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ANTIGENICITY AND IMMUNOGENICITY OF THE ENVELOPE GLYCOPROTEIN (GPG) OF VIRAL HAEMORRHAGIC SEPTICAEMIA RHABDOVIRUS (VHSV)

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PREFACE

Among the 5 structural proteins of the salmonid rhabdovirus viral haemorrhagic septicaemia virus (VHSV), the glycoprotein G (gpG) is a unique immunogenic (immune response) and antigenic (antibody) inducer. While recombinant gpG proteins had limited success as vaccines, their corresponding gpG DNA have been highly protective in different fish species (most probably because that is the best way to maintain their epitopes, disulphide bonds and glycosilation). Regarding their immunogenicity and antigenicity, T-cell or Mx and B-cell inducing epitopes, respectively, have been mapped in the gpG_{VHSV} amino acid sequence by using pepscan peptides and/or monoclonal antibody (MAb) resistant mutants. The recent elucidation of the crystal structure of gpG of vesicular stomatitis virus (VSV), a similar mammalian rhabdovirus, have allowed a first approximation to locate all those identified epitopes on the gpG_{VHSV} 3D modeled structure both at their physiological and low pH fusion-competent conformations. The availability of such abundant information on this model protein should help to understand further details of fish immunogenicity and antigenicity to improve fish DNA vaccines.

Chapter 1

IMPORTANCE OF RHABDOVIRAL DISEASES IN FARMED FISH

Intensive fish farming amplifies diseases caused by bacteria, viruses and parasites, causing severe losses. On the other hand, intensive farming also generates and spreads fish diseases to wild type populations, as shown by the increasing detection in captured ocean fish of viruses first isolated in fish farms. Therefore, intensive aquaculture is having an escalating impact on ecosystems which might be reducing their biocapacity and biodiversity.

Over-production beyond sustainable (long-term maintenance of wellbeing) disease levels must be counteracted with control prevention measurements. Strategies to prevent farmed fish diseases have mainly centered on treatment with antibiotics/chemicals. However, this strategy is both time intensive and requires continual monitoring. Pan use of these strategies can lead to developed resistance of the pathogens to antibiotics, and also their safety needs to be continuously assessed, including cumulative effects. In addition, this kind of treatments is ineffective against viral diseases and therefore, viral fish diseases have emerged as a serious double problem because they not only cause severe losses but also produce an enormous ecological impact. Thus, the prevention of viral diseases by means such as vaccination or immunostimulation has come to the fore (Biering *et al.*, 2005; Sommerset *et al.*, 2005). In an attempt to improve current vaccines, focus has shifted to increase our understanding both of the viral molecules involved in triggering the host

immune responses (immunogenicity and antigenicity) and of the viral-induced immune host responses.

Among the 9 notifiable fish diseases (diseases with great social and economic and/or public health repercussion or with present or potential risk for the aquaculture industry) appearing at the 2009 Aquatic Animal Health Code, the OIE (Office International des Epizooties, now the World Organization for Animal Health) (http://www.oie.int) include 7 caused by viruses. Three of them are caused by rhabdoviruses, thus showing the high risk of fish rhabdoviruses to worldwide aquaculture (Table 1). *Viral haemorrhagic septicaemia virus* (VHSV), *infectious hematopoietic necrosis virus* (IHNV) (both of them belonging to the *Novirhabdovirus* genus) and *spring viremia of carp virus* (SVCV) (*Vesiculovirus-like* genus) are the most important because of their highest economical impact on aquaculture (Table 2). Fish rhabdoviruses are commonly associated with frequent epizooties and are responsible for the greatest losses in aquaculture production since they not only affect fish at the early stages of development, but also produce a high percentage of mortality in adult fish with higher economic value.

Table 1. Fish viruses notifiable to the OIE in 2009

Genome	Virus	Family
RNA	*Viral haemorrhagic septicaemia virus* (VHSV)	*Rhabdoviridae*
	Infectious hematopoietic necrosis virus (IHNV)	
	Spring viremia of carp virus (SVCV)	
	Infectious salmon anaemia virus (ISAV)	*Orthomyxoviridae*
DNA	*Epizootic haematopoietic necrosis virus* (EHNV)	*Iridoviridae*
	Red sea bream iridoviral (RSIV)	
	Koi herpesvirus (KHV)	*Herpesviridae*

Table 2. Classification of rhabdoviruses affecting teleost fish (Essbauer & Ahne, 2001)

Genus	Virus
Novirabdovirus genus	*Viral haemorrhagic septicaemia virus* (VHSV)
	Infectious hematopoietic necrosis virus (IHNV)
	Hirame rhabdovirus (HIRRV)
Tentative Novirabdovirus genus	*Snakehead rhabdovirus* (SHRV)
	Eel virus B12 (EEV-B12)
	Eel virus C26 (EEV-C26)
Tentative Vesiculovirus genus	*Spring viremia of carp virus* (SVCV)
	Pike fry rhabdovirus (PFR)
	Eel Virus American (EVA)
	Ulcerative disease rhabdovirus (UDRV)

Chapter 2

VIRAL HAEMORRHAGIC SEPTICEMIA VIRUS (VHSV)

Among the most important fish rhabdoviruses, VHSV is one of the most important viral diseases of salmonids in european aquaculture (Olesen, 1998; Skall *et al.*, 2005). Mortality depends on the age of the fish but it is 100 % lethal in fry, and cause intermediate mortalities in older fish (Skall *et al.*, 2005). In addition, VHSV is spreading to wild ecosystems as it is being isolated from an increasing number of free-living marine fish species (Hopper, 1989; Isshik *et al.*, 2001; Meyers *et al.*, 1992; Schlotfeldt *et al.*, 1991).

The VHSV particle (Figure 1) has an average size of about 170 x 80 nm and its genome is a single RNA molecule of negative polarity of approximately 11 kbp (Hill *et al.*, 1975; Schutze *et al.*, 1999). The full genome sequence of VHSV is now known for several of their strains (Nishizawa, 2002; Schutze *et al.*, 1999). VHSV, together with other piscine rhabdoviruses, have been placed into the *Novirhabdovirus* genus because of the presence of an additional small gene encoding for a non-structural Nv protein in their genome, a gene not present in other rhabdoviruses (Basurco & Benmansour, 1995; Essbauer & Ahne, 2001; Schutze *et al.*, 1996). The VHSV genome codes for 6 different proteins, 5 of which are structural viral proteins (L, N, P, M and G proteins) and one is a non-structural protein (Nv protein). Inside the virus particle, the RNA genome is tightly packed by the nucleocapsid protein N (40 kDa), associated with the RNA-dependent RNA polymerase, L (190 kDa) and P (19 kDa) proteins, to form the replication complex. The viral envelope

is a lipid bilayer derived from the host cell containing approximately 400 trimeric transmembrane spikes consisting of the single viral glycoprotein G (gpG) (65 kDa). The matrix protein, M (25 kDa), is localized inside the viral envelope between the membrane and the nucleocapsid. The gene encoding the non-structural protein Nv (12 kDa) is localized between the gpG and L genes (3' N-P-M-G-Nv-L 5') (Thoulouze *et al.*, 2004).

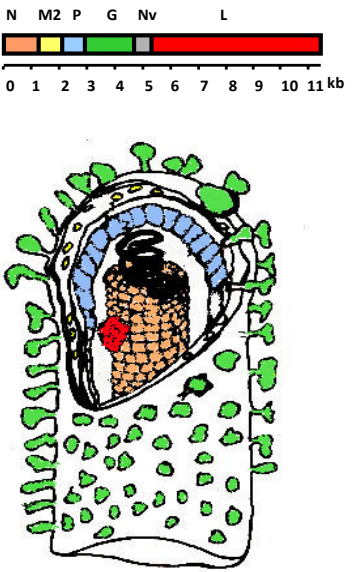

Figure 1. Scheme of a VHSV particle and their negative RNA genome.

The VHSV particle (170 x 80 nm) contains a single RNA molecule of negative polarity of ~ 11 kbp. The genome codes for 5 structural proteins (L, N, P, M and G) and a non-structural protein (Nv). The VHSV RNA-dependent RNA polymerase, L (190 kDa) is associated with the nucleocapsid (N 40 Kda) and P proteins (19 kDa) to form the replication complex. The viral envelope surrounds the replication complex and contains ~ 400 trimeric transmembrane spikes of glycoprotein G (gpG) (65 kDa). The different sequences of the gpG_{VHSV} can be grouped in 4 different neutralizable serotypes. Serotypes and sequence variations of hundreds of VHSV isolates have been deposited in a recent data base at the European Community Reference Laboratory for Fish Diseases located in the National Veterinary Institute in Aarhus Denmark (http://www. Fish pathogens.eu/).

The fin bases appeared as the main site of entry of rhabdoviruses into the fish (Harmache *et al.*, 2006). Once inside their fish host, rhabdoviruses spread throughout the body and produce a systemic infection, initially affecting lymphoid organs such as the head kidney and spleen, and later most other tissues (de Kinkelin *et al.*, 1977; Dorson *et al.*, 1995; Estepa & Coll, 1993; Estepa *et al.*, 1992; Tafalla *et al.*, 1998). After binding to its cellular receptor(s), VHSV enters into the host cells by endocytosis. The virions are then transported along the endocytic pathway towards lysosomes. Beyond early endosomes but prior to lysosomes, the acid pH triggers the fusion of the viral envelope with endosomal membranes, releasing the nucleocapsid into the cytosol, where transcription and replication of the viral genome occurs. Finally, new viral particles are assembled and released in a process known as budding. Both viral cell attachment and fusion are mediated by gpG.

