus containing a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) were coated overnight at 4° C with 100 µl per well of 3 mg wet weight *P. damselae* ssp *piscicida* ml^{-1} PBS obtained from the vaccine. Control wells coated with the BSA blocking solution only (see below) were used for checking specificity of the ASC. After washing (3 times, 200 µl/well) and immersion (5 min) in PBS, unbound sites were blocked by the addition of 200 µl PBS containing 1 % BSA per well for 2 h at room temperature. The blocking medium was removed and 100 µl of leukocyte suspensions were added to each well at four different concentrations (3-fold dilution) starting

at $5x10^4$ cells/well as the highest concentration. Duplicates were done in different plates to reduce the effect of plate variability. Secretion of antibodies was allowed to occur over 6-7 h at 25° C in a vibration-free place. After washing and immersion, as described above, 100 µl of a mixture of the mAbs to sea bass heavy (WDI1) and light (WDI3) Ig chains (dos Santos *et al.*, 1997) diluted 1:25 and 1:50, respectively, in PBS containing 0.1% BSA was added to each well and incubated overnight at 4° C. Subsequently, the nitrocellulose was removed from the plates and washed (3x) and immersed (5 min) in PBS-Tween (PBS + 0.05% of polyoxyethylene-sorbitan monolaurate) in a tray with shaking. The sheets were then incubated (shaking) for 1 h with goat anti-mouse Ig horseradish peroxidase (GAM-HRP, BIORAD) diluted 1:3000 in PBS-Tween containing 0.1% BSA. After washing in PBS-Tween (2x) and PBS (3x) the spots were developed for 10-15 min at room temperature by adding the chromogen solution containing 258 µg 3-amino-9-ethyl carbazole (Sigma) ml^{-1} and 0.04% H_2O_2 (30%) in 0.05M sodium acetate buffer pH 5.0 (Czerkinsky *et al.*, 1988). The reaction was stopped by washing with running tap water and the nitrocellulose allowed to dry before counting.

1.5. SPOT COUNTING AND STATISTICS

A grid was drawn in the nitrocellulose using a scalpel, which allowed the use of high magnification (40x) for counting real spots and the accurate counting of the maximum number of spots. All wells with more than 5 spots were considered for spot enumeration and the average number of spots was calculated for each treatment taking into account the corresponding number of cells per well. This allowed the reduction of any variation (dilution variability) between wells. The results were expressed as the average number of ASC from duplicated plates per 10^6 leukocytes.

Numbers of ASC in the spleen, head kidney, gut and gills were converted to non-zero format (+1) and transformed by conversion to $\log_e$ before comparison using two-way analysis of variance. Post-hoc comparisons were carried out using the Scheffé test in order to determine differences between organs and groups. P levels lower or equal to 0.05 were considered significant. The software STATISTICA 5.0 (STATSOFT INC., 1995) was used for all the statistical tests.

3. RESULTS

All control wells, coated only with the BSA blocking solution, were completely negative for ASC.

Figure 2 shows the kinetics of the ASC for each sampled organ in each group. Extremely high numbers of ASC were observed in the gills when compared with the other organs, especially in groups 2 (initial weight of 2 g) and 3 (initial weight of 5 g). Both primary and secondary responses in the gills showed significantly higher numbers of ASC compared to the respective responses in all the other organs within each group ($p \leq 0.0001$), with the exception of the primary response in the head kidney in group 1 (initial weight of 0.1 g) where a relatively low p level ($p = 0.059$) was obtained. Similar numbers of ASC were observed in the respective primary and secondary responses of all the other organs, which were not significantly different between each other ($p \geq 0.107$). The numbers of ASC in groups 2 and 3 showed sharp peaks on day 16 and day 8, respectively, although the data from group 2 suggests there was a biphasic response in the systemic organs. A more prolonged peak was evident in group 1, from about day 8-18. This was the case for both the primary and secondary responses. The kinetics of the responses appear to be similar between the organs, especially within groups 2 and 3 since the changes (increases and decreases) in numbers of ASC observed occurred at the same sampling points in all the organs. Nevertheless, this was only confirmed for group 2, where significant differences were not detected in time-organ interaction between all the sampled organs ($p \geq 0.067$), and for spleen, head kidney and gut of groups 1 and 3. No significant differences were detected between primary and secondary responses of any of the organs or groups ($p \geq 0.113$) although boosted fish always showed lower levels of ASC, than the primary response fish.

Comparisons between the different groups showed that both primary and secondary responses in the gills of groups 2 and 3 had significantly higher numbers of ASC than the respective responses in the gills of group 1 ($p \leq 0.004$) but were not significantly different from each other ($p \geq 0.658$). Concerning the other organs (gut, spleen and head kidney), significantly higher numbers of ASC were only detected in the primary response of the head kidney of fish in group 1 compared with group 3 ($p = 0.045$).

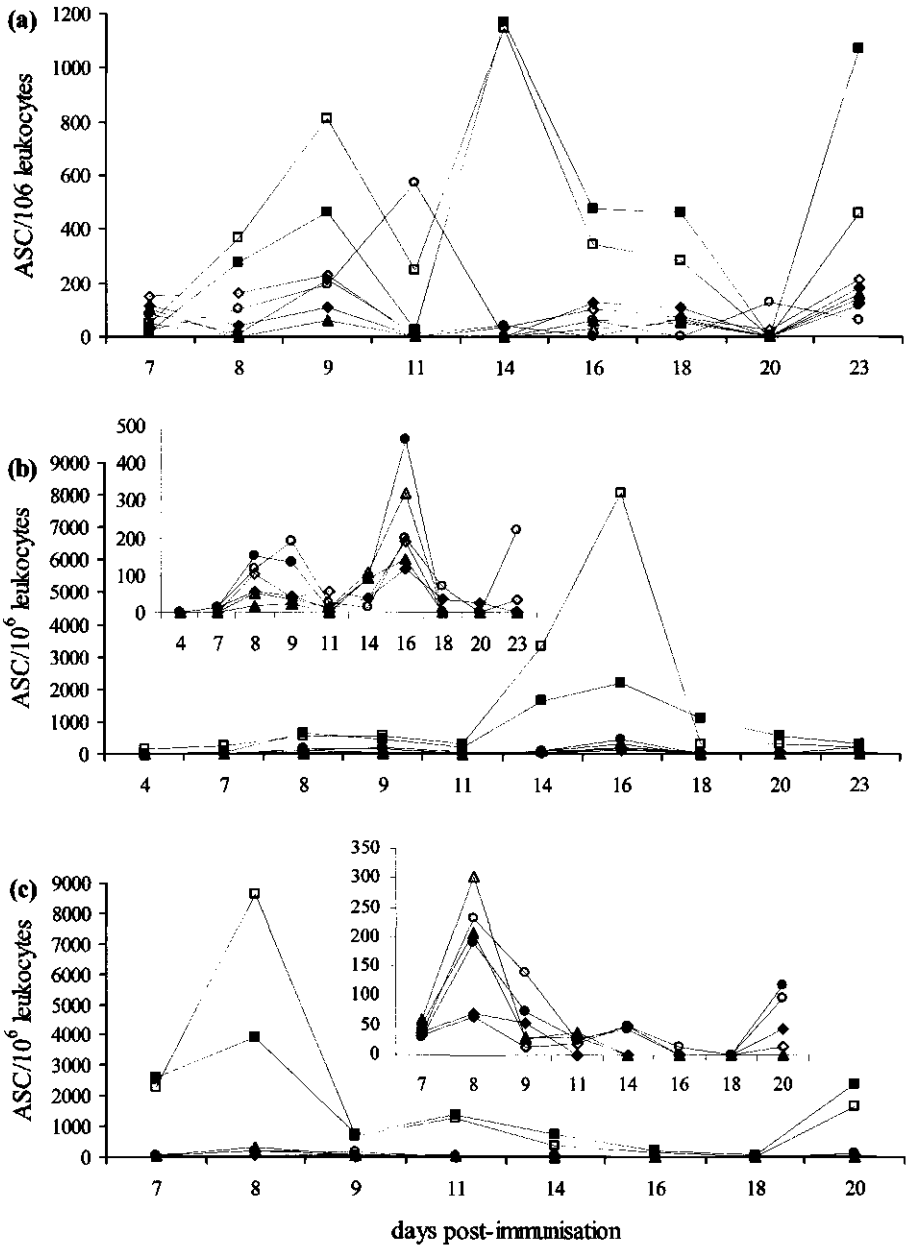

Fig. 2- Number of specific antibody secreting cells (ASC) in the head kidney, spleen, gut (magnified in the inserts) and gills after primary and secondary immersion immunisation of sea bass with *Photobacterium damselae* ssp. *piscicida* determined with the ELISPOT assay. Each point represents the mean number/10^6 leucocytes of N= 6, N= 4 or N= 2 fish (see Fig. 1), determined using duplicate counts of all wells with more than 5 spots. Standard error bars are omitted for clarity. Figure legend: spleen (primed), △; spleen (boosted), ▲; head kidney (primed), ™; head kidney (boosted), ◆; gills (primed) □; gills (boosted),

4. DISCUSSION

In this study, immersion immunisation of sea bass resulted in the development of an ASC response, especially in the gill, as measured by the ELISPOT assay. This response is considered to be specific because control wells coated with a heterologous antigen (BSA) were completely negative.

