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The specific immune response in rainbow trout: Somatic hypermutation and VH gene utilization

Teresa D. Lewis
College of William and Mary - Virginia Institute of Marine Science

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THE SPECIFIC IMMUNE RESPONSE IN RAINBOW TROUT:
SOMATIC HYPERMUTATION AND VH GENE UTILIZATION

A Dissertation
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Doctor of Philosophy

by

Teresa D. Lewis 2000
APPROVAL SHEET

This dissertation is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

The study of antibody responses in prominent aquaculture species such as the rainbow trout, *Oncorhynchus mykiss*, can facilitate vaccine development and contribute to producing useful paradigms of adaptive immunity in lower vertebrates. Thus, it is essential to identify genes responsible for antibody responses. In the mouse model, hybridoma technology allows for the association of monoclonal antibodies possessing various affinities for antigen with specific VH sequences, gene family utilization, and other molecular events (i.e. somatic hypermutation) that occur during the specific immune response. The absence of a comparable hybridoma technology in piscine systems has limited similar studies of fish immunogenetics to date. Molecular and serological experiments were performed in an attempt to obtain information regarding somatic mutation and VH gene utilization for trout antibodies without reliance on hybridoma technology. PCR primers recognizing consensus sequences of FR1 and FR3 were used to amplify antibody VH sequences from panned, antigen-specific B cells. To follow the development of the expressed VH repertoire, lymphocytes were obtained at weeks 0, 5, 10, and 20 post primary immunization with trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) or infectious hematopoietic necrosis virus (IHNV). Lymphocytes were also collected 10 weeks post secondary immunization (week 35). These studies were conducted in parallel with serological analyses of plasma antibodies obtained from the same sample in order to correlate molecular data with serological data from individual trout. Antigen-specific lymphocytes were processed to isolate RNA templates to produce cDNA which was cloned and sequenced. This sequence analysis allowed us to report, for the first time, the temporal accumulation of potential somatic variants that correlate to the development of new, high affinity antibody subpopulations during the immune response, some with the emergence of new antibody heavy chain isoelectropherotypes as identified by 2D-IEF/SDS-PAGE. Southern analysis and gene titration using various antigen-specific cDNA probes allowed us to correlate trout antibodies possessing various affinities for antigen with specific VH sequence and gene family utilization. Thus, trout Ig VH gene family utilization appears to follow the mouse model of differential use for specific immune response. These results reveal a capability for fine-tuning the piscine immune response previously not recognized. Furthermore, the identification of specific VH gene families used in generating antibodies to specific antigens will permit exhaustive germline sequence analysis in the future which is requisite for the absolute confirmation of these mutational processes.
THE SPECIFIC IMMUNE RESPONSE IN RAINBOW TROUT:
SOMATIC MUTATION AND VH GENE UTILIZATION
INTRODUCTION

Somatic mutation is the process whereby antibody genes are mutated within mature lymphocytes. These mutations are capable of creating new binding site variants from which higher affinity antibody producing cells may be preferentially expanded via antigen-driven selection. In essence, this is the primary process by which mammalian antibody, particularly IgG, IgA and IgE, responses develop their enhanced specificity post immunization. However, our mechanistic understanding of antigen selection and somatic mutation has yet to be fully developed, particularly in lower vertebrates.

Somatic mutation has been observed in the African clawed frog *Xenopus laevis* (Wilson et al. 1992) and the horned shark *Heterodontus francisci* (Hinds-Frey et al. 1993). However, such observed molecular changes have yet to be associated with either a specific immune response or a corresponding increase in affinity. In these studies, variation from germline sequences were identified in non-immunized animals, thus, no association with antigen-driven selection could be made. While the presence of somatically mutated VH genes could be taken as corroborative evidence of a past antigen-specific response, it is not conclusive. Such substantiation would require the observation of the emergence of somatic variants during a response to specific antigenic challenge.

Examination of the antigen-specific response in rainbow trout (*Oncorhynchus*...
mykiss) against two distinct protein antigens, the model antigen trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) from which an epitope-specific (TNP) specific response can be studied and the economically relevant viral antigen (infectious hematopoietic necrosis virus glycoprotein, IHNV gp) provides insight into the molecular processes occurring during the maturation of the specific antibody response in a teleost fish. Ultimately, this study may provide molecular evidence that contributes to the development of effective anti-viral vaccine strategies in teleost fish by identifying the conditions requisite for optimal affinity maturation of anti-viral antibodies. Also of potential importance is the role of somatic mutation in development of an anti-viral response in fish since many pathogens in aquaculture are viruses.

**Antibody structure**

Antibody (Ab) molecules, which are also termed immunoglobulin (Ig) molecules, are found in all jawed vertebrates. The structure of the Ig monomer consists of two heavy (H) chains and two light (L) chains, with each protein chain comprised of two principal regions: the N-terminal variable region (V) and the C-terminal constant region (C). Although separate genes encode the variable and constant regions, the two genes are never independently expressed and together they encode a single polypeptide product (Dryer and Bennett 1965).
In teleost fish, antibodies appear primarily as tetrameric molecules (termed IgM), composed of four monomeric subunits with the molecular weight of the monomer H chains approximately 72 kDa and L chains approximately 27 kDa (Kaattari and Piganelli 1996). There have been reports by other investigators of the monomeric form coexisting with a tetrameric form in sera (Acton et al. 1971; Clem and McClean 1975; Lobb and Clem 1981 a,b; Elcombe et al. 1985). Within the tetramer, the monomeric subunits have been found to associate either covalently via disulfide bonds, or non-covalently with one another (Evans et al. 1998). In mammals, the various associations of distinct monomeric Igs into different polymeric isotypes have been extensively described (reviewed in Max 1998).

The Ig binding site is generated by the association of the variable regions of the heavy (VH) and light (VL) chains (Fig. 1). A visual representation of the antibody monomer reveals a Y-shaped structure in which the arms of the Y are identical and each arm has a copy of the V region. These two arms join and extend downwards into the trunk of the Y shaped structure, which is composed of the constant regions of the heavy chains. Possession of many different V domains (sequences with different specificities) confers the ability of the system to respond to numerous different antigens. The total number of variable regions for either H or L chain proteins is in the hundreds in the mammalian system (Blackwell and Alt 1988). Enormous diversity in binding sites is not
Figure 1. Schematic of an antibody molecule. A. The association of heavy (H) and light (L) chain, each comprised of a variable (green) and constant (blue) region. H and L chain complementarity determining regions (CDRs) interact to form a functional antigen-binding site (indicated by colored circles). Two H chains and two L chains are linked by disulfide bonds (indicated by black lines) to form a monomeric antibody molecule. B. Teleost antibodies are tetrameric and consist of eight H chains and eight L chains linked by disulfide bonds.
surprising if one recognizes the necessity of producing antibodies capable of
accommodating the myriad number of possible foreign antigenic structures associated with
pathogens (Kaattari and Piganelli 1996).

Variability is particularly great within the short stretches of the VH and VL
domains called complementarity determining regions (CDRs). There are three such
CDRs in the VH (approximate location at amino acid positions 31-35, 50-65, and 95-102)
and three within the VL (approximate location at positions 24-34, 50-56, and 89-97) (Wu
and Kabat 1970). Flanking each CDR is a framework region (FR) which is relatively
invariant and maintains the CDRs in the requisite conformation for binding antigen.
Thus, if one aligns the sequences of heavy or light chains from a variety of antibodies,
one finds tremendous variability between all of the chains at the amino acid positions of
the CDRs, each reflecting the uniqueness of its own binding site for antigen.

In summary, a number of mechanisms have evolved to diversify the primary Ig
repertoire. That this is achieved by the rearrangement of variable, diversity (D), and
joining (J) segments of the variable region gene and the association of the variable region
gene with a constant region gene was first described by Tonegawa (1983) (Fig. 2). This
process wherein each B cell randomly splices V, D, and J together such that, throughout
the entire population of B cells, millions of possible antigen binding sites are formed
from relatively few VH gene segments is called somatic recombination. Additionally,
Figure 2. Ig H gene rearrangement. According to the translocon model of Ig gene arrangement, multiple VH gene segments are arranged 5' proximal to the group of multiple DH genes and JH genes with the CH genes at the 3' end. The number of VH, DH, JH, and CH genes is species-dependent. A single VH, DH, JH, and CH gene rearrange and are joined to produce the coding sequence for an individual antibody heavy chain protein. The VH segment encodes the FR1, CDR1, FR2, and CDR2 portion of the heavy chain and the DH and JH encode the CDR3 and FR3 portion. Other molecular processes allow for the insertion or deletion of additional nucleotides during this event but are not depicted in this simple schematic.
each B cell can form an antibody from the association of any of its possible H and L chain combinations (combinatorial association). This random selection of VL and VH further increases the heterogeneity of possible binding sites. Another mechanism of binding site diversification, called gene conversion, is by birds and occurs via non-reciprocal genetic recombination and generally employs a donor pool of V pseudogenes located upstream of the rearranged V gene (McCormack et al. 1991; Reynaud et al. 1987). Finally, the binding site, regardless of how it was generated, may be altered by the process of somatic mutation (Berek and Milstein 1987). This process modifies the sequence by the introduction of point mutations or insertions/deletions (Wilson et al. 1998; Ohlin and Borrebaeck 1998) in CDRs. In mice, these mutations are introduced during clonal proliferation, at an apparent rate of \(~10^{-3}\) base pair (bp)\(^{-1}\) generation\(^{-1}\) (Maizels 1995).

Different species use one or more of these diversification pathways. Humans and mice generate the majority of their antibody diversity through the rearrangement of multiple V(D)J gene segments (Alt et al. 1987; Max 1998). Additional diversity can be generated junctionally and through terminal deoxynucleotidyl transferase additions (Landau et al. 1987). Swine also appear to rely heavily on rearrangement and their Ig sequences are remarkably similar to humans (Butler et al. 1994). The best studied example of gene conversion as a primary mechanism of diversification is the chicken
(Reynaud et al. 1987). Other studies indicate that sheep use untemplated somatic point mutations to diversify light chain genes within the follicles of the ileal Peyer's patch (Reynaud et al. 1991). Rabbits have been shown to use a limited and preferential rearrangement strategy (Knight and Becker 1990) coupled with a combination of gene conversion and untemplated somatic point mutations (Weinstein et al. 1994) to diversify the primary repertoire. Ectothermic vertebrates such as shark (Greenberg et al. 1995; Marchalonis et al. 1993; Shamblott and Litman 1989), Xenopus (Hsu et al. 1989; Schwager et al. 1989), and teleost fishes (Warr 1995) rely on the same strategies used by endotherms to diversify their primary antibody repertoire.

The molecular organization of the Ig heavy chain locus has been reported in various species, from sharks and rays to mice and humans (reviewed in Pilstrom and Bengten 1996). All VH genes to date exhibit remarkable structural and organizational similarities in their coding regions as well as similarities in their non-coding regions (Litman et al. 1983, 1985; Schwager et al. 1989; Matsunaga et al. 1990; Wilson et al. 1991) including regulatory sequences and recombination sequences (Fig. 3). The unique organization of this locus in various taxa has been observed (reviewed in du Pasquier and Flajnik 1998).

In Chondrichthyes a multiclonal organization has been reported, with clusters of (VH-D-D-JH-CH)ₙ and (VH-D-JH-CH)ₙ repeated about 100 times in the genome.
Figure 3. Features common to most vertebrate VH genes.
Common features of Ig VH genes include:

5' promoter region which possesses a conserved octamer consensus sequence ATGCAAT and a TATA box. The only exception to this are the elasmobranchs.

N-terminal leader sequence consisting of an 18-20 amino acid hydrophobic coding region interrupted by an 80-120 base pair intron.

The variable region of approximately 98 codons which encodes 3 FRs and 2 CDRs, with FR1 and FR3 each containing a cysteine required for intra-chain disulfide bonding.

3' recombination signal sequences (RSS) responsible for recombination (VH-D joining) consisting of a heptamer motif, 22 or 23 bp spacer, and a nonomer motif.
All evidence suggests that rearrangement occurs within and not between these clusters, which has led some investigators to propose that diversity generated through somatic mutation preceded diversity obtained by combinatorial association of gene segments (Hinds-Frey et al. 1993). Teleosts have their H-chain encoding sequences organized in the translocon fashion (from which individual D. and J genes must rearrange to produce one V(D)J segment) and during which the D and J segments fuse to create the CDR3 and, thus, its uniquely high degree of variability. This translocon organization of Ig genes is a feature shared by teleosts, amphibians, and mammals, although each of these taxonomic classes possess varying numbers of V, D, J, and C genes (reviewed in du Pasquier and Flajnik 1998) (Fig. 4).

The two teleost species whose VH repertoires have been most extensively characterized are the rainbow trout (Oncorhynchus mykiss) and channel catfish (Ictalurus punctatus). These species have more than 100 VH genes as determined from Southern blot analysis (Andersson and Matsunaga 1995; Ghaffari and Lobb 1991; Roman and Charlemagne 1994; Ventura-Holman et al. 1994; Warr et al. 1991). The VH genes within a species are usually grouped into families of related sequences where a family is defined as sharing at least 80% nucleotide identity (Brodeur and Riblet 1984). In humans there are seven VH gene families (Matsuda et al. 1993; van Dijk et al. 1993), in the mouse fifteen (Tutter et al. 1991; Tutter and Riblet 1989), in rainbow trout at least eleven
Figure 4. Organization of Ig genes in various phylogenetic classes. Illustrated is the multicluster organization of the Elasmobranchs, compared to the translocon organization of Teleosts, Amphibians, and Mammals.
(Roman et al. 1996) and in channel catfish six VH gene families have been described (Ghaffari and Lobb 1991; Ventura-Holman et al. 1994; Warr et al. 1991). The similarity in the numbers of VH families among these phylogenetically distant groups (teleosts and mammals) is striking - why should fish have as many VH gene families as higher vertebrates to mediate what has been described as a restricted antibody response? Other teleosts have also been studied in this respect although less thoroughly. Atlantic cod (Gadus morhua) has at least three families with 10-20 members of each family (Pilstrom et al. 1996). The goldfish (Carassius auratus) has also at least three different families (Wilson et al. 1991) and the ladyfish (Elops saurus) has at least two families (Amemiya and Litman 1990).

**VH Gene Utilization During an Antigen-Specific Immune Response**

The generation of an effective antibody response, even with somatic mutational modifications is highly contingent on the germline composition of the VH complex. VH gene identification has been instrumental in the elucidation of the requirements for prophylactic immune responses (Kofler et al. 1992, Chung et al. 1995, Leclerq et al. 1997, Sun et al. 1999, Toran et al. 1999), the mechanisms of antibody-mediated immunopathology (Bona 1988, Spatz et al. 1990, Hoch and Schwaber 1996), and the ontological acquisition of antibody specificities (Perlmutter et al. 1985, Alt et al. 1987,
Jeong and Teale 1988). With respect to prophylactic immunity, for example, the use of a single human germline VH gene (DP50) appears to be responsible for generating the highest affinity antibodies to a critical antigen of HIV, gp 120 (Toran et al. 1999). These antibodies are thought to be responsible for long-term survival and protection against this virulent pathogen. Another important feature of VH genes is the role different allelic forms, or polymorphisms, can play in determining resistance or susceptibility to disease. Leclerq et al. (1997) demonstrated, through the use of isogenic and congenic mice, that the ability to produce a neutralizing antibody to the poliovirus capsid protein epitope (C3) is linked to loci within the VH complex. In these studies, mice bearing specific VH-associated haplotypes were unable to produce antibodies which could effectively bind this epitope and neutralize the virus. Thus, only certain alleles expressed at this locus can result in prophylactic antibodies, while others are ineffectual to the same pathogen. This polymorphic effect is likely to be of even more importance to a fish such as the rainbow trout, in which the antibody repertoire is known to be more limited than that of mice or humans (Wetzel and Charlemagne 1985, Cossarini-Dunier et al. 1986, Desvaux et al. 1987, Hastings and Ellis 1988).

These findings have serious implications for the development of fish vaccines. It is possible that certain vaccines, particularly subunit vaccines (Dertzbaugh 1998), may not be capable of effectively immunizing large, genetically diverse populations of fish if
the VH repertoire is small. If this proves to be the case, a more efficacious method of
vaccine development would be to first identify the specific VH alleles which can be
associated with a particular disease resistance and fashion probes for these VHs to
ascertain which populations could conceivably benefit from specific vaccines.
Additionally, such probes could be enormously valuable genetic markers to be used in the
breeding and development of disease resistant stocks.

The VH germline repertoire is also important in providing a unique substrate
from which certain specific mutations are possible. Such can be seen with the response
to an epitope, phosphorylcholine (PC), which relies on a very restricted VH family.
Phosphorylcholine is a primary epitope found in the polysaccharide antigens of a wide
variety of pathogens including *Streptococcus* (Bennett and Bishop 1977; Jennings et al.
1980; Claflin and Berry 1988), *Proteus* (Claflin et al. 1987), fungi, and parasitic
nematodes (Pery et al. 1979; Choy et al. 1991). Interestingly, in the mouse, antibody to
this critical epitope initially employs one gene family ($S107$) which contains only one
VH gene (Crews et al. 1981). Although the VH repertoire appears limited, anti-PC
antibodies can be the primary protection against diseases such as bacterial pneumonia
(Chen et al. 1995). However, considering the restricted repertoire, it would stand to
reason that any allelic polymorphism akin to that discussed above could result in global
differences in antibody reactivity to a variety of pathogens. This might be the case, were
it not for the process of somatic mutation which expands the antibody repertoire, often using this single gene as the substrate for the mutational process. As this is a genetically simple system, it has provided deep insights to the power of somatic mutation in molding the immune response capacity of an animal (Stenzel-Poore and Rittenberg 1989; Chen et al. 1995). Rittenberg and co-workers (1989) have elegantly demonstrated that although the early murine response is dominated by the progeny of the S107 VH gene, the eventual induction of mutations within an alternate lower affinity VH germline gene results in an even more aptly suited, high affinity antibodies late in the response. These progeny of low affinity progenitors eventually come to dominate the anti-PC response through antigen-driven selection via their newly mutated, high affinity receptors. Precise knowledge of the germline VH repertoire, thus, also becomes essential in determining if and when somatic mutation occurs and within which VH genes.

The developmental acquisition of antigen specificities has also been found to be contingent on VH gene family position and composition and specific VH gene family utilization against a number of antigenic epitopes have been identified in the murine model (Table I) (Crews et al. 1981; Fougereau et al. 1984; Dzierzak et al. 1985; Minoprio et al. 1989; Clarke et al. 1990a, b; Victor-Kobrin et al. 1990; Miller et al. 1996; Zinkernagel and Hengartner 1996; Clemons et al. 1998). Both in mice (Perlmetter et al. 1985; Alt et al. 1987; Jeong and Teale 1988) and humans (Schroeder and Wang 1990;
Table I. Antigen specificities which have been associated with specific VH gene family utilization in the murine model.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>VH family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylcholine</td>
<td>S107</td>
</tr>
<tr>
<td>β-2, 6 fructosan</td>
<td>X24</td>
</tr>
<tr>
<td>α-1, 3 dextran</td>
<td>J558</td>
</tr>
<tr>
<td><em>S. dysenteriae</em> O antigen</td>
<td>36-60</td>
</tr>
<tr>
<td><em>T. cruzi</em></td>
<td>7183, S107</td>
</tr>
<tr>
<td>Vesicular Stomatitis Virus</td>
<td>Q52</td>
</tr>
<tr>
<td>(primary response)</td>
<td></td>
</tr>
<tr>
<td>Vesicular Stomatitis Virus</td>
<td>Q52, 7183,</td>
</tr>
<tr>
<td>(secondary response)</td>
<td>J558</td>
</tr>
<tr>
<td>Influenza hemagglutinin</td>
<td>36-60</td>
</tr>
<tr>
<td>Trinitrophenyl (TNP)</td>
<td>36-60</td>
</tr>
<tr>
<td>GAT copolymer</td>
<td>J558</td>
</tr>
</tbody>
</table>
Gruwunder et al. 1995), the ability to employ specific VH genes is restricted ontologically to specific VH families. Studies thus far have indicated that murine VH families that are more DH proximal are incorporated earlier within the expressed antibody. Again, this phenomenon will likely have important implications for salmonid diseases such as IHNV where disease susceptibility is greatest in fry or young fish (Evelyn 1997). This susceptibility is not due to a general lack of immunocompetency as antibodies can even be produced in yolk sac fry (Castillo et al. 1993). Therefore, it is possible that IHNV susceptibility may be due to the selective early developmental acquisition of VH specificities which are non-essential to the anti-IHNV response.