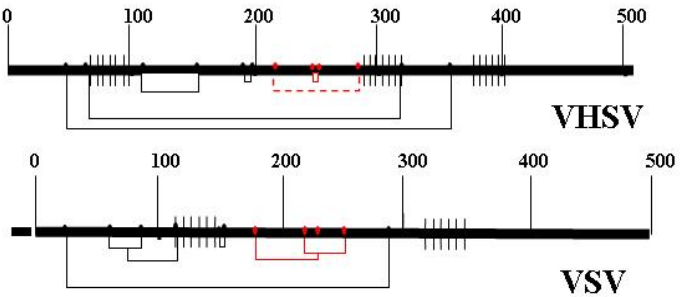

Figure 2. Comparison of the positions of the disulphide bonds and the hydrophobic heptad sequences in gpG_{VHSV} (Einer-Jensen *et al.*, 1998) and in gpG_{VSV} (Roche *et al.*, 2006).

Amino acid sequences (black horizontal lines) were numerated with the signal peptide (gpG_{VHSV}) or without the signal peptide (gpG_{VSV}). The gpG_{VSV} signal peptide is represented by a horizontal bar separated by an empty space before the mature protein. The cysteines are represented by dots along the black horizontal lines and their disulphide bonds by vertical and horizontal lines that link the cysteines. A series of 6-7 vertical lines on the black horizontal lines represent the localization of non canonical hydrophobic heptad repeats (Coll, 1995c). The alternative disulphide bonds located in the central part of the amino acid sequences are in red.

Chapter 3

THE STRUCTURE-FUNCTION RELATIONSHIPS IN THE GLYCOPROTEIN G OF VHSV (GPG_{VHSV})

The gpG_{VHSV} is 507 amino acid residues long. Its sequence has all the features described for mammalian rhabdovirus gpGs, including a signal peptide hydrophobic sequence (removed in the mature gpG), N-glycosilation sites, disulphide bonds, a transmembrane region, hydrophobic heptad repeat-like sequences and a carboxy-terminal cytoplasmic tail (Coll, 1995b) (Figure 2), despite fish and mammalian gpGs sharing only 18-26 % homology (Roche *et al.*, 2006; Walker & Kongsuwan, 1999). Among the VHSV proteins, gpG has probably received most interest due to their role at viral entrance, fusion and because is the only VHSV protein capable of inducing neutralizing antibodies (NAb) in the fish host during infection (Boudinot *et al.*, 1998; Lorenzen *et al.*, 1990). NAbs constitute one of the main components of the fish protective response against VHSV infections (Engelking & Leong, 1989; Lorenzen & Olesen, 1997) (see later).

With the elucidation of the crystal structure of gpG of the prototypical mammalian rhabdovirus *vesicular stomatitis virus* (VSV) both at physiological and fusion pHs (Roche *et al.*, 2006; Roche *et al.*, 2007), the tridimensional approximated location of many of their immunogenic and antigenic sites and their relationship to structural-functional features can be approximated by modelling the gpG_{VHSV} 3D structure by alignment to the gpG_{VSV} sequence (Figures 3, 4 and 5).

Despite the differences between the amino acid sequences of gpGs, the alignment of 14 representative sequences of 4 genus from the *Rhabdoviridae* family (*Vesiculovirus*, *Lisavirus*, *Efemerovirus* and *Novirhabdovirus*) (Walker & Kongsuwan, 1999) showed that the positions of 12 cysteine (C) residues were highly conserved and could be used as reporter sites to align the gpG_{VHSV} and gpG_{VSV} sequences and to model their 3D structure.

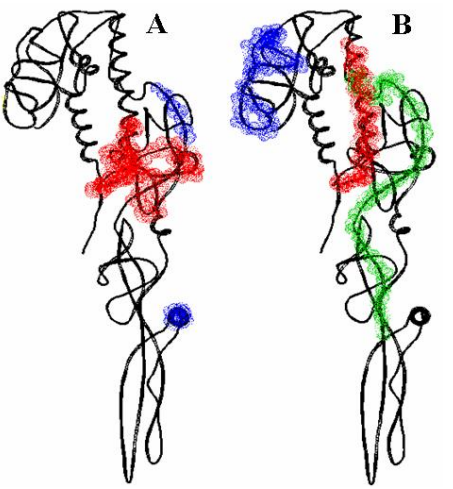

Figure 3. Positions in the gpG_{VHSV} 3D modeled structure of the highest variability regions (A) and the hydrophobic heptad repeats (B).

The tridimensional (3D) structure of the gpG_{VHSV} at low pH was developed after the X-ray structure of gpG_{VSV} (2CMZ accession number in the RCSB protein data bank at http://www.rcsb.org/pdb), the proposed amino acid sequence alignment (Roche *et al.*, 2006) and the modeling with the Swiss pdbv viewer (http://www.expasy.org/spdbv). Due to the existence of small gaps and different disulphide bonds, the resulting approximation has an estimated precision of ± 3 aminoacids. **A)** About 80 % of the amino acid variations of the gpG_{VHSV} are between the positions W245-S300 (highlighted in red) (Benmansour *et al.*, 1997). The only positions from the fusion region where amino acid sequence variations are found are highlighted in blue (79-82 and 135-139) (Rocha *et al.*, 2004). **B)** The non canonical hydrophobic heptad repeats of gpG_{VHSV} (Coll, 1995c) are highlighted in green (amino-terminal, 68-102), red (middle, 288-319) and blue (carboxy-terminal, 377-400).

Thus, the main 3D characteristics found in $gpG_{VSV/VHSV}$ were: i) Their overall shape at the low pH of fusion resembles an inverted cone of 125 Å identical to the size measured by electron microscopy (Gaudin *et al.*, 1993) and folding into 4 domains (DI, DII, DIII and DIV) distributed in a general shape of a head with a tail both at physiological and low pHs (Figure 3,4 and 5). ii) The top of the molecule of 60 Å of diameter is made by an external ß-sheet rich domain (310-384) and an internal alpha helix rich domain involved in trimerization (259 -310), iii) a neck domain homologous to pleckstrins (181-259) and iv) an elongated domain (51-181) located towards the same end of the transmembrane domain (Figure 5). The fusogenic motif is made of two protruding loops, each terminated by tips consisting of one hydrophobic amino acid. Membrane penetration of this bipartite fusion tip was calculated to be around 8.5 Å based in the presence of the hydrophilic small alpha helix located at positions 88-103 (blue circle at Figure 3A). The structure provided molecular-based explanations of the reversibility of the conformational changes at physiological pH and of the location of neutralizing monoclonal antibodies (NMAb) binding sites (Figure 5).

A comparison between the localization and connectivity of the C residues of gpG_{VHSV} and gpG_{VSV}, as they were deduced by mass spectrophotometry (Einer-Jensen *et al.*, 1998) and X-ray diffraction (Roche *et al.*, 2006; Roche *et al.*, 2007), respectively, is shown in Figure 2. In VHSV, 6 disulphide bonds involving 12 C were assigned. Two disulphide bonds formed two long-distance loops between C34-C344 and C49-C300. These bonds bend the lineal sequence bringing together the amino-terminal region and the central region of the gpG_{VHSV} molecule. Two other disulphide bonds formed shorter-distance loops located between 2 C inside the 2 longer loops. The loop C95-C137 (corresponding to the C68-C114 of the VSV) contains the region around the fusion domain and one of the epitopes of the MAb C10 MAR mutants (Bearzotti *et al.*, 1995). The loop C200-C270 contains the region where MAb 3F1A12 MAR mutants map (Figure 5). The 2 other disulphide bonds (C177-C182 and C236-C241) were only 5 amino acids apart.

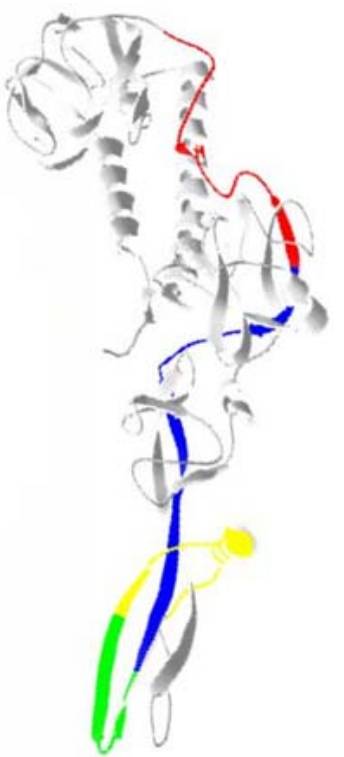

Figure 4. Localization in the gpG_{VHSV} modeled structure of the fusion related peptides p9, p2, p3, p4 and fr11.