Previous experiments have shown that in sea bass intraperitoneally immunised with Photogen® at 22±1° C, the peak ASC response in the head kidney occurred at about day 18-20 after primary (approximately 600 ASC/10^6 leukocytes) and about day 7-9 after secondary (approximately 2200 ASC/10^6 leukocytes) immunisation (dos Santos *et al.*, unpublished). Using fish of the same size (same batch) in the present work, immersion immunisation with Photogen® induced a faster (day 8) and higher (8800 ASC/10^6 leucocytes) ASC primary response in the gills. The immersion immunisation induced much lower numbers of ASC in the head kidney, spleen and gut (200-500 ASC/10^6 leucocytes) with peaks at the same time as in the gills. This indicates the importance of the gills in immersion vaccination. Specific antibodies have been detected in the gills of brook trout and rainbow trout following immersion infection or immunisation with *Flavobacterium branchiophilium*, respectively (Lumsden *et al.*, 1993, 1995). These authors showed that immersion vaccinated rainbow trout were better protected against experimental bath challenge than intraperitoneally immunised fish, and also had the highest gill-associated specific antibodies. Additionally, significant numbers of "constitutive" ASC were detected in the gills of dab (*Limanda limanda*), although the peak of specific anti-HGG ASC in intraperitoneally immunised or orally intubated dab accounted for less than 8% of the total ASC (Davidson *et al.*, 1997). The present data and that of Lumsden *et al.* (1993, 1995) suggest the importance of the route of immunisation to locally stimulate the production of specific ASC in the gill. Furthermore, they show the importance that the gills might have in specific antibody responses instead of being an organ better deployed in the production of a wide-ranging immunological barrier to infection in the form of "constitutive" antibody secretion as hypothesised by Davidson *et al.* (1997). The low ASC production in the head kidney and spleen suggests that the systemic compartment was only slightly stimulated upon immersion vaccination. It has been shown that the skin and the gills are the major sites of antigen uptake after immersion immunisation and the antigens remain in the site of uptake with only small amounts being transported to the kidney and the spleen (Ototake *et al.*, 1996; Moore *et al.*, 1998). This may explain the low numbers of ASC detected

here in the head kidney and spleen as well as the typically low to undetectable serum antibody titres reported after immersion vaccination (Nakanishi & Ototake, 1997). In this context, the present data strongly supports the idea that most antibodies present in mucosal tissues originate from local production and are not derived from serum transudation (Lobb & Clem, 1981; Rombout *et al.*, 1989; Zilberg & Klesius, 1997). In addition, since specific antibody stimulation in the skin has also been shown after immersion immunisation (Lobb, 1987; Loghothetis & Austin, 1994) and serum antibody levels frequently do not correlate with induced protection (Nakanishi & Otatake, 1997) it is likely that both the skin and the gills play the major role in the protection which has been reported following immersion vaccination.

It has been postulated that the mucosae of fish, like that of mammals, represents a single autonomous immune compartment and exposure of one mucosal tissue to antigen can lead to apparent immunological stimulation at another mucosal site (Kawai *et al.*, 1981; Rombout *et al.*, 1989; Davidson *et al.*, 1993). However, while high numbers of ASC were detected in the gills after oral immunisation of carp to *Vibrio anguillarum* (Joosten *et al.*, 1997), oral immunisation of dab with HGG failed to elicit high numbers of ASC in the gills (Davidson *et al.*, 1997). In the present study, although immersion immunisation induced extremely high numbers of ASC in the gills there was no response of comparable magnitude in the gut. However, Rombout *et al.* (1993) showed that only a limited number of Ig^+ lymphoid cells were freed from the gut by the isolation method used and, thus, it can not be excluded that the differences observed in the present study may be influenced by the efficacy of the ASC isolation from the gut.

A clear age effect was observed in the ASC response of the different groups, especially visible in the gills. Thus, significantly higher numbers of specific ASC were observed in the gills of the two oldest groups (immunised at 2 and 5 g body weight) compared with the youngest fish (immunised at 0.1 and 1 g body weight). A positive correlation between the size of the fish and vaccine uptake after immersion vaccination was reported by Tatner & Horne (1983a), which might explain the higher numbers of ASC observed in the older groups in the present study. These authors hypothesised that larger fish might have relatively more sites for antigen uptake, and hence would take up more vaccine. Additionally, a much faster response (shorter latent period) was observed in the oldest fish (immunised at 5 g) relative to fish immunised at 0.1 and 2 g. This is in agreement with studies where in older carp antibody production starts somewhat earlier than in younger fish (Manning *et al.*, 1982a). There was no evidence that direct immersion exposure to *P. damselae* ssp. *piscicida* at the earliest stages

used in the present study (0.1 g) is tolerogenic. Antibody responses to bacterial (T-independent) antigens have been reported to precede that elicited by protein (T-dependent) antigens (Manning *et al.* 1982a,b; Manning & Mughal, 1985; Zapata *et al.*, 1997) and many studies have shown that tolerance in young fish can only be induced to T-dependent antigens administered by injection but not to those administered by direct immersion (Zapata *et al.*, 1997). It has been postulated that fish might have a period of unresponsiveness and not tolerance to immersion vaccination in early stages due to a lack of uptake of antigens by the fry, as shown by Tatner & Horne, 1984. Thus, the immune response of young fish not only depends on the type of antigen but also on the route of its administration. This is further supported by the fact that rainbow trout fry were found to be susceptible to experimental infections of *Vibrio* anguillarum by the injection route before they were susceptible to infection by immersion (Tatner & Horne, 1983b).

A striking feature of the present results was the similar kinetics (time of peaking) of the ASC responses in both primary and secondary responses as well as the lower numbers of ASC in boosted fish in all groups of fish (although this was not significantly different, probably due to the high error variance). These results are in accordance with the lack of mucosal memory when rabbits were immunised by feeding dead bacteria, while those fed with the living organism elicited a good secretory IgA antibody response (Newby & Stokes, 1984). A similar kinetics for primary and secondary plaque forming cell response and a slightly reduced secondary plaque forming cell response was also reported in rainbow trout flush exposed to the O-antigen of *Yersinia ruckeri* (Anderson & Dixon, 1980). These authors hypothesised that the lack of immunological memory may have been due to the intrinsic nature of the response of the fish to that antigen. However, sea bass fry from the same batch as group 3 and reared under the same conditions showed enhanced production of ASC after intraperitoneal boosting using the same vaccine as in the present study. In addition, the time of peaking of the ASC responses were much faster in intraperitoneally boosted fish than in primed fish (dos Santos *et al.*, unpublished). Thus, it is unlikely that the lymphoid organs are not sufficiently mature to produce a secondary response but that the route of administration in addition to the nature of the antigenic stimulation significantly influences the systemic and gill antibody responses. In conclusion, the fast and similar time of peaking of the primary and secondary ASC response, in addition to the high levels of ASC observed in the gills of the immersion vaccinated fish suggests the importance of that organ and probably the skin in the immune protection of fish upon natural challenge.

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References

Anderson, D. P. & Dixon, O. W. (1980). Immunological memory in rainbow trout to a fish disease bacterin administered by flush exposure. In *Phylogeny of Immunological Memory* (M. J. Manning, ed.) pp. 103-111. Elsevier/North-Holland Biomedical Press, Amsterdam.

Candan, A., Ang Kucker, M. & Karatas, S. (1996). Pasteurollosis in cultured sea bass (*Dicentrarchus labrax*) in Turkey. *Bulletin of the European Association of Fish Pathologists* **16**, 150-153.

Czerkinsky, C. C., Moldoveanu, Z., Mestecky, J., Nilsson, L-Å. & Ouchterlony, Ö. (1988). A novel two colour ELISPOT assay; I. Simultaneous detection of distinct types of antibody-secreting cells. *Journal of Immunological Methods* **115**, 31-37.