Unfortunately, the current status of our knowledge of the trout VH genes and their encoded specificities is woefully insufficient to be of benefit to the aquaculture industry. Of critical relevance, no studies, to date, have attempted to determine the antigen specificity encoded by trout VH genes (Roman and Charlemagne 1994; Andersson and Matsunaga 1995; Roman et al. 1996; Widholm et al. 1999). All cloning and sequencing efforts have used primers derived from various consensus sequences, without regard to their probable specificity, and they are often based on sequences from phylogenetically distant species.
The Mechanism of Somatic Hypermutation

Although the process of somatic mutation clearly contributes to the generation of diversity of antibody structure, the actual mechanism has as yet to be described. In fact, there may be any number of enzymatic systems capable of introducing mutations. A system with G-C base pair preference that introduced mutations at a low frequency (10^{-3}) in a rearranged μ gene under the control of a 3’ cis-acting enhancer has been reported (Bachl and Wabl 1996). However, this mechanism would not account for the observed bias to replace purines or pyrimidines with purines (Neuberger and Milstein 1995; Dörner et al. 1997) or to explain the full spectrum of mutations noted. Two other models suggest either (1) a mechanism with strand polarity that targets random base pairs for mutations to a purine or (2) a mechanism that targets purines on both the sense and antisense strands. The first would better support the finding that both purine transitions and pyrimidine tranversions are favored. Indeed, the evidence cited in the literature is more consistent with a mutational mechanism that exhibits strand polarity (Both et al. 1990; Yélamos et al. 1995; Peters and Storb 1996). A reverse transcriptase (RT)-based mechanism was proposed by Steele and Pollard in 1987. The basic tenet of this model (the RT-mutatorsome model) is that the mRNA of the rearranged V(D)J is reverse transcribed in an error-prone manner to cDNA and then recombined homologously into the genome. This contrasts with the previously described alternative mechanisms which
are classed as DNA-based mechanisms. Steele et al. (1992) argues that a model involving reverse transcription best explains the experimental evidence of somatic mutation without the ad hoc assumptions which predicate the DNA-based mechanisms.

One impediment to the identification of the mechanism of somatic hypermutation has been the lack of an in vitro system to study this process. Recently, however, it has become possible to initiate somatic mutation in vitro by activating a Burkitt’s lymphoma cell line through cross-linking of the receptors. By co-culturing these cells with an activated T cell line, B cells will proliferate and accumulate somatic mutations (Källberg et al. 1996). Another strategy, described by Razanajaona and colleagues (1997), involves the co-culture of naive tonsillar B cells and activated T cells which also has been shown to induce in vitro triggering of somatic mutation. Another strategy to investigate the molecular mechanism underlying somatic mutation has been the analysis of somatic mutation in transgenic and transfected target genes (Storb et al. 1996).

**Essential Features of Somatically Mutated V(D)J**

Although somatic mutation has been identified in the horned shark, *Heterodontus franciscii* (Hinds-Frey et al. 1993) and *Xenopus laevis* (Wilson et al. 1992, 1995), basic research into this process as it occurs in lower vertebrates has not been investigated to the extent that it has been followed in mammals. In fact, many of the observed somatic
mutation patterns observed in ectothermic vertebrates contrast the patterns observed in mammals (Wilson et al. 1995). As summarized by Steele et al. (1997), there are essential features of somatically mutated V(D)J which have been identified in mouse and man (Table II).

These essential features, it must be emphasized, have been identified in warm-blooded vertebrate animal models and when common features of somatic mutation in cold-blooded vertebrates are considered a number of differences may occur. A survey of somatic mutational changes observed in *Xenopus* and horned shark reveal a striking preference for GC compared to AT bases, with a preference for transitions over transversions (du Pasquier and Robert 1992). *Xenopus* Ig genes, like their mammalian homologues, contain sequence motifs (A/G G C/T A/T) reported to target the mutational machinery (Schwager et al. 1989; Wagner and Neuberger 1996) (discussed in detail next section).

**Intrinsic vs. Antigen-Selected Mutational Hotspots**

Studies on the mechanism(s) of somatic mutation have been complicated by the fact that only those mutations that lead to high affinity antibodies will be selectively expanded upon interaction with antigen. This skews the profiles of the original or unselected/unbiased, mutated genes. To examine these original, unbiased mutations,
Table II. Features of somatically mutated V(D)J.

Unrearranged V elements do not undergo somatic mutation.

Isotype class switching is not required for somatic mutation to occur, although more mutations have been observed in V(D)J expressed as IgG or IgA.

The 5' boundary for somatic mutation is located at or near the transcription start site for VH and in the leader-VL intron for VL.

The 3' boundary for somatic mutation for VH and VL terminates in the J-C intron near the intron-enhancer/matrix attachment region (Ei/MAR).

Sequences in the J-C intron near the Ei/MAR appear necessary for somatic mutation to occur.

V segments in the V(D)J can be replaced by non-Ig DNA and still hypermutate.

The somatic point mutation rate is $10^{-3}$ bp$^{-1}$ generation$^{-1}$ with a frequency of 1-5% in the V(D)J target region: up to 1 nucleotide per 20 can be mutated.

Mutational bias is for transitions over transversions in the murine model.
without the influence of antigen-directed selection, non-productively transgenes were analyzed (Dörner et al. 1997). With the nonproductive rearrangements, antigen selection does not contribute to the proliferation of somatically mutated B cells, as the antibodies are not expressed on the lymphocyte surface. The results of these studies and others, including those of Betz et al. (1993, 1994) and Neuberger and Milstein (1995) clearly indicate the presence of commonly shared mutational hotspots. These hotspots are not at a conserved distance from the transcriptional start site, and seem to be a consequence of local DNA sequence. Many of these hotspots occur in serine AGC/T triplets, whether they are in frame or not. Serine AGC/T codons are almost exclusively targeted for somatic mutation in comparison to the more commonly occurring TCN serine codons (where N is any nucleotide) (Neuberger and Milstein 1995). One of the most commonly cited examples of this, by Betz et al. (1993), is the VkOx-1 Ser31 hotspot at the tip of a potential stem-loop structure TGTAA>G<TTACA. The involvement of secondary structure considerations such as this have also been reported by Golding et al. (1987) in which nearby direct repeats or palindromic sequences have been observed which could serve as potential templates for alteration.

The second base of the AGC/T codon has been seen to mutate to any of the other nucleotides, A/C/T, in a manner consistent with a ratio predicted by intrinsic substitution preferences (Betz et al. 1994). To contrast this with a commonly described antigen-
selected hotspot, the His34 (CAC) of VkOx-1 is characteristically mutated by
transversion to Asp (GAC). As previously noted, intrinsic point mutations show a bias
towards transition substitutions. Antigen-selected hotspots can generally be
discriminated from intrinsic hotspots by their tendency towards a mutation (Betz 1993)
which leads to a structurally improved area of contact for subsequent binding of antigen
and, thus, the selection and expansion of cells possessing this mutation when exposed to
this relevant antigen. Per the observations of Betz and others, CAGCT and AAGTT are
sequence motifs which are characteristic of intrinsic targets. Independent of these
findings by Betz, Rogozin et al. (1992) performed a statistical analysis of the sequences
of hypermutated antibodies in which they neither restricted the analysis to major hotspots
nor excluded the effects of antigen-selected mutations and found that the sequences TAA
and RGWY (R=purine, Y=pyrimidine, and W=A or T) are preferred sites of mutation.
The Betz sequences are more specific but both lie within the RGWY consensus sequence.
Most recently, Dörner et al. (1998) refuted the contention that the somatic mutational
machinery exhibits DNA strand polarity as analyses of the mutation rate of the inverse
repeat of RGWY, WRCY (and the inverse repeat of other mutational hotspot sequences)
revealed a similar mutation frequency.
The Role of Somatic Mutation in Affinity Maturation

The specificity of the antibody response to a pathogen ultimately determines the effectiveness of the response and, in many instances, whether an animal will be protected or succumb to the disease. As such, an antibody response, even a single antibody’s specificity, or affinity, for the specific antigen cannot remain a static feature. The expressed affinity of antibodies induced during an immune response is the product of a dynamic process termed affinity maturation (Eisen and Siskind 1964; Berek et al. 1985). Affinity maturation refers to the increase in overall affinity of serum antibodies in response to antigen over time. There are two distinct yet interactive processes which lead to the development of affinity maturation. First, upon exposure to antigen, B lymphocytes which have bound sufficient antigen via their surface Ig receptors are triggered to proliferate and secrete antibodies, possessing the same binding site as that expressed by the surface immunoglobulin (Burnet 1969; Siskind and Benacerraf 1969). During the immune response the initial \textit{in vivo} antigen concentration is relatively high and thus stimulates B lymphocytes possessing both high and low affinity antibody receptors. However, as the concentration of antigen declines over time, B cells with higher affinity receptors will be selectively stimulated as the low affinity B cells will be unable to bind sufficient antigen to trigger a proliferative response (Goidl et al. 1968).

This process is fundamentally antigen-driven. However, somatic mutation can
greatly enhance affinity maturation. In the most extensively studied species (mouse), it has been observed that towards the end of the primary antibody response, affinity increases surpass those observed during the primary response (Griffiths et al. 1984; Kocks and Rajewsky 1988). These increases are attributed to the accumulation of somatic mutations within the genes encoding the V regions of the antibody genes of activated B cells. Few mutations actually lead to a structural change in the binding site which generates an antigen receptor with increased affinity. However, when this occurs these higher affinity variants have a selective advantage in being triggered by antigen (Wysocki et al. 1986). Studies using syngeneic mouse strains, coupled with the use of hybridomas have elegantly demonstrated this phenomenon (Berek and Milstein 1987). In these studies the investigators primed and challenged mice with 2-phenyloxazolone and at various points during the response sacrificed individuals and fused the lymphocytes to produce hybridomas. These hybridomas provided the opportunity to analyze individual VH sequences responsible for the production of the specific monoclonal antibody. In this way the precise affinity of the monoclonal antibody was determined and could be associated with a specific VH sequence. These studies revealed a number of important features of this process. First, the number of hybridomas expressing specific monoclonal antibodies became skewed over time towards the higher affinity monoclonals. Further, the increases in affinity could be directly related to the appearance of hybridomas
expressing somatic variants. However, as the response matured the number of mutations
detected increased considerably, often in cells which had accrued very specific mutations
early in the response. The use of syngeneic animals and hybridomas has facilitated the
delineation of somatic mutation lineages and their effect on antibody affinity.

Unfortunately, the technology for the production of piscine hybridomas and monoclonal
antibodies does not exist and analysis of antigen-specific B cells in fish must involve
some means of immunopurification in order to derive Ig VH mRNA for subsequent
manipulation and analysis.

Affinity Maturation and Somatic Mutation in Xenopus and Shark

Antibody responses of ectothermic vertebrates do not mature in the same fashion
as responses analyzed in mice. This has been evidenced by either a total lack of affinity
maturation in some species or a lower increase in affinity in others. When it was
determined that all ectotherms studied possess a large number of V(D)J genes, and that
rearrangement processes to establish Ig repertoires are essentially the same as those in
mouse and human, it was theorized that poor immune responses in ectotherms could be
Alternatively, it was posed that somatic mutation arose late in vertebrate evolution, after
emergence of the rearrangement process that generates functional V genes (Matsunaga 1985).

*Xenopus* is the only ectothermic vertebrate in which the development of somatic mutations has been investigated during the course of an antigen-specific immune response (Wilson *et al.* 1995). The V heavy chain gene family involved in the anti-dinitrophenyl (DNP) response was well-known (VH1: Schwager *et al.* 1989) from previous sequencing of anti-DNP antibodies (Brandt *et al.* 1980); thus, one could monitor VH1 gene expression during an ongoing anti-DNP response in isogenic *Xenopus* tadpoles. All VH1 genes (32 in total) present in a single haplotype were sequenced and only a small number of mutations were noted (Wilson *et al.* 1992). On average, 1.6 mutations per gene was observed and there was no strong selection for mutations in the CDR1 and CDR2. Although CDR3 is generally the region most likely to accumulate replacement point mutations (Johnson and Wu 1998), virtually no point mutations were detected in the *Xenopus* CDR3. One theory proposed by Wilson in this study was that while the frequency of somatic mutations was lower than that found in antigen-specific mammalian B cells, quantitative differences would be equalized when one considers overall mutation rates. Taking the generation time of a *Xenopus* lymphoid tumor cell line as the basis of the calculation, the rate of somatic mutation appeared to be quite similar to that found in mammals during an antigen-specific response. Wilson’s observed *Xenopus*
IgM mutation rates of $2.5 \times 10^{-5}$ per bp per generation to $4.1 \times 10^{-5}$ per bp per generation are only four to seven times lower than the highest levels reported in hyperimmunized mice (du Pasquier and Robert 1992).

What, then, accounts for the relatively poor affinity maturation of the ectothermic vertebrate antibody response as compared to that observed in mammals? Although some differences in the essential features of somatic mutation are observed in ectothermic vertebrates, the primary difference may involve the selection process. It has been suggested (du Pasquier 1982; Wilson et al. 1992) that mutated B cells are poorly selected in the lymphoid organs of *Xenopus* and other ectothermic vertebrates because germinal centers (GCs), well known for their role in orchestrating the generation and selection of mutants in mouse and human (MacLennan 1994) are apparently absent in these species (Zapata et al. 1995). If true then *Xenopus* and horned shark somatic variants would likely bear the hallmarks of this poor selection. One strong indication that this might be the case was the observation that in spite of some of the clustering of the mutations in the CDRs, the rate of replacement to silent mutations (R:S) in *Xenopus* and horned shark Ig were not increased significantly in CDR over FR. However, in (non-immunized) rainbow trout the analysis of VH gene diversity in cDNA clones has shown that both replacement and silent mutations occur more frequently in CDRs (Matsunaga and Andersson 1997).
It is difficult to reconcile how germline V products can be selected in a specific primary response lacking a physical microenvironment such as that which occurs in GCs, but selection of *Xenopus* B cells appears obvious as a specific immune response can be engendered. This is evidenced by the differences observed in the *Xenopus* primary and secondary immune response. If the lack of a selective environment (i.e. germinal center) inhibits the recruitment of somatic mutants, why do Ig genes in *Xenopus* and horned shark (and by extension, all ectotherms) B cells mutate at all in the course of an antigen-specific response? One possibility is that the mutational mechanism originally arose in evolution to modify Ig genes that did not rearrange (du Pasquier 1982; Wagner and Neuberger 1996). In certain cases, such as with sheep, somatic mutation may simply be a mechanism by which the primary repertoire can be expanded, even from a single VL gene (Reynaud *et al.* 1991). Thus, in mature frog and shark B cells undergoing an antigen-specific response, the mutational mechanism may be expressed neotenically (in an evolutionary sense) perhaps in a similar fashion to re-expression of RAG genes in mouse germinal center B cells (Han *et al.* 1996). It is possible that the generation of mutants, in the absence of an efficient selection mechanism, may still provide animals with an expanded and potentially useful repertoire.

Indeed, if germinal centers play such a key role in selection, then GC-deficient mammals should not be capable of affinity maturation. However, this is not the case.
when examined experimentally. Two different GC-deficient murine models have been identified: lymphotoxin a (Lta) and CD19 knock-out (KO) mice. That these KO mice are GC-deficient was happenstance and not the original motivation for their production. In the Lta KO (Matsumoto et al. 1996) it was suggested that antibody affinity maturation occurs without germinal centers as the immunized mice had the same somatic mutations that are typically detected in responses of wild-type mice. and R:S ratios seemed, according to the authors, to indicate a selection of mutants in the CDR. However, given the number of mutants analyzed in these mice, the R:S ratio is not significantly different from that expected without selection (4.9 vs. 7.7, p > 0.3). Moreover, affinity maturation was estimated neither by equilibrium dialysis nor a step-wise titration of an inhibition of the antigen-antibody reaction. Instead, a rather imprecise estimation of low or high affinity was based on a four-fold level of hapten substitution of the test antigen only. In other words, the GC-deficient Lta KO model may have indeed recapitulated the situation in ectotherms. In contrast to the Lta KO mouse, the CD19 KO also lacked germinal centers but showed no evidence of affinity maturation (Rickert et al. 1995). Analysis of the Lta KO and wild-type mouse somatic mutations revealed that 65 out of 110 mutations (59%) affected a G or C in the wild-type mouse, whereas in the KO mouse the ratio was 32/41 (72%). This is a significant trend towards GC bias and is consistent with the somatic mutation patterns seen in ectothermic vertebrates. Thus, the GC bias that is not
generally observed in mammals may be a function of poor selection and bears additional investigation.

Perhaps trout do possess a localized microenvironment capable of selecting B cells possessing Ig receptors with increased affinity. A unique feature of piscine lymphomeloid tissue is the presence of melanomacrophage centers in the kidney, spleen, and liver (Kennedy-Stoskopf 1992). These melanomacrophage centers may be the forerunners of the germinal centers present in the spleen and lymph nodes of birds and mammals (Agius 1985). Both melanomacrophage centers and germinal centers are aggregates of reticular cells, lymphocytes, macrophages, and plasma cells. Espenes et al. (1995a) has suggested that ellipsoids found in the anterior kidney and spleen of rainbow trout may contribute to the processing of trapped immune complexes in these fish and others. Ultrastructural studies in trout (Espenes et al. 1995a) and enzyme histochemical studies in Atlantic salmon (Press et al. 1994) have demonstrated that salmonid ellipsoids contain macrophages that are active in phagocytosis of foreign particles. The observed immune-complex trapping in immunized trout suggests a specific role for splenic ellipsoids in rapid delivery of antigen to the peri-ellipsoidal leukocyte populations in a secondary immune response (Espenes et al. 1995b). Studies have indicated that the salmonid spleen contains a mature and heterogeneous B cell population (Irwin and Kaattari 1986) and a recirculating T cell population (Tatner and Findlay 1991). Efficient
delivery of antigen to these cells may contribute to the increased sensitivity to antigen observed in primed rainbow trout. However, further studies are needed to determine the contribution of immune-complex trapping in ellipsoids to the propagation and regulation of the piscine immune response.

**Affinity Maturation and Somatic Mutation in Teleost Fish**

Little work has been forthcoming on affinity maturation or somatic mutation in fish. Studies in our laboratory have demonstrated that the antibody response to TNP can increase over 10-fold (Kaattari and Shapiro 1994; Shapiro 1995; Shapiro *et al.* 1996; Khor 1996). Using a solid phase, affinity-based method (described below), not only can the average affinity of the serum antibodies be determined, but the development of specific high affinity subpopulations can be distinguished and quantified. This analysis revealed the emergence of new, high affinity subpopulations late in the immune response (Khor 1996, Fig. 5) This is in contrast to certain other studies in the catfish (Lobb, 1985), Atlantic salmon (Killie *et al.* 1991) and Pacific salmon (Voss *et al.* 1978). Unfortunately, in these latter studies, serum affinities were first determined 2 - 3 months post immunization. These relatively late sera were subsequently compared to even later sera to determine if affinity maturation had occurred. Studies performed by Ing Wei Khor, Haili Zhang, and others in our laboratory and in other laboratories (Cain *et al.* 2000;
Figure 5. Comparison of anti-TNP titers with average affinity sub-population values.

A. Mean antibody titer against TNP (units of activity μl⁻¹ plasma) is shown to increase between week 5 and 10 post primary immunization, then maintain at the same level at week 20 and start to decrease at week 25. B. While TNP titers begin to decrease at week 25 post primary immunization, the mean weighted average affinity (aK) is maintained at the same level at week 25 (Khor 1996).
Solem et al. 2000) have indicated that substantial affinity maturation does, in fact, occur between 5 and 10 weeks post primary immunization. Therefore, the earlier studies may have been attempting to detect an antibody affinity shift after it had already occurred. Our studies have revealed that the affinities measured by these investigators (\(K_a = 4.3 \times 10^5 \text{ M}^{-1}\) to \(8.4 \times 10^5 \text{ M}^{-1}\)) are not unlike average affinities which we observed at approximately the same time point, however we observe that \(K_a\) values prior to this time are considerably below \(1.0 \times 10^5 \text{ M}^{-1}\).

As a rule, little affinity maturation has been detected in fish, although some changes in fine specificities have been noted in trout (Arkoosh and Kaattari 1991). The diversity of binding sites has been addressed by a number of methods such as the use of Scatchard analysis of whole serum antibodies (Shankey and Clem 1980), relative specificity analysis (Makela and Litman 1980), IEF of immunopurified antibodies (Cossarini-Dunier et al. 1986; Wetzel and Charlemagne 1985) and anti-idiotypic analysis (Richter and Ambrosius 1988). In all cases the studies have indicated that little heterogeneity of binding sites exists among the fish species. However, limited heterogeneity is also true for mammalian IgM (Griffiths et al. 1984). These observations led to the intriguing speculation that the need for the expression of monomeric Ig may have led to the development of mechanisms to produce binding sites with high intrinsic affinity and perhaps a greater repertoire of binding sites. Supportive of this hypothesis is
the observation that sharks, which possess a monomeric form of Ig, appear to develop the same high intrinsic affinity in this class of Ig as do their monomeric mammalian counterparts (Voss and Sigel 1972).