The low pH fusion-related region of the gpG_{VHSV} was located by studying phosphatidyl serine (PS)-binding to synthetic peptides in solid-phase, binding of anti-p2 PAbs to p2 and purified VHSV only at low pH, inhibition of PS-binding to VHSV only at low pH and inhibition of VHSV-induced cell to cell low pH-induced fusion (Estepa & Coll, 1997b). P2 induced aggregation of lipid vesicles, closed apposition of membranes and destabilization of anionic phospholipid bilayers, most of the steps required for fusion (Estepa *et al.*, 2001; Nunez *et al.*, 1998). The frg11/p2 peptide acquired a ß-sheet structure at low pH to interact with anionic phospholipids, showed the highest anionic phospholipid-aggregation, induced non-infected cell-to-cell fusion and translocated PS from the inner to the outer leaflet of the plasma membrane. Site directed mutagenesis in the frg11 region affected fusion as detected by *in vitro* assays (Rocha *et al.*, 2004). p9 (red), p2 (blue), p3 (green) y p4 (yellow). frg11 is p9+p2.

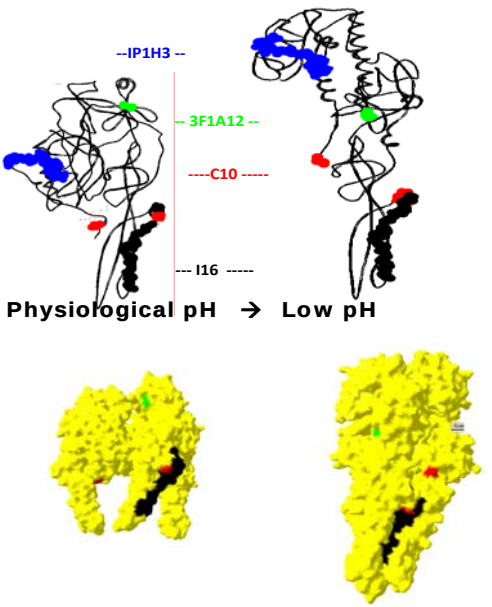

Figure 5. Approximated location of some neutralizing and lineal Mabs in the modeled structure of gpG_{VHSV} at physiological and fusion pHs.

B-cell lineal epitopes on the VHSV gpG were mapped by the use of overlapping 15-mer synthetic peptides allowing the identification of the binding sites of MAbs IP1H3 and I16 (Fernandez-Alonso *et al.*, 1998). Conformational (discontinuous) B-cell epitopes of neutralizing MAbs could only be mapped by sequencing MAb resistant mutants: C10 (Bearzotti *et al.*, 1994; 1995) and 3F1A12 (Lorenzen, personal communication). The locations of these sites on the 3D modeled structures of gpG_{VHSV} are shown according to the gpG_{VSV} RCSB pdb protein data bank (http://www.rcsb.org/pdb/home) accession numbers 2J67 at physiological pH and 2CMZ at low pH in both lineal and solid models.

The region of the gpG_{VHSV} sequence showing more amino acid variation was between the head of the molecule and the fusion double loop (Figure 3A). About 80 % of these variations were in the fragment W245-S300 (Benmansour *et al.*, 1997). The sequence variation of the gpG_{VHSV} from 74 isolates have been published (Einer-Jensen *et al.*, 2004).

Many surface glycoproteins from enveloped viruses contain hydrophobic heptad repeats (3-4 contiguous sequences of 7 amino acids: **a**bc**d**efg, hydrophobic amino acids in bold) forming amphipathic alpha

helixes and coiled coils polymers related to viral fusion (Skehel & Wiley, 1998). However, no coiled-coil motifs were predicted in the gpG of any rhabdovirus (Yao *et al.*, 2003). Only after arbitrarily considering in positions a-d all the hydrophobic amino acids, non-canonical hydrophobic heptad repeats could be detected in the gpG sequences of all rhabdoviruses in similar locations (Coll, 1995c). Only the 288-319 heptad repeat appears as an alpha helix in the gpG_{VHSV} model at low pH, just as predicted by their heptad repeat structure (Figure 3B).

The gpG regions interacting with phospholipids of the host membrane were identified by hydrophobic photolabeling approximately the first 200 amino acids in VSV and rabies virus (Durrer *et al.*, 1995; Gaudin *et al.*, 1995). Similarly that the phosphatidyl serine (PS)-binding to p2 (see Figure 4 for 3D location ~100) could be relevant to the earlier steps of the infective cycle of VHSV, was supported by the capacity of anti-p2 polyclonal antibodies (PAbs) to recognize both p2 and purified VHSV only at low pH, to inhibit the PS-binding to VHSV in solution only at low pH and to inhibit VHSV-induced cell to cell fusion (Estepa & Coll, 1997b). On the other hand, p2 induced aggregation of lipid vesicles, closed apposition of membranes and destabilization of anionic phospholipid bilayers, most of the steps required for fusion. Furthermore, similar pH-dependence profiles were shown by PS-binding to purified VHSV (Estepa & Coll, 1996) and by gpG_{VHSV} mediated membrane fusion (Lecocq-Xhonneux *et al.*, 1994). On the other hand, the frg11/p2 peptide (see Figure 4 for 3D location) acquired a ß-sheet structure at low pH to interact with anionic phospholipids. Of all the selected segments p9, p2, p3, p4 and frg11 (p9+p2) (Figure 4), frg11 showed the highest anionic phospholipid-aggregation specific activity and was the only one to induce non-infected cell-to-cell fusion and to translocate PS from the inner to the outer leaflet of the plasma membrane. Finally, site directed mutagenesis mutants in the above mentioned region affected fusion as detected by an *in vitro* assay (Rocha *et al.*, 2004), confirming the implication of these regions in viral fusion.

Chapter 4

THE IMPORTANCE OF THE GPG_{VHSV} IN VACCINATION AGAINST VHSV

The injection of fish with recombinant rhabdoviral gpG proteins produced in *E. coli* (Estepa *et al.*, 1994; Lorenzen *et al.*, 1993a), yeast (Estepa *et al.*, 1994) and/or baculovirus (Koener & Leong, 1990) did not obtain good protection levels despite good correlations between *in vitro* neutralization and *in vivo* protection in IHNV (Xu *et al.*, 1991) or VSHV (Estepa *et al.*, 1994; Lorenzen *et al.*, 1993b). In contrast, over 95 % protection have been obtained by injection of the gpG_{VHSV} gene (Anderson *et al.*, 1996a; Anderson *et al.*, 1996b; Fernandez-Alonso *et al.*, 2001; LaPatra *et al.*, 2001; Lorenzen, 2000; Lorenzen & LaPatra, 2005; Lorenzen *et al.*, 2001; Lorenzen, 1998; McLauchlan *et al.*, 2003). Efficient vaccination was only observed with the gpG gene since other rhabdoviral genes failed to induce protection (Corbeil *et al.*, 1999). DNA vaccines of their corresponding gpG genes have been proved effective for all fish rhabdovirus tested, VHSV, IHNV, SVCV (Kim *et al.*, 2000) and for the most exotic rhabdovirus such as hirame rhabdovirus (HIRRV) (Takano *et al.*, 2004) and snakehead rhabdovirus (SHRV) (Kim *et al.*, 2000; Kurath, 2008).

Although the protection conferred by DNA rhabdoviral vaccines is high and relatively long-lasting, these vaccines are still not being used worldwide due to safety issues, mostly concerning the viral promoter, and difficulties in the administration of the vaccine at a large scale in fingerlings (mass vaccination methods).

The study of the immune mechanism(s) responsible for protection is also actively studied because it is still largely unknown. In this respect, the recent availability of fish microarrays (Martin *et al.*, 2008) have offered a new possibility to further study fish immunological responses to rhabdoviral infection and vaccination in several rhabdovirus/fish models. Thus, rather than studying the transcript expression of a few previously selected genes, hybridization to microarrays allow the expression profiling of thousands of genes simultaneously. First microarray versions of the japanese flounder, trout, salmon and zebrafish genes have been used to identify genes induced by VHSV (Byon *et al.*, 2005; 2006; Encinas *et al.*, 2010), IHNV (MacKenzie *et al.*, 2008; Purcell *et al.*, 2006) and HRV (Yasuike *et al.*, 2007) infections and vaccinations.

Also, reverse transcriptase-quantitative-polymerase chain reaction (RT-Q-PCR) after DNA vaccination to fish rhabdoviruses (Kurath, 2008) in haematopoietic organs has allowed to quantitate gene transcription to characterize immunological responses (immunogenicity). Among the earliest responses identified, those of interferon inducible Mx (an indicator of activation of the interferon type I pathway) (Acosta *et al.*, 2005; Boudinot *et al.*, 1998; McLauchlan *et al.*, 2003; Purcell *et al.*, 2004; Robertsen, 2008; Tafalla *et al.*, 2007), virally-induced genes (*vig*) (Boudinot *et al.*, 1999; Boudinot *et al.*, 2001b), major hystocompatibility complex antigens (*mhc*) and T cell receptors (*tcr*) genes (Takano *et al.*, 2004) were the most often described.

According to the results commented above, non specific short-term protective immunity by vaccination with plasmid DNA encoding a gpG gene has been assumed to result from the early induction of interferon-related genes, while specific long-term protection is probably due to cellular (T-cell) and/or to antibody (antigenic) responses to the encoded gpG gene (Kurath *et al.*, 2007). Next we will briefly review these three subjects.

Chapter 5

EARLY GPG_{VHSV} IMMUNOGENICITY: ACTIVATION OF THE INTERFERON (IFN) SYSTEM

The innate immune system seems to play a greater role in the response to viral infections in fish than in mammals. Most probably that is due to the lower efficiency of the "naïve" temperature-dependent acquired immune response of poikilothermic fish (Magnadottir, 2006). In addition, fish have a more limited Ab response repertoire and slower lymphocyte proliferation rates than mammals (Du Pasquier *et al.*, 1998; Ellis, 2001; Magnadottir, 2006).