Davidson, G. A., Ellis, A. E. & Secombes, C. J. (1993). Route of immunization influences the generation of antibody secreting cells in the gut of rainbow trout, *Oncorhynchus mykiss*. *Developmental and Comparative Immunology* **17**, 373-376.

Davidson, G. A., Lin, S-H., Secombes, C. J. & Ellis, A. E. (1997). Detection of specific and "constitutive" antibody secreting cells in the gills, head kidney and peripheral blood leukocytes of dab (*Limanda limanda*). *Veterinary Immunology and Immunopathology* **58**, 363-374.

dos Santos, N. M. S., Taverne, N., Taverne-Thiele, A. J., de Sousa, M. & Rombout, J. H. W. M. (1997). Characterisation of monoclonal antibodies for sea bass (*Dicentrarchus labrax* L.) IgM indicates the existence of B cell subpopulations. *Fish & Shellfish Immunology* **7**, 175-191.

dos Santos, N. M. S., Taverne-Thiele, A. J., Barnes, A. C., Ellis, A. E. & Rombout, J. H. W. M. (2000). Kinetics of juvenile sea bass (*Dicentrarchus labrax*, L.) systemic and mucosal antibody secreting cell response to different antigens (*Photobacterium damselae* ssp. *piscicida*, *Vibrio anguillarum* and DNP). *Fish & Shellfish Immunology* **submitted**.

Joosten, P. H. M., Tiemersma, E., Threels, A., Caumartin-Dhieux, C. and Rombout, J. H. W. M. (1997). Oral vaccination of fish against *Vibrio anguillarum* using alginate microparticles. *Fish & Shellfish Immunology* **7**, 471-485.

Kawai, K., Kusudu, R. & Itami, T. (1981). Mechanisms of protection in ayu orally vaccinated for vibriosis. *Fish Pathology* 15, 257-262.

Kim, E-H. & Aoki, T. (1993). Drug resistance and broad geographical distribution of identical R plasmids of *Pasteurella piscicida* isolated from cultured yellowtail in Japan. *Microbiology and Immunology* **37**, 109-113.

Lobb, C. J. (1987). Secretory immunity induced in catfish, *Ictalurus punctatus*, following bath immunization. *Developmental and Comparative Immunology* **11**, 727-738.

Lobb, C. & Clem, W. (1981). The metabolic relationships of the immunoglobulins in fish serum, cutaneous mucus and bile. *Journal of Immunology* **127**, 1525-1529.

Loghothetis, P. N. & Austin, B. (1994). Immune response of rainbow trout (*Oncorhynchus mykiss*, Walbaum) to *Aeromonas hydrophila*. *Fish & Shellfish Immunology* **4**, 239-254.

Lumsden, J. S., Ostland, V. E., Byrne, P. J. & Ferguson, H. W. (1993). Detection of a distinct gill-surface antibody response following horizontal infection and bath challenge of brook trout *Salvelinus fontinalis* with *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease. *Diseases of Aquatic Organisms* **16**, 21-27.

Lumsden, J. S., Ostland, V. E., MacPhee, D. D. & Ferguson, H. W. (1995). Production of gill-associated and serum antibody by rainbow trout (*Oncorhynchus mykiss*) following immersion immunization with acetone-killed *Flavobacterium branchiophilum* and the relationship to protection from experimental challenges. *Fish & Shellfish Immunology* **5**, 151-165.

Magariños, B., Toranzo, A. E. & Romalde, J. L. (1996). Phenotypic and pathobiological characteristics of *Pasteurella piscicida*. *Annual Review of Fish Diseases* **6**, 41-64.

Manning, M. J., Grace, M. F. & Secombes, C. J. (1982a). Developmental aspects of immunity and tolerance in fish. In *Microbial Diseases of Fish* (R. J. Roberts, ed.) pp. 31-46. Academic Press, London.

Manning, M. J., Grace, M. F. & Secombes, C. J. (1982b). Ontogenetic aspects of tolerance and immunity in carp and rainbow trout: studies on the role of thymus. *Developmental and Comparative Immunology* **supplement 2**, 75-82.

Manning, M. J. & Mughal, M. S. (1985). Factors affecting the immune responses of immature fish. In *Fish and Shellfish Pathology* (A. E. Ellis, ed.) pp. 27-40. Academic Press, London.

Moore, J. D., Ototake, M. & Nakanishi, T. (1998). Particulate antigen uptake during immersion immunisation of fish: the effectiveness of prolonged exposure and the roles of skin and gill. *Fish & Shellfish Immunology* **8**, 393-407.

Nakanishi, T. & Ototake, M. (1997). Antigen uptake and immune responses after immersion vaccination. In *Fish Vaccinology* (R. Gudding, A. Lillehaug, P. J. Midtlyng and F. Brown, Eds.) pp. 59-68. Developments in Biological Standardization. Basel, Karger.

Newby, T. J. & Stokes, C. R. (1984). The intestinal immune system and oral vaccination. *Veterinary Immunology and Immunopathology* **6**, 67-105.

Ototake, M., Iwama, G. K. & Nakanishi, T. (1996). The uptake of bovine serum albumin by the skin of bath-immunised trout *Oncorhynchus mykiss*. *Fish & Shellfish Immunology* **6**, 321-333.

Rombout, J. W. H. M., Anja J. Taverne-Thiele & Maria I. Villena (1993). The gut-associated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): an immunocytochemical analysis. *Developmental and Comparative Immunology* **17**, 55-66.

Rombout, J. W. H. M., van den Berg, A. A., Berg, C. T. G. A., Witte, P. & Egberts, E. (1989). Immunological importance of the second gut segment of carp. III. Systemic and/or mucosal immune responses after immunization with soluble or particulate antigen. *Journal of Fish Biology* **35**, 179-186.

Tatner, M. F. & Horne, M. T. (1983a). Factors influencing the uptake of ^{14}C-labelled *Vibrio anguillarum* vaccine in direct immersion experiments with rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **22**, 585-591.

Tatner, M. F. & Horne, M. T. (1983b). Susceptibility and immunity to *Vibrio anguillarum* in post-hatching rainbow trout fry, *Salmo gairdneri* Richardson 1836. *Developmental and Comparative Immunology* **7**, 465-472.

Tatner, M. F. & Horne, M. T. (1984). The effects of early exposure to *Vibrio anguillarum* vaccine on the immune response of the fry of rainbow trout, *Salmo gairdneri* Richardson. *Aquaculture* **41**, 193-202.

Zapata, A. G., Torroba, M., Varas, A. & Jiménez, E. (1997). Immunity in fish larvae. In *Fish Vaccinology* (R. Gudding, A. Lillehaug, P. J. Midtlyng and F. Brown, Eds.) pp. 23-32. Developments in Biological Standardization. Basel, Karger.

Zilberg, D., & Klesius, P. H. (1997). Quantification of immunoglobulin in the serum and mucus of channel catfish at different ages and following infection with Edwardsiella ictaluri. *Veterinary Immunology and Immunopathology* **58**, 171-180.

CHAPTER 8

Summary and Conclusions

Samenvatting en Conclusies

Sumário e Conclusões

"The scientific mind does not so much provide the right answers as ask the right question." - Claude Levi-Strauss

In Mackay, A.L. (1991). A dictionary of scientific quotations. Adam Hilger, Bristol, 197 pp.

"The world is moved along, not only by the mighty shoves of its heroes, but also by the aggregate of the tiny pushes of each honest worker" – Helen Keller

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SUMMARY AND CONCLUSIONS

Research and knowledge on fundamental and applied immunology and immunopathology allows the development and/or improvement of vaccines, which can then be used successfully in the prophylaxis of important diseases. In fact, there are three major steps essential for the scientific development of an effective vaccine:

1. Knowledge of the mechanisms of pathogenicity and virulence factors of the infectious agent, which allows the selection of important molecules/epitopes essential for the pathogen's survival and their inclusion in the vaccine;
2. Knowledge of the host immune system, comprising its development, regulation/modulation, kinetics of the immune response and its local compartments, which allows distinguishing the best time and method to vaccinate.
3. Knowledge of the vaccine's effectiveness by studying the protection induced through experimental *in vivo* and/or *in vitro* challenges.

The fact that the sea bass culture has been increasing in the Mediterranean area and pseudotuberculosis has been spreading and causing mass mortalities in their culture initiated the development of an effective vaccine against *Photobacterium damselae* ssp. *piscicida* by several commercial vaccine producers. However, at the time of the first outbreaks very little was known about the sea bass immune system, and it became important to establish basic principles concerning their immunocompetent state and responsiveness to vaccination, which was the main goal of this thesis.