**Affinity Maturation and Development of an Effective Anti-Viral Response**

Protection against various viral diseases has been directly associated with the acquisition of high affinity antibodies to viral epitopes (Steward 1981; Steward et al. 1991; Brostrom et al. 1995; Chargelegeue et al. 1995). Of particular relevance to this study, which deals with a fish rhabdovirus (IHNV), is the dependence on mutationally-derived high affinity antibodies to vesicular stomatitis virus (VSV) (Kalinke et al. 1996) and rabies virus (Ikematsu et al. 1993). Such processes have also been noted in influenza responses (Clarke et al. 1990a, 1990b) and responses to hepatitis B virus (Andris et al. 1992). The requirement for high affinity antibodies to halt disease progression has been observed during human immunodeficiency virus (HIV) infections. Patients who demonstrate a marked progression of the disease lose high affinity antibodies to a critical neutralizing epitope of gp120. This occurs even though the overall antibody titer remains unchanged. Some investigators have postulated that this loss of high affinity antibodies could be due to a helper T cell dysfunction (Chargelegeue et al. 1995). The rapid generation of high affinity antibodies to cytomegalovirus in humans has also been shown...
to be impaired when patients are exposed to exogenous immunosuppressive therapy, indicating the critical role of T cells in the development of high affinity anti-viral antibodies (Lutz et al. 1994). Impairment of T helper cell (CD4) interactions with B cells when responding to antigen also leads to inability to undergo somatic mutation and express high affinity antibodies (Lane et al. 1994). Also, although severe DiGeorge syndrome patients exhibit comparable use of the germline VH genes as in normal individuals, they exhibit a lack of somatic mutation, implicating the need for T cells in this process (Haire et al. 1993). These studies emphasize that affinity maturation is a complex process that is highly contingent upon the T-dependency of the antigen. Thus, in order to provide the highest affinity antibodies, vaccines must not only provide B cell epitopes, but epitopes which will elicit strong T helper cell function which is necessary for somatic mutation. The ability to monitor the induction of somatic mutations and affinity maturation should, therefore provide vaccinologists with important tools to assess the capabilities of their vaccines. This is a very fertile area of comparative immunogenetics research, for which the trout model is most aptly suited.

The choice of IHNV as an immunogen for the current study arises from the large body of research characterizing fish immune responsiveness to this viral pathogen. IHNV is an aquatic rhabdovirus responsible for epizootics in wild and hatchery-reared salmonids (Groberg and Fryer 1983). It has recently been assigned a new genus

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(Novirhabdovirus) within the virus family Rhabdoviridae as it possesses a sixth viral
gene (the non-virion protein gene) which is not found in many other rhabdovirus
genomes (Kurath et al. 2000). This virus causes extensive necrosis of the blood-forming
tissues of kidney and spleen (Winton et al. 1988) and results in high mortality in juvenile
and yearling fish. The viral genome is 11,131 nucleotides in length and encodes six
structural proteins, including the viral envelope glycoprotein (Morzunov et al. 1995). A
cDNA of the IHNV glycoprotein has been cloned which is 1,609 bp in length and
encodes a protein 508 amino acids long (Koener et al. 1987). This glycoprotein is known
to elicit a neutralizing antibody response in rainbow trout (Engelking and Leong 1989).
RATIONALE AND OBJECTIVES

Recent experimental evidence on affinity maturation suggests that antigen specific somatic mutation may occur in teleost species. Further, the generation of somatic mutants has been observed in other lower vertebrates such as *Xenopus* and horned shark, however, these variants have not been correlated to the induction of a specific immune response. The development of affinity maturation and its promotion through the selection of somatic mutations during the immune response is a key factor in the development of a specific antibody response. Thus, it was the intent of this project to (1) assess the development of somatic mutation in trout during the antibody response against two different antigens and (2) ascertain their role in the affinity maturation of trout antibodies. Two dissimilar antigens, TNP and a synthetic 20-mer peptide epitope of IHNV glycoprotein, were used to assess the specificity of induction of the somatic mutational process.

Previous experiments (Khor 1996; Zhang 1999) demonstrated the induction of high affinity antibody variants to TNP during the late antibody response to TNP-KLH. Whether or not these were produced by somatic mutation had yet to be established. IHNV is a devastating pathogen in salmonid populations and may quickly evolve resistance to an antibody response by the selection of glycoprotein variants within immune fish (Winton et al. 1988). In response to this evolution of the virus, the host
itself must demonstrate plasticity in its ability to generate a neutralizing antibody response. Thus, somatic mutation could be critical to this counter-evolution of the immune response in the host. Based on these observations, the use of both antigens in this study would provide insight about the molecular processes occurring during the development of a piscine immune response.

In order to examine this process, individual trout with the highest antibody affinity shift response from each immunization group were identified by serological methods. cDNA sequences were then obtained from these individual responders to examine the antibody (H chain) response on the molecular level over time. This was done to limit the expense of obtaining cDNA sequences (ideally, 10 per each time point per individual). As the characterization of an antigen-specific immune response correlating serologic and molecular results had never before been attempted in a teleost species, any evidence supporting the occurrence of somatic mutation would be considered sufficient / informative for the purpose of this study.

Thus, the objectives of this study may be summarized as follows:

- Immunize trout with TNP-KLH or IHNV glycoprotein peptide conjugated to BSA and identify antibody affinity shift responders by ELISA. Include as a control trout immunized with PBS to identify possible adjuvant effects.
• Immunoselect high affinity B cells from hyperimmune fish during the time course of the experiment.

• Procure cDNA sequences and identify any somatic mutations occurring during the generation of the specific immune response.

• Correlate molecular results with serological results to determine whether antigen specific somatic mutations occur and accumulate in the trout B cell repertoire.
MATERIALS AND METHODS

Animals

Fish in these experiments were between 250-500 g and 1+ years prior to immunization and sampling. They were maintained in 370 or 1500 L tanks with recirculating systems supplied with biological and UV-filtered well-water. Freshwater exchange was approximately 2% per day with 75% of the volume recirculated through the filter system every hour. Water temperature was maintained at 12° C and photoperiod adjusted to match seasonal changes. Fish were fed a dry pellet diet (Trout Grower High Fat, Zeigler Brothers, Gardners, PA) and the water quality monitored daily for pH, alkalinity, ammonia, and nitrite levels. For identification purposes, each trout was implanted with a 12 mm passive radio-frequency identification tag (PIT tag) (Biomark, Boise, ID) which transmits a unique identification number for each animal (Table III). The PIT tag was implanted with a hand-held injection device which delivers the tag to the inter-muscular area centered approximately 1 cm below the dorsal fin.

Antigen Preparation

TNP Preparations

Trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) was prepared as
Table III. Identification of rainbow trout used in experiment.

<table>
<thead>
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<td>414C73642E</td>
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</tr>
<tr>
<td>918</td>
<td>(none - from H. Zhang study)</td>
<td>TNP-KLH</td>
</tr>
</tbody>
</table>
described by Rittenberg and Amkraut (1966) for use as an immunogen (Fig. 6A).

Briefly, 5 ml of a 15 mg ml\(^{-1}\) solution of KLH (Sigma, St. Louis, MO) was dialyzed against three changes of 0.28 M sodium cacodylate buffer. After dialysis the KLH solution was stored in a foil-covered tube. Trinitrophenylation of KLH was accomplished by dissolving 16.2 picrylsulfonic acid (Sigma) in 1.5 ml of cacodylate buffer. This solution was added dropwise to the KLH and the solution was mixed on a rotator-mixer for 1 h at room temperature. The solution was dialyzed against four one liter exchanges of 0.7 M phosphate buffered saline (PBS), pH 7.2. Once produced, the TNP-KLH conjugate was analyzed spectrophotometrically to determine the haptenization ratio of TNP:KLH monomer (Garvey et al. 1977). The TNP-KLH prepared for these experiments was determined to be approximately 75 TNP per 400,000 Da monomer (373 TNP per KLH). After preparation, the solution was filter-sterilized using a 0.22 μm filter (Pall Gelman Laboratory, Ann Arbor, MI) and stored in a sterile stoppered serum bottle at 4°C pending use. Trinitrophenylated-bovine serum albumin (TNP-BSA) was prepared essentially in the same manner as TNP-KLH, however, the haptenation ratio was 8 TNP per BSA molecule. TNP-BSA was used as the plate coating antigen for the ELISAs of plasma anti-TNP titration and affinity analysis.
Figure 6. Antigens used to immunize trout. (A) TNP-KLH with a haptenation ratio of 373 TNP per KLH. (B) A synthetic 20-mer protein epitope of IHNV glycoprotein (ASESREECLEAHAEIISTNS) (IHNV gp peptide-BSA) with a ratio of 40:1 peptide per BSA (Genosys, Woodlands, TX).
A. haptenation ratio 373:1 (TNP:KLH)

B. conjugation ratio 40:1 (peptide:BSA)
Alternate Production of IHNV Glycoprotein Antigen

In order to assess the trout immune response to a single IHNV gp epitope, a 20-mer peptide was synthesized (Genosys, Woodlands, TX) based on epitope analysis performed by Mourich and Leong (1991). These investigators mapped the immunogenic regions of the IHNV gp using trpE fusion proteins, containing various regions of the IHNV gp gene, expressed in Escherichia coli. Although multiple regions of the glycoprotein stimulated a response in the mouse, only one of the glycoprotein regions and more specifically the 20-mer peptide region located at gp amino acid residues 321 to 340 (ASESREECLEAHAEIISTNS) stimulated a measurable antibody response in trout. This synthetic peptide was received lyophilized (conjugated to BSA) and was reconstituted in PBS, pH 7.2, and filter sterilized (0.22 µm) prior to use for primary immunization of trout (Fig. 6B).

IHNV Purification

IHNV (Hagerman Valley isolate, strain 039-82) was obtained from Dr. Sandra Ristow of Washington State University and propagated in the chinook salmon embryo (CHSE-214; American Type Culture Collection cell repository line CRL 181) cell line as described by Leong et al. (1981). In brief, CHSE cells were grown to confluency in

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tissue culture flasks in RPMI-1640 medium supplemented with L-glutamine, sodium bicarbonate, 10% fetal bovine serum (FBS: Life Technologies, Inc.) and antibiotics (penicillin 100 U ml\(^{-1}\), streptomycin 100 \(\mu\)g ml\(^{-1}\)) (media and supplements from Sigma unless otherwise noted). Culture media was removed by pipetting and the monolayer coincubated with IHNV for one hour with gentle rocking on a platform (Bel-Art. Pequannock, NJ). After one hour the medium containing free virus was removed by pipetting and replaced with fresh RPMI medium supplemented as above, with 2% FBS. Cells were incubated at 17°C in ambient air conditions until the observed viral cytopathic effect (CPE) was 100%. To purify IHNV for subsequent use as an antigen, IHNV infected cells and supernatant were harvested and cellular debris removed by low speed centrifugation. The supernatant from this procedure was centrifuged in a Beckman SW 28 rotor onto a glycerol cushion for 90 min at 90,000 x \(g\). Virus pellets were resuspended in STE buffer, pH 7.0, and centrifuged through a discontinuous sucrose gradient comprised of 50%, 35%, and 20% sucrose layers (sucrose diluted in STE) as above. The purified virus was retrieved from the 20% and 35% sucrose interface after centrifugation. This layer was removed and transferred onto a continuous gradient of 5% to 35% sucrose in STE which was centrifuged to further purify the virus in a Beckman SW 41 rotor for 30 min at 55,000 x \(g\). The resultant purified virus was collected and the protein
concentration was determined using a modified Lowry protein assay procedure and stored in aliquots at -80°C. Confirmation of the recovery/purity of IHNV was done by analysis on a 12% SDS-PAGE mini-slab gel (Bio-Rad, Hercules, CA) with comparison to an aliquot of previously purified virus.

Electrophoretically separated virus protein bands were visualized by silver stain using a commercial kit manufactured by Sigma. The virus was also titrated using a sandwich ELISA technique (Ristow et al. 1993) and an immunoblot performed to assess reactivity against a panel of IHNV monoclonal antibodies. Finally, the purified virus was formalin-killed and extensively dialyzed against PBS prior to use as an immunogen (for secondary immunization). Virus used as the coating antigen for ELISA was not formalin-killed.

Immunization and Sample Collection

Rainbow trout were anesthetized by immersion in 3-aminobenzoic acid ethyl ester (MS-222) (0.2 g l⁻¹) and approximately 3 ml of blood was collected from the caudal vein in heparinized Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) at each time interval and tubes were immediately placed on ice to minimize cell mortality. Primary immunization was performed by intraperitoneal (i.p.) injection with 100 μg TNP-KLH or
IHNV gp peptide-BSA, or with PBS, pH 7.2, emulsified 1:1 in Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, MI). Antigen was injected in a final volume of 0.05 ml. Trout received a secondary immunization in the same manner (i.p.) with 20 μg of antigen emulsified in Freund's incomplete adjuvant (FIA; Difco) 25 weeks post primary injection in a final volume of 0.05 ml. The IHNV group received formalin-killed whole virus for the secondary immunization instead of the synthetic glycoprotein epitope injected for the primary immunization. Each fish was bled at week 0, 5, 10, and 20 post primary and week 10 post secondary immunization.

**Immunoselection of Antigen-Specific B Cells**

 Peripheral blood leukocytes were isolated by a standard leukocyte isolation procedure (Kaattari *et al*. 1986). Briefly, whole blood was centrifuged at 500 x g for 15 min at 4°C and the plasma removed and stored in aliquots at -80°C until analyzed. All subsequent centrifugations were also at 500 x g and 4°C. The cell pellet was resuspended in RPMI-1640 (Sigma)(1:8 dilution) and the suspension layered over an equal volume of Histopaque 1077 (Sigma). The cells were centrifuged for 45 min and the leukocytes collected from the interface above the Histopaque layer. These cells were washed an additional two times in RPMI-1640 for 10 min each and the final cell pellet resuspended
in RPMI-1640. Viability of lymphocytes and cell count was determined by trypan blue exclusion.

Uncoated magnetic beads (DynaBeads M-450, Dynal, Lake Success, NY) were coated with IHNV or TNP-BSA for immunomagnetic separation of specific B cells based on their surface receptor affinity for the specific coating antigen per the manufacturer’s instructions. Beads were coated by the addition of 9-15 μg protein 100 μl⁻¹ of beads suspended in 0.1 M sodium phosphate buffer, pH 7.4. This suspension was incubated on an end-over-end tube rotator for 24 h at 4° C. Afterwards the unbound protein was removed by placing the tube into a Dynal magnetic particle concentrator (MPC) and aspirating the supernatant. The coated beads were washed three times in PBS containing 0.1% BSA, pH 7.2. The first two washes were performed for 5 min each at 4° C and the last wash was done overnight at 4° C.

A minimum ratio of 4 beads per lymphocyte was co-incubated at 4° C with end-over-end mixing and the bead-rosetted cells isolated by placing the test tube in the MPC for 2-3 minutes per manufacturer’s direction. The supernatant was removed by pipetting and antigen-specific cells were washed twice in PBS, pH 7.2, containing 0.1% BSA. The cells were detached by repeated pipetting of the rosetted beads and the magnetic beads removed with the MPC. Confirmation of recovery and quantitation of the cells was performed by trypan blue exclusion of the panned, flow-through, and bead fractions.
generated in this manner. Panned cells were examined microscopically to confirm the presence of surface Ig by labelling with a fluorescent mAb (Warr's L-14; DeLuca et al. 1983) which recognizes an Fc epitope on the trout B cell receptor (BCR). Antigen-specific cells were then immediately processed for extraction of total RNA using TriZol (Life Technologies, Inc., Gaithersberg, MD) and the RNA was stored at -80°C until week 10 samples had been collected and individuals evidencing a shift in antibody affinity were identified.

**Production and Sequencing of cDNA Clones**

Total RNA from antigen-specific B cells was used as template for first strand synthesis of cDNA and subsequent RT-PCR (reverse transcriptase-polymerase chain reaction). All PCR reactions were performed in a MJ DNA Engine Peltier thermal cycler (MJ Research Inc., Watertown, MA) using thin-walled 0.2 ml PCR tubes (individual or 8 per strip) and a high fidelity Taq polymerase (PLATINUM Taq High Fidelity polymerase; Life Technologies, Inc.) to minimize the introduction of PCR artifact point mutations. An anchor primer and a forward primer devised from the relatively invariant framework regions of the VH antibody gene (Hansen et al. 1994) were used to amplify the specific intervening CDR2 region of the trout by PCR. Hansen et al. designed these primers by aligning several amino acid and nucleotide sequences from various vertebrate VH genes
to develop the following degenerate primers for V region genes: Sense FR2 = 5’- CCISAIMGNCCTGYAMAGCCTCYDDITT-3’ and antisense FR3 = 5’- CCGGATCCGGACAITAATAA VNYGCNGTGTCYTC-3’. The FR3 primer was designed to take advantage of the fact that most VH genes end in the conserved amino acid motif YYCAR. Amplification of the expected PCR product, approximately 240 bp, was confirmed on a 1.2% agarose gel by standard DNA electrophoresis methods (Sambrook et al. 1989) and subsequently cloned into the TA cloning vector (Invitrogen, Carlsbad, CA). Clones containing the putative CDR2 insert were selected by blue/white screening on LB agar plates containing ampicillin (amp) and 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal) (per formulation in Sambrook et al. 1989). Plasmid DNA was isolated using a CONCERT plasmid DNA purification kit (Life Technologies, Inc.) and presence of insert confirmed by digestion with the restriction enzyme Eco RI (Promega, Madison, WI). Analysis of the digestion products was done on a 1.2% agarose gel as previously described. Plasmid preps shown to possess the appropriate size insert were sequenced using a NEN Global IR² automated DNA Sequencer (LI-COR, Lincoln, NE) using a Thermo Sequenase™ cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Cycle sequencing reactions were set up employing a protocol provided by LI-COR utilizing 50 fmol of plasmid DNA template. Sequencing alignments were performed using MacVector v. 7 (Oxford Molecular Group,
Molecular format and BLAST sequence similarity searches (Altschul et al. 1997) were performed to confirm sequence identity with published trout Ig VH sequences (GenBank, National Center for Biotechnology Information, Bethesda, MD).

Additional sense primers were employed later in the study (Table IV) (generously provided by Dr. Greg Warr from the Medical University of South Carolina), used in combination with an antisense primer that annealed to sequence in the constant region (CH1 = 5'-GGGAGCGGAGATATGATGA-3') to ensure there was no skewing of selection of VH amplicons based on primer bias (Fig. 7). These PCR products (insert range ~245 - 450 bp) were cloned and sequenced as described above.

**Genomic DNA Isolation**

Extraction of genomic DNA (gDNA) was performed as described by Spruell et al. (1994) for fin clip samples, otherwise routine genomic isolation using trout RBCs was done per Sambrook et al. (1989). Briefly, fin clip samples approximately 0.7 cm² were excised and stored at -80°C in 95% (v/v) ethanol until processed. Samples were incubated in digestion buffer for 4 - 5 h in a 55°C waterbath with gentle shaking. An equal volume of phenol:chloroform: isoamyl alcohol (PCI) (25:24:1) was added and the sample mixed gently by inversion. The aqueous layer containing DNA was recovered by
Table IV. Sequence of primers provided by Dr. G. W. Warr (Medical University of South Carolina).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>G-844</td>
<td>ACTGTCMTGTACTGTGTMGGWTT</td>
</tr>
<tr>
<td>G-845</td>
<td>CATCTCCTGCAAATTCCTGGGTT</td>
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<tr>
<td>G-847</td>
<td>TCTGTACMRCYTCTGGSTTCACATT</td>
</tr>
<tr>
<td>G-848</td>
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Figure 7. PCR Primer Strategy. A suite of primers was employed to amplify trout Ig VH cDNA sequence encompassing VH gene encoding the FR1 (partial), CDR1, FR2, and CDR2. In some instances a 3' primer was utilized with various FR1 primers which allowed for the amplification of FR1 (partial), CDR1, FR2, CDR2, FR3, CDR3, FR4, and into the Cμ 1 domain. 5' primers G-843 - G-853 were designed by Dr. G. W. Warr (Medical University of South Carolina) and FR1 was designed by Hansen et al. 1994. The constant region primers CH1-CH3 were designed in our laboratory by Dr. A. Dacanay.
centrifugation of the samples for 15 min at 12,000 x g and extracted a second time with chloroform: isoamyl alcohol (24:1). Genomic DNA was precipitated by the addition of 0.1 vol 3 M ammonium acetate, pH 5.2, and 2.5 vol of 100% ethanol and then collected by spooling on sterile glass rods. The DNA was washed with 70% ethanol and dialyzed at 4°C with three buffer exchanges of Tris-NaCl-EDTA (STE) buffer, pH 8.0, in 3500 MWCO dialysis tubing (Spectrum Laboratories, Houston, TX). This step was included to remove residual contamination from the samples prior to restriction enzyme digestion and assayed for purity spectrophotometrically (A_{260}/A_{280} > 1.6). Integrity of high molecular weight gDNA was examined by electrophoresis on a 0.8% agarose gel.