Nevertheless, the fish innate immune responses against pathogens also comprise cellular and humoral components, including pattern-recognition receptors which are capable of recognizing pathogen-associated molecular patterns (PAMPs), just as it occurs in mammals. Similarly, once the PAMPs are recognized, type I interferons (IFNs) (IFN-α/ß) are induced and have a central role in innate antiviral immune responses by establishing an intracellular antiviral state that prevents viral replication and restricts viral spread (McFadden, 2009; Randall & Goodbourn, 2008; Stark *et al.*, 1998). IFN-mediated antiviral mechanisms respond rapidly to viral infections during their early stages, suggesting that IFN responses provide some degree of protection until the slower specific immune defences are prepared. Moreover, IFN synthesis is increased *in vivo* with increasing water temperature and VHSV virulence (Dorson & DeKinkelin, 1974). Recent progress in

genome sequencing has identified type I IFN-like gene sequences in several fish species including zebrafish *Danio rerio* (Altmann *et al.*, 2003), channel catfish *Ictalurus punctatus* (Long *et al.*, 2004), Atlantic salmon *Salmo salar* (Robertsen, 2006), puffer fish *Fugu rubripes* (Zou *et al.*, 2005) and rainbow trout *Oncorhynchus mykiss* (Zou *et al.*, 2007).

Type I IFNs induce different cell signalling pathways leading to transcription of specific sets of interferon stimulated genes (ISGs). The best characterized ISGs encode the myxovirus resistance proteins (Mx proteins) (Bazzigher *et al.*, 1992; Haller *et al.*, 2007; Staeheli, 1993), the dsRNA-dependent protein kinase (PKR) (Garcia *et al.*, 2007; Samuel, 2001), the oligoadenylate synthase (OAS) proteins (Ghosh *et al.*, 1991; Hovanessian & Justesen, 2007), etc. Among them, Mx proteins have been the most thoroughly studied in different fish species. The Mx proteins comprise GTPases with homology to the dynamin superfamily (Haller & Kochs, 2002; Haller *et al.*, 2007), some of them showing antiviral activity.

Most studies on the type I IFN-mediated responses induced by viruses have focused on the viral genomes and their replication intermediates as the more relevant stimulus for these responses. However, other viral molecules, such as gpG, also induce type I IFN-mediated responses (Seeds *et al.*, 2006) and inactivated virus as well as fixed virus-infected cells have been shown to be able to trigger a type I IFN-mediated antiviral response in many different cell types (Ito, 1994; Miller, 2003). Furthermore, the fact that fish immunized with a plasmid encoding gpG_{VHSV} showed high stimulation of type I IFN responses (Boudinot *et al.*, 1998; Chico *et al.*, 2009; Kim *et al.*, 2000; McLauchlan *et al.*, 2003; Tafalla *et al.*, 2007), suggest that gpG_{VHSV} plays a direct role on type I IFN induction. In this respect, a collection of 60 overlapping synthetic 20-mer peptides covering the full-length of the gpG_{VHSV} were recently used in an *in vitro* assay of Mx3 induction (Chico *et al.*, 2010). In that study, the major Mx3 inducing region was located at aa 340-470 of the gpG_{VHSV} sequence (Figure 6). The peptide included the ^{356}Arg-Gly-Asp or RGD motif tripeptide (a functional domain related to integrin-binding), suggesting that gpG_{VHSV} might interact with integrins on the surface of infected cells and that this interaction might trigger innate responses such as it was demonstrated in human cells (Boisvert *et al.*, 2007; Higuchi, 2003). Other of the identified peptides expanded the amino acids 280 to 310, a similar region earlier implicated in the

induction of *in vitro* anamnestic trout leucocyte proliferation (Lorenzo *et al.*, 1995a; Lorenzo *et al.*, 1995b).

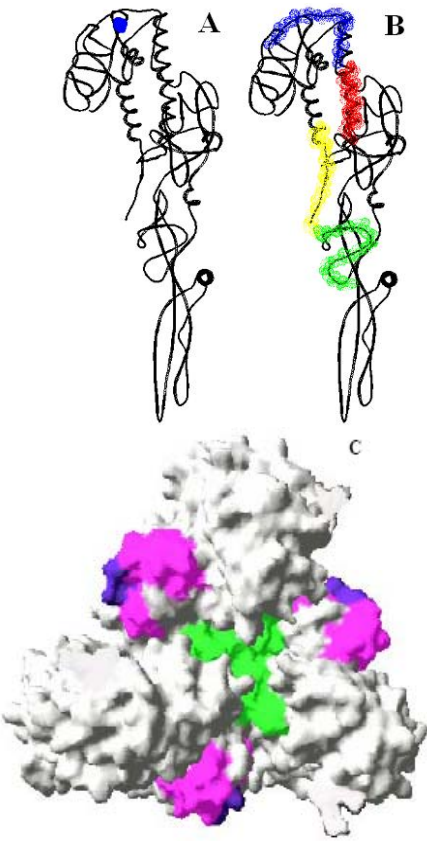

Figure 6. Localization in the gpG_{VHSV} modeled structure of the RGD motif (A), the peptides that bind fibronectin in rainbow trout (B) and the major Mx3-inducing regions (C).

A) The RGD motif (blue) is found at R356 in the gpG_{VHSV}. **B)** The main peptides of gpG_{VHSV} that bind rainbow trout fibronectin are C49-R63 (red), E189-G203 (green), F299-T313 (blue) and M419-I433 (yellow) (unpublished). **C)** Mx3-inducer peptides mapped onto the surface viewed from above of the pre-fusion conformation of the VHSV gpG trimer (physiological pH). p31 (residues 280 to 310), green; p33 (residues 340 o 370), magenta; RGD motif (residues 356 to 358), purple (Chico *et al.*, 2010).

Chapter 6

LONG-TERM GPG IMMUNOGENICITY: INDUCTION OF T CELLULAR RESPONSES

Specific late protection to VHSV infection can be mediated by a delayed cellular memory consisting in both helper and/or cytotoxic activities (T-like cell responses). Most of the studies on this topic have been restricted to *in vitro* estimation of leucocyte proliferative responses (lymphoproliferation assays). Those have been performed by adding polyclonal mitogens (Chilmonczyk, 1978a; Estepa & Coll, 1992a; b), whole inactivated rhabdovirus (Chilmonczyk, 1978b), isolated rhabdoviral proteins (Estepa, 1992), recombinant rhabdoviral protein fragments (Estepa *et al.*, 1994) or pepscan peptides derived from the gpG_{VHSV} (Lorenzo *et al.*, 1995a; Lorenzo *et al.*, 1995b). Stimulation of *in vitro* lymphoproliferative responses resulted only when leucocytes were obtained from trout surviving VHSV infection but not when obtained from healthy trout (Estepa *et al.*, 1994).

On mammal/virus models, leucocyte proliferative responses defining T-cell responses, occurred after presentation of a limited number of short viral protein peptides in the membrane of the host infected cells in the same MHC context, a mechanism reinforced in anamnestic responses. Leucocytes from trout surviving VHSV infections were capable of *in vitro* proliferation when cultured in the presence of 15-mer synthetic peptides designed from the gpG_{VHSV} (Lorenzo *et al.*, 1995a; Lorenzo *et al.*, 1995b). The recognition of each of the peptides varied within individuals from an outbred trout population. Thus, T cell epitopes mapped by pepscan lymphoproliferation in 12 trout showed peptides

299-323 (7 trout), 339-393 (4 trout) and other peptides (1-3 trout) to stimulate lymphoproliferation (Lorenzo *et al.*, 1995b). In contrast, no significant proliferative responses were obtained for the above mentioned peptides when leucocytes were obtained from either non-infected or genetically VHSV-resistant trout.

Head kidney cultures obtained from trout resistant to VHSV infections could be maintained during more than a year retaining their initial capacity of purified recombinant gpG-dependent lymphoproliferation when incubated with autologous macrophages pulsed with purified recombinant gpG_{VHSV}. The long-term proliferating cell lines had the morphology of lymphocytes, cell surface TcR staining, expression of both α and ß *tcr* chains mRNA sequences and secreted immunostimulating molecules (Estepa *et al.*, 1999; Estepa *et al.*, 1996; Estepa & Coll, 1997a). Because the *in vitro* cell immunological memory to gpG_{VHSV} exposure lasted during more than a year (Estepa *et al.*, 1994; Lorenzo *et al.*, 1995b) in contrast with the 4-6 months of most NAbs, lymphoproliferation memory to gpG_{VHSV} could be one of the most important mechanisms for long-term protection.