Monoclonal antibodies (mAb) have proven to be extremely useful tools in studies of fish immunology. *The characterisation of the sea bass immunoglobulin (Ig) and the production of mAb against the heavy (H; WDI 1) and light (L; WDI 2 and WDI 3) chains were successfully performed. These have proved to be useful for measuring specific immune responses and studying the ontogeny of the B lymphocyte lineage.*

Sea bass Ig

Teleosts generate considerable structural diversity in their immunoglobulins by nonuniformly cross-linking subunits ("halfmers", monomers, dimers and trimers) within the tetrameric molecule. *In sea bass, nonuniform disulphide cross-linking of monomeric (M_r ±210 kDa) and dimeric (M_r ±420 kDa) subunits appears to occur with no joining chain involved in the polymerisation of the tetrameric (M_r ±840 kDa) form.* However, no clear physiological significance for this structural diversity has yet been shown. Additionally, as for other species,

it was shown that each monomeric unit is composed of two H (M_r ±78 kDa) and two L (M_r ±28 kDa) polypeptide chains, bound together by a cysteine bridge between the L chain constant domain and the first constant domain of the H chain. Further characterisation of the sea bass Ig was performed by sequencing its L chain. This revealed the same cluster-like genomic organisation as the Ig L chain of other teleosts. *All characteristic segments (V, J and C) coding for the V and C domains of the Ig L chain could be deduced, with the V domain showing the three complementarity determining regions (CDRs) and the four framework regions (FRs) on either side of the CDRs.* The typical cysteine residues that probably form the intra- and inter-chain interactions were also conserved in the sea bass. In addition, the deduced sequence could be considered as a L1/G isotype, which has been suggested to be closely related to the mammalian κ isotype.

Sea bass B cells

As in "higher" vertebrates, fish B cells express Ig on their surface, where it acts as specific antigen receptor. All mAb against sea bass Ig produced in the present study were reactive with a subpopulation of lymphocytes, which in ultrastructural studies appeared with clustered surface Ig on the cell membrane, as shown for other fish species. Plasma cells showed less reaction at their surface than found in carp and more closely resemble mammalian plasma cells, which lose their surface Ig completely during differentiation. Most importantly, the results strongly indicated the existence of B cell heterogeneity in sea bass, based on the expression of at least two different H and L chains. The existence of IgM diversity in other teleost species, and more recently, isotypic forms of the H and L chains, have been reported. However, whether this heterogeneity has functional expression in the physiological context remains to be investigated. The percentage of B cells in the different immunological organs was studied using all the mAb, and the combination of WDI 1 and WDI 3 appears to label all or almost all B cells. As in other teleost species, sea bass head kidney appears to be the primary organ for B-lymphocyte differentiation. *The proportion of sIg^+ (B) cells in the different organs increased during the ontogenetic development being higher at early stages in the head kidney followed by spleen, PBL, gut and thymus. This sequential order changed during ontogeny until the adult distribution is reached (PBL, spleen, head kidney, gut and thymus). As reported for rainbow trout, sea bass cytoplasmic Ig^+ (cIg^+) cells (pre-B cells) were detected earlier in ontogeny than sIg^+ cells, which is in agreement with the results reported for amphibians and mammals.* It is worth noticing that the sea bass cIg^+ cells were detected using the L chain mAb, which may suggest that the L chain might be transcribed in

high amounts during early stages of B cell development. Whether there is co-expression of H and L chain Ig in fish pre-B cells or that the expression of the H chain precedes the expression of the L chain remains to be investigated. However, germline joining-constant (JC) IgL2 transcripts have been found in the rainbow trout, suggesting the existence of a surrogate JLC2 protein, which may be expressed on the surface of pre-B cells in combination with the H chains, as happens in mammals. Thus, it is possible that cIg^+ cells could have been detected even earlier in the sea bass if the mAb against the H chain had been used. Cytoplasmic Ig^+ plasma cells appear later in ontogeny and might be indicative of immunological maturation, regarding the onset of adaptive humoral immune responses. In addition, the results presented strongly suggest that, at least in sea bass, age is the most important factor relating to the development of immunocompetence. Although stated by some that in fish weight might better correlate with immunocompetence than age, this might only be true when time, in hours, days, months, years or other, is taken as the measuring unit. However, since fish are poikilothermic and temperature regulates directly or indirectly all physiological reactions, degree-days are undoubtedly the best way for measuring physiological time/age, allowing in addition direct comparison between equivalent physiological age of different species.

Sea bass T cells

Although, T cell receptor (TCR)-α and -β chains gene sequences have recently been described in teleosts, only a very limited number of specific mAb have been obtained, which has limited functional studies. However, a mAb (DLT 15) to sea bass thymocytes and peripheral T-cells has been produced and used in studies that strongly suggested its specificity. Additional information was obtained in the present thesis relating to the ontogenetic development of sea bass T cells. In sea bass, as in other fish species, the thymus is the major site for T cell differentiation. However, in the present thesis, early T cells could be detected using DLT15 before the thymus became lymphoid, suggesting that they might originate in a different compartment and further mature in thymus as happens in mammals. After thymic differentiation, T lymphocytes appear to migrate to peripheral lymphoid organs, as suggested by their high percentage in PBL early in the development. *During the ontogenetic development, the proportion of T cells increases in the thymus and the gut but decreases in PBL, head kidney and spleen until around 137 days post-hatch, when adult levels are reached.*

Sea bass humoral immunity

Sea bass Ab effector mechanisms have been shown during the experiments performed in the present thesis. Agglutination was observed in tests using sera from sea bass immunised with a *P. damselae* ssp. *piscicida* bacterin when testing the immunisation and vaccine specificity. The fact that a few macrophages were specifically stained by the mAb might indicate the presence of Ig receptors on their surface, which are important for mediating the binding of Ab-opsonised antigens promoting their phagocytosis. Neutralisation by specific Ab is also suggested, since heat-inactivated antiserum against *P. damselae* ssp. *piscicida* reduced the ability of the bacteria to invade tissue culture cells, presumably by blocking the surface receptors involved in adhesion and/or invasion.

In this thesis it was clearly demonstrated that sea bass is able to mount a specific antibody response to different antigens: *Vibrio anguillarum*, *P. damselae* ssp. *piscicida* and DNP. Moreover, memory formation [faster response and higher numbers of antibody secreting cells (ASC)] was clearly shown in the head kidney and spleen after intraperitoneal (ip) immunisation. On the other hand, some evidence of immunosuppression is available for the immune response in the gut. As demonstrated for other teleost species, the head kidney is the main ASC-producing organ after ip immunisation. However, when fish from the same size (same batch) as the ones immunised ip were directly immersed in the *P. damselae* ssp. *piscicida* bacterin, a faster and higher ASC primary response was observed in the gills, with much lower numbers of ASC in the head kidney, spleen and gut. Surprisingly, a similar peak day of ASC and even lower numbers of ASC were observed following a second immersion. The decreased level of the secondary ASC response observed might be due to a lower antigen uptake in the boosted fish caused by combined effect of immunological and non-immunological components of the mucosal barrier, similar to that which occurs in the gut of mammals. Antibody present in the gills (and probably in the skin) from the primary response could interact with the antigen to form complexes, thereby preventing or decreasing the binding of antigen to, and subsequent pinocytosis by, gill (and skin) epithelial cells. In addition, increases in both discharge of globlet cell mucus and proteolytic activity has been shown to occur in the gut of immunised mammals and it is likely that similar phenomena may also occur in the fish mucosal system. A clear age effect was observed in the ASC responses with significantly higher numbers of ASC and faster ASC responses in the oldest groups. More importantly, direct immersion exposure to *P. damselae* ssp. *piscicida* at the earliest stages used in the present study (0.1g) did not result in immunosuppression, which is in agreement with what has been reported for other bacterin immersed fish species of

comparable size. *In conclusion, the importance of the route of vaccination for the local stimulation of Ab production, especially in gills but probably also skin is clearly demonstrated and this mucosal response probably plays a role in the protection against the disease.*

Vaccination

As mentioned before, knowledge of the mechanisms of pathogenicity and virulence factors of a pathogen will allow the selection of important molecules/epitopes for inclusion in the vaccine. In the present thesis, the kinetics and specificity of the invasion ability of *P. damselae* ssp. *piscicida* was studied and confirmed that there was an intracellular phase to the life-cycle of the bacterium. In addition to the intracellular phase, the presence of a weakly immunogenic capsule conferring resistance to serum killing and decreasing the phagocytosis index, may help the pathogen to escape the host's cellular and humoral defences. Thus, identification of the components and mechanisms involved in the internalisation process may provide a good antigen for inclusion in an effective vaccine. The fact that heat-inactivated antiserum obtained from sea bass immunised with the *P. damselae* ssp. *piscicida* bacterin has reduced the invasiveness of the bacteria, suggests that the bacterin may have contained some of the factors responsible for the adhesion and invasion capacity of the pathogen. In addition, other effective antigen targets for a vaccine formulation might be the proteins involved in the iron acquisition system [iron regulated outer membrane proteins (IROMPs) and siderophore] as well as some factors present in the extra-cellular products (ECP), which have been shown to be promising in other bacterial vaccine formulations. However, the fact that both intramolecular- and intermolecular-induced immune suppression occurs in some fish species raises the question of how this might influence the specificity of immune responses against vaccine antigens and induction of immune protection, and the need for a better understanding how the fish immune response is regulated. *In the present thesis, at least intramolecular-induced suppression appears to play an important role in the regulation of the antibody response in sea bass, which was suggested by the extremely low variability observed in the Ig L chain sequences obtained from DNP_{494}-KLH immunised sea bass.* The potentially immunodominant determinant located in one part of a molecule may induce intramolecular suppression of the antibody response to other epitopes present on the same antigen which could be important for immune protection.