**VH Gene Identification**

*Probe Construction and Southern Blotting*

To determine the copy number of Cμ genes in the trout genomic DNA, an additional set of primers was designed (sense CH2 = 5' - CATCAACTGCTCCGACTTTGTTC-3' and antisense CH3 = 5'-CGCCTCGTCATTCCATTTG-3') and used to generate a plasmid construct containing a 130 bp insert that would be used in experiments described below. Probes based on cDNA sequences diagnostic for individual VH gene families and the Cμ gene were produced by labeling via random priming in the presence of [α-33P] dATP (NEN Life Science Products, Boston, MA) and used to screen genomic
DNA using a standard Southern blot technique (Sambrook et al. 1989). Briefly, 10 µg gDNA from individual trout were digested with Eco RI restriction endonuclease and then electrophoresed on 0.8% agarose gels. A DNA molecular weight marker (1 Kb DNA ladder; Life Technologies, Inc.) was also run on the gel. The gel run was considered complete once the first loading buffer dye front (bromphenol blue) had run to the end of the gel. The lane containing the molecular weight ladder was removed and stained with ethidium bromide for visualization via UV transillumination of the migration of the individual bands to provide a calibration curve. Gels were subjected to two denaturation washes and two neutralization washes (20 min each at room temperature on an orbital shaker) and then equilibrated in 10x SSC which was used as the transfer buffer to blot the gel onto a 0.45 µm pore sized nylon membrane (MagnaGraph, Osmonics Inc., Minnetonka, MN) by capillary transfer. The DNA was fixed to the membrane by exposure to 120,000 µJ of UV energy (UV Stratalinker 1800, Stratagene, La Jolla, CA). To hybridize the probe to the membrane, the probe was first denatured by incubation at 95°C then added to PerfectHyb Plus (Sigma) hybridization buffer which co-incubated with the blotted membrane overnight in hybridization bottles at 68°C in a waterbath with a rotissier. Low stringency (at room temperature) and high stringency (at 68°C) washes were performed. The membrane was wrapped in clear plastic and exposed to Kodak Biomax MS film (Fisher Scientific) with an intensifying screen at -70°C for 5 days.
before development using a Konica SRX-101 medical film processor (Konica Corporation, Tokyo, JPN).

Gene Titration

Plasmid and genomic DNA concentrations were determined fluorometrically (DynaQuant, Hoefer Scientific Instruments, San Francisco, CA). The DNA per haploid genome per trout erythrocyte has been calculated to be 2.4 pg (Ohno and Atkin, 1966), thus 10 µg of trout DNA contains $4.26 \times 10^6$ haploid genomes. This number of Eco RI linearized plasmids was loaded onto a 0.8% agarose gel to yield one genome equivalent (GE) and multiples of this number were loaded to provide a range of genomic equivalents (GE = 1, 3, 5, 10 for Cμ1 probe); GE = 1, 5, 10, 15, 20, 30, 40, 50 for VH family probes). Blots were developed as described above and the relative densitometry of the bands was assessed for the number of VH or Cμ gene copies present.

Determination of Antibody Titers and Affinity

Specific Antibody Titration

The titration of specific anti-IHNV gp or TNP antibody in the plasma was determined by an antigen-capture enzyme-linked immunosorbent assay as previously described (Ristow et al. 1993; Shapiro et al. 1996). Briefly, wells of a 96-well microtiter
plate were coated overnight at 4°C with 50 μl well⁻¹ of 2 μg ml⁻¹ antigen (TNP-BSA or IHNV) in coating buffer. Afterwards, the unbound protein was washed from the plate by washing three times with Tris-buffered saline with 0.1% Tween 20 (TTBS). The wells were blocked with 200 μl well⁻¹ TTBS containing 1% bovine serum albumin (BSA; Sigma) for one h at room temperature in a humidified chamber. The wells were washed again with TTBS three times and serial dilutions of the plasma samples added (50 μl well⁻¹). For anti-TNP, dilutions were made in TTBS containing 1% BSA ranging from 1:25 to 1:1353125 using a five-fold serial dilution scheme. For anti-IHNV, dilutions were made in TTBS containing 1% BSA ranging from 1:30 to 1:14580 using a three-fold serial dilution scheme. The plate was incubated again at room temperature for one h as described above and then washed three times. Fifty μl well⁻¹ of a 1 mg ml⁻¹ biotinylated mouse anti-trout Ig mAb (Warr's 1-14; de Luca et al. 1983) diluted 1:1000 in TTBS was added to each well and the plate was incubated and washed as above. Strepavidin-horseradish peroxidase (SA-HRPO; Sigma) was added to the wells in the same volume as above, using a 1:1000 dilution of 125 μg ml⁻¹ SA-HRPO. After a final one h incubation the plate was washed and 50 μl well⁻¹ of ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) developer solution (Sigma). The plate was covered with foil for 15 - 20 min to allow chromogen development, then the optical density at 405 nm (OD₄₀₅) was measured using a Titertek Multiskan MCC/340 microplate reader (Titertek, Chantilly).
VA). The volume of plasma equivalent to one unit of antibody activity (defined as the amount of antibody required to produce a color intensity of half the maximum OD) was determined from a plot of OD<sub>405</sub> versus plasma volume (Arkoosh and Kaattari 1990).

**TNP Affinity ELISA**

Briefly, for the anti-TNP affinity measurements graded concentrations of the antigen TNP-BSA were coated onto an ELISA microtiter plate. To each well, a constant concentration of antibody activity (one unit - defined above) was added together with graded concentrations of the hapten inhibitor TNP-L-lysine (ICN Biochemicals, Cleveland, OH). The lowest coating antigen concentration captures the highest affinity antibodies within the volume of serum containing a standard unit of antibody activity and the simultaneous inhibition analysis using varying concentrations of hapten at each coating concentration provides an estimate of K (Keq or aK) of that antibody subpopulation (Fig. 8). Therefore, the affinity of each of these subpopulations is defined by the equation:

\[
K = - \log\left(\frac{1}{[H]_{50}}\right)
\]

where K is the affinity constant or the measure of the degree of binding between an antibody and its specific antigen and [H]<sub>50</sub> is the concentration of hapten (TNP-lysine) required to cause 50% inhibition of the maximum OD for that coating concentration.
Figure 8. Schematic of anti-TNP affinity ELISA plate set-up. The coating antigen (TNP-BSA) is added to the plate in graded concentrations with the lowest concentration in row B, with 1:3 dilutions of antigen in increasing concentrations loaded from row B to row A. Row A is coated with coating buffer without antigen. Graded concentrations of the inhibitor, TNP-lysine, in TTBS containing 1% BSA is added with the lowest concentration in columns 3,4 and 1:5 serial dilutions added in duplicate to the other columns such that the highest concentration of inhibitor is in columns 11,12. No inhibitor is added to columns 1,2 (diluent buffer without inhibitor was added). After the inhibitor is added the plasma sample is added at 2 units specific anti-TNP activity per well, also diluted in TTBS containing 1% BSA. The graded green circles illustrate a representative affinity response.
consistent over plate
A weighted average affinity of all the antibody subpopulations was then calculated from the K values and the proportion of antibody in each subpopulation using the equation:

\[
\text{weighted average affinity} = K \times P
\]

where \( P \) = fraction of antibodies in the mixture which possess a particular K value (Nieto et al. 1984). Using a MicroSoft Excel software macro, the affinity and proportion of antibody in each subpopulation as well as the weighted average affinity (\( aK \)) for a complex mixture of antibodies was easily obtained. The subpopulation distribution for each antiserum was plotted using DeltaGraph 4.5 (SPSS Inc., Chicago, IL) with the \( aK \) values along the abscissa (x-axis) and proportion of antibodies within the sub-population on the ordinate (y-axis).

**Diethylamine Shift Assay**

The affinity of the antibodies produced to the IHNV gp was determined using a solid phase assay that employs diethylamine (DEA; Sigma) as a denaturant which decreases the binding of lower affinity virus-specific antibodies (Thomas and Morgan-Capner 1991). Using the DEA ELISA method, assay plates were coated with IHNV as described for the antibody titration ELISA and all other steps of the assay were the same, excluding the dilution of samples (Fig. 9). In the DEA denaturant method,
Figure 9. Schematic of DEA shift ELISA plate set-up. The plate is coated with a uniform concentration of antigen and then plasma is serially diluted in TTBS containing 1% BSA (indicated in odd numbered columns) or TTBS containing 1% BSA and 35 mM DEA. As indicated by graded green circles, a sample with low affinity antibodies is inhibited from binding the coating antigen in the presence of DEA. Plasma possessing high affinity / avidity antibodies will still react with the antigen coated on the plate in the presence of 35 mM DEA in buffer.
<table>
<thead>
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<tr>
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<td>E</td>
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<tr>
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<td>G</td>
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<table>
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<th>coating Ag</th>
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<tbody>
<tr>
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<table>
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<th>wk 5</th>
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<tbody>
<tr>
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<td>+ DEA</td>
<td>- DEA</td>
<td>+ DEA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>negative control</th>
<th>positive control</th>
</tr>
</thead>
</table>

- no affinity shift |
- affinity shift
three-fold dilutions of plasma were made in TTBS containing 1% BSA with and without 35 mM DEA to yield a dilution range of 1:30 to 1:14580). The plasma dilutions were incubated for 2 h at room temperature in a humidified chamber. Once the ABTS developer was added and plates read at OD₄₀₅, two dilution curves were plotted for each sample: one for the plasma diluted in TTBS containing 1% BSA without DEA and one for the plasma diluted in TTBS containing 1% BSA and 35 mM DEA. Plasma antibodies which were not inhibited in the presence of DEA for binding the coating Ag compared to their non-DEA diluted counterpart possessed higher affinity for the coating antigen.

**Immunopurification of Antibodies**

**TNP-Specific Antibodies**

Immunoadsorbents for the affinity purification of the trout antibodies consist of CNBr-activated Sepharose beads conjugated with either TNP-BSA or TNP-lysine as per the manufacturer's instructions (Sigma). Prior to immunoadsorption of the antibodies, plasma was diluted 1:2 in PBS, pH 7.2 and added to a slurry of coupled beads placed in Bio-Spin columns (Bio-Rad). The suspension was mixed overnight at 4ºC and then anti-TNP antibodies were eluted using modified procedures of Harlow and Lane (1988). Non-adsorbed material was eluted from the column using PBS until the OD₄₀₅ was equivalent to background. TNP-lysine (1mM) in PBS was added to the column and...
allowed to mix for 2 hr at 4°C on an end-over-end mixer (Lab Industries Inc., Berkeley, CA). One column volume of eluate was collected and this step was repeated 3 times with the last mixing step occurring overnight at 4°C. Acid and base elutions were performed respectively with a single 2 hr mixing step prior to each fraction collection. One hundred percent recovery of the anti-TNP activity from each sample was achieved.

**IHNV-Specific Antibodies**

Anti-viral antibodies were purified from immune trout plasma by immunoprecipitation. Purified virus was added to 75 µl of each serum in 10 µg aliquots and gently stirred with a pipette tip. This suspension was incubated for an additional 5 minutes at 17°C with gentle agitation on a rotary shaker. A total of 200 - 300 µg of virus was added to the plasma. The precipitate was isolated by centrifugation at 12,000 x g for 5 minutes, gently washed once with PBS and isolated by centrifugation as before. The adsorbed antibody was isolated from the precipitate by vigorous resuspension of the precipitate in 100 µl of 100 mM glycine, pH 2.5. Antibody was further dissociated by incubating the precipitate in the glycine for 20 minutes at 17°C with agitation on a rotary table (300 rpm). The volume was increased to 400 µl with PBS and the viral proteins were removed by centrifugation at 130,000 x g for 90 minutes at 4°C. The solution was dialyzed against three exchanges of PBS in 12-14000 MWCO dialysis tubing (Spectrum...
Laboratories). Confirmation of binding specificity was conducted by determining that whole, isoelectrically-focused antibodies bound IHNV gp by Western blot analysis (after Harlow and Lane 1988).

**IEF/2D-SDS PAGE Analysis of Antibodies**

Whole plasma samples or immunopurified antibodies from plasma evidencing a shift to higher affinity specific antibodies were subjected to 2-dimensional IEF electrophoresis in an attempt to isolate putative newly emergent antigen-specific heavy chains in these samples compared to those from week 0 or week 5 not possessing unique isoelectropherotypes in similar studies performed in the laboratory. Quantitative densitometry of the focused H chains with an Alpha Imager 2000 Documentation and Analysis System was performed using Alpha Imager version 4.0 software (Alpha Innotech Corp., San Leandro, CA). Briefly, 5 μg of individual samples were subjected to isoelectric focusing per Harlow and Lane (1988) using a Hoefer Mighty Small tube IEF electrophoresis system per manufacturer's protocol (Amersham Pharmacia Biotech Inc.) for the first dimension electrophoresis (to separate by pl) and the Bio-Rad mini-Protean electrophoresis system for the second dimension (to separate by molecular weight). Visualization of 2-D protein spots was done by silver staining the second dimension acrylamide gels (Sigma silver stain kit) which also had a molecular weight marker well in
which 3 μg of purified trout antibody or pI markers (Sigma) were electrophoresed for comparison.

Variability Analysis

Ig VH cDNA sequence was obtained as described above using a variety of primers. If the sequence amplified by these primers included CDR3 or CH sequence, the sequence was truncated for variability analysis. Additional amino acid sequences were obtained from the Kabat database (Johnson et al. 2000) or the GenBank database (NCBI, Bethesda, MD) for analysis of amino acid variability. All Kabat database sequences were obtained from the July 2000 dataset using the variability search tool available at http://immuno.bme.nwu.edu/variability.html. Per Wu and Kabat (1970), variability was determined by dividing the total number of different amino acids that occur at a single position of the Ig VH sequence by the frequency of occurrence of the most common amino acid at that position. The results were plotted as a histogram with amino acid position along the abscissa (x-axis) and variability on the ordinate (y-axis) using DeltaGraph 4.5.
N-Terminal Sequencing of Specific Antibody Heavy Chains

For protein sequencing, the second dimension gel (from above) was transblotted onto an Immobilon-P (Millipore, Bedford, MA) transfer membrane using the Bio-Rad mini-Protean II transblot apparatus (Bio-Rad). The membrane blot was stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol (v/v) which had been diluted in ultrapure water. The membrane was destained with a 50% methanol-10% acetic acid-40% ultrapure water solution which allowed the visualization of the stained protein "spots". The membrane was equilibrated in ultrapure water and air-dried. Isolated heavy chain proteins determined to be unique to the plasma samples collected late in the specific immune response were excised and sent to the Louisiana State University Medical Center protein sequencing facility (New Orleans, LA) for N-terminal sequencing. Based on analysis of existing Genbank database sequences, five to fourteen amino acid residues require sequencing to prove diagnostic in terms of trout VH gene family usage (Zhang 1999). This sequence analysis was performed to provide additional support that the family specific sequences we might identify in our cDNA analyses would be the ones being used to produce the newly emergent higher affinity antibodies post-immunization.
RESULTS

Trout Antibody Response to the Defined Hapten TNP

Serological Analyses

Antibody Titers / Affinity

Anti-TNP antibody titers of rainbow trout immunized with TNP-KLH were measured using standard ELISA techniques. In the TNP-KLH immunized group, all animals developed an antibody response to the hapten (ranging from 0.5 to 3 units activity µl⁻¹ at week 0 to 237 to 940 units activity µl⁻¹ plasma by week 10) (Fig. 10) and the control samples did not develop positive titers to TNP or IHNV (all samples < 3 units activity µl⁻¹. A comparison of week 5 and week 10 affinity ELISA aK profiles allowed identification of the two trout (#9 and #10) with the highest affinity subpopulation shifts as measured by the change in the weighted average aK and the development of populations with uniquely high aK's at the later timepoint. The weighted average aK at week 5 was 4.77, 4.45, 5.36, 4.59, and 4.62 for trout # 6-10, respectively and increased by week 10 to 5.46, 5.22, 5.41, 5.18, and 5.3, respectively. Individual aK profiles for the two time points are provided in figure 11. Upon secondary immunization with TNP-KLH trout #9 was shown to possess additional newly emergent subpopulations of higher affinity antibodies (Fig. 12) and, thus, this individual was selected for the molecular analysis of the antibody response. Affinity ELISA measurements of trout #10 week 35
Figure 10. Anti-TNP titers. Graph of anti-TNP titers of individual trout immunized with TNP-KLH at weeks 0, 5, and 10. The x-axis denotes the time point of sample collection (week 0, 5, 10) and the y-axis denotes the units of specific anti-TNP antibody activity μl⁻¹ plasma.
Figure 11. Affinity profiles of anti-TNP plasma for individual trout. These profiles illustrate affinity shifts between week 5 (blue bars) and week 10 (green bars) for five trout (#6 - #10). The aK range of each histogram is denoted on the x-axis and percent of total antibody represented by that affinity subpopulation is given on the y-axis.
Figure 12. Affinity profile of anti-TNP plasma from trout #9. This profile illustrates a shift in affinity subpopulations at week 5 (blue), 10 (green), 20 (red), and 35 (yellow). The aK range of each subpopulation is depicted on the x-axis, the percent of the antibody pool possessing that affinity on the y-axis, and time post-immunization on the z-axis.
plasma did not provide a definitive measure of aK, although the assay was repeated, thus a measure of the secondary TNP response (e.g. week 35) for this individual was not obtained (Fig. 13).

**IEF of TNP-Specific H Chains**

Trout antibody against TNP was purified by adsorbing plasma from TNP immunized trout on an affinity column containing TNP-coupled Sepharose as described in the Methods section (Fig. 14). The primary purpose of purification was to isolate samples for N-terminal sequencing of TNP-specific heavy chains (see section below). Antibody purification also allowed for easy reference of trout antibody heavy and light chains during the 2D-IEF/SDS-PAGE analysis of non-purified trout plasma samples when purified Ab was included on the gel as a molecular weight marker.

Once plasma samples had been collected from all time points, 2D-IEF/SDS-PAGE was performed which revealed newly emergent heavy (and light) chain proteins from the trout which underwent an antibody affinity shift. Trout #9 2D antibody profiles (Fig. 15) from week 5, 20 and 35 reveal the presence of new heavy chain isoelectrophereotypes which accumulate temporally over the course of the immune response. Densitometric analysis of the week 5 sample is characterized by heavy chain pIs of 5.5 - 5.6, and another single spot at 6.2 (Fig. 16A). By week 20 the profile shifts with new isoelectropherotypes...
Figure 13. Affinity profile of anti-TNP plasma from trout #10. This profile illustrates a shift in affinity sub-populations between week 5 (blue) and week 10 (green) and illustrates the accumulation of new, increased affinity sub-populations through week 20 (red). The aK range of each subpopulation is depicted on the x-axis, the percent of the antibody pool possessing that affinity on the y-axis, and time post-immunization on the z-axis.
Figure 14. Purified trout antibody protein profile for trout #9. Silver stained 12% SDS-PAGE gel illustrating the protein profile (A) before and (B) after affinity chromatography purification of trout plasma. Molecular weight markers are indicated in the far left lane.
Figure 15. Two dimensional IEF / SDS-PAGE gels of plasma from trout #9 which was immunized with TNP-KLH emulsified in Freund's complete adjuvant and shown to possess increased anti-TNP affinity over the course of the experiment as measured by affinity ELISA. (A) Five µg of plasma from week 5 run out on a gel and silver stained. (B) An equivalent amount of plasma from week 20 and (C) week 35 reveals a heavy chain protein profile possessing new H chain isoelectropherotypes compared to the earlier profile (indicated by circles). Purified trout antibody (H chain 72 kD, L chain 25 kD) is included on the gels as a molecular weight marker.
Figure 16. Densitometric profile of silver stained 2D IEF / SDS-PAGE gels at 72 kD. (A) Profile of anti-TNP trout #9 plasma week 5. (B) Profile for same trout at week 20 and (C) week 35. This trout did evidence a shift in antibody affinity profiles as measured by affinity ELISA which correlates to the emergence of new H chain isoelectrophoretotypes. The x-axis represents the pI range and the y-axis denotes optical density (OD).
at 5.1 - 5.3 and a loss of the heavy chain protein spot of pI 6.2 (Fig. 16B). Week 35 plasma which corresponds to the secondary antibody profile in trout #9 revealed the emergence of even more new IEF spectrotypes, with these new heavy chains possessing pIs in the range of 5.7 - 5.8 and 6.1 - 6.3 (Fig. 16C). As all fish in the TNP immunized group evidenced an antibody affinity shift, plasma samples from a TNP-KLH immunized trout that developed a TNP titer without undergoing an affinity shift was included in the 2D-IEF/SDS-PAGE analyses (from Zhang 1999). The sample for trout #918 did not evidence a change in heavy chain isoelectrophoretotypes between week 5 and week 20 (Fig. 17 - 18), and neither did the profile for the PBS control trout #1 (Fig. 19 - 20). This lack of profile change within non-affinity shift trout samples emphasizes that immunization of these animals with adjuvant was not sufficient to elicit any measurable change in the antibody heavy chain composition.