Identification and separation of potentially existing T cell subsets is critical for the continuation of the study of lymphoproliferative responses (cytotoxic or helper) to rhabdoviral antigens, but production of MAbs to T cell markers have met with difficulties (Nakanishi *et al.*, 1999). On the other hand, other assays are beginning to be used to study cellular memory to rhabdoviral antigens. Thus, up-regulation of *mhcII* expression (another sign of T-cell activation) was observed in trout immunized with the gpG_{VHSV} gene (Boudinot *et al.*, 1998) and the gpG_{VHSV} was shown to be the target of most of the public anti-VHSV T cell response, suggesting that T helper cells probably contribute to the Ab response (Boudinot *et al.*, 2004). VHSV infection induced modifications of the TcR repertoire from polyclonal to oligoclonal as studied by spectratyping (methodology that delivers a global view of the ß-TcR repertoires by showing the size distribution of the variable V region of the TcR) (Bernard *et al.*, 2006). Specific VßJß rearrangements were amplified among spleen T cells in response to injection with the gpG gene from VHSV (Boudinot *et al.*, 2001a). Sequencing of cloned VßJß PCR products corresponding to spectratypes with reduced number of peaks (oligoclonal), identified recurrent sequences corresponding to the expanded clones. Interestingly, the sequence SSGDSYSE (amino acids in single letter code) was the

most expanded in the spleen public T cell response to the gpG_{VHSV} (Boudinot *et al.*, 2004) and was also amplified in gut intraepithelial lymphocytes (IELs) from VHSV infected trout (Bernard *et al.*, 2006).

Despite all the studies mentioned above, the possible role of different gpG_{VHSV} epitopes in inducing VHSV protective cellular memory remains to be fully characterized.

Chapter 7

LONG-TERM GPG_{VHSV} ANTIGENICITY: ANTIBODY RESPONSES

Long-term NAbs to VHSV only recognized gpG_{VHSV} (Bearzotti *et al.*, 1995; Engelking & Leong, 1989; Lorenzen *et al.*, 1993a; Lorenzen *et al.*, 1990) and injection of the gpG_{VHSV} and other VHSV genes which confirmed that only gpG_{VHSV} induced NAbs (Boudinot *et al.*, 1998; Lorenzen *et al.*, 1990).

Long-term resistance of trout surviving VHSV infection to re-infection had been long observed by trout farmers (de Kinkelin *et al.*, 1995), together with failure to detect *in vitro* NAbs in their sera (Vestergaard-Jorgensen, 1974), until complement was included (Dorson & Torchy, 1979; Olesen & Vestergard-Jorgensen, 1986). After VHSV infections: i) *in vitro* NAbs could only be detected in approximately 50-60 % of the survivor fish (Olesen *et al.*, 1991; Olesen & Jorgensen, 1991), ii) in contrast, most survivors also resisted a second VHSV infection (Dorson *et al.*, 1995) and iii) after the second infection there was no detectable increase in the NAbs levels (Olesen *et al.*, 1991; Olesen & Jorgensen, 1991). One of the possible explanations for the existence of VHSV resistant trout without *in vitro* detectable NAbs (Jorgensen *et al.*, 1991) could be the presence of *in vitro* non neutralizing but *in vivo* protective anti-gpG Abs as demonstrated with some MAbs (Lorenzen *et al.*, 1990). Alternatively, since protection lasted more than NAbs presence (McLauchlan *et al.*, 2003), it could also be posible that: i) the *in vitro* technique did not detect all NAbs, because of their low sensitivity and/or dependence on the VHSV isolate used for the assay

(Fregeneda-Grandes & Olesen, 2007), ii) *in vitro* non NAbs also mediated protection *in vivo* as mentioned above, or iii) there were cellular mechanisms (i.e.: those detected by lymphoproliferation) other than Abs involved in protection. On the other hand, at least other isotype (IgT) found abundantly in fish mucus, have been discovered in trout (Hansen *et al.*, 2005) and other fish (Danilova *et al.*, 2005) but their participation on VHSV resistance is yet untested. Mucus Abs could also explain some of the failures to detect NAbs in VHSV survivors.

Most NAbs, as well as NMAbs, seem to recognize discontinuous conformational epitopes rather than lineal continuous epitopes on the gpG_{VHSV} (Bearzotti *et al.*, 1995; Lorenzen *et al.*, 1993a; Lorenzen *et al.*, 1990). Consequently, NMAbs could not be mapped by using overlapping 15-mer synthetic peptides from the gpG_{VHSV} (Fernandez-Alonso *et al.*, 1998). Only some MAbs recognizing lineal epitopes could be mapped by using pepscan techniques (Figure 5). Among the epitopes recognized by hyperimmunized fish sera, frg11 (56-110) was recognized by 40 % of the trout Abs on pepscan peptides of the gpG_{VHSV} (Fernandez-Alonso *et al.*, 1998; Rocha *et al.*, 2002), probably because frg11 locates in the exterior of the gpG_{VHSV} molecule (Figure 4). There are only 2 VHSV conformational NMAbs well characterized: C10 (Bearzotti *et al.*, 1994; 1995) and 3F1A12 (Lorenzen, personal communication). By sequencing MAb resistant mutants, the NMAb C10 was mapped simultaneously to positions 140 y 433 (Bearzotti *et al.*, 1995) and the 3F1A12 to position 253 (Lorenzen, personal communication) (Figure 5). Our own attempts to obtain more NMAbs to VHSV among those selected by flow cytometry screening (to maintain gpG conformation) failed (unpublished). The difficulties to obtain more NMAbs to VHSV could be due to an easy loss of the gpG conformation at the elevated temperature of the mice (37 ºC) (Coll, 1995a; Lorenzen *et al.*, 1990).

REFERENCES

[1] Acosta, F., Petrie, A., Lockhart, K., Lorenzen, N. & Ellis, A. E. (2005). Kinetics of Mx expression in rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (Salmo salar L.) parr in response to VHS-DNA vaccination. *Fish Shellfish Immunol*, *18*, 81-89.

[2] Altmann, S. M., Mellon, M. T., Distel, D. L. & Kim, C. H. (2003). Molecular and Functional Analysis of an Interferon Gene from the Zebrafish, Danio rerio. *J Virol*, *77*, 1992-2002.

[3] Anderson, E. D., Mourich, D. V., Fahrenkrug, S. C., LaPatra, S., Shepherd, J. & Leong, J. A. (1996a). Genetic immunization of rainbow trout (Oncorhynchus mykiss) against infectious hematopoietic necrosis virus. *Mol Mar Biol Biotechnol*, *5*, 114-122.

[4] Anderson, E. D., Mourich, D. V. & Leong, J. C. (1996b). Gene expression in rainbow trout (Onchorynchus mykiss) following intramuscular injection of DNA. *Mol Mar Biol Biotechnol*, *5*, 105-113.

[5] Basurco, B. & Benmansour, A. (1995). Distant strains of the fish rhabdovirus VHSV mantain a sixth functional cistron which codes for a nonstructural protein of unknown function. *Virology*, *212*, 741-745.

[6] Bazzigher, L., Pavlovic, J., Haller, O. & Staeheli, P. (1992). Mx genes show weaker primary response to virus than other interferon-regulated genes. *Virology*, *186*, 154-160.

[7] Bearzotti, M., Monnier, A. F., Vende, P., Grosclaude, J., DeKinkelin, P. & Benmansour, A. (1994). Molecular aspect of antigenicity and pathogenicity of viral hemorrhagic septicemia

virus (VHSV), a fish rhabdovirus. *IX International Conference on Negative Strand Viruses EstorilPortugal*, Oct. 2-7, *242*.

[8] Bearzotti, M., Monnier, A. F., Vende, P., Grosclaude, J., DeKinkelin, P. & Benmansour, A. (1995). The glycoprotein of viral hemorrhagic septicemia virus (VHSV): antigenicity and role in virulence. *Vet Res., 26*, 413-422.

[9] Benmansour, A., Basurco, B., Monnier, A. F., Vende, P., Winton, J. R. & de Kinkelin, P. (1997). Sequence variation of the glycoprotein gene identifies three distinct lineages within field isolates of viral haemorrhagic septicaemia virus, a fish rhabdovirus. *J Gen Virol*, *78(Pt 11)*, 2837-2846.

[10] Bernard, D., Six, A., Rigottier-Gois, L., Messiaen, S., Chilmonczyk, S., Quillet, E., Boudinot, P. & Benmansour, A. (2006). Phenotypic and functional similarity of gut intraepithelial and systemic T cells in a teleost fish. *J Immunol*, *176*, 3942-3949.

[11] Biering, E., Villoing, S., Sommerset, I. & Christie, K. E. (2005). Update on viral vaccines for fish. *Dev Biol (Basel)*, *121*, 97-113.

[12] Boisvert, M., Gendron, S., Chetoui, N. & Aoudjit, F. (2007). Alpha2 beta1 integrin signaling augments T cell receptor-dependent production of interferon-gamma in human T cells. *Mol Immunol*, *44*, 3732-3740.

[13] Boudinot, P., Bernard, D., Boubekeur, S., Thoulouze, M. I., Bremont, M. & Benmansour, A. (2004). The glycoprotein of a fish rhabdovirus profiles the virus-specific T-cell repertoire in rainbow trout. *J Gen Virol*, *85*, 3099-3108.

[14] Boudinot, P., Blanco, M., de Kinkelin, P. & Benmansour, A. (1998). Combined DNA immunization with the glycoprotein gene of viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific response in rainbow trout. *Virology*, *249*, 297-306.

[15] Boudinot, P., Boubekeur, S. & Benmansour, A. (2001a). Rhabdovirus Infection Induces Public and Private T Cell Responses in Teleost Fish. *J Immunol*, *167*, 6202-6209.

[16] Boudinot, P., Massin, P., Blanco, M., Riffault, S. & Benmansour, A. (1999). vig-1, a new fish gene induced by the rhabdovirus glycoprotein, has a virus-induced homologue in humans and shares conserved motifs with the MoaA family. *J Virol*, *73*, 1846-1852.