Since most disease outbreaks may occur at very early stages of fish development and injection of the vaccines is not possible at these ages, alternative methods of vaccination must

be taken into account. Although oral immunisation would be ideal for mass vaccination, the need for protecting the antigens from degradation in the anterior/gastric segment of the gut might increase the costs of the vaccine. In addition, the dose and period of administration must be clearly known for each stage of development, in order to avoid induction of tolerance to protein antigens. In this context, immersion vaccination appears as a valuable alternative to oral immunisation, since in young fish small amounts are needed for vaccination without any risk of tolerance induction.

Final conclusion and perspectives

It can be stated that the bacterin used in the presented thesis contains immunogenic determinants, which appear to be important for protection. In addition, sea bass can be vaccinated against P. damselae ssp. piscicida from at least 137-145 days post-hatch (~1g), regardless of the route of administration, or from at least 80-100 days post-hatch (~0.1g) if immersion vaccination is performed.

Isolating and characterising important target antigens, studying their inclusion in a vaccine formulation in terms of immune regulation as well as the best way to deliver them, are important processes in the development of new fish vaccines and vaccination methods. Presently, research towards the characterisation of vaccine components and mechanisms involved in their uptake will continue. Testing different vaccine formulations through *in vitro* and *in vivo* challenge studies, in addition to field tests, are also required in this process of vaccine development. The possible inclusion and effect of adjuvants in immersion vaccination might also be an important item to study, especially with respect to the duration of protection. Concerning sea bass immunity, basic and applied research/knowledge about the cell-mediated immune responses is limited and the future development of vaccines against many other pathogens could be highly stimulated by knowledge in this area.

SAMENVATTING EN CONCLUSIES

Fundamenteel en toegepast immuno(patho)logisch onderzoek is van grote betekenis voor de ontwikkeling en verbetering van vaccins tegen belangrijke visziektes. Er kunnen drie fases onderscheiden worden in de ontwikkeling van een effectief vaccin:

1. Kennis van het pathologische proces en de virulentiefactoren van het pathogeen, waardoor belangrijke moleculen of delen daarvan (epitopen) geïdentificeerd en vervolgens gebruikt kunnen worden bij de bereiding van een vaccin;
2. Gedetailleerde kennis van het immuunsysteem van de vis, o.a. de ontwikkeling, regulatie, modulatie en kinetiek van de immuunrespons op diverse locaties (intern versus extern), waardoor min of meer vastgesteld kan worden vanaf wanneer en met welke methode de vissen het beste gevaccineerd kunnen worden.
3. Kennis van de effectiviteit van het vaccin, waarbij de mate van bescherming tegen virulente pathogenen getest kan worden.

Als gevolg van de toenemende zeebaarskweek in de mediterrane landen is pseudotuberculose een belangrijke visziekte geworden, die massale sterfte veroorzaakt. Diverse producenten hebben dan ook geprobeerd een effectief werkend vaccin tegen het pathogeen *Photobacterium damselae* ssp. *piscicida* te ontwikkelen. Echter toen de ziekte een steeds groter probleem werd was er nog maar weinig bekend van het immuunsysteem van de zeebaars. Het onderzoek beschreven in dit proefschrift heeft tot doel de basale principes van de immunocompetentie en de respons na een vaccinatie vast te stellen.

Monoclonale antilichamen (mAb) hebben bewezen zeer bruikbaar te zijn in de studie van het immuunsysteem van vissen. *De karakterisering van het immunoglobuline (Ig) van de zeebaars en de productie van mAb tegen zowel zware (H; WDI 1) als lichte (L; WDI 2 and WDI 3) ketens zijn met succes uitgevoerd. Deze mAb zijn onmisbaar gebleken bij het meten van de specifieke immuunrespons en het bestuderen van de ontwikkeling van B-cellen.*

Zeebaars Ig

Beenvissen vertonen een aanzienlijke structurele diversiteit in hun Ig: half-, mono-, di-, tri- en (de meest voorkomende) tetrameren. *Zeebaars Ig kan via disulfide-bruggen monomeren (M_r ± 210 kDa) en dimeren (M_r ± 420 kDa) laten polymeriseren tot tetrameren (M_r ± 840 kDa) zonder dat een verbindende J-keten daarbij nodig is.* Er zijn echter geen duidelijke aanwijzingen dat deze verschillende vormen ook andere fysiologische functies hebben. Zoals bij andere vissoorten bestaat ieder monomeer uit twee H- (M_r ± 78 kDa) en twee L- (M_r ± 28

kDa) ketens, die via een cysteïne-brug tussen het constante deel van de L-keten en het constante deel van de H-keten bij elkaar gehouden worden. Tevens is er in dit proefschrift aandacht besteed aan de DNA-sequentie van de L-keten, die een vergelijkbare cluster-achtige genomische organisatie heeft als de L-keten van andere beenvissen. *Alle karakteristieke segmenten (V, J en C) coderend voor de V- en C- domeinen van de Ig L-keten zijn gevonden; het V- domein met zijn drie "complementarity determining regions" (CDRs) en de vier "framework regions" (FRs) aan elke zijde van de CDRs.* De karakteristieke cysteïne-residuën die waarschijnlijk de intra- en inter-keten interacties verzorgen zijn ook aantoonbaar. De gevonden sequentie kan het beschouwd worden als een L1/G isotype, dat het sterkst gerelateerd is aan het zoogdier κ isotype.

Zeebaars B-cellen

Net als bij zoogdieren brengen de B-cellen van vissen Ig op hun celoppervlak tot expressie, waar het functioneert als een specifieke antigeenreceptor. Alle in deze studie geproduceerde mAb tegen zeebaars Ig zijn reactief met een subpopulatie van lymfocyten. Ultrastructureel blijken de Ig-moleculen, net als bij andere vissen in clusters op de celmembraan voor te komen. Plasmacellen vertonen minder Ig aan hun oppervlak, vergelijkbaar met zoogdier plasmacellen die ook hun Ig verliezen wanneer ze uit B-cellen differentiëren. De gevonden resultaten zijn een sterke indicatie voor B-cel heterogeniteit in zeebaars, hetgeen gebaseerd is op de expressie van minstens twee verschillende H- en L-ketens. IgM-diversiteit gebaseerd op H- en L-isotypes is ook in andere beenvissen beschreven, echter een verschil in functie is tot nu toe niet aangetoond. Ook het percentage B-cellen in de verschillende immunologische organen is bestudeerd, waarbij de combinatie van de mAb WDI 1 en WDI 3 alle of bijna alle zeebaars B-cellen lijkt te labelen. Net als in andere beenvissen blijkt de kopnier het primaire orgaan voor B-cellen te zijn. *De percentages Ig^+ B-cellen in de verschillende lymfoïde organen neemt tijdens de ontogenie toe, waarbij het in vroege stadia hoger is in kopnier, gevolgd door milt, bloed, darm en thymus. Deze volgorde veranderde gedurende ontogenie tot uiteindelijk de volwassen volgorde werd bereikt: bloed, milt, kopnier, darm en thymus. Vergelijkbaar met de forel worden cellen die cytoplasmatisch Ig^+ zijn (pre-B-cellen) eerder waargenomen dan de membraan Ig^+ cellen, hetgeen overeen komt met de resultaten beschreven voor amphibiën en zoogdieren.* Opvallend is dat de cytoplasmatisch Ig^+ cellen met het L-keten-specifieke mAb aangetoond zijn, suggererend dat de L-keten al vroeg in de B-cel ontwikkeling tot expressie komt. Het is nog niet duidelijk of het hier gaat om een co-expressie van H- en L-keten in zeebaars pre-B-cellen of dat de expressie van de H-keten er toch aan