**Molecular Analyses**

**Panning for TNP-Specific B Cells**

At each sampling time point, peripheral blood lymphocytes were isolated, or "panned", so temporal changes in VH gene sequence could be monitored. This process was accomplished by co-incubation of peripheral blood leukocytes with TNP-BSA conjugated magnetic beads in the presence of saturating concentrations of BSA in PBS.
Figure 17. Two dimensional IEF / SDS-PAGE gels of plasma from trout # 918 that was immunized with TNP-KLH emulsified in Freunds complete adjuvant and did not evidence an antibody affinity shift over the course of the experiment as measured by affinity ELISA. (A) Five μg of plasma from week 5 run out on a gel and silver stained. (B) An equivalent amount of plasma from week 20 reveals a heavy chain protein profile which is unchanged relative to the earlier profile. Purified trout antibody is included on the gels as a molecular weight marker, of which only the H chain (72 kD) is visible.
Figure 18. Densitometric profile of silver stained 2D IEF / SDS-PAGE gels at 72 kD. (A) Profile of anti-TNP trout # 918 plasma week 5. (B) Profile for same trout at week 20. This trout did not evidence a shift in antibody affinity as measured by affinity ELISA. The x-axis represents the pI range and the y-axis denotes optical density (OD).
Figure 19. Two dimensional IEF / SDS-PAGE gels of plasma from trout #1 immunized with PBS emulsified in Freund's complete adjuvant. A. Five µg of plasma from week 5 run out on a gel and silver stained. B. An equivalent amount of plasma from week 20 reveals a heavy chain protein profile which is unchanged compared to the earlier profile. Purified trout antibody was included on the gels as a molecular weight marker, of which only the H chain is visible.
Figure 20. Densitometric profile of silver stained 2D IEF / SDS-PAGE gels at 72 kD. (A) Profile of PBS control trout # 1 plasma week 5. (B) Profile for same trout at week 20. The x-axis represents the pI range and the y-axis denotes optical density (OD).
These adherent lymphocytes were detached from the beads via physical disruption by repeated pipetting. Both adherent (Fig. 21A) and non-adherent lymphocytes (Fig. 21B) were stained with FITC conjugated TNP-BSA to determine their antigen specificity using fluorescent light microscopy (40x). As can be seen, virtually 100% of the panned lymphocytes recognize TNP, whereas 100% of the non-adherent cells do not recognize the hapten. Similar results were seen when panning with IHNV-coated beads.

Typically, 3 ml of heparinized, whole blood yielded approximately $10^8$ leukocytes at each sampling. After panning, approximately $0.5 - 1 \times 10^9$ antigen-specific cells were recovered for isolation of total RNA.

cDNA Sequences

Primers which successfully amplified TNP-specific template were all family V specific (Table V) and there was one primer pair, capable of amplifying family I and family V homologous sequence, which also amplified TNP-specific template but the sequence itself was a family V sequence. The deduced amino acid sequence of TNP-specific cDNA (using the MacVector protein translation tool) revealed replacement mutations which accrued in the majority of clone sequences from week 10 through week 35 (Table VI) occurring primarily in CDR2. Three replacement mutations were observed to occur in FR, Ile 37 → Val 37 in FR2, Thr 48 → Val 48, and Ile 81 → Ser 81 in FR3.

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Figure 21. Immunofluorescence demonstrating efficiency of immunobead purification of antigen-specific trout lymphocytes. Peripheral blood lymphocytes were 'panned' using TNP-BSA conjugated beads in the presence of saturating concentrations of BSA. These lymphocytes, detached from the beads (A), and the non-adherent lymphocytes (B) were stained with FITC conjugated TNP-BSA (40x). As can be seen, virtually 100% of the panned lymphocytes recognize TNP, whereas 100% of the non-adherent cells do not recognize the hapten. Similar results were seen when panning with IHNV-coated beads.
Table V. Primers which amplified TNP-specific trout Ig cDNA.

<table>
<thead>
<tr>
<th>primer i.d.</th>
<th>most homologous BLAST specificity</th>
<th>amplify Ag-specific template?</th>
</tr>
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<tbody>
<tr>
<td>G-843/CH1</td>
<td>family V</td>
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</tr>
<tr>
<td>G-844/CH1</td>
<td>family IX</td>
<td>-</td>
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<td>FR1/FR3</td>
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Table VI. Representative TNP-specific VH cDNA from trout #9 and controls - deduced amino acid translations.

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<tr>
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Table VII. TNP-Specific VH cDNA from #9 highlighting mutations. Consensus sequences are denoted by an asterisk (*). Replacement mutations are indicated by a red nucleotide. Replacement mutations that occur to sequence previously mutated are indicated in blue. The majority of sequences after wk 5 accrued replacement mutations in CDR2. Additionally, there were three mutations which became fixed in FR sequences later in the response (wk 10+: Ile 37 → Val 37, Thr 48 → Ile 48; wk 20+: Ile 81 → Ser 81).
wk 0
(n=10)
ACCTGCACCCGCCTCCGAGTTCCACATTTCACGCAGCTACTACATGGCGTGGAT

CAGACAGGCTCTGGGAAAGGACTGGAGTGGACCGCTTCTTAGTTATAGTA

CTGCTACATATTATTCCCAAGTCAGTCAGGGTAGATTTACCACATCTCCAGA

GATGACTCAAGGATCTAAGCTGCTACAGATGAACATTCTGAAAGTCGGA

GGACACCGGCTTCATTACTGTCGAGA

wk 5
(n=10)
ACCTGCACCCGCCTCCGAGTTCCACATTTCACGCAGCTACTACATGGCGTGGAT

CAGACAGGCTCTGGGAAAGGACTGGAGTGGACCGCTTCTTAGTTATAGTA

CTGCTACATATTATTCCCAAGTCAGTCAGGGTAGATTTACCACATCTCCAGA

GATGACTCAAGGATCTAAGCTGCTACAGATGAACATTCTGAAAGTCGGA

GGACACCGGCTTCATTACTGTCGAGA

wk 10
(n=10)
ACCTGCACCCGCCTCCGAGTTCCACATTTCACGCAGCTACTACATGGCGTGGAT

CAGACAGGCTCTGGGAAAGGACTGGAGTGGACCGCTTCTTAGTTATAGTA

CTGCTACATAATATTCCCAAGTCAGTCCAGGGTAGATTTACCACATCTCCAGA

TCCAGAGATGACTCAAGGATCTAAGCTGCTACAGATGAACATTCTGAAAGTCGGA

GGACACCGGCTTCATTACTGTCGAGA

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wk 20  ACCTGCACCCGCTCCGAGTTCACTCAGCAGCTACTACATGGCGTGGAT
(n=9)  ******************************************G*
CAGACAGGCTCCTGGGAAAGGACTGAGTGGACCGCTTCTAGTTATAGTA
******************************************T******A**T**C**---
CTGCTAC--------ATATTATTCAGTCAGTCAGGAGTATTTACCAC
***T*****CTCTAT ******************************************
TCCAGAGATGACTCAAGATCTAAGCTGTACCTACAGATGAACATTCTGAA
******************************************TC********
GTCGGAGGACACCGCGCCCTTTATTACTGTCGAGA
******************************************

wk 35  ACCTGCACCCGCTCCGAGTTCACTCAGCAGCTACTACATGGCGTGGAT
(n=3)  ******************************************G*
CAGACAGGCTCCTGGGAAAGGACTGAGTGGACCGCTTCTAGTTATAGTA
******************************************T******A**T**C**---
CTGCTAC--------ATATTATTCAGTCAGTCAGGAGTATTTACCAC
***T*****CTCTAT ******************************************
TCCAGAGATGACTCAAGATCTAAGCTGTACCTACAGATGAACATTCTGAA
******************************************TC********
GTCGGAGGACACCGCGCCCTTTATTACTGTCGAGA
******************************************
Residue 37 is adjacent to the highly conserved Trp 35 which, by definition, demarcates the boundary between CDR1 and FR2. Residue 48 is also located on the boundary between FR2 and CDR. Residue 81 is a known intrinsic (non-Ag selected) mutational hotspot in mammals and this appears to hold true with fish antibodies (reviewed in Milstein and Neuberger 1996). There were two separate sets of insertions/deletions observed to occur in CDR2, as well as mutation of Ser 51 → Ile 51. Residue 51 has not been described as a mutational hotspot in mammalian H chains, thus mutation noted at this position may be considered extrinsic. Although point mutations were observed to occur in a temporal fashion in the majority of sequences from trout #9 (the trout with the highest TNP antibody affinity shift) (Table VII), sequences derived from PBS control and naive trout cDNA samples did not possess any replacement mutations and few silent mutations compared to #9 sequences.

Over the course of the anti-TNP response the utility of the Hansen et al. (1994) primers became limited in the ability to amplify sequence. This may be indicative of an additional shift in the response which we were not able to address as none of the primers we were using amplified sequence from the majority of template available from this time point. It is still questionable as to whether we have primers capable of amplifying cDNA for all trout Ig VH (Table VIII).
Table VIII. Primers which amplified non-antigen selected trout Ig cDNA.

<table>
<thead>
<tr>
<th>primer i.d.</th>
<th>most homologous BLAST specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-843/CH1</td>
<td>family V</td>
</tr>
<tr>
<td>G-844/CH1</td>
<td>family IX</td>
</tr>
<tr>
<td>G-845/CH1</td>
<td>family VIII, III</td>
</tr>
<tr>
<td>G-847/CH1</td>
<td>family V</td>
</tr>
<tr>
<td>G-848/CH1</td>
<td>family V, I</td>
</tr>
<tr>
<td>G-849/CH1</td>
<td>family V</td>
</tr>
<tr>
<td>G-850/CH1</td>
<td>family VII</td>
</tr>
<tr>
<td>G-851/CH1</td>
<td>family V</td>
</tr>
<tr>
<td>G-852/CH1</td>
<td>family IV</td>
</tr>
<tr>
<td>G-853/CH1</td>
<td>family V, I</td>
</tr>
<tr>
<td>FR1/FR3</td>
<td>family V, I</td>
</tr>
</tbody>
</table>
Additional cDNA sequences were also obtained for trout #10 and support the observation that replacement mutations also occurred in the other trout that underwent a significant TNP antibody affinity shift during the primary antibody response against TNP (Appendix II).

PCR amplification of cDNA produced from non-antigen selected RNA template produced amplicons which, when cloned, represented the majority of trout Ig VH families (Table VIII). However, no representatives of Ig VH family II, VII, IX or X were ever amplified in this study. Of these, only family VII is represented by a single reference sequence in GenBank (accession number L28747) and the others are represented by two to four reference sequences each (family II, accession numbers X65262, X81512, X81509; family IX, X81505, X81504, X81507, X81506; family X, X81508, X81489) all of which were identified in European stocks of rainbow trout (Lee et al. 1993; Andersson and Matsunaga 1995). This was an unexpected result as it was anticipated that without selection, members from all trout Ig VH families would be amplified to some degree.

VH Gene Utilization and Gene Titration

Based on our cDNA studies, Family V appears to be used to generate most of the anti-TNP response, with some shift occurring during the secondary response. Primers

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were capable of amplifying more Ig VH sequences early in the response than at later time points and there was difficulty retrieving more than a few clone sequences late in the response (Table VII). Whether this is indicative of a shift in VH gene utilization to one of the families for which we were unable to obtain sequence due to possible primer constraints, or whether this indicates that there are VH gene families yet to be identified, awaits additional experimentation and/or the improved design of PCR primers such that all VH gene families can be amplified (at least, in naive fish). Another possibility is that relatively less clonal expansion occurred later in the response for this trout (# 9) and there were fewer recombinant clones containing VH-specific DNA from which sequence could be obtained.

To gain an estimate of the actual size of the gene family it is necessary to use gene titration analysis, as VH family size can be quite variable (1-500 or more in the mouse model) (Livant et al. 1986); thus simple Southern analysis of positive restriction fragments would be insufficient. Our analysis of the size of an anti-TNP coding VH family was conducted by gene titration analysis using the probe generated from a PCR-amplified sequence which is homologous to Ig VH family V by BLAST analysis (# 9 week 35 consensus sequence). The decision to use a late response, mutated sequence for probe production was made in an attempt to identify VH sequences with the greatest sequence homology to the VH family utilized and the germline gene whose mutated
product was selected for the anti-TNP response. As can be seen in figure 22, family V appears to demonstrate that minimally 5 VH genes may exist in this family, however, family composition analysis must await sequencing of the individual probe-reactive bands. As a control, a gene titration experiment was conducted using a probe generated to the Cμ1 sequence (which should only possess one copy per haploid genome). As can be seen in figure 23, the degree of reactivity is commensurate with one gene copy per haploid genome.

N-Terminal Sequencing

As observed in a previous section, newly emergent Ig heavy chain spots were identified that were coincident with observed TNP affinity shifts which occurred at weeks 10 - 35. Immunopurified plasma antibodies from trout #9 (week 35) were electrophoresed and transblotted to PVDF membranes in order to isolate distinct H chains for N-terminal sequencing. The most abundant of the newly emergent H chain spots (pI 6.1) was excised from Coomassie stained, transblotted membranes and sent to the protein sequencing core facility at Louisiana State University Medical Center (Fig. 24). It was expected that such sequences would be diagnostic as each VH family N-terminus was unique (Table IX) and allow for clear identification of the VH gene family utilized for the isolated antibody heavy chain. Initial sequence results, however, indicated a
Figure 22. Quantitative Gene Titration - *IgVH* Family V probe. Genomic DNA (gDNA) (10 μg) from an outbred Shasta strain rainbow trout hybridized with a trout Ig VH family V (anti-TNP) cDNA probe labelled with $^{32}$P-dATP. Titrated amounts of plasmid DNA containing a range of genomic equivalents (GE) was also digested and run out to quantitate the VH gene copy number (range 1 - 50 GE). Restriction enzymes used to digest the gDNA are annotated on the autoradiograph. The genomic equivalent plasmid DNA was digested with *Eco RI*.
Figure 23. Quantitative Gene Titration - $C\mu 1$ probe. Genomic DNA (5 µg) from two outbred Shasta strain rainbow trout hybridized with a trout Ig $C\mu 1$ cDNA probe labelled with $^{33}$P-dATP. Titrated amounts of plasmid DNA containing a range of genomic equivalents (GE) was also digested and run out to quantitate the VH gene copy number (range 1 - 10 GE). *Eco RI* was used to digest the gDNA and plasmid genomic equivalent DNA.
Figure 24. Resolution of discrete H chain products from immunopurified anti-TNP (trout #9 week 35). A 2D-IEF / SDS-PAGE gel was transblotted onto PVDF membrane and stained with Coomassie blue. Individual 'spots' were excised from the membrane and sent out for N-terminal amino acid sequencing. Circle indicates the heavy chain protein (pI 6.1) that initial sequencing attempts indicated was blocked at the N-terminus. H chain molecular weight is 72 kD and L chain molecular weight is 25 kD.
Table IX. Figure N- terminal sequences of trout antibody V_H families based on translation of cDNA sequences from the GenBank database. Family designations follow that of Roman et al. 1996. **Bold** text indicates a diagnostic sequence which may be used to differentiate families.

<table>
<thead>
<tr>
<th>Family</th>
<th>N-terminal Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>family I</td>
<td>QTLTE SGPVV KKPLE AHKLT</td>
</tr>
<tr>
<td>family V</td>
<td>QTLTE SGPVV KITGE AHKLT</td>
</tr>
<tr>
<td>family II</td>
<td>QSLES IPSSP</td>
</tr>
<tr>
<td>family III</td>
<td>QTLED QFGSL</td>
</tr>
<tr>
<td>family IV</td>
<td>DEMDO SPSQV</td>
</tr>
<tr>
<td>family VI</td>
<td>VELOS PASMT</td>
</tr>
<tr>
<td>family VII</td>
<td>LELOS PNNMV</td>
</tr>
<tr>
<td>family VIII</td>
<td>QIVLT QAESV</td>
</tr>
<tr>
<td>family IX</td>
<td>QSLTS SESV</td>
</tr>
<tr>
<td>family X</td>
<td>DIETI QSGPV</td>
</tr>
<tr>
<td>family XI</td>
<td>I5LTS SPAQL</td>
</tr>
</tbody>
</table>

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blocking group was impeding the identification of N-terminal sequence and other investigators attempting to obtain Ig VH N-terminal sequence have encountered this problem (Scott et al. 1988). This result led to the implementation of a variety of procedural changes as suggested by the sequencing facility to improve the chance of successfully identifying the five to fourteen N-terminal amino acid residues required for assignment of the specific protein heavy chain to its VH gene family. If, in fact, the isolated H chain is a member of VH family V, the N-terminus is a glutamine residue which could inherently possess a pyroglutamyl blocking group (Tsunasawa et al. 1998). As such, while no definitive evidence of family V assignment has be obtained, the result of a blocked N-terminus for this protein may corroborate the molecular data that indicates the anti-TNP response engenders trout Ig VH family V utilization.

Modifications that were made to the original protocol to blot individual H chain "spots" to a PVDF membrane for N-terminal sequencing included changing the transblot buffer solution from a general purpose buffer containing glycine to CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer which does not contain glycine. This was done to alleviate the possible introduction of pyroglutamyl blocking groups as a procedural artifact and to decrease the stain/detain incubation times which has been shown to introduce N-terminal blocking groups (pers. comm. Dr. Jim Carlton. Louisiana State University Protein Sequencing facility). Additionally, the amount of protein
submitted for sequencing was greatly increased from 10 pmol to mmol amounts, which generally translated to eight to ten protein "spots" from an initial sample concentration of 40 µg protein. However, to date, all attempts to obtain the N-terminal protein sequence were unsuccessful and no definitive evidence supporting the molecular identification of Ig VH gene utilization during the antigen-specific immune response was collected.

**Trout Antibody Response to IHNV**

**Serological Analyses**

**Antibody Titers / Affinity**

The animals that were immunized with IHNV gp peptide-BSA produced IHNV specific antibodies (Fig. 25). The titers of these antibodies, however, would not be considered high if compared to animals immunized with whole virus, as the coating antigen in the assay is whole IHNV, yet the antibodies produced during this study were to one antigenic epitope within the viral glycoprotein's exposed surface.

As hapten-inhibitor methods cannot be used to assess antibody affinities to protein antigens, a denaturant based assay was applied. This assay, termed the diethylamine (DEA) shift assay, has been used in measuring avidity differences in human antibody responses to the rubella virus (Thomas and Morgan-Capner 1991). However, prior to executing these studies the validity of the method was tested using our anti-TNP...
Figure 25. Anti-IHNV titers. This graph illustrates anti-IHNV ELISA results of individual trout immunized with an IHNV 20-mer gp epitope conjugated with BSA at weeks 0, 5, 10, 20, and 35. The x-axis denotes the time of sample collection and the y-axis denotes the units of specific anti-IHNV antibody activity µl⁻¹ plasma.
plasma. Testing these samples permitted us to determine if a direct correlation existed between DEA shifts and absolute affinity changes, thus verifying this assay for use with trout plasma. The results obtained from anti-TNP plasma samples having previously been shown to possess low, medium, and high affinity subpopulations substantiated the assay's utility. Plotting the percent stable antibodies (those which did not shift in OD$_{405}$ in the presence of DEA in the dilution buffer) against the average mean affinity for each subpopulation illustrates that greater shifts occur with lower affinity antibodies (Fig. 26). The only drawback in comparison between the two assays is that the TNP affinity ELISA provides a quantitative measure of the various subpopulations of antigen-specific antibodies whereas the DEA shift ELISA can only be semi-quantitative. Nevertheless, this assay provides definitive identification of samples possessing increased affinity and was sufficient for our purpose of identifying the individual trout which expressed affinity shifts.