[17] Boudinot, P., Salhi, S., Blanco, M. & Benmansour, A. (2001b). Viral haemorrhagic septicaemia virus induces vig-2, a new interferon-responsive gene in rainbow trout. *Fish Shellfish Immunol*, *11*, 383-397.

[18] Byon, J. Y., Ohira, T., Hirono, I. & Aoki, T. (2005). Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (Paralichthys olivaceus) following DNA vaccination. *Fish Shellfish Immunol*, *18*, 135-147.

[19] Byon, J. Y., Ohira, T., Hirono, I. & Aoki, T. (2006). Comparative immune responses in Japanese flounder, Paralichthys olivaceus after vaccination with viral hemorrhagic septicemia virus (VHSV) recombinant glycoprotein and DNA vaccine using a microarray analysis. *Vaccine*, *24*, 921-930.

[20] Coll, J. (1995a). Mapeo de epitopos neutralizantes contra el rabdovirus de la septicemia hemorrhagica virica (SHV) de salmonidos utilizando mutantes resistentes a anticuerpos monoclonales. Aplicaciones en la prevencion y diagnostico de la enfermedad. *Investigacion Agraria*, *10*, 41-52.

[21] Coll, J. M. (1995b). The glycoprotein G of rhabdoviruses. *Arch Virol*, *140*, 827-851.

[22] Coll, J. M. (1995c). Heptad-repeat sequences in the glycoprotein of rhabdoviruses. *Virus Genes*, *10*, 107-114.

[23] Corbeil, S., Lapatra, S. E., Anderson, E. D., Jones, J., Vincent, B., Hsu, Y. L. & Kurath, G. (1999). Evaluation of the protective immunogenicity of the N, P, M, NV and G proteins of infectious hematopoietic necrosis virus in rainbow trout oncorhynchus mykiss using DNA vaccines. *Dis Aquat Organ*, *39*, 29-36.

[24] Chico, V., Martinez-Lopez, A., Ortega-Villaizan, M., Falco, A., Perez, L., Coll, J. M. & Estepa, A. (2010). Pepscan mapping of Viral Haemorrhagic Septicaemia virus (VHSV) glycoprotein G major lineal determinants implicated in triggering host cell antiviral responses mediated by type I IFN. *J Virol doi:0101128/JVI00023-10*.

[25] Chico, V., Ortega-Villaizan, M., Falco, A., Tafalla, C., Perez, L., Coll, J. M. & Estepa, A. (2009). The immunogenicity of viral haemorragic septicaemia rhabdovirus (VHSV) DNA vaccines can depend on plasmid regulatory sequences. *Vaccine*, *27*, 1938-1948.

[26] Chilmonczyk, S. (1978a). In vitro stimulation by mitogens of peripheral blood lymphocytes from rainbow trout (Salmo gairdneri). *Ann Immunol*, *129*, 3-12.

[27] Chilmonczyk, S. (1978b). Stimulation specifique des lymphocytes de truites arc-en-ciel (Salmo gairdneri) resistantes a la septicemie hemorragique virale. *CR Acad Sci Paris*, *287*, 387-389.

[28] Danilova, N., Bussmann, J., Jekosch, K. & Steiner, L. A. (2005). The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin *Z*. *Nat Immunol*, *6*, 295-302.

[29] de Kinkelin, P., Baudouy, A. M. & Le Berre, M. (1977). [Response of the brown trout (Salmo trutta, L. 1766) and rainbow trout (Salmo gairdneri Richardson, 1836) to infection by a new rhabdovirus]. *C R Acad Sci Hebd Seances Acad Sci D*, *284*, 401-404.

[30] de Kinkelin, P., Bearzotti, M., Castric, J., Nougayrede, P., Lecocq-Xhonneux, F. & Thiry, M. (1995). Eighteen years of vaccination against viral haemorrhagic septicemia in France. *Vet Res*., *26*, 379-387.

[31] Dorson, M. & DeKinkelin, P. (1974). Mortalitet production d'interferon circulan chez la truite arc-en-ciel apres infection experimentale avec le virus d'Egtved:Influence de la temperature. *Ann Rech Vet*, *5*, 365-372.

[32] Dorson, M., Quillet, E., Hollebecq, M. G., Torhy, C. & Chevassus, B. (1995). Selection of rainbow trout resistant to viral haemorrhagic septicaemia virus and transmission of resistance by gynogenesis. *Vet Res*., *26*, 361-368.

[33] Dorson, M. & Torchy, C. (1979). Complement dependent neutralization of Egtved virus by trout antibodies. *J Fish Dis*., *2*, 345-347.

[34] Du Pasquier, L., Wilson, M., Greenberg, A. S. & Flajnik, M. F. (1998). Somatic mutation in ectothermic vertebrates: musings on selection and origins. *Curr Top Microbiol Immunol 229*, 199-216.

[35] Durrer, P., Gaudin, Y., Ruigrok, R. W., Graf, R. & Brunner, J. (1995). Photolabeling identifies a putative fusion domain in the envelope glycoprotein of rabies and vesicular stomatitis viruses. *J Biol Chem*., *270*, 17575-17581.

[36] Einer-Jensen, K., Ahrens, P., Forsberg, R. & Lorenzen, N. (2004). Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *J Gen Virol*, *85*, 1167-1179.

[37] Einer-Jensen, K., Krogh, T. N., Roepstorff, P. & Lorenzen, N. (1998). Characterization of Intramolecular Disulfide Bonds and Secondary Modifications of the Glycoprotein from Viral Hemorrhagic Septicemia Virus, a Fish Rhabdovirus. *J Virol*, *72*, 10189-10196.

[38] Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Dev Comp Immunol*, *25*, 827-839.

[39] Encinas, P., Carbajosa, S., Rodriguez-Milla, M. A., Novoa, B., Estepa, A., Figueras, A. & Coll, J. M. (2010). Zebrafish immune-related fin gene expression shortly after viral haemorrhagic septicemia rhabdovirus infection by immersion. A proteomic and transcriptomic approach. *BMC Genomics Submitted*.

[40] Engelking, H. & Leong, J. C. (1989). The glycoprotein of Infectious Hematopoitic Necrosis Virus eluciting antibody and protective responses. *Virus Res.*, *13*, 213-230.

[41] Essbauer, S. & Ahne, W. (2001). Viruses of lower vertebrates. *J Vet Med B Infect Dis Vet Public Health*, *48*, 403-475.

[42] Estepa, A. (1992). Estudios de immunización con proteinas electroeluidas y clonadas del virus de la septicemia hemorragica virica de la trucha. *University of Madrid Spain Doctoral Thesis* PHD Thesis, *243*.

[43] Estepa, A., Alvarez, F., Ezquerra, A. & Coll, J. M. (1999). Viral-antigen dependence and T-cell receptor expression in leucocytes from rhabdovirus immunized trout. *Vet Immunol Immunopathol*, *68*, 73-89.

[44] Estepa, A., Alvarez, F., Villena, A. & Coll, J. M. (1996). Morphology of antigen-dependent haematopoietic cells from trout surviving rhabdoviral infections. *Bull Eur Assn Fish P*, *16*, 203-207.

[45] Estepa, A. & Coll, J. M. (1992a). In vitro immunostimulants for optimal responses of kidney cells from healthy trout and from trout surviving viral haemorrhagic septicaemia virus disease. *J Fish Shellfish Immunol*, *2*, 53-68.

[46] Estepa, A. & Coll, J. M. (1992b). Mitogen-Induced proliferation of trout kidney leucocytes by one-step culture in fibrin clots. *Vet Immunol Immunopathol*, *32*, 165-177.

[47] Estepa, A. & Coll, J. M. (1993). Enhancement of fish mortality by rhabdovirus infection after immunization with a viral nucleoprotein peptide. *Viral Immunol*, *6*, 237-243.

[48] Estepa, A. & Coll, J. M. (1996). Pepscan mapping and fusion-related properties of the major phosphatidylserine-binding domain of the glycoprotein of viral hemorrhagic septicemia virus, a salmonid rhabdovirus. *Virology*, *216*, 60-70.

[49] Estepa, A. & Coll, J. M. (1997a). An in vitro method to obtain T-lymphocyte-like cells from the trout. *J Immunol Methods*, *202*, 77-83.

[50] Estepa, A. & Coll, J. M. (1997b). Temperature and pH requirements for viral haemorrhagic septicemia virus induced cell fusion. *Dis Aquat Organ*, *28*, 185-189.

[51] Estepa, A., Frias, D. & Coll, J. M. (1992). Susceptibility of trout kidney macrophages to viral haemorrhagic septicaemia virus. *Viral Immunol*, *5*, 283-292.

[52] Estepa, A., Thiry, M. & Coll, J. M. (1994). Recombinant protein fragments from haemorrhagic septicaemia rhabdovirus stimulate trout leukocyte anamnestic responses in vitro. *J Gen Virol*, *75(Pt 6)*, 1329-1338.

[53] Estepa, A. M., Rocha, A. I., Mas, V., Perez, L., Encinar, J. A., Nunez, E., Fernandez, A., Gonzalez Ros, J. M., Gavilanes, F. & Coll, J. M. (2001). A Protein G Fragment from the Salmonid Viral Hemorrhagic Septicemia Rhabdovirus Induces Cell-to-Cell Fusion and Membrane Phosphatidylserine Translocation at Low pH. *J Biol Chem.*, *276*, 46268-46275.