vooraf gaat. Echter in de forel zijn er indicaties dat een surrogaat JLC2-eiwit geproduceerd wordt en tot expressie zou kunnen komen op de membraan van pre-B-cellen in combinatie met de H-keten, zoals ook bij zoogdieren het geval is. Met andere woorden het is mogelijk dat de cytoplasmatisch Ig^+-cellen in de zeebaars eerder gedetecteerd hadden kunnen worden wanneer het mAb tegen de H-keten was gebruikt. Cytoplasmatisch Ig^+ plasmacellen verschijnen later en zijn een indicatie voor de immunologische maturatie. *Onze resultaten suggereren bovendien dat de leeftijd van een zeebaars belangrijker is dan het gewicht bij de ontwikkeling van immunocompetentie, hoewel door anderen ook beweerd wordt dat het gewicht belangrijk is.* Omdat vissen koudbloedig zijn en temperatuur direct of indirect alle fysiologische reacties reguleert, is het werken met dag-graden onbetwist de beste manier om de fysiologische leeftijd van dieren uit te drukken, hetgeen deze verwarring kan voorkomen.

Zeebaars T-cellen

Hoewel de T-cel-receptor(TCR)-α en –β keten gensequenties recent beschreven zijn in beenvissen, is er slechts een beperkt aantal T-cel-specifieke mAb beschikbaar. Voor zeebaars is mAb DLT 15 beschikbaar en reactief met thymocyten and perifere T-cellen. Hiermee is in dit proefschrift ook informatie verkregen over de ontwikkeling van T-cellen. Net als in andere vissen blijkt de thymus de belangrijkste plaats voor T-cel-differentiatie te zijn. Echter in dit proefschrift staan aanwijzingen dat vroege T-cellen aantoonbaar zijn met DLT15 voordat de thymus lymfoïd wordt, een indicatie dat deze cellen voortkomen uit een ander compartiment en afrijpen in de thymus, zoals ook voor zoogdieren beschreven is. Na de maturatie in de thymus blijken T-lymfocyten naar de perifere lymfoïde organen te migreren, hetgeen het hoge percentage T-cellen in het bloed vroeg in de ontwikkeling kan verklaren. *Tijdens de ontogenie neemt het percentage T-cellen af in thymus en darm, maar tot 137 dagen na hatching toe in bloed, kopnier en milt, wanneer de adulte niveaus bereikt zijn.*

Zeebaars humorale immuniteit

Zeebaars antilichaam-effector mechanismen worden in de diverse experimenten uit dit proefschrift beschreven. Agglutinatie is waargenomen in testen met sera van de dieren geïmmuniseerd met een *P. damselae* ssp. *piscicida* bacterin. Het feit dat sommige macrofagen specifiek reageerden met het anti-IgM mAb is een indicatie dat er Ig receptoren op hun oppervlak aanwezig zijn, die van belang kunnen zijn om effectief antigeen-antilichaam-complexen te kunnen fagocyteren. Neutralisatie van pathogenen door specifieke antilichamen is ook aantoonbaar, omdat het hitte-geïnactiveerd antiserum tegen *P. damselae* ssp. *piscicida*

het vermogen van bacteria om cellen binnen te dringen reduceerde, waarschijnlijk door het blokkeren van oppervlaktemoleculen betrokken bij adhesie en/of invasie.

In dit proefschrift is ook duidelijk aangetoond dat zeebaars in staat is een specifieke antilichaamrespons te geven tegen verschillende antigenen: *Vibrio anguillarum*, *P. damselae* ssp. *piscicida* en het hapteen DNP. Bovendien is ook geheugenvorming (snellere en sterkere respons) aantoonbaar in kopnier en milt na een intraperitoneale (ip) immunisatie. Daarentegen is er ook een indicatie voor het optreden van immuunsuppressie in de darm. Zoals ook voor andere visoorten aangetoond is de kopnier het belangrijkste orgaan voor de vorming van antilichaam-secreterende cellen (ASC) na een ip-immunisatie. Echter wanneer vissen van dezelfde groep een immersie-immunisatie met het *P. damselae* ssp. *piscicida* bacterin krijgen is er een snellere en hogere primaire ASC-respons aantoonbaar in de kieuwen in vergelijking met kopnier, milt of darm. Opmerkelijk is het feit dat er een vergelijkbare piekdag en zelfs lagere aantalen ASC gevonden worden na een tweede immersie. Dit kan mogelijk verklaard worden door de vorming van antigeen-antilichaam-complexen in de kieuwen, maar mogelijk ook in de huid, waardoor minder antigeen wordt opgenomen. In oudere dieren werd een significant hogere en snellere ASC-respons gevonden dan in jongere dieren. Voor immersie-vaccinatie is het in ieder geval belangrijk te constateren dat er in de jongste dieren (0.1g) geen sprake was van immuunsuppressie. *Samenvattend blijkt de route van vaccinatie van belang voor de locale antilichaam-productie in de kieuwen, maar mogelijk ook in de huid. Deze mucosale responsen verklaren waarschijnlijk de waargenomen bescherming tegen de ziekte.*

Vaccinatie

Zoals eerder gemeld zal de kennis over het mechanisme van pathogeniciteit en van virulentiefactoren van een pathogeen, de selectie van belangrijke moleculen/epitopen mogelijk maken. In dit proefschrift zijn de kinetiek en specificiteit van de invasie van *P. damselae* ssp. *piscicida* bestudeerd en is vastgesteld dat er een intracellulaire fase is in de levenscyclus van de bacterie. Ook de aanwezigheid van een zwak immunogeen kapsel kan het pathogeen helpen de cellulaire en humorale afweersystemen te ontduiken. Daarom is het belangrijk de componenten en mechanismen betrokken bij het internalisatie proces te kennen. Het feit dat hitte-geïnactiveerd antiserum van een met *P. damselae* ssp. *piscicida* bacterin geïmmuniseerde zeebaars de invasie van de bacterie kan remmen suggereert dat het bacterin enkele van de bovengenoemde componenten van belang voor adhesie en invasie bevat. Ook eiwitten die van belang zijn voor het verkrijgen van ijzer ("iron regulated outer membrane proteins" (IROMPs) en siderofoor), maar ook extracellulaire producten (ECP) kunnen

veelbelovend zijn in een andere vaccinsamenstelling. Echter bij de keuze van deze producten moet men rekening blijven houden met de mogelijke inductie van intramoleculaire en/of intermoleculaire immunosuppressie. Een beter inzicht in hoe het immuunsysteem van vissen wordt gereguleerd is daarom noodzakelijk. *Dit proefschrift bevat aanwijzingen voor intramoleculair-geïnduceerde suppressie in zeebaars, zoals gesuggereerd wordt door de extreem lage variabiliteit in de Ig L-keten-sequenties van met DNP_{494}-KLH geïmmuniseerde zeebaars.* De potentiële immunodominante determinant op het molecuul zou intramoleculaire suppressie kunnen induceren van de antilichaam-respons tegen andere epitopen op hetzelfde antigeen, die juist van belang zouden kunnen zijn voor het verkrijgen van bescherming.

Omdat veel ziektes uitbreken bij zeer jonge vissen en vaccinatie via injectie dan nog niet mogelijk is zijn alternatieve vaccinatiemethodes noodzakelijk. Hoewel orale vaccinatie ideaal zou zijn voor de vaccinatie van grote groepen jonge dieren is op dit moment de bescherming van het vaccin tegen vertering in de darm nog een kostbare zaak. Bovendien moet voor elke vissoort en leeftijd de dosis en toedieningsperiode vastgesteld worden om het risico van tolerantie-inductie te vermijden. Daarom is immersie-vaccinatie een waardevol alternatief voor orale immunisation, omdat voor jonge vissen slechts kleine hoeveelheden vaccin nodig zijn met een geringer risico van tolerantie-inductie.

Eindconclusie en verder onderzoek

Geconcludeerd kan worden dat het gebruikte bacterin immunogene determinanten bevat, die belangrijk zijn voor bescherming tegen het pathogeen. Bovendien kan zeebaars tegen P. damselae ssp. piscicida gevaccineerd worden van 137-145 dagen na "hatching" (~1g) via diverse toedieningsroutes en vanaf 80-100 dagen na "hatching" (~0.1g) wanneer de immersiemethode gebruikt wordt.