Using the DEA shift assay, only one individual (trout #13) was shown to produce antibodies possessing increased / high affinity receptors for IHNV as measured by the stability of IHNV-specific antibody titers in the presence of 35 mM DEA (Fig. 27). Thus, RNA samples from this fish were selected for subsequent molecular analyses after week 10 DEA assay results were obtained.
Figure 26. Comparison of average weighted affinity (aK) and DEA percent stable values for trout anti-TNP plasma samples. This was done with TNP samples to validate the assay for use in measuring antibody affinity / avidity shifts in anti-IHNV plasma samples. As illustrated, high affinity antibodies are increased in percent stability in the presence of DEA compared to antibodies of medium or low TNP affinity.
Figure 27. Diethylamine (DEA) shift ELISA reveals that of the five rainbow trout immunized with an IHNV 20-mer gp epitope conjugated with BSA, only one individual (#13) maintains affinity for IHNV in the presence of 35 mM DEA
IEF of IHNV-Specific H Chains

Isoelectric focusing of whole plasma from trout #13, followed by second dimension SDS-PAGE revealed production of a few new Ig heavy chains over the course of the immune response monitored in this study (Fig. 28). There was a dramatic increase in production of the Ig heavy chain (pI 6.4) at week 20 compared to the week 5 profile and additional heavy chain IEF spectrotypes were observed, one at pI 5.4, another between 5.4 - 5.5, and two more between 5.7 - 5.8, which may have been present in diminished concentration at week 5 (Fig. 29). Additional light chain pIs were also observed to emerge over the course of the experiment for this individual although densitometry was not undertaken. In contrast, in trout #11 which possessed a similar anti-IHNV titer but did not develop antibodies with increased affinity for IHNV, no apparent change in Ig heavy chain isoelectropherotypes was observed (Fig. 30) and this result was confirmed densitometrically (Fig. 31).

Initial experimental design was to immunoprecipitate IHNV-specific antibodies for the 2D IEF / SDS-PAGE experiments. However, as revealed by analysis of the unpurified plasma samples from affinity shift responders versus non-responders, we were able to identify H chain profiles without the costly purification step. This was supported by the result that 2D IEF / SDS-PAGE of non-affinity shift responders from TNP-KLH or
Figure 28. Two dimensional IEF / SDS-PAGE gels of plasma from trout #13 which was immunized with IHNV-gp peptide-BSA emulsified in Freunds complete adjuvant and shown to possess an antibody affinity shift as measured by the DEA affinity shift assay over the course of the experiment. A. Five μg of plasma from week 5 run out on a gel and silver stained. B. An equivalent amount of plasma from week 20 reveals a heavy chain protein profile possessing new H chain isoelectropherotypes compared to the earlier profile (indicated by circles). Purified trout antibody (H chain 72 kD, L chain 25 kD) is included on the gel as a molecular weight marker.
A.

B.

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Figure 29. Densitometric profile of silver stained 2D IEF / SDS-PAGE gels at 72 kD. 
(A) Profile of anti-IHNV trout # 13 plasma week 5. (B) Profile for same trout at week 20. 
This trout did evidence a shift in antibody affinity profiles as measured by the DEA 
shift ELISA which correlates to the emergence of new H chain isoelectropherotypes. The 
x-axis represents the pI range and the y-axis denotes optical density (OD).
Figure 30. Two dimensional IEF / SDS-PAGE gels of plasma from trout #11 immunized with IHNV gp peptide-BSA emulsified in Freund's complete adjuvant which did not evidence an antibody affinity shift as measured by the DEA affinity shift assay. A. Five μg of plasma from week 5 run out on a gel and silver stained. B. An equivalent amount of plasma from week 20 reveals a heavy chain protein profile which is unchanged compared to the earlier profile. Purified trout antibody was included on the gels as a molecular weight marker, of which only the H chain (72 kD) is visible.
A.

MW

pI 4.5 5.2 5.5 5.9 6.6 7.0 8.5

H chain

B.

MW

pI 4.5 5.2 5.5 5.9 6.6 7.0 8.5

H chain
Figure 31. Densitometric profile of silver stained 2D IEF / SDS-PAGE gels at 72 kD. (A) Profile of anti-IHNV trout # 11 plasma week 5. (B) Profile for same trout at week 20. This trout did not evidence a shift in antibody affinity as measured by the DEA shift ELISA. The x-axis represents the pI range and the y-axis denotes optical density (OD).
IHNV gp peptide-BSA did not possess new IEF spectrotypes (Fig. 17, 30) and neither did the PBS injected control (Fig. 19).

**Molecular Analyses**

**Panning for IHNV-Specific B Cells**

At each sampling point, peripheral blood lymphocytes were panned in an attempt to monitor temporal changes in VH gene sequence as described above with the following modification. Peripheral blood leukocytes were co-incubated with IHNV conjugated magnetic beads instead of TNP-BSA coated beads. Virtually 100% of the panned lymphocytes recognize IHNV, whereas the non-adherent cells did not recognize the viral glycoprotein epitope(s).

**cDNA Sequences**

Total RNA was isolated from panned, IHNV-specific B cells from each fish and stored at -80°C until at least one fish (# 13 in this study) was identified as exhibiting a significant Ab affinity shift. cDNA clones were then produced for sequence analysis. Table X illustrates the primers which amplified IHNV-specific template from this individual as well as from trout # 14, a non-affinity shift responder. Both individuals produced clones that were homologous to trout VH family IV and the deduced amino
acid sequence of the IHNV-specific DNA with representative sequence at each sampling time point was determined (Table XI). No replacement mutations were observed to occur at any time point for trout # 13 in the CDRs and few silent mutations were observed in the nucleotide sequence (Table XII).

**VH Gene Utilization and Gene Titration**

Family IV appears to be utilized for most of the anti-IHNV response generated, based on the homology of amplified cDNA sequences compared with GenBank archived trout Ig VH cDNA sequences. To gain an estimate of the actual size of this gene family, an anti-IHNV coding VH cDNA probe (from #13 week 35) was used to conduct gene titration analysis as previously described. As evidenced by the results shown in figure 32, genomic DNA digested with *Eco RI, Eco RV*, or *Hind III* and probed with the Ig VH family IV probe demonstrates that many more VH genes may exist in this family compared to that of family V, perhaps as many as 30 gene copies. This will make characterization of the individual family repertoire and absolute confirmation of somatic mutation more complicated than that of the family V repertoire as more sequencing of genomic DNA probe reactive fragments will have to be performed. However, as no replacement mutations appear to have been generated, verification of the possible requirement for somatic mutation to generate a high affinity response is moot.
Table X. Primers which amplified IHNV-specific trout Ig VH cDNA.

<table>
<thead>
<tr>
<th>primer i.d.</th>
<th>most homologous BLAST specificity</th>
<th>amplify Ag-specific template?</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-843/CH1</td>
<td>family V</td>
<td>-</td>
</tr>
<tr>
<td>G-844/CH1</td>
<td>family IX</td>
<td>-</td>
</tr>
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<td>family VIII, III</td>
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</tr>
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<td>family V</td>
<td>-</td>
</tr>
<tr>
<td>G-848/CH1</td>
<td>family V, I</td>
<td>-</td>
</tr>
<tr>
<td>G-849/CH1</td>
<td>family V</td>
<td>-</td>
</tr>
<tr>
<td>G-850/CH1</td>
<td>family VII</td>
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<td>G-851/CH1</td>
<td>family V</td>
<td>-</td>
</tr>
<tr>
<td>G-852/CH1</td>
<td>family IV</td>
<td>+ IHNV</td>
</tr>
<tr>
<td>G-853/CH1</td>
<td>family IV</td>
<td>+ IHNV</td>
</tr>
<tr>
<td>FR1/FR3</td>
<td>family IV</td>
<td>+ IHNV</td>
</tr>
<tr>
<td>wk</td>
<td>FR1 CDR1 FR2 CDR2 FR3</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>-----------------------</td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>LSCKISGFDMTNYMHIRQKPGKALEWIGRINSGSTAPVYSDSLKGQFTLTEDVSTSTQSEAKSLGSED SAVY YCAR</td>
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</tr>
<tr>
<td>10</td>
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<td></td>
</tr>
<tr>
<td>20</td>
<td>LSCKISGFDMTNYMHIRQKPGKALEWIGRINSGSTAPVYSDSLKGQFTLTEDVSTSTQSEAKSLGSED SAVY YCAR</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>LSCKISGFDMTNYMHIRQKPGKALEWIGRINSGSTAPVYSDSLKGQFTLTEDVSTSTQSEAKSLGSED SAVY YCAR</td>
<td></td>
</tr>
</tbody>
</table>
Table XII. IHNV-Specific VH cDNA highlighting mutations. Consensus sequences are denoted by an *. Replacement mutations are indicated by a red nucleotide. Of all the cDNA sequences in this table, only one had a replacement mutation in a CDR (wk 10, CDR2. Pro 59 → Ser 59) and three late sequences had replacement mutations in a FR (wk 35, FR3, Ser 81 → Phe 81 and Gly 88 → Ser 88).
wk 0 (n=10)  
AAACTATTACATGCACTGGATAAGACAGAAACCAGGCAAAGCTCTGGAGT  
GGATTGGGAGAATTAACTCTGTTCAAACAGATGCTCCAGTCTACTCAGAC  
TCCCTGAAAGGCCAGTTACCTGACCAGGGAGCTCTGCTGTTTATT  
ACAGTCCCTAGGGCAAGAGCTCTGGGTTCAGAGGACTCTGCTGCTGCTGAGA  

wk 5 (n=10)  
AAACTATTACATGCACTGGATAAGACAGAAACCAGGCAAAGCTCTGGAGT  
GGATTGGGAGAATTAACTCTGTTCAAACAGATGCTCCAGTCTACTCAGAC  
TCCCTGAAAGGCCAGTTACCTGACCAGGGAGCTCTGCTGTTTATT  
ACAGTCCCTAGGGCAAGAGCTCTGGGTTCAGAGGACTCTGCTGCTGCTGAGA  

wk 10 (n=10)  
AAACTATTACATGCACTGGATAAGACAGAAACCAGGCAAAGCTCTGGAGT  
GGATTGGGAGAATTAACTCTGTTCAAACAGATGCTCCAGTCTACTCAGAC  
TCCCTGAAAGGCCAGTTACCTGACCAGGGAGCTCTGCTGTTTATT  
ACAGTCCCTAGGGCAAGAGCTCTGGGTTCAGAGGACTCTGCTGCTGCTGAGA  

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wk 20  (n=8)  
AAAATTTACATGCACTGGATAAGACAGAAACCAGGCAAAGCTCTGGAGT
GGATTGGGAGAATTAACTCTGGTTCAACAGATGCTCCAGTCTACTCAGAC
TTCCTGAAGGCACTTCAACCTGGAGGGAGCTCTCTCTAACGACACACA
ACAGTCCTTAGGCAAGAGCTTGGCTGGTTCAGGACTCTGCTGTTTATT
ATTGTGCTCGAGA

wk 35  (N=9)  
AAAATTTACATGCACTGGATAAGACAGAAACCAGGCAAAGCTCTGGAGT
GGATTGGGAGAATTAACTCTGGTTCAACAGATGCTCCAGTCTACTCAGAC
TTCCTGAAGGCACTTCAACCTGGAGGGAGCTCTCTCTAACGACACACA
ACAGTCCTTAGGCAAGAGCTTGGCTGGTTCAGGACTCTGCTGTTTATT
ATTGTGCTCGAGA
Figure 32. Quantitative Gene Titration - IgVH Family IV probe. Genomic DNA (gDNA) (10 μg) from an outbred Shasta strain rainbow trout hybridized with a trout Ig VH family IV (anti-IHNV) cDNA probe labelled with \(^{33}\)P-dATP. Titrated amounts of plasmid DNA containing a range of genomic equivalents (GE) was also digested and run out to quantitate the VH gene copy number (range 1 - 50 GE) Restriction enzymes used to digest the gDNA are annotated on the autoradiograph. The genomic equivalent plasmid DNA was digested with Eco RI.
N-Terminal Sequencing

N-terminal sequencing of the antibody heavy chain spot (pI 6.4) that was observed to markedly increase in production during the anti-IHNV immune response is awaiting the refinement of the transblot procedure as determined to be required after attempts to obtain anti-TNP heavy chain N-terminal sequence proved unsuccessful (see earlier section). An additional modification to the procedure employed for obtaining IHNV specific heavy chains was necessitated due to the expense of the original purification method. In the original experimental design it was believed that immunoprecipitation of virus specific antibodies by co-incubation of IHNV and trout plasma from late in the response, followed by elution of these antibodies by a standard acid/base elution procedure (Harlow and Lane 1988) would be required to provide an immunopurified sample for subsequent 2D-IEF/SDS-PAGE. However, purification of the mg quantities of IHNV that are required became too costly and time consuming. This step will be eliminated in future attempts to isolate IHNV specific heavy chains as these studies have demonstrated they can be isolated and visualized readily by 2D-IEF/SDS-PAGE of unpurified plasma (Fig. 28).

Variability Analysis

The variability plot for rainbow trout Ig VH sequences archived in the Kabat
Database (July 2000 dataset) revealed limited diversity of CDRs (filled bars) (Fig. 33) especially when compared to the variability plots for mammalian IgG (Fig. 34) and IgM (Fig. 35) archived within the same dataset. The inclusion of our cDNA sequences and additional sequences from GenBank brings the variability characteristics of trout CDR closer to the pattern illustrated on the overall variability for Ig VH (Fig. 36). This is especially true for CDR2 particularly and for residue 56 which now appears quite mutable. This position is generally considered to be an extrinsic hotspot for mutation in the mouse (Milstein and Neuberger 1996), meaning this position has been shown to be an antigen-selected hot spot, and the observation that trout also possess this particular hotspot illustrates that trout VH are more similar to the model of CDR variability than previously recognized. It is now felt that the observation that trout VH possessed less variability in their CDRs as evidenced in figure 32 is more the result of having a limited number of trout VH sequences and (until now) no antigen-selected sequences for comparison with the sizeable database of available, antigen-selected Ig sequences from other species.

Analysis of the variability of previously archived trout Ig indicated CDR1 was relatively more variable than CDR2, with the amino acid at position 33 being the most mutable. This is one of three positions of increased variability in the comparison of IgG/IgM Ig variability plots (along with amino acid residues 31 and 35) but in these plots...
Figure 33. Variability plot of all rainbow trout Ig VH region sequences in the Kabat Database (from the July 2000 dataset, n = 45). CDRs indicated by filled bars, FRs by clear bars. on the histogram.
Figure 34. Variability plot of all IgG VH region sequences in the Kabat Database (from the July 2000 dataset, n = 2343). CDRs are indicated by filled bars, FRs by clear bars, on histogram.
Figure 35. Variability plot of all IgM VH region sequences in the Kabat Database (from the July 2000 dataset, n = 2597). CDRs are indicated by filled bars, FRs by clear bars, on histogram.
Figure 36. Variability plot of all rainbow trout Ig VH region sequences in the GenBank Database combined with additional Ig VH cDNA sequences obtained in our laboratory (n = 117). This dataset is truncated due to experimental design and illustrates the variability from amino acid position 27 to 94 (encompasses partial FR1 and all of CDR1, FR2, CDR2). CDRs indicated by filled bars, FRs by clear bars, on the histogram.
CDR2 is overall more variable than CDR1. Combining our trout cDNA sequences with the original database sequences for trout strongly indicates a similarity in variability between CDR1 and CDR2, with heightened amino acid variability at position 56.

Although not included in the variability plots presented herein, the CDR3 sequences amplified in our study possessed the most variability of all trout CDR regions. This result is not unexpected as molecular characterization of trout DH and JH cDNA sequences (reviewed in du Pasquier and Flajnik 1998) indicates the complexity of teleost CDR follows the mammalian paradigm and CDR3 is known to possesses the greatest capacity for fine tuning the antigen specificity of individual heavy chains (Johnson and Wu 1998).
DISCUSSION

Evidence for Somatic Hypermutation in Trout Ig VH

As previously demonstrated (Zhang 1999; Khor 1997), fish immunized with TNP-KLH produced a specific, high-titered antibody response to TNP. The titers in these animals increased over 2000-fold during the first ten weeks post immunization. As the goal of this research was to determine if the accrual of somatic variants could be observed during a phase of affinity maturation, peripheral blood lymphocytes were isolated for analysis of VH cDNA sequences, while the plasma from these same samples were analyzed for increases in affinity and spectrotype changes. Thus, it could be determined not only whether somatic variants arise during the immune response, but if the induction of these variants correlated with affinity increases and physical changes in antibody structure. In the present study, accumulation of variants were identified in TNP immunized trout which correlated to the emergence of new H chains and an affinity shift in these fish.

Both Khor (1997) and Zhang (1999) observed that the majority of TNP-KLH immunized trout underwent affinity maturation; however, in a number of cases the individual antibody subpopulations never exceed the highest affinity exhibited initially at week 0. Therefore the possibility existed that although the overall affinity (e.g. the weighted average) may consistently increase over time, there may have simply been an
antigen-driven selection of pre-existent high affinity clones. Thus affinity maturation could simply be the skewing, or preferential induction, of primarily the highest affinity clones which were innately available. However, there were select, specific cases wherein new and higher affinity subpopulations would arise only late in the response (≥ 10 weeks) and this prompted the consideration that high affinity somatic variants may only arise post antigen-induction, then selectively induced with the diminishing concentration of antigen over time (Zhang 1999).

One objective of this study was the generation of sufficient cDNAs from antigen-specific lymphocytes to analyze the expressed repertoire. The experimental strategy used was to periodically bleed the fish and test their plasma, while preserving the cDNA for each animal at that specific phase of the response until the affinity shift responders could be clearly identified by serological assay. At the completion of the sampling regime, the individuals that demonstrated the greatest degree of affinity maturation, those possessing late appearing, higher affinity subpopulations and unique antibody heavy chain isoelectropherotypes, were selected for molecular characterization of the antibody response as these individuals would be the most likely candidate(s) for somatic mutation. Thus, the total cDNA was screened via the use of primers specific for each of seven of the eleven known VH families.

Of the five individuals immunized with TNP-KLH, trout #9 and #10
demonstrated the greatest increase in affinity (Fig. 12, 13), with the appearance of unique high affinity subpopulations later in the response. These affinity subpopulations ranged in $aK$ from 3.8 to 5.6 at week 5 and 4.4 to 6.2 at week 20 for #9 and from 4.2 to 5.6 at week 5 and 4.6 to 6.6 at week 20 for #10, respectively, whereas the highest affinity observed at week 35 was for trout #9 with an $aK$ of 6.6 - 6.8. The mean percentage of antibodies (of trout #9 and #10 comparing weeks 5, 10, and 20) with these new, higher affinities for the primary TNP response was 46.7\% (Fig. 37). The secondary TNP response measured for week 35 trout #9 revealed an additional percentage of almost 10\% for the subpopulation possessing the $aK$ of 6.6 - 6.8 (Fig. 12). Thus, this immunochemical analysis strongly suggested that unique higher affinity, antigen-specific B lymphocytes were emerging late in the response and becoming increasingly dominant within the individual. As further evidence that unique antibody species were being produced at this point, isoelectric focusing of non-purified plasma as well as immunopurified antibodies was performed. Coincident with increase in affinity, these individuals expressed new antibody heavy and light chains. Thus, the late emerging antibodies not only possess uniquely increased affinities but also physically distinct antibodies. Again, this finding is consistent with the induction of somatic hypermutation. The appearance of these new isoelectropherotypes was unique to the trout demonstrating affinity maturation, as trout which did not exhibit affinity maturation (non-affinity shift
Figure 37. Combined TNP affinity profile averaging the aK for trout #9 and #10 for week 5, 10, and 20 to illustrate the temporal accumulation of new, increased affinity subpopulations. The aK range of each subpopulation is depicted on the x-axis and the percent of the antibody pool possessing that affinity on the y-axis.
individuals) (Fig. 17, 30) or fish injected with PBS emulsified in adjuvant (Fig. 19) revealed no altered isoelectrophoretotypes.

The anti-IHNV response, however, seemed to use a different mechanism of affinity maturation than that of the anti-TNP response. Whereas the TNP individual possessed somatic variation in the CDRs which was coincident with the development of new IEF spectrotypes, the IHNV response appears to have been simply an antigen-driven selection of pre-existent high affinity clones. This has been noted to occur in the murine immune response against other viruses, such as vesicular stomatitis virus, which has also been shown to possess an innate subpopulation of high affinity VSV neutralizing antibodies (Andersson et al. 1998) that is stimulated to proliferate after exposure to antigen. Optimal protection against pathogenic Streptococcus pneumoniae has also been traced back to germline T15 anti-phosphorylcholine antibodies which do not undergo somatic mutation to further increase antigen specificity (Briles et al. 1982). Thus, affinity maturation in fish may also be a product of skewing, or preferential induction, of primarily the highest affinity clones which were innately available and this is not necessarily to be construed as a more primitive antibody immune response.

On the basis of this immunochemical analysis, cDNAs generated from lymphocytic RNA isolated from antigen-specific B lymphocytes were analyzed using primer sets designed to recognize known trout Ig VH families. Whereas unselected
lymphocytes produced cDNA reactive with the majority of VH primers in our possession (Table X), these same primers identified cDNA from TNP-panned lymphocytes as being family V sequences (Table V) and those from IHNV-panned lymphocytes as being family IV (Table XI). Dr. Trond Jorgensen's research group (University of Tromso, Tromso, Norway) generated a recombinant phage expression library of Atlantic salmon VH genes, and in testing the reactivity to TNP of antibodies derived from these constructs, found that family V was also used (Solem et al. 2000). As Atlantic salmon and rainbow trout possess homologous family specific sequence designations, the utilization of family V for the anti-TNP response was indirectly corroborated by this study. These results have revealed, for the first time, which specific VH gene family is used by trout to produce two different antigen-specific responses.