[54] Fernandez-Alonso, M., Lorenzo, G., Perez, L., Bullido, R., Estepa, A., Lorenzen, N. & Coll, J. M. (1998). Mapping of the lineal antibody epitopes of the glycoprotein of VHSV, a salmonid rhabdovirus. *Dis Aquat Organ*, *34*, 167-176.

[55] Fernandez-Alonso, M., Rocha, A. & Coll, J. M. (2001). DNA vaccination by immersion and ultrasound to trout viral haemorrhagic septicaemia virus. *Vaccine*, *19*, 3067-3075.

[56] Fregeneda-Grandes, J. M. & Olesen, N. J. (2007). Detection of rainbow trout antibodies against viral haemorrhagic septicaemia

virus (VHSV) by neutralisation test is highly dependent on the virus isolate used. *Dis Aquat Organ*, *74*, 151-158.

[57] Garcia, M. A., Meurs, E. F. & Esteban, M. (2007). The dsRNA protein kinase PKR: Virus and cell control. *Biochimie*, *89*, 799-811.

[58] Gaudin, Y., Ruigrok, R. W., Knossow, M. & Flamand, A. (1993). Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. *J Virol*, *67*, 1365-1372.

[59] Gaudin, Y., Ruigrok, R. W. H. & Brunner, J. (1995). Low-pH induced conformational changes in viral fusion proteins: implications for the fusion mechanism. *J Gen Virol*, *76*, 1541-1556.

[60] Ghosh, S. K., Kusari, J., Bandyopadhyay, S. K., Samanta, H., Kumar, R. & Sen, G. C. (1991). Cloning, sequencing, and expression of two murine 2'-5'-oligoadenylate synthetases. Structure-function relationships. *J Biol Chem.*, *266*, 15293-15299.

[61] Haller, O. & Kochs, G. (2002). Interferon-Induced Mx Proteins: Dynamin-Like GTPases with Antiviral Activity. *Traffic*, *3*, 710-717.

[62] Haller, O., Staeheli, P. & Kochs, G. (2007). Interferon-induced Mx proteins in antiviral host defense. *Biochimie*, *89*, 812-818.

[63] Hansen, J. D., Landis, E. D. & Phillips, R. B. (2005). Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proc Natl Acad Sci U S A*, *102*, 6919-6924.

[64] Harmache, A., LeBerre, M., Droineau, S., Giovannini, M. & Bremont, M. (2006). Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. *J Virol*, *80*, 3655-3659.

[65] Higuchi, A., Takanashi, Y., Tsuzuki, N., Asakura, T., Cho, C. S., Akaike, T. & Hara, M. (2003). Production of interferon-beta by fibroblast cells on membranes prepared with RGD-containing peptides. *Journal Biomed Mater Res-A*, *65(3)*, 369-3678.

[66] Hill, B. J., Underwood, B. O., Smale, C. J. & Brown, F. (1975). Physico-chemical and serological characterization of five rhabdoviruses infecting fish. *J Gen Virol*, *27*, 369-378.

[67] Hopper, K. (1989). The isolation of VHSV from salmon at Glenwood Springs, Orcas Island, Washington. *American Fish Society Fish Health Newsletter*, *17*, 1.

[68] Hovanessian, A. G. & Justesen, J. (2007). The human 2'-5'oligoadenylate synthetase family: Unique interferon-inducible enzymes catalyzing 2'-5' instead of 3'-5' phosphodiester bond formation. *Biochimie*, *89*, 779-788.

[69] Isshik, T., Nishizawa, T., Kobayashi, T., Nagano, T. & Miyazaki, T. (2001). An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder Paralichthys olivaceus in Japan. *Dis Aquat Organ*, *47*, 87-99.

[70] Ito, Y., Bando, H., Komada, H., Tsurudome, M., Nishio, M., Kawano, M., Matsumura, H., Kusagawa, S., Yuasa, T. & Ohta, H. (1994). HN proteins of human parainfluenza type 4A virus expressed in cell lines transfected with a cloned cDNA have an ability to induce interferon in mouse spleen cells. *J Gen Virol*, *75*, 567-572.

[71] Jorgensen, P. E. V., Olesen, N. J., Lorenzen, N., Winton, J. R. & Ristow, S. (1991). Infectious hematopoietic necrosis (IHN) and viral haemorragic septicaemia (VHS): detection of trout antibodies to the causative viruses by means of plaque neutralization, immunofluorescence, and enzyme-linked immunosorbent assay. *J Aquat Anim Health*, *3*, 100-108.

[72] Kim, C. H., Johnson, M. C., Drennan, J. D., Simon, B. E., Thomann, E. & Leong, J. A. (2000). DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish. *J Virol*, *74*, 7048-7054.

[73] Koener, J. F. & Leong, J. C. (1990). Expresion of the glycoprotein gene from a fish rhabdovirus by using baculovirus vectors. *J Virol*, *64*, 428-430.

[74] Kurath, G. (2008). Biotechnology and DNA vaccines for aquatic animals. *Rev Sci Tech*., *27*, 175-196.

[75] Kurath, G., Purcell, M. K. & Garver, K. A. (2007). Fish rhabdovirus models for understanding host response to DNA vaccines. *CAB reviews*, *2*, 1-12.

[76] LaPatra, S. E., Corbeil, S., Jones, G. R., Shewmaker, W. D., Lorenzen, N., Anderson, E. D. & Kurath, G. (2001). Protection of rainbow trout against infectious hematopoietic necrosis virus four

days after specific or semi-specific DNA vaccination. *Vaccine*, *19*, 4011-4019.

[77] Lecocq-Xhonneux, F., Thiry, M., Dheur, I., Rossius, M., Vanderheijden, N., Martial, J. & DeKinkelin, P. (1994). A recombinant viral haemorrhagic septicaemia virus glycoprotein expressed in insect cells induces protective immunity in rainbow trout. *J Gen Virol*, *75*, 1579-1587.

[78] Long, S., Wilson, M., Bengten, E., Bryan, L., Clem, L. W., Miller, N. W. & Chinchar, V. G. (2004). Identification of a cDNA encoding channel catfish interferon. *Dev Comp Immunol*, *28*, 97-111.

[79] Lorenzen, E., Einer-Jensen, K., Martinussen, T., LaPatra, S. E. & Lorenzen, N. (2000). DNA vaccination of rainbow trout against viral hemorrhagic septicaemia virus: a dose-response and time-course study. *J Aquat Anim Health*, *12*, 167-180.

[80] Lorenzen, N. & LaPatra, S. E. (2005). DNA vaccines for aquacultured fish. *Rev Sci Tech.*, *24*, 201-213.

[81] Lorenzen, N., Lorenzen, E. & Einer-Jensen, K. (2001). Immunity to viral haemorrhagic septicaemia (VHS) following DNA vaccination of rainbow trout at an early life-stage. *Fish Shellfish Immunol*, *11*, 585-591.

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

[83] Lorenzen, N. & Olesen, N. J. (1997). Immunization with viral antigens: viral haemorrhagic septicaemia. *Dev Biol Stand*, *90*, 201-209.

[84] Lorenzen, N., Olesen, N. J., Jorgensen, P. E., Etzerodt, M., Holtet, T. L. & Thogersen, H. C. (1993a). Molecular cloning and expression in Escherichia coli of the glycoprotein gene of VHS virus, and immunization of rainbow trout with the recombinant protein. *J Gen Virol*, *74(Pt 4)*, 623-630.

[85] Lorenzen, N., Olesen, N. J. & Jorgensen, P. E. V. (1993b). Antibody response in rainbow trout to VHS virus proteins. *Fish Shellfish Immunol*, *3*, 461-473.

[86] Lorenzen, N., Olesen, N. J. & Vestergaard-Jorgensen, P. E. (1990). Neutralization of Egtved virus pathogenicity to cell cultures and

fish by monoclonal antibodies to the viral G protein. *J Gen Virol*, *71*, 561-567.

[87] Lorenzo, G., Estepa, A., Chilmonczyk, S. & Coll, J. M. (1995a). Mapping of the G and N regions of viral haemorrhagic septicemia virus (VHSV) inducing lymphoproliferation by pepscan. *Vet Res*., *26*, 521-525.

[88] Lorenzo, G. A., Estepa, A., Chilmonczyk, S. & Coll, J. M. (1995b). Different peptides from haemorrhagic septicemia rhabdoviral proteins stimulate leucocyte proliferation with individual fish variation. *Virology*, *212*, 348-355.

[89] MacKenzie, S., Balasch, J. C., Novoa, B., Ribas, L., Roher, N., Krasnov, A. & Figueras, A. (2008). Comparative analysis of the acute response of the trout, O. mykiss, head kidney to in vivo challenge with virulent and attenuated infectious hematopoietic necrosis virus and LPS-induced inflammation. *BMC Genomics*, *9*, 141.

[90] Magnadottir, B. (2006). Innate immunity of fish (overview). *Fish Shellfish Immunol*, *20*, 137-151.

[91] Martin, S. A. M., Collet, B., Mackenzie, S., Evensen, O. & Secombes, C. J. (2008). Genomic tools for examining immune gene function in salmonid fish. *Rev Fish Sci*., *16*, 112-118.

[92] McFadden, G., Mohamed, R. M., Rahman, M. M. & Bartee, E. (2009). Cytokine determinants of viral tropism. *Nature Reviews Immunology*, *9*, 645-656.