De isolatie en karakterisering van voor een vaccin geschikte antigenen, maar ook de keuze van de methode om ze toe te dienen, zijn belangrijke stappen in de ontwikkeling van nieuwe visvaccins en vaccinatiemethodes. Veel onderzoek moet dan ook gericht worden op de karakterisering van vaccincomponenten en de opnameroute in de vis. Het testen van verschillende vaccins via *in vitro* en *in vivo* "challenge" studies naast veldstudies is uiteraard ook essentieel voor het proces van vaccinontwikkeling. Verder dient aandacht besteed te worden aan de toevoeging van geschikte adjuvantia. Extra kennis van het immuunsysteem van de zeebaars, met name met betrekking op het cellulaire immuunsysteem, kan ook van groot belang zijn voor het ontwikkelen van betrouwbare vaccins.

SUMÁRIO E CONCLUSÕES

A investigação e o conhecimento em imunologia e imunopatologia fundamental e aplicada deverão conduzir em princípio ao desenvolvimento e/ou melhoramento de vacinas, as quais poderão ser usadas na profilaxia de importantes doenças. Do ponto de vista científico, podem considerar-se três passos essenciais para o desenvolvimento de uma vacina efectiva:

1. Conhecimento dos mecanismos de patogenecidade e factores de virulência do agente infeccioso, o que permite a selecção de importantes moléculas/epitopos essenciais para a sobrevivência do patogénio, e a sua inclusão na vacina;
2. Conhecimento do sistema imune do hospedeiro, incluindo o seu desenvolvimento, regulação/modulação, cinética da resposta imune e sua localização compartimental, o qual permite escolher o melhor momento e método para vacinar;
3. Conhecimento da eficiência da vacina através de estudos da protecção induzida, após infecções experimentais *in vivo* e/ou *in vitro*.

O facto da cultura de robalo ter vindo a aumentar na zona Mediterrânica e a pseudotuberculose ter vindo a aumentar e a causar mortalidade maciça na sua cultura, despolotou o interesse no desenvolvimento de uma vacina efectiva contra *Photobacterium damselae* ssp. *piscicida* por parte de várias empresas produtoras de vacinas. Contudo, aquando dos primeiros surtos muito pouco se conhecia acerca do sistema imune do robalo. Tornou-se portanto importante conhecer os princípios básicos de imunocompetência e de capacidade de resposta do robalo à vacinação. Foram esses os objectivos principais desta tese.

Os anticorpos monoclonais (mAb) têm-se mostrado como ferramentas extremamente úteis em estudos de imunologia de peixes. *A caracterização da imunoglobina (Ig) de robalo e a produção de mAb contra as cadeias pesada (H; WDI 1) e leve (L; WDI 2 e WDI 3) foram realizadas com sucesso. Estes provaram ser úteis na monitorização de respostas imunes especificas e em estudos de ontogenia da linhagem dos linfócitos B.*

Ig em robalo

Os teleósteos geram uma considerável diversidade estrutural nas suas imunoglobulinas através de interligações entre subunidades não-uniformes ("halfmers", monomeros, dimeros e trimeros) dentro da molécula tetramérica. *No robalo, parecem ocorrer interligações disulfido de subunidades não-uniformes monoméricas (M_r ±210 kDa) e diméricas (M_r ±420 kDa) sem o envolvimento de uma cadeia de ligação na polimerização da forma tetramérica.* No entanto, ainda não se conhece qual o significado fisiológico desta diversidade estrutural. Tal

como em outras espécies, cada unidade monomérica é composta por duas cadeias polipeptídicas H (M_r ±78 kDa) e duas cadeias polipeptídicas L (M_r ±28 kDa), interligadas por uma ponte de cisteina entre o domínio constante da cadeia L e o primeiro domínio constante da cadeia H. Foi realizada igualmente a caracterização da Ig de robalo sequenciando-se a sua cadeia L. Esta revelou a mesma organização genómica "tipo-cacho" que a cadeia L de outros teleosteos. *Todos os segmentos característicos (V, J and C) que codificam para os domínios V e C da cadeia L da Ig poderam ser deduzidos, com o domínio V a mostrar as três "complementarity determining regions" (CDRs) e as quatro "framework regions" (FRs) em ambos os lados das CDRs.* Os resíduos de cisteina típicos que provavelmente formam as interacções intra- e inter-cadeias foram igualmente conservados no robalo. A sequência deduzida pode ser considerada como um isotipo L1/G, o qual pode estar relacionado com o isotipo κ dos mamíferos.

Células B em robalo

Tal como nos vertebrados superiores, as células B dos peixes expressam Ig na sua superfície, onde actua como receptor antigénico específico. Todos os mAb contra a Ig de robalo produzidos no presente estudo reagiram com uma subpopulação de linfócitos, os quais em estudos ultra-estruturais apresentaram a Ig agrupada na membrana celular, tal como descrito em outras espécies de peixes. Os plasmócitos apresentaram uma reacção menor à superfície do que a encontrada em carpa, lembrando mais os plasmócitos dos mamíferos, que perdem completamente a Ig de superfície durante a sua diferenciação. Os resultados indicaram também a existência de heterogeneidade de células B no robalo, baseado na expressão de pelo menos duas cadeias H e L diferentes. A existência de diversidade de IgM em outras espécies de teleosteos, e mais recentemente, formas isotípicas de cadeias H e L têm sido relatadas. Contudo, permanece ainda por investigar se esta heterogeneidade tem expressão funcional no contexto fisiológico. A percentagem de células B nos diferentes órgãos imunológicos foi estudada usando todos os mAb. A combinação de WDI 1 e WDI 3 parece marcar todas ou quase todas as células B. Tal como em outras espécies de teleosteos, o rim parece ser no robalo o órgão primário para a diferenciação de linfócitos B. *A proporção de células (B) Ig^+ de superfície (sIg^+) nos diferentes órgãos aumentou durante o desenvolvimento ontogenético sendo mais elevada em estágios precoces no rim anterior seguido pelo baço, leucócitos sanguíneos periféricos (PBL), intestino e timo. Esta ordem sequencial modificou-se durante a ontogenia até ser atingida a distribuição adulta (PBL, baço, rim anterior, intestino e timo). Tal como referido para a truta arco-íris, as células*

(pré-B) Ig$^+$ citoplasmática (cIg$^+$) foram detectadas mais cedo na ontogenia do que as células sIg$^+$, o que está de acordo com as observações feitas em anfíbios e mamíferos. É de realçar o facto das células cIg$^+$ terem sido detectadas usando o mAb para a cadeia L, o que pode sugerir que a cadeia L poderá ser transcrita em elevadas quantidades durante os estágios precoces do desenvolvimento das células B. O facto de poder haver co-expressão das cadeias H e L da Ig em células pré-B de peixes ou que a expressão da cadeia H preceda a expressão da cadeia L permanece por investigar. No entanto, em truta arco-íris foram encontrados transcriptos germinais "ligação-constante" (JC) de IgL2, sugerindo a existência de uma proteína *JLC2*, que pode ser expressa na superfície das células pré-B em combinação com a cadeia H, tal como ocorre nos mamíferos. Assim, é possível que as células cIg$^+$ pudéssem ter sido detectadas ainda mais cedo no robalo se o mAb contra a cadeia H tivesse sido usado. Os plasmócitos cIg$^+$ aparecem mais tarde na ontogenia e poderão ser indicativos de maturação imunológica, tendo em conta o desencadear das respostas imunes humorais adaptativas. Mais ainda, os resultados apresentados sugeriram fortemente que, pelo menos no robalo, a idade é o factor mais importante no que respeita ao desenvolvimento the imunocompetência. Embora defendido por alguns autores que o peso dos peixes poderá corelasionar-se melhor com o estado de imunocompetência do que a idade, isto poderá ser verdade unicamente quando o tempo, em horas, dias, meses, anos ou outro, é tido como unidade de medida. No entanto, uma vez que os peixes são poiquilotermicos, e, a temperatura regula directa ou indirectamente todas as reacções fisiológicas, os graus-dia são induvitavelmente a melhor forma the medição do tempo/idade fisiológica, permitindo adicionalmente a comparação directa entre diferente espécies com idades fisiológicas equivalentes.