As can be seen from Table VII, the representative deduced anti-TNP amino acid sequences through week 5 are the same as the (banked) germline sequences. However, by week 10, a time at which affinity maturation is detectable by serological assay, potential somatic mutations begin to accrue. There are a number of essential features that should be noted and are consistent with the induction of somatic mutations in mammals. First, the mutations occur almost exclusively within the CDR regions (primarily CDR2). Second, the majority of observed mutations are replacement mutations and not silent mutations. Finally, the fact these variants can be easily detected from the random
cloning of only seven to ten cDNA sequences infers that the lymphocytes bearing these variants have come into clonal dominance.

Thus, although the process of somatic mutation is somewhat random, yielding both silent as well as replacement mutations (Storb 1996), the fact that replacement mutations in the antigen contact regions of trout VH are coming into dominance is indicative that they are being selectively expanded. In the mammalian system, germinal centers have been identified as the physical microenvironment in which antigen selection occurs (MacLennan 1994). Germinal centers have not been identified in rainbow trout, however, the results indicate antigen selection of pre-existent high affinity B cell clones and antigen-driven somatic hypermutation does occur in fish. This suggests that the localized microenvironment of germinal centers is either (1) not entirely requisite for the antigen selection of high affinity B cell clones to occur or (2) this microenvironment has yet to be identified in teleost fish.

The variability plot combining new and banked trout VH (deduced) sequences illustrates what is felt to be a more realistic estimate of the variability of CDRs, particularly the CDR2, over what was indicated by previous variability analysis. Additional methods such as Shannon entropy analysis have been used by others investigating Ig variability in fish, including analysis of sturgeon (Lundqvist et al. 1998) and Atlantic charr (Widholm et al. 1999). Although not included in our variability plots,
additional trout Ig VH cDNA sequences were amplified that confirm that trout CDR3 variability is greater than that of CDR1 and CDR2, which follows the mammalian model of hypervariable regions and increased plasticity in CDR3 over other CDRs.

Although the variability plot of all trout VH sequences to date (inclusive of this study and GenBank contributions) revealed a CDR variability more consistent with that observed for mammalian Ig in the Kabat database. However, other investigators have argued that variability plots may not be the best method available to characterize the variability of low sample size Ig VH sequences. Per Stewart et al. (1997), the use of Shannon entropy analysis is an improved method for this type of study in that variability plots have a primary limitation and account for only the most common one or two amino acids in estimating diversity. This may lead to an underestimate of low diversity or an exaggeration of high diversity in instances where overall sample number is low. Additionally, Gex-Fabry et al. (1989) also argues against the use of Wu and Kabat criteria for identification of CDRs when only a small number of sequences are available for analysis. These authors believe that using the Wu and Kabat method with a small sample number inherently leads to flawed identification of CDRs when originally distinguishing CDRs from FRs in newly described Ig or Ig-related molecules. They, therefore, have developed a method which takes into account the type of substitution which occurs, coupled with a statistical criterion for classifying a site as CDR or FR.
VH Genes and Gene Families

The findings described in the previous section are very supportive of the induction of somatic mutation. However, absolute confirmation requires that all possible germline sequences of family V be identified and sequenced. To date, this has been a weak area of comparative molecular immunology as 53 of the 56 archived VH gene sequences are cDNA (GenBank). The studies reported here represent an initial step in characterizing the trout Ig VH gene repertoire. Gene titration experiments were executed using cDNA clone sequences from late in the TNP or IHNV response for family specific probe production. Late response cDNA sequences were selected in an attempt to hybridize germline sequences closest to these antigen-selected sequences in the family repertoire. Examination of these blots reveals that minimally 1 - 5 germline genes may exist for family V but that the family IV repertoire is much more complex (Fig. 22, 32).

The complexity contrasted between the two trout VH families is reflective of the dissimilarity of VH gene repertoire size in both the mice and humans. The mouse has been shown to possess 15 VH gene families, and it is believed that all murine VH gene families have been identified since Southern blot experiments screening 2,000 cDNA clones against Cμ and JH probes revealed that all possessed VH regions with sequence homology that could be traced to the described 15 VH regions (Mainville et al. 1996).
The family number, name, and complexity (in terms of estimated murine germline VH sequences in each family) is presented in Table XIII. A similar table for human VH region families is also provided for comparison (Table XIV) and illustrates that there are seven human VH families identified to date and that the complexity of these families, while less, overall, than that of murine VH families (mouse complexity ranges from 1 germline sequence identified for the VH12 and VH13 families, ranging up to as many as 1000 germline sequences for the VH1 family (Kofler et al. 1992). Human VH complexity ranges from 1 germline sequence for the VH6 family up to 46 germline sequences identified for VH3 (Cook and Tomlinson 1995). Thus, the gene titration analysis for trout clearly illustrates that the inherent complexity of trout VH gene families is on the level of that observed in higher vertebrates.

An additional question which must be addressed for trout VH is the question of how germline VH genes are arrayed along the chromosome(s). In the murine system, it has been observed that adjacent VH genes are generally members of the same VH gene family, generally found in a clustered arrangement, and are oriented in the same direction 5' → 3' (Kemp et al. 1979; Bothwell et al. 1981). They are also spaced about seven to fifteen Kb apart along the chromosome (Givol et al. 1981). This differs in humans, in that studies using phage and cosmid clones of human VH genes demonstrated extensive interdigitation of human VH gene families (Walter et al. 1990, 1991) as opposed
Table XIII. Murine Ig VH gene families. Although presented with the VH# designation foremost, murine VH gene families are more commonly referred to by the "name" designation in the middle column. This "name" designation is a carryover from early identification of families by hybridization against various myeloma VH sequence probes and is not a classification used when identifying human Ig VH genes (Brodeur and Riblet 1984).

<table>
<thead>
<tr>
<th>family number</th>
<th>name</th>
<th>complexity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH1</td>
<td>J558</td>
<td>6 - 1000</td>
</tr>
<tr>
<td>VH 2</td>
<td>Q52</td>
<td>15</td>
</tr>
<tr>
<td>VH 3</td>
<td>30-60</td>
<td>5 - 8</td>
</tr>
<tr>
<td>VH 4</td>
<td>X-24</td>
<td>2</td>
</tr>
<tr>
<td>VH 5</td>
<td>7183</td>
<td>12</td>
</tr>
<tr>
<td>VH 6</td>
<td>J606</td>
<td>10 - 12</td>
</tr>
<tr>
<td>VH 7</td>
<td>S107</td>
<td>3</td>
</tr>
<tr>
<td>VH 8</td>
<td>3609</td>
<td>7 - 10</td>
</tr>
<tr>
<td>VH 9</td>
<td>VGAM3-8</td>
<td>5 - 7</td>
</tr>
<tr>
<td>VH 11</td>
<td>CP3</td>
<td>1 - 6</td>
</tr>
<tr>
<td>VH 12</td>
<td>CH27</td>
<td>1</td>
</tr>
<tr>
<td>VH 13</td>
<td>3609N</td>
<td>1</td>
</tr>
<tr>
<td>VH 14</td>
<td>SM7</td>
<td>3 - 4</td>
</tr>
<tr>
<td>VH 15</td>
<td>VH15</td>
<td>2</td>
</tr>
</tbody>
</table>
Table XIV. Human Ig VH gene families. The complexity refers to how many individual genes have been identified for the family (Cook and Tomlinson 1995).

<table>
<thead>
<tr>
<th>family number</th>
<th>complexity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH1</td>
<td>14</td>
</tr>
<tr>
<td>VH2</td>
<td>4</td>
</tr>
<tr>
<td>VH3</td>
<td>43</td>
</tr>
<tr>
<td>VH4</td>
<td>9</td>
</tr>
<tr>
<td>VH5</td>
<td>2</td>
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<tr>
<td>VH6</td>
<td>1</td>
</tr>
<tr>
<td>VH7</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 38. Map of the murine VH loci. Depicted are approximate map positions for the fifteen known mouse VH gene families. Each rectangle represents a gene cluster and illustrates the general tendency towards a clustering of genes within each family, although some interdigitation between families occurs (after Kemp et al. 1979; Bothwell et al. 1981).
to the clusters of discrete family-specific VH loci in mice (Fig. 39). Knowledge of the mouse and human arrangement reveals differences in organization and it would be of interest to see whether trout are more like man or mouse in this regard. If affinity maturation is possible, as was demonstrated by the anti-TNP response, a lack of affinity maturation may indicate that the germline repertoire is better adapted or more extensive than one which undergoes affinity maturation. Thus, a VH family which is capable of fine tuning an antibody response through the process of somatic mutation might be less likely to maintain a large germline repertoire. This would support the observation that the trout anti-TNP response accumulated somatic variants over time and utilized VH family V, with ≤ 5 genes in the germline. If an animal possesses VH gene family(s) with a larger germline repertoire, such as trout VH family IV, the increased repertoire may preclude the need for somatic mutation in response to complex antigen(s) (i.e. pathogens). This would corroborate the observation that the trout anti-IHNV VH sequences did not accumulate somatic variants over time.

Indeed, the murine anti-VSV response presented earlier in this Discussion as an example of an antigen-driven selection of pre-existent high affinity clones may also strengthen this argument. Mouse anti-VSV antibodies possess high binding avidities (~ 10⁹ M⁻¹), are devoid of somatic mutation, and utilize the Q52 VH family for production of virus-specific H chains (Kalinke et al. 1996). Q52 VH is possesses the
Figure 39. Map of the human VH loci. Depicted are the approximate map positions for the seven known human VH gene families. Each bar represents an individual gene of a prototypic haplotype (after Cook and Tomlinson 1995) and illustrates the interdigitation observed within families.
second largest germline repertoire of murine VH families, with 15 individual genes (Kofler et al. 1992).

The Use of Model Antigens vs. Complex Antigens

The most informative studies of the immune response have used defined antigens such as TNP (Haines and Siskind 1980; Arkoosh and Kaattari 1991), PC (Chang and Rittenberg 1981; Clarke et al. 1983), arsonate (Rothstein and Gefter 1983), or oxazolone (Griffiths et al. 1984; Berek and Milstein 1987). Even in the trout response, studies describing affinity maturation have only been addressed using the hapten TNP (Khor 1997; Zhang 1999). This has been problematic in characterizing the underlying immune processes occurring to relevant, pathogen-associated antigens in teleosts. We attempted to address this issue by measuring the immune response against a complex viral antigen, IHNV, using a synthetic gp epitope identified as being immunogenic in trout by Mourich and Leong (1991). There are still aspects of this approach that need to be resolved, one of which is the ability to measure affinity shifts and antibody titers with more complex antigens. We were able to illustrate the applicability of the DEA shift assay as a means of identifying trout undergoing an affinity maturation response but this is not a quantitative assay, at best semi-quantitative, and strategies must be devised to address this constraint when investigating the specific
immune response with a view to developing diagnostic and/or prophylactic applications.

Additionally, the experimental design was complicated by our decision to boost the trout with formalin-killed IHNV for the secondary immunization since plasma IHNV titers were not increasing as high as originally expected. In hindsight, this decision should not have been made as we were not able to measure a true secondary antibody response (the trout were undergoing a primary response to other glycoprotein epitopes not contained in the initial immunization) which is the reason only results for the anti-IHNV gp response through week 20 are reported within this manuscript.

Future Studies

In conclusion, the novelty of the approach used in this dissertation to investigate the antigen-specific immune response, compared to that previously attempted in other laboratories, allows the first description of the molecular basis of the trout immune response against a defined antigen and a relevant pathogen. The results obtained reveal that the piscine antibody response is much more sophisticated than previously described, with a capability to fine tune the specific immune response on the molecular level, as observed in mammals. However, there are still technical issues to overcome which need to be addressed for future studies.

One such issue is the N-terminal protein sequencing of the trout heavy chain
iso-electropherotypes for verification of VH family usage. For anti-TNP, it is essential to
determine whether the sequence is, in fact, inherently blocked due to pyroglutamyl
blocking groups which may occupy the N-terminus of family V heavy chains or whether
we introduced a blocking group artificially sometime during the isolation process to
obtain individual heavy chain "spots" for sequencing. If it is a technique-related issue,
this must be rectified and additional experiments to refine the technique are underway.
If the blocking group is inherent, as may be indicated by the N-terminal glutamine
residue identified in family V VH sequences identified to date, protein sequencing
experiments should still be pursued as we may still be able to obtain N-terminal sequence
for the anti-IHNV gp peptide heavy chain with the case of the family IV VH sequences,
an aspartate residue should exist at the N-terminus and, thus, might prove more amenable
to the repeated electrophoretic manipulations required to isolate individual heavy chains.
One final means of identifying the specific VH family employed for the response against
either of these antigens was proposed by Zhang (1999) by which cyanogen bromide
would be used to cleave trout H chains. Cyanogen bromide treatment, which specifically
cleaves proteins after methionine residues, would produce an electrophoretic profile that
would permit family identification based on the size of individual heavy chain fragments
produced (see figure, appendix III).

Another important requirement for the continuation of this type of investigation is
the identification and sequencing of germline sequences for as many of the representative
Ig VH families as possible. There are currently only three germline sequences for trout.
This is a problem which many comparative immunologists will encounter when
exploring immunoglobulin binding site production. This problem is not restricted to
Salmonids, nor teleosts alone as there are few non-mammalian Ig germline sequences
archived to date. Another avenue ripe for investigation is the inclusion of experiments
utilizing isogenic strains of rainbow trout and other species for studies of somatic
mutation as well as for identification of germline Ig sequences.

As new technologies are developed that measure antibody responses, the
requirement to apply these techniques in comparative studies is becoming more evident.
Recently, additional evidence for the anti-TNP affinity maturation response in trout was
presented in which investigators used commercially available biosensor equipment
(BIAcore biosensor; Pharmacia) to measure off-rates of antibodies for TNP (Cain et al.
2000). These investigators reported affinity kinetics quite similar to those we measure by
affinity ELISA. The biggest drawback to employing these types of cutting edge
technology is the cost and lack of reagents developed for use with lower vertebrate
animal models.
SUMMARY

This is the first comparative immunogenetics study which illustrates the accrual of somatic mutations temporally over the course of an antigen-specific immune response in rainbow trout. This accumulation of somatic variants, primarily in the CDR2 region of the Ig VH, corresponds with the emergence of new heavy chain isoelectropherotypes and affinity maturation. Thus, the trout's specific immune response to a defined hapten, TNP, clearly reveals an adaptability of the teleost immune response which was heretofore characterized as quite restricted. New antibody heavy chain isoelectropherotypes were also observed to come into dominance during the anti-TNP response and an isolated heavy chain (pI 6.1) has been identified as a suitable candidate to provide for N-terminal sequencing in an effort to provide corroborative data to support putative Ig VH gene utilization during the TNP antibody response.

Analysis of the response against the IHNV glycoprotein epitope revealed no replacement mutations in the VH over the course of the study but apparently a pre-existing antibody subpopulation was stimulated to proliferate upon immunization against this antigen. This was illustrated by densitometric analysis of the 2D-IEF/SDS-PAGE profile of antibodies sampled over the course of the experiment which revealed marked increase in production of an individual antibody heavy chain (pI 6.4) later in the response over the other heavy chain components of the plasma sample in a trout identified as an
affinity shift responder. Additional antibody heavy chain IEF spectrotypes were also produced during the anti-IHNV response but whether these proteins were produced by the same Ig VH family as that which was isolated and cloned during the study awaits completion of N-terminal protein sequencing of these individual heavy chains.

Germinal centers have not been identified in rainbow trout, however, our results indicate selection of pre-existent high affinity B cell clones and antigen-driven somatic hypermutation does occur in fish and is suggestive that the localized microenvironment of germinal centers is either (1) not entirely requisite for the antigen selection of high affinity B cell clones to occur or (2) this microenvironment has yet to be identified in teleost fish.

The DEA shift assay was validated for use in the study of antibody affinity / avidity shifts against complex antigens when a traditional inhibitor assay is unavailable and should be considered for use by other investigators. Although it is clearly easier to demonstrate the molecular and serological processes occurring during an antibody response when using defined antigens such as TNP, one important aspect of studying antigen-specific immune responses lies within the context of being able to identify and/or engender antibody responses that positively impact an animal's health and well-being.

Finally, this study provides the first correlative data of specific Ig VH gene family utilization with antigen-specific immune responses in lower vertebrates with quantitative
results regarding the gene copy number for these VH gene families in individual, outbred Shasta strain rainbow trout. Preliminary results indicate that trout VH gene families are no more, or less, complex in germline repertoire than the mouse or humans, and that their specific immune response is no less restricted than either of these higher vertebrate species. The application of Ig VH gene titration methods such as those employed in our laboratory to the study of isogenic strains of trout will provide additional insight into the complexity of the various trout Ig VH families described to date. The technique is also an important tool with application for assessing the pre-existing capacity for an antigen-specific immune response in various trout populations.
LITERATURE CITED


Clemons, A., A. Rademaekers, C. Specht, and E. Kolsch. 1998. The J558 $V_H$ CDR3 region contributes little to antibody avidity; however it is the recognition element for cognate T cell control of the $\alpha(1 \rightarrow 3)$ dextran-specific antibody response. Int. Immunol. 10:1931-1942.


200

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201

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Tsunasawa, S., S. Nakura, T. Tanigawa, and I. Kato. 1998. Pyrrolidone carboxyl peptidase from the hyperthermophilic Arachaeon *Pyrococcus furiosus*: cloning and


Note: Ultrapure water produced by a NANOPURE ultrapurification system manufactured by Barnstead is used for all buffer and reagent formulations below.

ABTS (2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) Developer Solution

Citrate Buffer

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<th>Amount</th>
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</thead>
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</tr>
<tr>
<td>H₂O</td>
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</tr>
<tr>
<td>HCl</td>
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<tr>
<td>H₂O</td>
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ABTS solution

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<td>H₂O</td>
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</tbody>
</table>

30 % H₂O₂
Make immediately prior to use by combining 4.8 ml citrate buffer with 200 µl ABTS solution and 5 µl H₂O₂ for each ELISA plate to be developed.

Cacodylate Buffer (0.28 M)

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<td>H₂O</td>
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<tr>
<td>HCl</td>
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<tr>
<td>H₂O</td>
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CAPS Buffer for Electroblotting Proteins

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<td>10X CAPS, pH 11</td>
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<tr>
<td>MeOH</td>
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<tr>
<td>H₂O</td>
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</table>

ELISA Coating Buffer

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<tr>
<td>Na₂HCO₃</td>
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<td>NaHCO₃</td>
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<tr>
<td>H₂O</td>
<td>to 450 ml</td>
</tr>
<tr>
<td>NaOH</td>
<td>to pH 9.6</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 500 ml</td>
</tr>
</tbody>
</table>
IEF Buffers

**Cathode (Upper chamber) Buffer**
NaOH 0.2 g
H₂O to 250 ml
Chill to 4° C, then de-gas.

**Anode (Lower chamber) Buffer**
H₃PO₄ 0.34 ml
H₂O to 500 ml
Chill to 4° C before use.

**IEF Lysis Buffer**
Urea 5.7 g
20% NP-40 1.0 ml
Ampholytes (pI range 3-10) 0.5 ml
2-Mercaptoethanol (w/v) 0.45 ml
H₂O to 10 ml

Mix all ingredients until dissolved and aliquot into microfuge tubes. Store at -20° C until use.

**IEF Sample Buffer for Second Dimension SDS-PAGE**
Tris base 0.15 g
SDS 0.4 g
2-Mercaptoethanol 1.0 ml
Glycerol 2.0 ml
H₂O to 7 ml
Bromophenol Blue 0.02 g
H₂O to 10 ml

Filter through a 0.22 µm syringe filter and aliquot. Store at -20° C until use.
IEF Tube Gel Equilibration Buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>65.0 mg</td>
</tr>
<tr>
<td>SDS</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>~ 2 mg</td>
</tr>
<tr>
<td>Tris base</td>
<td>0.76 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>30 ml</td>
</tr>
<tr>
<td>6N HCl</td>
<td>to pH 6.8</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 50 ml</td>
</tr>
</tbody>
</table>

Mix all ingredients until dissolved and store at 4° C until use.

IEF Tube Gels

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Urea</td>
<td>1.9 g</td>
</tr>
<tr>
<td>Ampholytes (pI range 3-10)</td>
<td>58 µl</td>
</tr>
<tr>
<td>Ampholytes (pI range 5-7)</td>
<td>0.117 ml</td>
</tr>
<tr>
<td>30% Acrylamide, 5% C</td>
<td>0.365 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>1.13 ml</td>
</tr>
<tr>
<td>20% NP-40</td>
<td>0.35 ml</td>
</tr>
</tbody>
</table>

Mix all ingredients until dissolved, then add the following:

10% APS    25 µl
10% TEMED  25 µl

Swirl gently to mix and immediately pour into the tube gel caster per manufacturer's protocol.