[93] McLauchlan, P. E., Collet, B., Ingerslev, E., Secombes, C. J., Lorenzen, N. & Ellis, A. E. (2003). DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection-early protection correlates with Mx expression. *Fish Shellfish Immunol*, *15*, 39-50.

[94] Meyers, T. R., Sullivan, J., Emmeneger, E., Follet, J., Short, S., Batts, W. N. & Winton, J. R. (1992). Identification of viral haemorrhagic septicemia virus isolated from pacific cod, Gadus Macocephalus in prince William Sound, Alaska, USA. *Dis Aquat Organ*, *12*, 167-175.

[95] Miller, J. L. & Anders, E. M (2003). Virus-cell interactions in the induction of type 1 interferon by influenza virus in mouse spleen cells. *J Gen Virol*, *84*, 193-2002.

[96] Nakanishi, T., Aoyagi, K., Xia, C., Dijkstra, J. M. & Ototake, M. (1999). Specific cell-mediated immunity in fish. *Vet Immunol Immunopathol*, *72*, 101-109.

[97] Nishizawa, T., Iida, H., Takano, R., Isshiki, T., Nakajima, K. & Muroga, K. (2002). Genetic relatedness among Japanese, American and European isolates of viral hemorrhagic septicemia virus (VHSV) based on partial G y P genes. *Dis Aquat Organ*, *48*, 143-148.

[98] Nunez, E., Fernandez, A. M., Estepa, A., Gonzalez-Ros, J. M., Gavilanes, F. & Coll, J. M. (1998). Phospholipid interactions of a peptide from the fusion-related domain of the glycoprotein of VHSV, a fish rhabdovirus. *Virology*, *243*, 322-330.

[99] Olesen, N. (1998). Sanitation of viral haemorrhagic septicaemia (VHS). *J Appl Ichthyol*, *14*, 173-177.

[100] Olesen, N., J, Lorenzen, N. & Vestergaard-Jorgensen, P. E. (1991). Detection of rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and plaque neutralization tests (50% PNT). *Dis Aquat Organ*, *10*, 31-38.

[101] Olesen, N. J. & Jorgensen, P. E. V. (1991). Rapid Detection of Viral Hemorrhagic Septicemia Virus in Fish by Elisa *J Appl Ichthyol*, *7*, 183-186.

[102] Olesen, N. J. & Vestergard-Jorgensen, P. E. (1986). Detection of neutalizing antibody to Egtved virus in Rainbow trout (Salmo gairdneri by plaque neutralization test with complement addition. *J Appl Icthyol*, *2*, 33-41.

[103] Purcell, M. K., Kurath, G., Garver, K. A., Herwig, R. P. & Winton, J. R. (2004). Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. *Fish Shellfish Immunol*, *17*, 447-462.

[104] Purcell, M. K., Nichols, K. M., Winton, J. R., Kurath, G., Thorgaard, G. H., Wheeler, P., Hansen, J. D., Herwig, R. P. & Park, L. K. (2006). Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. *Mol Immunol*.

[105] Randall, R. E. & Goodbourn, S. (2008). Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol*, *89*, 1-47.

[106] Robertsen, B. (2006). The interferon system of teleost fish. *Fish Shellfish Immunol*, *20*, 172-191.

[107] Robertsen, B. (2008). Expression of interferon and interferon-induced genes in salmonids in response to virus infection, interferon-inducing compounds and vaccination. *Fish Shellfish Immunol*, *25*, 351-357.

[108] Rocha, A., Fernandez-Alonso, M., Mas, V., Perez, L., Estepa, A. & Coll, J. M. (2002). Antibody response to a fragment of the protein G of VHS rhabdovirus in immunised trout. *Vet Immunol Immunopathol*, *86*, 89-99.

[109] Rocha, A., Ruiz, S., Tafalla, C. & Coll, J. M. (2004). Conformation- and fusion-defective mutations in the hypothetical phospholipid-binding and fusion peptides of viral hemorrhagic septicemia salmonid rhabdovirus protein G. *J Virol*, *78*, 9115-9122.

[110] Roche, S., Bressanelli, S., Rey, F. A. & Gaudin, Y. (2006). Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. *Science*, *313*, 187-191.

[111] Roche, S., Rey, F. A., Gaudin, Y. & Bressanelli, S. (2007). Structure of the prefusion form of the vesicular stomatitis virus glycoprotein G. *Science*, *315*, 843-848.

[112] Samuel, C. E. (2001). Antiviral Actions of Interferons. *Clin Microbiol Rev.*, *14*, 778-809.

[113] Schlotfeldt, H. J., Ahne, W., Vestergard-Jorgensen, P. E. & Glende, W. (1991). Occurrence of viral haemorrhagic septicaemia in turbot (Scophthalmus maximus)-A natural outbreak. *Bull Eur Assn Fish P*, *11*, 105-107.

[114] Schutze, H., Enzmann, P. J., Mundt, E. & Mettenleiter, T. C. (1996). Identification of the non-virion (NV) protein of fish rhabdoviruses viral haemorrhagic septicaemia virus and infectious haematopoietic necrosis virus. *J Gen Virol*, *77(Pt 6)*, 1259-1263.

[115] Schutze, H., Mundt, E. & Mettenleiter, T. C. (1999). Complete genomic sequence of viral hemorrhagic septicemia virus, a fish rhabdovirus. *Virus Genes*, *19*, 59-65.

[116] Seeds, R. E., Gordon, S. & Miller, J. L. (2006). Receptors and ligands involved in viral induction of type I interferon production by plasmacytoid dendritic cells. *Immunobiology*, *211*, 525-535.

[117] Skall, H. F., Olesen, N. J. & Mellergaard, S. (2005). Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming--a review. *J Fish Dis.*, *28*, 509-529.

[118] Skehel, J. J. & Wiley, D. C. (1998). Coiled coils in both intracellular vesicle and viral membrane fusion. *Cell*, *95*, 871-874.

[119] Sommerset, I., Krossoy, B., Biering, E. & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Rev Vaccines*, *4*, 89-101.

[120] Staeheli, P., Pitossi, F. & Pavlovic, J. (1993). Mx proteins: GTPases with antiviral activity. *Trends Cell Biol.*, *3*, 268-272.

[121] Stark, G. R., Kerr, I. M., Williams, B. R. G., Silverman, R. H. & Schreiber, R. D. (1998). How Cells Respond To Interferons. *Annu Rev Biochem*, *67*, 227-264.

[122] Tafalla, C., Chico, V., Perez, L., Coll, J. M. & Estepa, A. (2007). In vitro and in vivo differential expression of rainbow trout (Oncorhynchus mykiss) Mx isoforms in response to viral haemorrhagic septicaemia virus (VHSV) G gene, poly I:C and VHSV. *Fish Shellfish Immunol*, *23*, 210-221.

[123] Tafalla, C., Figueras, A. & Novoa, B. (1998). In vitro interaction of viral haemorrhagic septicaemia virus and leukocytes from trout (Oncorhynchus mykiss) and turbot (Scophthalmus maximus). *Vet Immunol Immunopathol*, *62*, 359-366.

[124] Takano, T., Iwahori, A., Hirono, I. & Aoki, T. (2004). Development of a DNA vaccine against hirame rhabdovirus and analysis of the expression of immune-related genes after vaccination. *Fish Shellfish Immunol*, *17*, 367-374.

[125] Thoulouze, M. I., Bouguyon, E., Carpentier, C. & Bremont, M. (2004). Essential role of the NV protein of Novirhabdovirus for pathogenicity in rainbow trout. *J Virol*, *78*, 4098-4107.

[126] Vestergaard-Jorgensen, P. E. (1974). Indirect fluorescent antibody techniques for demonstration of trout viruses and corresponding antibody. *Acta Vet Scand*, *15*, 198-205.

[127] Walker, P. J. & Kongsuwan, K. (1999). Deduced structural model for animal rhabdovirus glycoproteins. *J Gen Virol*, *80*, 1211-1220.

[128] Xu, L., Mourich, D. V., Engelking, H. M., Ristow, S., Arnzen, J. & Leong, J. C. (1991). Epitope mapping and characterization of the

infectious hematopoietic necrosis virus glycoprotein, using fusion proteins synthesized in Escherichia coli. *J Virol*, *65*, 1611-1615.

[129] Yao, Y., Ghosh, K., Epand, R. F., Epand, R. M. & Ghosh, H. P. (2003). Membrane fusion activity of vesicular stomatitis virus glycoprotein G is induced by low pH but not by heat or denaturant. *Virology*, *310*, 319-332.

[130] Yasuike, M., Kondo, H., Hirono, I. & Aoki, T. (2007). Difference in Japanese flounder, Paralichthys olivaceus gene expression profile following hirame rhabdovirus (HIRRV) G and N protein DNA vaccination. *Fish Shellfish Immunol*, *23*, 531-541.

[131] Zou, J., Carrington, A., Collet, B., Dijkstra, J. M., Yoshiura, Y., Bols, N. & Secombes, C. (2005). Identification and bioactivities of IFN-gamma in rainbow trout Oncorhynchus mykiss: the first Th1-type cytokine characterized functionally in fish. *J Immunol*, *175*, 2484-2494.

[132] Zou, J., Tafalla, C., Truckle, J. & Secombes, C. J. (2007). Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates. *J Immunol*, *179*, 3859-3871.

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