Células T em robalo

Embora a sequência genética das cadeias α e β do receptor de células T (TCR) tenha sido recentemente descrita em teleosteos, apenas um número muito limitado de mAb específicos têm sido obtidos, o que tem limitado os estudos funcionais. No entanto, um mAb (DLT 15) contra timócitos e células T periféricas de robalo foi produzido e usado em estudos que sugerem fortemente a sua especificidade. Informação adicional foi obtida na presente tese relacionada com o desenvolvimento ontogenético das células T de robalo. No robalo, como em outras espécies de peixes, o timo é o local principal para a diferenciação de células T. Contudo, na presente tese, poderam ser detectadas células T precoces usando o DLT 15 antes do timo se tornar linfóide, sugerindo que se poderão originar num compartimento diferente e migrar posteriormente para o timo, tal como acontece em mamíferos. Após diferenciação

tímica, os linfócitos T parecem migrar para os órgãos linfóides periféricos, tal como é sugerido pela sua elevada percentagem em PBL durante a fase precoce do desenvolvimento. *Durante o desenvolvimento ontogenético, a proporção de células T aumenta no timo e no intestino mas decresce nos PBL, rim anterior e baço até aproximadamente ao dia 137 pós-eclosão, quando os níveis adultos são atingidos.*

Imunidade humoral em robalo

Durante as experiências realizadas na presente tese foram evidenciados mecanismos efectores dos Ab de robalo. Foi observado o mecanismo de aglutinação em testes usando soro de robalos imunizados com a bacterina de *P. damselae* ssp. *piscicida* quando se ralizaram ensaios de imunização e de avaliação de especificidade da vacina. O facto de alguns macrófagos terem sido marcados especificamente pelos mAb poderá indicar a presença de receptores de Ig na sua superfície, os quais são importantes na mediação da ligação de antigénios opsonisados por Ab, promovendo assim a sua fagócitose. Também é sugerido o mecanismo de neutralização por Ab específicos, uma vez que anti-soro contra *P. damselae* ssp. *piscicida* termicamente inactivado reduziu a capacidade da bactéria invadir células de tecido em cultura, provavelmente por bloqueamento dos receptores de superfície envolvidos na adesão e/ou invasão.

Na presente tese foi claramente demonstrado que o robalo é capaz de montar uma resposta especifica de anticorpos contra diferentes antigénios: *Vibrio anguillarum*, *P. damselae* ssp. *piscicida* e DNP. Além disso, foi claramente demonstrada a formação de memória [resposta mais rápida e número mais elevado de células secretoras de anticorpos (ASC)] no rim anterior e baço após uma segunda imunização intraperitoneal (ip). Por outro lado, os resultados sugeririam o desenvolvimento de imuno-supressão da resposta imune no intestino. Tal como demonstrado em outras espécies de teleosteos, o rim anterior é o principal órgão produtor de ASC após imunização ip. No entanto, quando peixes do mesmo tamanho (mesmo lote) que os imunizados ip foram imersos directamente na vacina de *P. damselae* ssp. *piscicida*, foi observada uma resposta primaria mais rápida e mais elevada na brânquia, com números de ASC muito mais baixos no rim anterior, baço e intestino. Surpreendentemente, foi observado um dia-pico de ASC semelhante, e mesmo um menor numero de ASC, após uma segunda imersão. Estes resultados sugerem fortemente que estavam presentes Ab na brânquia (e provavelmente na pele) resultantes da resposta primaria, os quais poderiam ter complexado o antigénio e prevenido ou diminuído subsequentemente a sua internalização pelas células epiteliais da brânquia (e pele). Adicionalmente, foi observado um efeito claro da idade nas

respostas de ASC com números significativamente mais elevados e respostas mais rápidas de ASC nos grupos mais idosos. De realçar é o facto de a exposição por imersão directa em *P. damselae* ssp. *piscicida* nos estágios mais precoces usados no presente estudo (0.1 g) não terem resultado em imuno-supressão, o que está de acordo com o que tem sido referido para outras espécies de peixes de tamanho comparável quando igualmente imersos em bacterinas. *Em conclusão, foi claramente demonstrada a importância do método de vacinação para a estimulação local da produção de Ab, especialmente na brânquia mas provavelmente também na pele e que esta resposta tem possivelmente uma função na protecção contra a doença.*

Vacinação

Tal como referido anteriormente, o conhecimento dos mecanismos de patogenecidade e factores de virulência do patogénio permitirá a selecção de moléculas/epitopos importantes para inclusão na vacina. Na presente tese, foi estudada a cinética e especificidade da capacidade invasiva de *P. damselae* ssp. *piscicida* o que confirmou a existência de uma fase intracelular no ciclo de vida da bactéria. Para além da fase intracelular, a presença de uma capsula fracamente imunogénica conferindo resistência à morte pelo soro e um diminuído índice de fagocitose, pode ajudar o patogénio a escapar às defesas humoral e celular do hospedeiro. Assim, a identificação dos componentes e mecanismos envolvidos no processo de internalização pode fornecer um bom antigénio para inclusão numa vacina efectiva. O facto da exposição ao anti-soro obtido a partir de robalos imunizados com a bacterina de *P. damselae* ssp. *piscicida* e inactivado termicamente ter reduzido a capacidade invasiva da bactéria, sugere que a bacterina pode ter incluídos alguns factores responsáveis pela capacidade de adesão e invasão do patogénio. Adicionalmente, outros antigénios alvo efectivos poderão ser as proteínas envolvidas no sistema de aquisição de ferro [proteínas de membrana externa reguladas pelo ferro (IROMPs) e sideroforos] assim como alguns factores presentes nos produtos extra-celulares (ECP), os quais têm sido descritos como promissores em outras formulações de vacinas bacterianas. No entanto, o facto de em algumas espécies de peixes ocorrer supressão imune induzida por interacções entre epitopos dentro da mesma molécula (supressão intramolecular) e entre diferentes moléculas (supressão intermolecular), levanta a questão de como é que este facto poderá influenciar a especificidade das respostas imunes contra antigénios presentes em vacinas, e consequente indução de protecção imune. *Na presente tese, parece que pelo menos a supressão induzida intramolecularmente tem um papel importante na regulação da resposta de anticorpos em robalo. Esta conclusão foi sugerida pela muito baixa variabilidade observada nas sequências da cadeia L da Ig obtidas*

a partir de robalos imunizados com DNP_{494}-KLH. Assim, um determinante potencialmente imuno-dominante localizado numa parte de uma molécula pode induzir supressão intramolecular da resposta de anticorpos a outros epitopos presentes no mesmo antigénio que poderiam ser importantes para a protecção imune.

Uma vez que a maioria dos surtos de doenças podem ocorrer durante estágios muito precoces do desenvolvimento dos peixes e a injecção de vacinas não é possível nessas idades, têm de ser tidos em conta métodos alternativos de vacinação. Embora a imunização oral seja ideal para a vacinação em massa, a necessidade de proteger os antigénios da degradação no segmento anterior/gástrico do intestino poderá aumentar o custo da vacina. Além do mais, a dose e período de administração têm de ser claramente conhecidos para cada estágio de desenvolvimento, de modo a evitar a indução de tolerância a antigénios de origem proteica. Neste contexto, a vacinação por imersão aparece como uma alternativa valiosa à imunização oral, uma vez que em peixes jovens são necessárias pequenas quantidades para a vacinação e não existe o risco de indução de tolerância.

Conclusão final e perspectivas

A bacterina utilizada na presente tese contém determinantes imunogénicos, os quais parecem ser importantes para conferir protecção. Os robalos podem ser vacinados contra P. damselae ssp. piscicida a partir de pelo menos o dia 137 pós-eclosão (~1 g), independentemente do método de administração, ou a partir de pelo menos o dia 80-100 pós-eclosão (~0.1 g) se for realizada a vacinação por imersão.

O isolamento e caracterização de antigénios alvo importantes, o estudo da sua inclusão numa formulação de vacina em termos de regulação imune assim como o melhor método de os administrar, são processos indispensáveis ao desenvolvimento de novas vacinas para peixes e novos métodos de vacinação. Presentemente, continuarão a ser efectuados estudos direccionados para a caracterização de componentes de vacinas e dos mecanismos envolvidos na sua internalização. Ensaios com diferentes formulações de vacinas através de estudos de infecção *in vivo*, adicionalmente a testes de campo em larga escala, são igualmente necessários neste processo de desenvolvimento de vacinas. A possível inclusão e efeito de adjuvantes na vacinação por imersão poderá igualmente ser um importante item de estudo, especialmente no que respeita à duração da protecção. No que respeita à imunidade em robalo, o conhecimento/investigação básico e aplicado acerca de respostas imunes mediadas por células é ainda limitado. Assim, o futuro desenvolvimento de vacinas contra muitos

outros patogénios poderá ser altamente estimulada pelo aprofundar do conhecimento nesta área.

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Curriculum vitae

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