LB Medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 1 L</td>
</tr>
</tbody>
</table>

For agar plates, add 15 g L⁻¹. Heat to dissolve all components and autoclave for 15 min at 250 psi.
10X Phosphate Buffered Saline (PBS)

- NaCl: 80 g
- KCl: 2 g
- Na₂HPO₄ • 7H₂O: 26.8 g
- KH₂PO₄: 2.4 g
- H₂O: to 800 ml
- HCl: to pH 7.4
- H₂O: to 1 L

SDS-PAGE Gel Solutions

<table>
<thead>
<tr>
<th>Component</th>
<th>Separating Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide (2.7% C)</td>
<td>3.3 ml</td>
<td>0.67 ml</td>
</tr>
<tr>
<td>1.5 M Tris, pH 8.8</td>
<td>2.5 ml</td>
<td>**</td>
</tr>
<tr>
<td>0.25 M M Tris, pH 6.8</td>
<td>**</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.10 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.10 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 μl</td>
<td>3 μl</td>
</tr>
</tbody>
</table>

Swirl to mix all ingredients and then add TEMED and APS. Make and cast the separating gel first and allow it to polymerize approx. 1 h. Then make and cast the stacking gel.

SDS-PAGE Running Buffer (Laemmli Buffer)

- Glycine: 14.4 g
- Tris base: 3.03 g
- 10% SDS solution (w/v): 10 ml
- H₂O: to 800 ml
- HCl: pH to 8.8
- H₂O: to 1 L

20X SSC

- NaCl: 175.3 g
- Na₃ citrate • 2H₂O: 88.2 g
- H₂O: to 800 ml
- 1M HCl: to pH 7.0
- H₂O: to 1 L

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STE
Tris-HCl
NaCl
EDTA
H₂O
HCl to pH 8.0
H₂O

10X TBE Buffer
Tris base 108 g
Boric acid 55 g
0.5 M EDTA, pH 8.0 40 ml
H₂O to 1 L

TE Buffer
1 M Tris, pH 8.0 10 ml
0.5 M EDTA, pH 8.0 2 ml
H₂O to 1 L

Transblot Buffer
Glycine 43.2 g
Tris base 9.09 g
MeOH 600 ml
H₂O to 3L

Tris-Buffered Saline containing Tween 20 (TTBS)
Tris base 121.4 g
EDTA 8.18 g
NaCl 174 g
H₂O to 15 L
HCl pH to 8
Tween 20 20 ml
H₂O to 20 L

Wash Buffer for Southern Blots
Low Stringency  High Stringency
2X SSC  0.5X SSC
0.1% SDS 0.1% SDS

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APPENDIX II

cDNA SEQUENCES
Trout #10 cDNA sequences

10 week 0
AGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGC
AGAATTCCGCTTCCGAGCGACCTGTACAGCCTCCGAGTTCACATTCAGTAGCTACTAC
ATGGCTTGGATCGAGACAGGCTCCGGGGAAAGGACTGGAGTGGACTGCTTCTAGTTATAG
TACTGTCACTATTATTCTCCAGTCAGTCAGGAGGTAGATTCACCATTCTCCAGAGATGACT
CCAGAGTAAGCTGTACCTACAGATGAACATTCTGAAGAGTGAAGACTGACTGCCACCTCT
ATTACTGTGCGATCCAGCAAATTTGGAATTTGACCTCTAGATGCATGCTCGAGCGGCCGC
CAGTGTGATGGATATCTGCAGAATTCGGCTTCCGGAGCGACCTGTACAGCCTCCGAGTT
CACATTCAGGTAGCTACTACATGGCTTGGATCAGACAGGCTCCGGGGAAAGGACTGGAGT
GACTGCTTCTATGTATTATGACTGCTACATATTATTTACACTCCAGTCAGTCGAGGTAGATT
ACCATCTCCAGAGATGACTCCAGAAGTGAAGCTGTACCTACAGATGAACATTCTGAAGAG
TGAAGACACCGGCCTTTATTACTGTGCGATCCAGGAAATTCCAGACACTCTCCAGGAAAG
ACTTGGAA

10 week 10
TAATATAGACTCAGCTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGC
AGTGTGATGGATATCTGCAGAATTCGGCTTCTGGTAGTTGTATGTTCCTTGATGTATGTGAT
ACTGCTTCTATGTATTATGACTGCTACATATTATTTACACTCCAGTCAGTCGAGGTAGATT
ACCATCTCCAGAGATGACTCCAGAAGTGAAGCTGTACCTACAGATGAACATTCTGAAGAG
TGAAGACACCGGCCTTTATTACTGTGCGATCCAGGAAATTCCAGACACTCTCCAGGAAAG
ACTTGGAA

10 week 10
TATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCAGTGTGATGGATATCTGC
AGTGTGATGGATATCTGCAGAATTCGGCTTCTGGTAGTTGTATGTTCCTTGATGTATGTGAT
ACTGCTTCTATGTATTATGACTGCTACATATTATTTACACTCCAGTCAGTCGAGGTAGATT
ACCATCTCCAGAGATGACTCCAGAAGTGAAGCTGTACCTACAGATGAACATTCTGAAGAG
TGAAGACACCGGCCTTTATTACTGTGCGATCCAGGAAATTCCAGACACTCTCCAGGAAAG
ACTTGGAA

10 week 10
GTAATACGACGCTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCAGTGTG
AGTGTGATGGATATCTGCAGAATTCGGCTTCTGGTAGTTGTATGTTCCTTGATGTATGTG
ACTGCTTCTATGTATTATGACTGCTACATATTATTTACACTCCAGTCAGTCGAGGTAGATT
ACCATCTCCAGAGATGACTCCAGAAGTGAAGCTGTACCTACAGATGAACATTCTGAAGAG
TGAAGACACCGGCCTTTATTACTGTGCGATCCAGGAAATTCCAGACACTCTCCAGGAAAG
ACTTGGAA

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Additional "representative" sequences (does not include all sequences obtained during the study - additional sequences will be archived in GenBank).

1 week 0 (also replicated by other control fish at this time point)
ACCTGTACAGGCTCTGGGATGTCGTGCTATGGATCTGAGAATTCGGCTTCTGTACAGGCTCTGGGAAAGGATTGGAGTGGGTTGGAA
ATGTATATTCTGGAGACACACGATACAAGGATTCACTGAAGAACAAGTTCAGCCTCTCA
GTAGACTCTAGCAGTAATACTGTGTTTTTAAAAGGGCAGAACCTTCAGACTGAAGACTC
AGCTGTGTATTATTGTGCCTAC

1 week 5 (also replicated by other control fish at this time point)
ACCTGTACAGGCTCTGGGATGTCGTGCTATGGATCTGAGAATTCGGCTTCTGTACAGGCTCTGGGAAAGGATTGGAGTGGGTTGGAA
ATGTATATTCTGGAGACACACGATACAAGGATTCACTGAAGAACAAGTTCAGCCTCTCA
GTAGACTCTAGCAGTAATACTGTGTTTTTAAAAGGGCAGAACCTTCAGACTGAAGACTC
AGCTGTGTATTATTGTGCCTAC

6 week 0
TGGATATCTGCAGAATTCGGCTTTCTGGGCATCATCAGATAGGATGCTATGGATCTGAGAATTCGGCTTCTGTACAGGCTCTGGGAAAGGATTGGAGTGGGTTGGAA
ATGTATATTCTGGAGACACACGATACAAGGATTCACTGAAGAACAAGTTCAGCCTCTCA
GTAGACTCTAGCAGTAATACTGTGTTTTTAAAAGGGCAGAACCTTCAGACTGAAGACTC
AGCTGTGTATTATTGTGCCTAC

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8 week 0
AATTGTAATACGACTCACTATAGGGGCGAATTGGGCCGCTGCTAGATGCTGAGCGGCGGCGCAGGTGTAATGGATAGTCTATCCAGAGTGACATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTAGACCTGTACAGCCTCCGAGTTCTCAACTGGGAGCGGAGATATGATGAAAGCCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATBCGAGCTCGGWACCAAGCTTGGCGTAATCATGGTC

10 week 0
TACACTACTTATAGGGGGAATTGGGCCCTCTAGATGCTGAGCGAGCGGCCGCCAGTGTGAATGGATATCTGCAGAATTCGGCTTTCAGTGAAACTATCTTGCCAAGTCTCTGGCTATGTCCTGACAGACTATGGCACAGGTTGGATACGACAGCAGCCAGGGAAAACACTGGAGATGACACACAGCTGTGATTATTGTGCAAAAACGTGAGCGCGGCGAGGCTTTTGGAGACTACTGGGGAAAGGCACAATGGTTACCGTTCATCAGCCTCATCAACTGCTCCGACTTTGTTCCCTCTTGCGCAATGTGGCTCCGGGAGCGGAGATATGATGAAAGCCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGCGTTCTCTCCTGTAAGGATCTGGGTATTCTTTAACCAGTACCAGCTACTGTACAGGTTGGGTACGACAGCCTGCAGGAAAAGCACTGGAGTGGGTTGGATACATGTGTGGCAGTGSTAACAATGACTACAGTGATACGCAATGCTGAGTGTTGGGATACATGTGTCAGGTGGGATCATCTGGGGTGAGGGGAAAGGGACCATGGTGACCGTGTCCACAACCTCACTCAACTGCTCACSACTTGGTTCCCTCTTGCGCAATGTGGCTCCGGGAGCGGAGATCTGAAAGCCGAATTCCAGCACACGGCTYCTTTACTAGTGATCCGAGCTCGCACGCTAGATCATGCATCTAYGAGGGGCCAA

10 week 0
TCTCTCCCTGAAGGTATCCTGGGTATTCTTTAACCAGTACCAGCTACTGTACAGGTTGGGTACGACAGCCTGCAGGAAAAGCACTGGAGTGGGTTGGATACATGTGTGGCAGTGSTAACAATGACTACAGTGATACGCAATGCTGAGTGTTGGGATACATGTGTCAGGTGGGATCATCTGGGGTGAGGGGAAAGGGACCATGGTGACCGTGTCCACAACCTCACTCAACTGCTCACSACTTGGTTCCCTCTTGCGCAATGTGGCTCCGGGAGCGGAGATCTGAAAGCCGAATTCCAGCACACGGCTYCTTTACTAGTGATCCGAGCTCGCACGCTAGATCATGCATCTAYGAGGGGCCAA

10 week 0
GGCTCCTGGGAAAGCACTGCAGTGGATTGCTTATATTAGCACACAGAGTAATCCAATCTTATTCCCAGTCAGTCCAGAGTAGATTCACTATCTCCAGAGATAACTCTAGCAGTAAGCTGTACCTACAGATGAACAGTCTGAGGAGTGAAACAGCAGTATATTACTGTGCTAGAGAGAACTACGCCCGTTTTGACTACTGGGGGAAAGAGACAATGGTTACTGTTTCATCAGCCTCATCAACTGCTCCGACTTTGTTCCYTCTTGCGCAATGTGGCTCCGGGAGCGGAGATCTGAAAGCCGAATTCCAGCACACGGCTYCTTTACTAGTGATCCGAGCTCGCACGCTAGATCATGCATCTAYGAGGGGCCAA

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11 week 0
CGCCAAAGCTTTGTAACCGAGCTCGCATCCACATACTAGTAACGGCCGCCAGTGTGCTGGAATTC
GGCTTCCGGATCCGGACAGTAATAAGCTGCTGTGTCCTCCTCTCTCAGACTGTGCC
GTAGGTGTAGCTGCTGAGCTACCTCTCTGTGGAATGTGAAATCTCAGAGGCTG
TGAGAGTAAAGAGATGAGAGCTACTACATATATATGCAATTAAGCAGCCCTCCCAGCTC
11 week 0
ATTGTCATGTAAAATCTCTGGGTTTGATATGACAAACTATTACATGCACTGGATAAGAC
AGAAACCAGGCAAAGCTCTCGGAGTGGATGGAATTGGAGATTAAACTCTGGTTCAACAGATGCT
CCAGTCATCTCGGCAGCTCCCTCTGAAAGGGCAGTTCACTCCCTGAGAGCTCCTACTACAAG
CAGAACTCTTTAGGAGGCAAGAGCTCGAGAGTTACAGCTTGGCTGTGGTTTATATATGCTG
CTCAGAGAGGGAATGGGGTGGGGAGCGGAGATATGATGAAAGCCGAATTCCAGCACACTGGCGCCG
15 week 5
CGGCCAGTGGAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCTCTAGATGCATGC
TCGAGCCGCGCCAGTGTGATGGATATCTGCACTGTGTAATATGCTGTCCTCGGCTCAGATGCT
CTCAGGTGAGGTTGGAATAGAAGTCTCCTGGCTTCAACAGATGCTCAGTCTACACTCAGACT
CCCTGAAAGGCGAGGTCCCTGGCAGAGCTCCTCTACAGATGGTACCTCACCACAGGCAAGG
15 week 5
CGCCCAAGCTTTGTAACCGAGCTCGCATCCACATACTAGTAACGGCCGCCAGTGTGCTGGAATTC
GGCTTCCGGATCCGGACAGTAATAAGCTGCTGTGTCCTCCTCTCTCAGACTGTGCC
GTAGGTGTAGCTGCTGAGCTACCTCTCTGTGGAATGTGAAATCTCAGAGGCTG
TGAGAGTAAAGAGATGAGAGCTACTACATATATATGCAATTAAGCAGCCCTCCCAGCTC
15 week 5
ATTGTCATGTAAAATCTCTGGGTTTGATATGACAAACTATTACATGCACTGGATAAGAC
AGAAACCAGGCAAAGCTCTCGGAGTGGATGGAATTGGAGATTAAACTCTGGTTCAACAGATGCT
CCAGTCATCTCGGCAGCTCCCTCTGAAAGGGCAGTTCACTCCCTGAGAGCTCCTACTACAAG
CAGAACTCTTTAGGAGGCAAGAGCTCGAGAGTTACAGCTTGGCTGTGGTTTATATATGCTG
CTCAGAGAGGGAATGGGGTGGGGAGCGGAGATATGATGAAAGCCGAATTCCAGCACACTGGCGCCG
15 week 35
ATTGTCATGTAAAATCTCTGGGTTTGATATGACAAACTATTACATGCACTGGATAAGAC
AGAAACCAGGCAAAGCTCTCGGAGTGGATGGAATTGGAGATTAAACTCTGGTTCAACAGATGCT
CCAGTCATCTCGGCAGCTCCCTCTGAAAGGGCAGTTCACTCCCTGAGAGCTCCTACTACAAG
CAGAACTCTTTAGGAGGCAAGAGCTCGAGAGTTACAGCTTGGCTGTGGTTTATATATGCTG
CTCAGAGAGGGAATGGGGTGGGGAGCGGAGATATGATGAAAGCCGAATTCCAGCACACTGGCGCCG

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* Kabat definition is based on sequence variability.

**CDR1** - start is approximately residue 26.
Kabat definition starts 5 residues after CYS.
Residues before are always CYS-XXX-XXX-XXX-XXX.
Residue after always TRP. Typically TRP-VAL but may also be TRP-ILE, TRP-ALA.
Length 10 - 12 a.a.

**CDR2** - start is always 15 a.a. after the end of CDR1.
Residues before are typically LEU-GLU-TRP-ILE-GLY.
Residues after are LYS/ARG-LEU/ILE/VAL/THR/ALA-THR/SER/ILE/ALA.
Length 16 - 19 a.a.

**CDR3** - start is always 33 a.a. after the end of CDR2 (always 2 residues after a CYS).
Residues after are always TRP-GLY-XXX-GLY
Length 3 - 25 a.a.

Antibody heavy chain CDR definitions per the mammalian model.
<table>
<thead>
<tr>
<th>VH Family</th>
<th>Accession #</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><em>Onmy VH I</em></td>
<td>M57442</td>
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<td>X92501</td>
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<td></td>
<td>S63348</td>
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<td><em>Onmy VH II</em></td>
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<td><em>Onmy VH III</em></td>
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<td>X81509</td>
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<td><em>Onmy VH IV</em></td>
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<td><em>Onmy VH VI</em></td>
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<td>X81486,X81485, X81495,X81481</td>
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<td><em>Onmy VH VII</em></td>
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<td><em>Onmy VH VIII</em></td>
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<td>X81483</td>
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<tr>
<td><em>Onmy VH IX</em></td>
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<tr>
<td><em>Onmy VH X</em></td>
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<tr>
<td><em>Onmy VH XI</em></td>
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Representative trout Ig VII family sequences archived in GenBank database.

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<th>CDR1 FR2</th>
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<th>FR3</th>
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<tr>
<td>I QT-LTESGPVVK-ITGEPHVCTASGFTFS----YYSOSVQGRFTISRDNSKQQVYLQMNSLKTEDSAVYYCAR</td>
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<td>II QVVTQTEQSVQGTPAGSLKLTACSGFTLS----STNMHWIRQAPGKLEWIYYYSDSKSN--APVQVQGRFTASKDSRN--FYLHMSQLKPEDSAVYYCAR</td>
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<tr>
<td>III QT-ELTPQSLTLQPQGQLTSLCQNGSGLS----STSYCTGWVRQPAGKALEWGVYMCQSNI--YYSKDKNKFSTISRDTSSTTVTQLQSTQEDTVYYCAR</td>
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<td>IV DE-MDQSFSQVK-PKGDKFKLSCQGSDMT----NYYKHWIRQKPQGKLEWGVINSQTDAYSDSLKQGFPLTEDVSTSEQFLEAKSLSEDASAVYYCAR</td>
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<tr>
<td>V QT-LTESGPVVK-KPGESHLKLTACGFTFS----SYAMGNVRFAPGKLEWGVVINSQTDAYSDSLKQGFPLTEDVSTSEQFLEAKSLSEDASAVYYCAR</td>
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<tr>
<td>VI E--ELTPASMTQSVQSGPLTISCKVSV-YSVG--T--YITTAWIRQPAKTEIWIGNY-TGGT--HKDSKLNKFSLTVDSSNTVTQLQLQTEDTAVYYCAR</td>
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<tr>
<td>VII GGELEQPNMVVEKPGESLSVTCPVSGVYSGDSSIFATGVWIRQAPGKAMEWISHWDNQDI--YKMDALKNKFSISTASSNSVSLQGKSLQPEDTAVYYCAR</td>
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<tr>
<td>VIII QYVLTQASQP--GTPAGSLKLTACGFTLS----RGNMHWIRQAPGKLEWIYYYSDSKSN--APVQVQGRFTASKDSRN--FYLHMSQLKPEDSAVYYCAR</td>
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<td></td>
</tr>
<tr>
<td>IX QS-LTSESVPVVK-RPSEVTSLTCTVSFGSNGS----YWWQHWIRQPKPQKLEWIGFDGTTT--AQAQSLQGQFTIKDTSKNQLYELMNSKLRTEDSAVYYCAR</td>
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<td></td>
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<tr>
<td>X D-IEIQSGPVVIEPGVSFGSISCKFSGSIS----SYDLGWIRQAEKALWEVGTVCSST-T--YTVDSLKSITSTRDSSSTVFLQGNNQFEDTAVYYCAR</td>
<td></td>
<td></td>
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<tr>
<td>XI IS-LTSSPAQLK-PPGEVKSNCQNVCGYTEL--SGTGWIRQAPKTEIWIGISWSSIES--GASFRRFTISRD-SSNVLYLTDILTQLAEDTAVYYCAR</td>
<td></td>
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</tbody>
</table>
Predicted SDS-PAGE banding patterns of CNBr-digested trout heavy chains (Zhang 1999). The red bands represent the theoretical products generated by the CH portion. The blue bands represent the unique products of VH digestion. The Roman numerals designate the VH families (I-XI) and the Arabic, distinct VH sequences within each family group. The sequence data was obtained from GenBank.
VITA

TERESA DAWN JENKINS LEWIS

Born in Bethesda, MD. Earned a B. S. (Biology) from Christopher Newport University in 1993. Entered the doctoral program at the College of William and Mary, School of Marine Science, in 1995.

Professional Memberships:

International Society of Developmental and Comparative Immunology
American Association of Immunologists
American Fisheries Association
International Association of Genetics in Aquaculture

Offices:

President of the Graduate Council. College of William and Mary May 1998 - May 1999
Virginia Institute of Marine Science Graduate Student Association
  President May 1998 - May 1999
  Treasurer May 1996 - May 1997

Presentations:

Eighth Congress of the International Society of Developmental and Comparative Immunology, July 2 - 6, 2000. Cairns, QLD, AUS.
Seventh Congress of the International Society of Developmental and Comparative Immunology, Jul. 21 - 27, 1997. Williamsburg, VA.
Twenty-second Annual Eastern Fish Health Workshop, Mar. 18 - 20, 1997. Atlantic Beach, NC.

Publications: