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Investigations into DNA vaccination against Channel Catfish Virus

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INVESTIGATIONS INTO DNA VACCINATION AGAINST CHANNEL CATFISH VIRUS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences through the
Department of Pathobiological Sciences

By
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B. Sc. Texas A&M University, 1998
May, 2004
ACKNOWLEDGEMENTS

The past five years would have been unbearable without the help and support of many people. To these many people who have kept me from despairing and kept me laughing, I give my undying gratitude. My family, foremost, I could not have done without. My parents and sister, who have always been by my side, have always been willing to listen (even when they didn’t understand what I was talking about), and kept me going when I wanted to give up, were the biggest source of strength that I could have asked for. I can never express enough how much all of the words of encouragement and unconditional love meant. Secondly, I would like to express thanks to someone who is one of the best friends I have ever had and a person who I worked with most intimately, Karen Plant. Not only did you get me through the day-to-day celebrations and sorrows, but you helped me gain the confidence to stand by my opinion. My research life became infinitely better when you joined our lab, and I am glad to have been able to have you for a friend. I’d also like to thank the lab - Ron Miller (my first commiserator), Tara Landrum, Ahmad El-Kamel, Maria Kelley-Smith, Denise Fernandez, Matt Rogge, Natha Booth, Judy Wiles, and more – for helping me when they could, but mostly for being there to listen when I needed it, and supporting me when I needed it. Thank you to the department, those of you that listened when things went wrong and those of you that provided unique services – Thaya Geudry for the sequencing and Michael Kearney for the statistical services. I’d like to give a special thanks to two members of my committee that took a special interest in me, D. Allen Rutherford and, my new member, John P. Hawke. I would like to thank my committee as a whole, who individually and collectively helped steer me along the best path available. Thank you for all of your help and support. And lastly, I’d like to thank my major advisor, Ron Thune.
It’s been quite an experience for us, and in the end I’d like to say that it’s been good to know you. Thank you for all of your guidance, troubleshooting advice, and support.
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Figure 3.5. Serum neutralization titers. Fingerlings (average weight 1 g) were vaccinated with pORF59, pORF6, pORF59+6HH, KN59, KN6, KN59+6, pcDNA, and TE. Three fish were sampled at day 35 for neutralizing antibodies and subjected to the varying serum/constant virus serum neutralization test. Results shown are the average of the 3 fish where the titer represented is the reciprocal of the dilution at which no CPE was detected.
ABSTRACT

The leading viral killer of commercially produced channel catfish is Channel Catfish Virus (CCV). Studies conducted to evaluate DNA vaccination against CCV compared encoded gene, dose, multiple DNA vaccines, and immune response to vaccination. Genes were selected (ORFs 1 and 3 [immediate early genes], ORFs 6, 19, and 46 [membrane genes], and ORF59 [putative major envelope glycoprotein gene], cloned into a plasmid, and expressed in mammalian and fish cell culture to detect predicted molecular weight proteins. Plasmid vaccines were injected into fish muscle in doses of 50 µg, 25 µg, 5 µg, or 1 µg and efficacy was evaluated upon challenge. Immune responses were measured by Mx gene expression (α/β interferon indicator), serum neutralization, specific antibody stimulation (ELISA), and DNA vaccine specific expression. Comparisons were made between published live attenuated CCVTK-, DNA vaccines (KN59 and KN6), and DNA vaccines tested in this study. In all experiments, no protection was observed. Multiple groups of vaccines delivered per fish were tested for efficacy and protection was not observed. The live attenuated CCVTK- and published CCV DNA vaccines (KN59 and KN6) were not protective. Mx gene expression in response to DNA vaccination showed positive expression profiles at all time points, but most often at day 3 in all experiments. In the multiple group vaccination, Mx gene expression was detected at a higher level overall and at day 35, indicating that multiple antigens induce a stronger innate response with longer duration. In all experiments, serum neutralizing titers were low in most treatments (< 10), but weakly reactive in the multiple group vaccination (3 groups with titers > 10) and the comparison vaccination (4 groups with titers > 10). ELISA of vaccinated fish sera detected low reactivity, but significant levels of reactivity were observed between sera from pORF46 and pORF3 and the negative control. In the comparison vaccination, DNA vaccine specific gene expression was detected in
all groups and at most time points, indicating the DNA vaccines were transcriptionally functional. A protective vaccine against CCV is a goal to be striven for, because currently available vaccines may not be suitable for commercial use.
CHAPTER 1. LITERATURE REVIEW

Channel Catfish Virus

Viral Characteristics and Classification. Commercial channel catfish (*Ictalurus punctatus*) production is the predominant finfish aquaculture industry in the United States. This industry produced 630 million pounds of fish in 2002, valued at $3 billion dollars in gross national product annually (USDA Aquaculture Outlook Report, 2003). The leading viral killer of catfish is Channel Catfish Virus Disease (CCV), caused by *Ictalurid herpesvirus 1*. Fijan, Wellborn, and Naftel (1970) originally isolated Channel catfish virus, a fish herpesvirus, from five epizootics involving young channel catfish. CCV is a devastating, highly-communicable disease affecting fry and fingerling channel catfish primarily in the southern United States commercial catfish industry. Maximum losses usually occur in the warmer months of the year, June to September, due to the resultant warmer water temperature (Plumb 1978). Channel Catfish Virus is an alphaherpes virus with a 134 kilobase (kb), double stranded DNA genome that is encapsulated by an icosahedral envelope (Davison 1992). Electron microscopy showed that the nucleocapsid is approximately 100 nm in diameter and negative staining showed that the complete virion is about 175-200 nm in diameter, comprised of 162 capsomeres (Wolf and Darlington 1971). CCV is distantly related to human Herpes Simplex Virus 1 (HSV-1) based on virion morphology (Wolf and Darlington 1971; Davison 1998). CCV has a capsid that is smaller than the capsid of HSV-1, however, the genome, which is 12% smaller, is contained within the capsid at approximately the same average density as HSV-1 (Booy et al. 1996). The capsid of CCV is 20% thinner than that of HSV-1 and the icosahedrons are flatter (Booy et al. 1996). The basic architecture of the viron is, however, very well conserved with HSV-1 in that the triangulation number is the same as HSV-1 (T=16), the pentons and hexons have a chimney-like
protrusion that contains an axial channel through all capsomeres, and at sites of local threefold symmetry there are triplexes at the outer surface (Booy et al. 1996). In spite of the remarkable structural similarities to HSV-1, due to incompatibilities in primary nucleic and amino acid sequence, CCV is unable to be accommodated in current taxonomy (Davison 1998). The CCV genome is comprised of a 95 kb unique region flanked by a direct repeat of 18 kb on each side (Chousterman et al. 1979). The genome has a molecular weight of $85 \pm 4 \times 10^6$ and is linear and nonpermutated (Dixon and Farber 1980). There are 77 genes present, with 14 located in the repeat portion of the genome (Chousterman et al. 1979; Davison 1992). Thirty-two polypeptides were identified as being virus-specific, with 18 of those identified as structural components of virons (Dixon and Farber 1980). Viral replication occurs via a rolling circle mechanism and viral DNA of one unit length is separated from head to tail concatamers by excision (Cebrian et al. 1983). Replication of the virus occurs from 10 to 35°C, with most rapid replication at 35°C and maximum replication at 30°C (Bowser and Plumb 1980). Best growth was seen from 25-33°C (Wolf and Darlington 1971). The cycle of CCV replication at 30°C takes place over a period of approximately 8 hours, during which viral DNA synthesis could be initially detected between 2 and 2.5 hours, rose to a maximum at 5 hours, and decreased to a low level at 8 hours (Dixon and Farber 1980). This enveloped virus replicates in the cell nucleus and acquires its envelope at the nuclear membrane and in cytoplasmic vacuoles (Wolf and Darlington 1971).

**Transmission.** Channel Catfish Virus is host specific to the channel catfish, *Ictalurus punctatus* (Boon et al. 1988). The blue catfish (*Ictalurus furcapus*) can be experimentally infected but does not exhibit signs of disease (Plumb and Chappel 1978). The african catfish (*Clarias gariepinus*) and the asian catfish (*Clarias batrachus*) have been tested for susceptibility and have been found to be resistant to CCV infection (Boon et al. 1988). Plumb et al. (1975)
tested eight strains of 1-3 month old CCV-free channel catfish fingerlings (Falcon, Kentucky, Marion, Tennessee, Warrior, Yazoo, Warrior-Yazoo, and Yazoo-Tennessee) for susceptibility to wild-type CCV. Following lethal challenge with CCV, significant differences were observed between strains of fish in mortality. The Falcon strain suffered 71% mortality, which was significantly higher than the rest. The least susceptible strains were the Yazoo and the Tennessee, experiencing 12% mortality, and the hybrids of these strains (Warrior-Yazoo and Yazoo-Tennessee) experienced 10% mortality. Plumb et al. (1975) attribute the wide range of susceptibility of strains to CCV as familial genetic resistance to disease, supported by the inability they experienced in isolating virus from experimentally challenged fish. Transmission has been shown to be horizontal (through the environment) and vertical (through reproductive products) from fish to fish. Nusbaum and Grizzle (1987) showed that when in small volumes, CCV rapidly associated with sperm, slowly associated with channel catfish cells, and did not associate with channel catfish eggs. Association with sperm, however, did not occur in larger volumes of water. Plumb et al. (1981) tested spawning fish with a known history of CCV at the time of spawning. Spawning fish were injected with an immunosuppressant and tested for detection of CCV in parent tissue, eggs, and fry, as well as by indirect fluorescent antibody (IFA) tests on frozen parent tissue sections. They found that although serum neutralizing antibodies did not increase and CCV could not be isolated from internal organs, eggs, or fry, the IFA tests on frozen spent ovarian tissue was positive for CCV. This indicates the female fish as the parent responsible for vertical transmission. Currently, the reproductive product responsible and method of vertical transmission of CCV is unclear but, transfer from latently-infected adult catfish to offspring has been demonstrated by polymerase chain reaction (PCR) (Wise et al. 1985; Wise et al. 1988; Boyle and Blackwell 1991; Gray et al. 1999; Gray et al. 1999b).
Horizontal transmission is thought to occur when a stressed, latently infected fish sheds virus into the water by excretion of feces and urine and is taken up through the skin, olfactory canals, gut, or gills of a naive fish (Nusbaum and Grizzle 1987). The mode of horizontal transmission and exact point of entry into the fish is still unresolved.

**Pathology.** Channel Catfish Virus Disease is a hemorrhagic viremia that results in death of fry and fingerlings and resolves in adult catfish (Noga 1996). High mortality rates caused by this disease (>90%) has occurred in fry or fingerlings smaller than 5-10 cm and less than five months old (Plumb 1978). Size and age of fish are not the only determinant, stress, such as low dissolved oxygen levels, crowding, high temperatures, handling stress, and bad water quality, can trigger outbreaks. Water temperature at or above 25°C is optimal for CCV disease. Diminished mortalities at 20°C have been observed (Plumb 1978). Clinical signs of CCV include: reddening of the body, base of fins, and gills due to hemorrhaging; hanging tail-down in the water; disorientation; corkscrew swimming; abdominal distension; yellow fluid in the peritoneal cavity (ascites); exophthalmos; lethargy; and punctate hemorrhaging of the viscera. The viremia affects all major organs causing a focal and/or diffuse necrosis of the kidney, hematopoietic tissue, excretory tissue, liver, spleen, gastrointestinal tract, pancreas, and skeletal muscle (Fijan et al. 1970; Plumb 1971). The primary site of viral presence or replication in infected fish is the kidney, with lower counts of virus also present in the gut, intestine, spleen, liver, and tail (Plumb 1971b; Nusbaum and Grizzle 1987). The virus damages the central nervous tissue and can be isolated from the brain of infected fish (Noga 1996). CCV also interacts with the cytoskeleton, causing the formation of syncytia, which appear as giant multi-nucleated cells (Pope and Scheetz 1998). Mortalities can occur as soon as one day after infection (Noga 1996). Within days after an epidemic of CCV ends, virus is difficult to isolate from the
fish population (Wolf 1988). The virus is unstable in the environment, but can persist for one to two months depending on water temperature (Noga 1996). There is a 50% reduction in viral recovery from fish which are kept at 20°C for 100 days and a 90% reduction from fish that are on ice for three days. The virus can survive for three days in dead fish that are kept at room temperature (Plumb 1973). Once infected with CCV, the fish population is susceptible to secondary infections, such as *Aeromonas*, which makes diagnosis of the primary cause of death difficult (Noga 1996).

**Diagnostics.** Diagnosis of Channel Catfish Virus has been accomplished by isolation in cell culture, neutralization of virus with immune sera, fluorescent antibody techniques, nucleic acid probing, and polymerase chain reaction to date.

**Cell Culture and Serum Neutralization.** Channel Catfish Virus can be grown in Channel Catfish Ovary cells (CCO) or Brown Bullhead cells (BB) for diagnostic purposes (Bowser and Plumb 1980). Viral replication is identified by the typical cytopathic effect (CPE), where infected cells fuse with neighboring cells, producing giant multi-nucleated cells, or syncytia. Plaques (zones of clearing) in the cell monolayer result from cell lysis due to release of CCV viral particles (Buck and Loh 1985). Syncytial plaque formation can be detected as early as two hours post-infection, and viral particles are visible in the nucleus after four hours (Wolf and Darlington 1971). Serum collected from fish exposed to CCV naturally or experimentally can be used to neutralize virus for diagnostic purposes, however with inconsistent results (Plumb1973b; Arkush, McNeill, and Hedrick 1992).

**Fluorescent Antibody and Nucleic Acid Probe.** The fluorescent antibody technique was used by Plumb et al. (1981), not in a diagnostic capacity, but to identify channel catfish tissues that harbor the virus. A nucleic acid probe method was developed by Wise and Boyle (1985),
with an *EcoRI* fragment of the CCV genome in a Southern hybridization technique. DNA isolated from the heart, liver, spleen, stomach, intestine, posterior kidney, gonads, blood, skeletal muscle, dorsal fin, and parts of the brain from fish injected with $1 \times 10^{7.5}$ TCID$_{50}$ CCV/ml were probed with the fragment to diagnose CCV. The sensitivity of this test was determined to be one viral DNA per cell. Seven of eleven tissues tested positive for CCV with this technique, however not all fish were positive (Wise and Boyle 1995). In another experiment, a group of 22 channel catfish that were asymptomatic for the virus were all positive in liver tissue with the probing technique (Wise et al. 1985). Ten out of 22 of these fish were previously identified as positive for latent CCV by co-cultivation of their leukocytes with CCO cells (Bowser et al. 1985). Additionally, in a group of 14 asymptomatic fish, 11 were identified as positive by the nucleic acid probe method after testing seven organs (Wise et al. 1985). These data indicates that DNA hybridization is much more sensitive than the leukocyte co-cultivation in detecting exposure to CCV.

**Polymerase Chain Reaction.** Another technique for detecting CCV, especially in latently infected animals, is polymerase chain reaction (PCR). This technique has been used extensively to identify CCV-free fish and broodstock (Boyle and Blackwell 1991; Tham and Moon 1996; Gray et al. 1999; Gray et al. 1999b). The thymidine kinase gene of CCV, encoded by open reading frame (ORF) 5, was used for diagnosis of clinical samples of suspected CCV infected fish when a PCR detection assay was developed by Tham and Moon (1996). The sensitivity of their assay was determined to be 3 picograms (pg) of viral DNA. A PCR method utilizing primers to ORF8 (early gene) and ORF59 (late gene) was developed by Gray et al. (1999) to detect acute CCV infection in channel catfish tissues (brain, blood, intestine, kidney, and liver). The sensitivity of this test was determined to be 100 femptograms (fg) of CCV DNA and
increased 10-fold compared to catfish DNA when detected by Southern blot. CCV was detected by PCR in the blood, brain, intestine, kidney, and liver in acutely infected fish. Various methods have been developed to detect latent CCV by PCR. In 1991, primers constructed from the R band of an EcoRI digest of CCV DNA were created and used to detect CCV DNA to a sensitivity of 0.1 pg (Boyle and Blackwell 1991). Two asymptomatic fish were screened with this detection method and one fish tested positive, indicating a latent carrier of CCV. This technique was determined to be more rapid and more sensitive than previous nucleic acid probing methods (Boyle and Blackwell 1991). A nested PCR technique was developed to detect latent CCV in blood samples of healthy broodfish to standardize testing of broodfish and reduce vertical transmission to progeny (Baek and Boyle 1996). The DNA polymerase gene (ORF57) was targeted where one set of primers amplified a product of 694 bp and the second set of primers (the “nest”) amplified a 251 bp fragment from the previous 694 bp product. Nested PCR is used in this study because it increases sensitivity (Baek and Boyle 1996), which was 1 fg or about 10 copies of CCV DNA. Gray et al. (1999b) tested blood, brain, intestines, kidney, liver, and peripheral blood leukocytes from fingerling channel catfish 20 weeks after experimental infection with CCV by a PCR method with primers to ORF8. These are the same primers used in the acute infection study (Gray et al. 1999). In this study, however, an internal set of primers was chosen to increase sensitivity and another set was chosen to detect circular or concatemeric structures of CCV DNA. The second set of primers were chosen so that amplified products could only be derived from adjacent genomes, circular DNA, or concatemeric intermediates. Infectious virus could not be cultivated 20 weeks after challenge. Standard PCR of ORF8 did not detect latent CCV in any tissues. Nested PCR of ORF8, however, detected latent CCV in blood, brain, gastrointestinal tract, kidney, and liver. CCV DNA was detected when pooled
Peripheral blood leukocytes were subjected to the nested ORF8 PCR (Gray et al. 1999b). When testing for concatemeric structures, nested PCR amplified the expected product, implicating a circular or concatemeric structure of CCV (Gray et al. 1999b). The detection of CCV latency in leukocytes in this study suggests their role in harboring latent virus, which was previously reported by Chinchar et al. (1993), who discovered that a cloned catfish B cell line and two other populations of non-cloned macrophages and putative T-cells could be productively infected with CCV.

**Treatment.** Once a positive diagnosis is achieved, chemical treatment of CCV-infected fish is not possible. Phosphonoacetic acid has been shown to inhibit CCV replication up to 95% at a dose of 1000 µg/ml in cell culture (Komet 1978). This is impractical for commercial use. However, acyclovir, a known HSV-1 inhibitor, was tested as a CCV inhibitor and reduced plaque numbers by 95% at a concentration of 2 µm in cell culture (Buck and Loh 1985b). Acyclovir did not reduce plaque numbers late in CCV infection. Chinchar et al. (2001) investigated the ability of two antimicrobial peptides isolated from frog skin to inactivate Frog Virus 3 and CCV. They found that the peptides esculentin-2P and ranatuerin-2P directly inactivate CCV, instead of inhibiting replication in infected cells. Antimicrobial peptides are thought to act by creating pores or disrupting bacterial or viral outer membranes, which might be the case in this study (Chinchar et al. 2001). The only reported control measure for CCV, once an epizootic has begun, is to lower the temperature. Plumb (1973) reduced the temperature in 24 hours from 28°C to 18°C resulting in a reduction of mortality from 95 to 24%. At the onset of clinical signs, reducing the temperature reduced mortality from 95% to 58-78%.
**Immune Responses.** The immune response of channel catfish elicited by CCV is not well characterized. Adult fish that survived a natural infection as juveniles have been documented to possess anti-CCV neutralizing antibody in the serum up to two years following exposure (Hedrick et al. 1987). Upon a second exposure, the neutralizing activity did not increase. In naïve fish, neutralizing antibody was detected 1 week post-infection with CCV and persisted for 9 weeks. Increasing the temperature from 18°C to 27°C had a negative effect on the titer of neutralizing sera and on the number of fish seropositive for anti-CCV antibodies (Hedrick et al. 1987). According to Plumb (1973b), neutralizing titers peaked at 60 days post-experimental infection. Bowser and Munson (1986) investigated anti-CCV neutralization titers of convalescent channel catfish in different seasons of the year. They found that significantly lower antibody titers were detected in colder months (January and April) than were detected in hotter months (July and October). The increase in the neutralizing titers of fish sera in hotter months might correspond with shedding from carriers in the population, stimulating the secondary immune response and therefore increasing titers, which does not agree with the results of Hedrick et al. (1987). This neutralization activity is transferable to naïve juveniles who subsequently survived experimental challenge with CCV (Hedrick and McDowell 1987).

Cellular immunity to CCV has not been extensively investigated. However, Hogan et al. (1996) demonstrated that channel catfish possess two populations of cytotoxic cells. One set lyses allogenic targets (cells from a same species individual who is not genetically identical) and the other set lyses channel catfish virally infected cells. Moreover, this team showed that early viral gene products were sufficient to allow the cytotoxic cell to identify and lyse that cell as virally infected.
Characterization of CCV Gene Function. The development of a vaccine against CCV has been an elusive goal for researchers. The problems are multi-fold. CCV is related to HSV-1, but not closely enough to apply knowledge of immunodominant antigens from one to the other. For example, CCV does not encode genes analogous to gB and gD, popular genes for use in HSV-1 vaccination (Davison, 1992). Davison (1992) sequenced the CCV genome and postulated the roles of potential genes. There was no clear list of major glycoproteins, major antigens, or receptors used by the virus for attachment. Davison (1992) postulated that ORFs 72, 73, 14, 15, and 16 are sets of protein kinases, ORFs 9, 11, and 12 are potential zinc binding proteins, ORF 25 is a superfamily I helicase, ORF 57 is the DNA polymerase, ORF 49 is a deoxyuridine triphosphatase (dUTPase), and ORF 5 is the thymidine kinase. He also postulated that ORF 46, due its size, hydrophobicity, and N-linked glycosylation sites, is the best choice for a major envelope glycoprotein. Davison and Davison (1995) characterized some of the genes by mass spectroscopy. ORF 59 encodes four products, each weighing about 8 kDal. ORFs 60 and 30 encode tegument proteins, weighing about 185 kDal and 28 kDal respectively. ORFs 72, 73, 74, and 15 encode tegument associated proteins, weighing about 150, 97, 68, and 44 kDal respectively. ORFs 39, 53, 27 encode capsid proteins, weighing about 115, 34, 30, and 40 kDal respectively. Furthermore, gene products from ORFs 39, 53, and 27 show analogy to HSV-1 proteins from genes UL19, UL38, and UL18, respectively, which encode proteins VP5, VP19C, and VP23, three of the five major capsid proteins of HSV-1. Proteins corresponding to proteins VP24 and VP26 were not identified in CCV (Davison and Davison 1995).

Vaccine development for CCV has been hindered because the major antigen of CCV has not been demonstrated. Davison and Davison (1995) lists these ORFs as potential membrane associated genes: 6, 7, 8, 10, 19, 46, 51, and 59. Kucuktas, Brady, and Tuzun (1998) tested
ORFs 46 and 59 with a baculovirus expression system but did not detect expression of the gene product of ORF 46 with polyclonal CCV antiserum. They did detect expression of ORF 59 and recognition of that protein by polyclonal CCV antiserum. When the ORF 59 gene product was added to cells prior to infection, infection as shown by syncytia and plaque formation was inhibited. Thus, ORF 59 is thought to be the major envelope glycoprotein of CCV and is possibly the immunodominant antigen of the virus (Davison and Davison 1995; Kucuktas, Brady, and Tuzun 1998). Products of ORF 59 are present in at least five forms by mass spectroscopy (38 kDal each) and have at least three N-linked glycosylation sites on the external surface of the virion (Davison and Davison 1995).

Temporal regulation of the expression of the gene products, in addition to the arrangement of the genome containing a unique region flanked by two direct repeats, closely affiliates the channel catfish virus with the alphaherpesvirus family (Dixon and Farber 1980; Silverstein et al. 1995; Huang and Hanson 1998). From the direct repeat region, Silverstein et al. (1995) isolated two immediate early transcripts, a bicistronic transcript of ORF 8a and 9, and ORF 9 alone. The mRNA from ORF 8a and ORF 9 encodes a very hydrophobic protein, which indicated a potentially membrane associated protein. The transcript from ORF 9 alone is predicted to be a zinc-binding domain and therefore a probable transcription factor. These two transcripts, ORF 8a and 9 and ORF9 alone, were later characterized to have kinetics of early or late genes, in contrast to earlier findings (Silverstein et al. 1998). A further immediate early transcript was identified and is thought to encode the product of ORF 12, which is proposed to have a consensus RING finger metal binding motif (Huang and Hanson 1998). Zinc metal binding motifs are involved in protein-protein interactions and in DNA and RNA binding. Interestingly, HSV-1 has two immediate early proteins (ICP0 and ICP27) that contain zinc metal
binding motifs. The RING finger binding domain is similar to ICP0 and has homologs in other alphaherpesviruses (Everett et al. 1993; Silverstein et al. 1995). A study utilizing metabolic inhibitors analyzed the 14 genes in the directly repeated regions and elucidated ORFs 1 and 3 as immediate early genes; ORFs 2, 5, 6, 8, 9 and ORFs 11-14 as early genes; and ORFs 4, 7, and 10-13 as late transcripts (Stingley and Gray 2000). Silverstein et al. (1998) describe the ORF3 transcript as an immediate early transcript that has a very short half-life and an mRNA destabilization motif. ORF 5 is postulated to be an early gene, and encodes the thymidine kinase gene (Huang and Hanson 1998; Hanson et al. 1995). Interruption of the ORF 5 gene confers resistance to Ara-T and attenuates CCV while retaining the ability to infect (Hanson et al. 1994; Zhang and Hanson 1995). ORFs 38, 39 and 46 were identified as true late class genes and ORF 39 was postulated to be the major capsid gene (Huang and Hanson 1998; Davison and Davison 1995). ORF 50 is postulated to be a secreted glycoprotein weighing over 200 kDa (Vanderheijden et al. 1999). It possesses a hydrophobic N-terminal leader sequence, no membrane anchor sequence, 35 potential N-glycosylation sites, and many O-glycosylation sites which implies a mucin-like glycoprotein (Vanderheijden et al. 1996). Further characterization of the molecular and functional products of the ORFs of CCV has not been investigated.

**The Fish Immune System**

**Innate Immunity.** The fish immune system is not considered as evolutionarily advanced as the mammalian system. However, the immune response of a fish is strong and measurable. The non-specific, innate arm of the immune system exists to either prevent pathogens from entering the host or prevent multiplication and colonization of the microbe within the host. If a pathogen multiplies/colonizes a host and creates disease, the specific/adaptive arm of the immune system becomes primed and functions in resolving the infection (Kuby 1997). Innate
immunity comprises four types of defensive barriers. Anatomic barriers consist of the skin, a physical barrier to infection with an acidic environment (pH 3-5) that retards growth of microbes. Mucous membranes also provide a physical barrier, trapping microbes and using cilia to propel invaders out of the body. Mucous also competes with pathogens for attachment sites and nutrients (Kuby 1997).

Innate physiological barriers consist of temperature, low pH, and chemical mediators. Normal body temperature and fever inhibits the growth of some microbes. Low pH of the stomach kills ingested pathogens. Chemical mediators include lysozyme, interferon, and complement. Lysozyme can cleave the peptidoglycan of bacteria, interferon induces an antiviral state in uninfected cells, and complement lyses microorganisms and facilitates phagocytosis (Kuby 1997).

Phagocytic and endocytic barriers consist of cells that phagocytose or endocytose foreign macromolecules and break them down, and specialized phagocytic cells (monocytes, macrophages, and neutrophils). Inflammatory barriers involve inflammatory mediators that promote leakage of serum proteins with bactericidal activity and movement of phagocytes into areas of tissue damage, promoting the initiation of an immune response (Kuby 1997).

Nonspecific defense mechanisms of fish consist of physical barriers, ie. skin, mucous, and complement, and nonspecific internal effectors, ie. macrophages, innate cytokines such as Interleukin-1 (IL-1), Tumor Necrosis Factor (TNF), antibacterial peptides, acute phase proteins, Nonspecific Cytotoxic Cells (NCC), and granulocytes. Antibacterial peptides in fish include defensins (circular molecules that can form channels in bacterial membranes) and lysozyme (hydrolytic enzyme in mucous and tears, capable of cleaving the peptidoglycan layer of bacterial cell walls) (Kuby 1997). Fish lysozymes have a broader function than mammalian, with activity
against gram-negative and gram-positive bacteria (Grinde et al. 1988). Lysozyme is produced in different amounts in response to different diseases, and has therefore been used as a nonspecific immune indicator. In mammalian systems, insult to the organism by injury results in a cytokine cascade wherein tumor necrosis factor α (TNFα) is released, followed closely by interleukin 1β, and then IL-6 (Secombes et al. 2001). IL-1 is known to activate lymphocytes, and IL-1, IL-6, and TNFα promote fever in mammals. TNFα is known to kill a variety of cells, including tumor cells in mammalian systems (Kuby 1997). Some of these cytokines have been identified in fish, including IL-1β, and TNF-α. TNF-α and the TNF-α receptor have been sequenced in the Japanese flounder (Paralichthys olivaceus) and rainbow trout, and transcripts have been found in peripheral blood leukocytes, as well as gill and kidney (Hirono et al. 2000; Hirono et al. 2000b; Laing et al. 2001). A second TNF gene has been cloned and sequenced in rainbow trout that is more akin to mammalian TNF-α than TNF-β. After stimulating macrophages with lipopolysaccharide (LPS) the TNF-α2 gene showed stronger expression, which was detected earlier and lasted longer than the TNF-α1 gene (Zou et al. 2002). IL-1β has been cloned in rainbow trout and carp, and genes have been sequenced in seabream (Sparus aurata L.), seabass (Dicentrarchus labrax), and dogfish (Scyliorhinus caniculus) (Zou et al. 1999; Fujiki et al. 2000; Scapigliati et al. in press; Pelegrin et al. 2000; Bird et al. 1999). LPS has been shown to induce IL-1β expression as well as other immunostimulatory functions. IL-1β exerts its effects by binding to a type I IL-1 receptor (IL-1R), which uses an accessory molecule to signal called IL-1RacP (IL-1 Receptor Accessory Protein). Another IL-1R exists, IL-1RIII, which acts as a decoy as it has no intracellular signaling domain. Both IL-1RI and IL-1RIII have been cloned in salmonids (Auron 1998; Sangrado-Vegas et al. 2000; Subramaniam et al. 2002).
Interferon (IFN) is widely known as an anti-viral substance in mammals and as part of both innate and adaptive immunity. In the innate immune response, IFN can inhibit viral replication, induce an anti-viral state in cells, activate macrophages and natural killer cells, and many other functions. In the adaptive immune response, IFN can further activate macrophages and push a cellular immune response toward the T-helper type 1 response (Kuby 1997). Bony fish have been shown to have interferon type I (IFNα and β) which inhibits virus replication, among other functions (Alexander and Ingram 1992). Channel catfish, zebrafish, and Atlantic salmon are three species of fish in which the sequence of the interferon gene has been identified (Altmann et al. 2003; Robertsen et al. 2003; Long et al. 2004). The sequence from the zebrafish interferon gene was used to search the database of the *Fugu* (Japanese pufferfish), resulting in the identification of a putative interferon gene in that species (Altmann et al. 2003). The catfish interferon gene was discovered by BLASTX screening of a catfish EST library created from a mixed lymphocyte culture (Long et al. 2004). Upregulation of catfish interferon mRNA was detected when CCO cells were treated with UV-inactivated catfish reovirus and double stranded RNA. The cDNA from the catfish interferon was cloned and expressed in COS-7 cells. This recombinant factor was shown to have antiviral activity on CCO cells, supporting its’ α interferon-like activity (Long et al. 2004).

Several anti-viral interferon induced molecules have been discovered in fish. Mx genes, vig-1 and 2, and interferon regulatory factors have been found in trout. Mx proteins are anti-viral and were originally found to confer resistance against orthomyxoviruses (Lindenmann 1962). The fact that Mx gene expression is induced by type I interferons was discovered by Haller et al. (1979), leading to their use as an interferon indicator due to the fact that fish type I interferons had not been purified to date. Mx genes have been cloned in the rainbow trout,
*Oncorhynchus mykiss* (Trobridge and Leong 1995); Atlantic salmon, *Salmo salar* (Robertson et al. 1997); Atlantic halibut, *Hippoglossus hippoglossus* (Jensen and Robertson 2000); Japanese flounder, *Paralichthys olivaceus* (Lee et al. 2000); the Fugu, *Takifugu rubripes* (Yap et al. 2003); and the channel catfish (Plant and Thune 2003). An Mx gene has also been cloned in channel catfish by Plant and Thune (2003). The Mx gene was elicited in channel catfish after injection of poly I:C and CCV and had between 71-74% identity to the rainbow trout and Atlantic salmon Mx genes. This suggests that the catfish Mx gene should not only have conserved structure but function with the trout and salmon Mx genes. There is evidence that 3 Mx genes exist in channel catfish (Karen Plant, personal communication). The first fish interferon has been cloned and characterized in the zebrafish (*Danio rerio*) (Altmann et al. 2003). Interferon regulatory factor 1 and 2 have been cloned and sequenced in rainbow trout by Collet et al. (2003). Interferon regulatory factor 1 is expressed in response to DNA vaccination against viral hemorrhagic septicemia (VHSV) and in response to poly I:C. Interferon regulatory factor 2 was upregulated in response to poly I:C, not DNA vaccination. The *vig-1* and 2 genes are directly induced by VHSV and have an unknown function. The *vig* genes are upregulated in rainbow trout when vaccinated with DNA against VHSV and are induced by an interferon-like factor (Boudinot et al. 1999). A molecule analogous to STAT (signal transducers and activators of transcription) has been identified in the channel catfish using a monoclonal antibody against human STAT6 (Rycyzyn et al. 1998). It is approximately 100 kDal and becomes activated and translocates to the nucleus after growth factors and mitogen stimulation of catfish leukocytes is performed. It binds to the mammalian interferon γ activation site, which further suggests that this molecule is a catfish STAT molecule that could be important in interferon γ related responses (Rycyzyn et al. 1998). The leukocyte common antigen (CD45) was identified in the Pacific hagfish, *Eptatretus*
stoutii. This antigen is a prototypical transmembrane protein tyrosine phosphatase that functions in signal transduction through T and B cell receptors. The discovery of this antigen indicates that an adaptive immune response (maturation through receptor signaling) is present in an animal previously assumed to be without the capacity for an adaptive humoral immune response (Rycyzyn et al. 1998). Recently, it has been discovered that CD45 has an additional function in innate immunity by negatively regulating signaling through cytokine receptors (Nagata et al. 2002).

Chemokines. Chemokines, which are small, secreted cytokines that direct the migration of cells to sites of infection, are classified in two classes, CXC and CC, depending on the arrangement of the first two cysteine residues in their structure. Chemokines are chemotactic for leucocytes, and direct immunoregulatory or inflammatory cells to the site of injury (Kuby 1997). Only a few chemokines have been identified in fish (Secombes et al. 2001). A CC chemokine, CK1, has been identified in rainbow trout (Dixon et al. 1998). Although functional studies have not been conducted, analysis showed that the genomic construction is more similar to the CXC family, perhaps suggesting a primordial cytokine (Dixon et al. 1998). Another chemokine of the CXC family that resembles IL-8 has been identified in the Japanese flounder, but further studies are needed to fully characterize this molecule (Nam et al. 2000). A CXC chemokine, called scyba, with homology to mammalian BRAK chemokines was identified in zebrafish (Long et al. 2000). It is suggested that this chemokine functions in nonhematopoietic cells of the central nervous system and has a possible role in the development of the acoustico-lateralis system. Several chemokine receptors have been sequenced in rainbow trout that are widely expressed in tissues. A receptor with identity to mammalian CXCR4 has been identified, as well as a receptor with identity to CCR7 (Daniels et al. 1999).
**Acute Phase Proteins.** Cytokine and chemokine molecules upregulate acute phase proteins in the liver, which minimize host damage or act directly on the pathogens (Secombes et al. 1999). C-reactive protein, serum amyloid A and P, and other acute phase proteins have been identified in fish (Jensen et al. 1997). C-reactive protein is produced in the liver and binds to the C-polysaccharide cell wall of bacteria and fungi, activating complement for lysis and opsonization (Kuby 1997). Despite their many anti-pathogenic properties, acute phase proteins seem to have other functions in some fish species. For example, the level of C-reactive protein decreased in response to some infections in channel catfish and salmonids (Szalai et al. 1994). Complement proteins C3 and C4 are present in fish and are classified as acute phase reactants because their synthesis is upregulated during inflammation (Watts et al. 2001). Complement has proinflammatory, chemotactic, lytic, and opsonic activities, that are involved in phagocytic functions (Watts et al. 2001). In the classical complement pathway, antibodies bound to an antigen bind complement and begin the complement cascade. This pathway depends on antibodies specific to an antigen, and depends on the adaptive immune system. The alternate pathway begins with complement component C3, which can bind to pathogens and begin the complement pathway independent of an adaptive immune response. In this aspect, complement acts in an innate defense mechanism (Kuby 1997). Complement is very important in the inflammatory reaction of mammals and is also found in fish serum and mucus (Harrell et al. 1976). Most fish species have some sort of complement lysis system similar to mammals which can operate in the classic or alternate pathways. Although fish complement has not been completely characterized, it is postulated to be active in hemolysis, bacterial destruction, and viral inactivation. Opsonic activities are also achieved by complement in fish, but a receptor analogous to mammalian C3b has not been identified (Sakai 1992). An interesting difference
between mammalian and teleost C3 is that teleost C3 occurs in a number of different isoforms that have different binding efficiencies to their respective targets, and therefore, possibly different functions. Rainbow trout have four isoforms, gilthead seabream (Sparus aurata) have five, carp have eight, and medaka (Oryzias latipes) have three (Sunyer and Lambris 1998). Complement component 5 (C5) has been cloned in the rainbow trout system from a liver cDNA library. Functional studies have not been performed (Franchini et al. 2001). Recently, complement regulatory factor I has been identified from the common carp (Cyprinus carpio) as a novel serine protease that regulates complement activation (Nakao et al. 2003).

Granulocyte Development. Granulopoiesis in fish occurs in the spleen, kidneys, epigonal organ, organ of Leydig, and other specialized tissues depending on the species of fish (Ainsworth 1992). After generation, granulocytes are found mostly in the peripheral blood and organs. The identification and classification of granulocytes in fish has proven difficult because they do not share all of the same characteristics with mammalian cells, leading to classification wherein cells that share two characteristics with mammalian cells are therefore labeled the same type of granulocyte as the mammalian cell (Ainsworth 1992). Mammalian neutrophils have a multi-lobed nucleus (polymorphonuclear cells) and both basic and acidic granules, are phagocytic, have Fc receptors, and functions in ADCC (antibody dependent cell cytotoxicity). Neutrophils are the most numerous granulocytes in the circulation, at about 50-70% (Kuby 1997). Putative neutrophils have been identified in the channel catfish (Cannon et al. 1980). However, histochemical staining of catfish neutrophils shows an eccentric round-to-oval nucleus, instead of the mammalian characteristic multi-lobed nucleus (Ainsworth and Dexiang 1990; Finco-Kent and Thune 1987). Neutrophils are shown to be phagocytic cells in catfish. Phagocytic activities have been shown against bacteria, specifically Aeromonas hydrophila and Edwardsiella ictaluri,
in the channel catfish (Ainsworth and Dexiang 1990; Finco-Kent and Thune 1987). Chemotactic activity of neutrophils has been exhibited in fish species, but in the channel catfish chemotactic activity has been difficult to demonstrate (Ainsworth, unpublished data). Beta 2, or CD18, a molecule that functions in adhesion of mammalian leucocytes (neutrophils and macrophages) in areas of inflammation has been identified in channel catfish (Qian et al. 1999). Channel catfish neutrophils contain a variety of enzymes and lytic peptides that function in pathogen destruction, including a recently discovered neutrophil collagenase (Noya et al. 1999). This enzyme has been shown to degrade type I collagen. This feature is shared in mammalian neutrophils as an innate destructive mechanism. In fish without neutrophils, eosinophils have been shown to be phagocytic (Ainsworth 1992). In striped bass, however, both neutrophils and eosinophils have been shown to be phagocytic, instead of the eosinophil taking a phagocytic role because of the lack of neutrophils (Bodammer 1986). Eosinophils are found in some fish species, including different isoforms in found in sturgeon and dogfish (Hine and Wain 1988; Marrow and Pulsford 1980). Mammalian eosinophils are highly granular, somewhat phagocytic, have acidic granules, have IgE receptors, and function in anti-nematode, anti-parasitic capacity. They are rarer in the circulation than neutrophils, at about 1-3% of the granulocytes (Kuby 1997). Eosinophil function in fish that possess eosinophils, aside from some phagocytic activity, has been shown to be anti-parasitic, similar to the mammalian eosinophils. In goldfish infected with trematodes, eosinophils adhere to the parasite and function in parasite killing, neutralization of parasitic products, and attraction of leukocytes to the area (Huizinga 1980). Basophils are not found in most species of fish that have two or more characteristics in common with mammalian basophils (basic granules and multiple nuclear lobes) (Ainsworth 1992). Mammalian basophils are nonphagocytic, are heavily granulated with basic granules, have Fc IgE receptors that can be
cross-linked to effect degranulation of the cell, which releases chemoattractants and other chemical mediators (Kuby 1997). Barber and Mills-Westermann (1978) postulate that the periodic acid-Schiff-positive granular leukocytes in *Catostomus commersoni* are an evolutionary precursor to mammalian basophils. Due to the difficulties in identification of teleost basophils, most studies have been morphologic instead of functional. Therefore, it is unknown what function teleost basophils may have (Ainsworth 1992).

Macrophages. Macrophages are important cells in the innate and the specific immune response. In the innate response, mononuclear cells (immature macrophages) circulate in the peripheral blood and eventually enter the tissues to become tissue macrophages. When monocytes become a macrophage, they enlarge 5-10 fold, the organelles increase and become more complex, it increases in phagocytic ability, and it secretes more immuno-regulatory factors. Activated macrophages have more MHCII molecules on their surface and secrete complement proteins, TNFα, lysozyme and other factors (Kuby 1997). Monocytic cells were first detected in the thymus of catfish fry, indicating the organ of cellular genesis, as in mammalian systems (Petrie-Hanson and Ainsworth 2001). In adult fish, monocytic cells are concentrated in lymphoid tissues, such as the head kidney (Petrie-Hanson and Ainsworth 2001). Upon encountering a foreign pathogen or particle, the mononuclear cells function to phagocytose the foreign object and at that point, begin secreting cytokines as well as respond to cytokines secreted by neutrophils, which are active in the initial phases of inflammation. At this point, the mononuclear cell becomes an activated macrophage, a cell primed for phagocytosis and killing of those phagocytosed pathogens by degrading enzymes (proteases, nucleases, phosphatases, esterases, lipases, antimicrobial peptides), decreasing the pH in phagolysosomes, and releasing reactive oxygen and nitrogen intermediates (also known as the respiratory burst) (Kuby 1997).
These actions will directly kill a phagocytosed pathogen unless the pathogen has evolved mechanisms to avoid or neutralize these actions.

After nonspecific phagocytosis of foreign antigens occurs (bacteria and virus particles adhere well and are easily endocytosed), the particles reside in a phagosome and enters the endocytic processing pathways. The phagosome fuses with a lysosome which contains hydrolytic enzymes such as lysozyme, defensins, reactive oxygen intermediates, and reactive nitrogen intermediates (both together involved in respiratory burst), that destroy the pathogen. The degraded material then may be complexed, if complementary, in an MHC class II molecule in the phago/lysosome or be exocytosed and removed. At this point, the MHCII receptor will traffic through the cell and be expressed on the outer membrane where it can stimulate CD4+ T-cells by antigen recognition and thus begin the adaptive immune response (Kuby 1997).

Macrophages are well established members of the fish immune cell repertoire and can be differentiated from neutrophils with a Sudan black B stain identifying neutrophils and a nonspecific esterase and peroxidase identifying activity in the macrophages (Ellsaesser et al. 1985). Channel catfish monocyte-like cell lines can be established with LPS induction and calcium ionophore/phorbol ester stimulation (Vallejo et al. 1991; Lin et al. 1992). These cell lines secrete high levels of high and low molecular weight forms of catfish interleukin-1, which reacts with channel catfish and mouse T-cells (Vallejo et al. 1991). The cells are phagocytic and can present antigen to peripheral blood leukocytes, resulting in a mixed lymphocyte reaction, but not in specific antibody responses (Vallejo et al. 1991).

Channel catfish macrophages have increased killing activity against *E. ictaluri* after immersion exposure (Shoemaker et al. 1997). Macrophages from catfish immune to *E. ictaluri* demonstrated increased bacterial activity to immune-serum opsonized *E. ictaluri* than to non-
serum opsonized bacteria. Moreover, exoantigen from *E. ictaluri* reportedly induces an increase in chemotactic responses from macrophages (Klesius and Sealey 1996). Observed differences in catfish responses to *E. ictaluri* antigens are postulated to be due to differences in sensitivity of different channel catfish strains and families (Wolters and Johnson 1994). Plumb et al. (1975) observed similar strain susceptibility differences between eight strains of channel catfish challenged with CCV. A wide range from 71%-10% mortality was observed, indicating that these previously disease free fish had genetic resistance characteristics that resulted in the wide range of mortality. In mammals, a gene designated as Nramp 1, natural resistance associated macrophage protein 1, has been shown to function in genetic control of macrophage function and has been identified in catfish (Chen et al. 2002). NrampC is expressed in macrophage-rich catfish tissues and cell lines after exposure to LPS and therefore, might be a potential marker for disease resistance or innate immune responses.

**Nonspecific Cytotoxic Cells.** Nonspecific cytotoxic cells (NCC) of catfish are thought to be analogous to mammalian NK cells and are able to kill a variety of target cells, including allogeneic and xenogeneic tumor cells, virally infected cells, and protozoan parasites (Evans et al. 1988; Jaso-Friedmann et al. 2001). NKs are non-T and B cells that have large cytoplasmic granules, but catfish NCCs are agranular and have a pleomorphic nucleus (Evans and Jaso-Friedmann 1992). NCC cells are homologous to mammalian NK cells based on a cross reactivity of a monoclonal antibody (mab 5C6) with mammalian NK receptor and the NCC cell receptor protein-1 (NCCRP-1) (Evans and Friedmann 2000). The NCCRP-1 receptor is a type III membrane protein and has a role in antigen recognition to initiate target cell lysis and intracellular signaling involving JAK kinases and a STAT6 molecule to initiate cytokine production and release (Jaso-Friedmann et al. 2001). Like NK cells, NCCs require cell contact
with the target cell to kill. These cells are able to spontaneously kill a variety of xenogenetic targets, which includes fish parasites and traditional mammalian NK targets (Graves et al. 1984; Evans and Jaso-Friedmann 1992). NCC cells also exhibit all of the pathognomonic characteristic of an apoptosis-inducing cell in target cells: chromatin condensation, nuclear peripheralization, the formation of apoptotic bodies, and the appearance of compact electron dense nuclei. These changes take place in the absence of morphological changes, a hallmark of apoptosis vs. necrosis (Evans et al. 2001). Mammalian NK cells are known to induce apoptosis using receptors, Fas and Fas-L, that signal between cells (Fas-L on T cells and Fas expressed on a target cell).

Activation induced cell death (AICD) is a phenomenon wherein a cell that has been activated after recognizing a dangerous signal is induced into a “suicide” pathway. Fas and Fas-L are common receptors in this pathway. NCCs from tilapia kill targets via a Fas-L pathway and catfish NCCs have been shown to produce cytosolic Fas-L (Jaso-Friedmann et al. 2000).

Gilthead seabream (*Sparus aurata* L.) produce cytosolic Fas-L in leucocytes (lymphocytes and monocyte-macrophages) in the head kidney, spleen, blood, and peritoneal exudates (Cuesta et al. 2003). After exposure to stress, an immediate observed response of NCC is the secretion of apoptosis regulatory factors or stress activated serum factors into the peripheral blood. These factors activate NCCs by protecting them from AICD by downregulating Fas or blocking other receptors (Evans 2001). In some studies, surface IgM has been identified on cytotoxic cells that retain cytotoxic activity. Because those cells do not express message for the Ig heavy or light chains, it is assumed that some channel catfish cytotoxic cells “arm” themselves with circulating IgM via an Ig heavy chain receptor (FcμR) present on the cytotoxic cell (Shen et al. 2002). Because of this arming, these cells may kill via ADCC (antibody dependent cell-mediated cytotoxicity), which is reminiscent of mast cells binding IgE to facilitate killing of antibody specified targets (Shen et al. 2002).
In classical ADCC, the bound Ig recognizes a target on an antigen and signals through the bound Fc receptor to initiate degranulation, phagocytosis, or other killing functions (Kuby 1997). Shen et al. (2003) analyzed catfish peripheral blood leucocytes (PBL) by flow cytometry and identified 4-8% of the PBLs that were positive for spontaneous killing of allogenic targets were also positive for presence of the Ig light chain type F or G on the surface of their cells. Long term cultures of allogen stimulated PBLs were surface IgM positive and cytotoxic in the absence of message for Igµ, TCRα, β, or δ chains (Shen et al. 2003). This study supports the assumption that NK-like cells (not NCC) participate in ADCC in catfish. Cell lines of channel catfish cytotoxic cells established with weekly alloantigen stimulation may represent the first ectothermic cytotoxic cell culture, but the lack of ability to distinguish between TCRα/β negative cells and TCRγ/δ negative cytotoxic cells (γ/δ chains have since been sequenced in the Japanese flounder by Nam et al. in 2003) precludes the definitive designation of the cell line as an NCC cell line. The TCRα/β- cells of some lines react with the NCC defining mAb 5C6, the mAb against the NCCR-1 molecule, supporting the fact that since it exhibits granularity, it is most likely a type of granular NCC line (Shen et al. 2002). The inhibition of allogeneic killing in this cell line by calcium chelation suggests also that these channel catfish cytotoxic cells kill by a secretory calcium dependent “perforin/granzyme”-like pathway to trigger apoptosis (Hogan et al. 1999). This cell line might represent a cytotoxic cell of fish that is similar to catfish NCCs, although NCCs are not granular. Shen et al. (2004) further characterized this cell line as a TCR α, β, γ negative granular cytotoxic line that kill allogenic and nonspecific targets by a perforin/granzyme apoptotic pathway. This line was also negative for staining with mAb 5C6, and was negative for neutrophil and macrophage staining. Flow cytometry identified a Fcµ
receptor, a LFA-1 like molecule, a putative T-cell antigen, and MHCI on the surface of the cells, which also reacted with a polyclonal antibody against catfish B2 microglobulin. This cell line is characterized as a granular line that is similar to NCCs but distinct from NCCs. NCC cells have also been shown to become activated by synthetic oligodeoxynucleotides and bacterial genomic DNA, especially CpG motifs known to be an inflammatory adjuvant in DNA vaccination studies. Eukaryotic DNA had no effect on cytotoxicity (Oumouna et al. 2002).

**Specific Immunity.** Specific defense mechanisms consist of antibodies, cellular immunity, interferons, enzymes, and complement. Animals use the nonspecific defense system to prevent infectious organisms from gaining access to the body or establishing infection. They use the specific defense systems to combat infectious organisms once they have entered the body (Kuby 1997). The four characteristics of adaptive immunity are antigenic specificity, diversity, immunologic memory, and self/nonself recognition. Basically, the adaptive immune response enables an organism to respond specifically to one antigen (via antibody binding or MHC/TCR recognition), the diversity to accommodate a large number of antigens, the ability to respond more quickly to an antigen previously encountered, and the ability to differentiate between a self antigen and a nonself antigen to prevent autoimmunity (Kuby 1997). Based on histology and cytology, the organs involved in lymphogenensis in teleosts are thought to be the thymus and the kidney (the equivalent to mammalian bone marrow). The teleost kidney is broken into two segments, the pronephros (head kidney) and the mesonephros (trunk kidney) with the former very active in lymphoid proliferation and the latter functioning in excretion. Antibodies are produced from both segments, but mostly from the head kidney (Rijkers et al. 1980).

**Humoral Responses.** Fish antibodies are produced by a B type lymphocyte, which has been defined as a surface Ig positive cell (sIg+ or sIgM+). In mammalian systems, B cell
development occurs in the bone marrow (Kuby 1997). In fish it is accepted that lymphocyte
development occurs in hematopoietic tissue, in the head kidney (pronephros). In zebrafish, early
development of lymphopoiesis indicates that no sites for B cell differentiation are observed until
three weeks of development. Rearrangement of genes encoding B-cell receptors can be detected
as transcripts by day four, as well as recombination activation gene 1 or RAG1. Interestingly,
these transcripts were detected in the pancreas, an organ not normally associated with
lymphopoiesis (Danilova and Steiner 2002). After testing fry exposed to *E. ictaluri*, Petrie-
Hanson and Ainsworth (1999) could detect bacterium specific antibodies in fry only at day 21
post-hatch or older, asserting that previous to this time point, a detectable specific antibody
response is not produced. Macrophages play key roles in the development and differentiation of
B lymphocytes in mammalian systems and are thought to play a similar role in the catfish, as
they are present in the renal interstitial tissue before mature Ig positive lymphocytes. Ig positive
lymphocytes were detected on day 7, 10, and 14 in the anterior renal hematopoietic tissue,
thymus, and spleen respectively. Mature B lymphocytes were present in the anterior renal
hematopoietic tissue before any other lymphoid tissue which indicates that this tissue is the site
of generation and maturation of B lymphocytes in the catfish. These authors postulate that the
lack of detectable antibody production until day 21 in the catfish coincides with the point at
which thymic regionalization and splenic lymphoid tissue organization were observed (Petrie-
Hanson and Ainsworth 2001). Also, at day 21, the kidney separates into the head kidney and
trunk kidney at which time the head kidney takes on the sole hematopoietic role (Petrie-Hanson
and Ainsworth 2001).

Antibody producing cells consist of two populations, those with membrane-bound Ig, and
surface-bound Ig (Lobb and Clem 1982). After development and maturation, the antibody
produced is IgM-like, which in teleosts is a high molecular weight tetramer with eight binding sites. A monomeric form occurs in some species, and a dimer is found in secretions in some species (Wilson and Warr 1992). Recently, IgD has been described in the channel catfish, Atlantic salmon, and brown trout (Salmo trutta) with undetermined, possibly evolutionary function (Miller et al. 1998; Hordvik et al. 1999). Stenvik et al. (2001) suggest that the equal numbers of IgD positive and IgM positive B cells in tissues, suggest that the IgD molecule may be the B-cell receptor, as is the case in mammals. The IgM molecule is composed of equimolar amounts of heavy (H) (70-81 kDal) and light (L) (22-32 kDal) chains which, like mammals, form pairs that are held together by disulphide bonds. A pair of H-L chains bound to another pair of H-L chains form the basic monomeric unit. Fish B cells show Ig H gene rearrangement, allelic exclusion, and produce both membrane and secreted forms of antibody (Wilson and Warr 1992). A difference from mammalian IgM lies in the absence of a J-chain linking the monomers together. This is accomplished by a noncovalent bond, unlike the mammalian IgM. Heterogeneity in the J and L chains has been described based on charge, reactivity with monoclonal antibodies, and molecular weight. For example, channel catfish have been shown to produce different H and L isotypes early in the immune response. The Japanese flounder (Paralichthys olivaceus) has been shown to shift to a lower molecular weight IgM during infection with Edwardsiella tarda (Lobb and Olson 1988; Bang et al. 1996). The lack of the J-chain and the existence of different molecular weight IgM forms might make for a more diverse repertoire of Ig subclasses and might provide for enhanced ability to bind epitopes or to accommodate more epitopes by greater flexibility (Kaattari et al. 1998). The δ chain of the catfish IgD was characterized as a chimeric molecule containing seven domain encoding exons, and a transmembrane tail. It was originally thought that the secreted and membrane forms of
catfish IgD were a result of alternative splicing from one domain. However, Bengten et al. (2002) indicate that the secreted and membrane forms of IgD are not a result of alternative splicing, but products expressed from separate δ domain genes. Catfish membrane IgD transcripts are thought to come from gene IGHD1 whereas secreted IgD transcripts are thought to come from gene IGHD3 (Bengten et al. 2002). Electrophoretic separation of DNA, transfer to a membrane, and hybridization (Southern hybridization) of a long term channel catfish B cell line revealed multiple µ-chain gene rearrangements and parental cell lines transcribed mRNA from all of the six known channel catfish variable heavy chains (Miller et al. 1993). The mRNA expression also revealed expression of both of the secreted and membrane forms of the µ-heavy chain producing both secreted and membrane forms of IgM (Miller et al. 1993). Antibody is important in fish for neutralization of virus, precipitation, agglutination, opsonization, and complement activation (Haattari and Piganelli 1996).

Recombination activator gene 1 (RAG-1), has been cloned in zebrafish (Danio rerio) (Greenhalgh and Steiner 1995). RAG genes encode enzymes that are required for V(D)J rearrangements and are expressed in maturing and differentiating B and T cells. RAG1 was detected on the fourth day of development in the thymus (Willett et al. 1997). Genes encoding a T cell receptor subunit, TCRα, were detected at day four in the thymus as well as some developing lymphoid cells. However, RAG transcripts were not detected at any site, nor were lymphoid cells observed in the pronephros (Willett et al. 1999). Danilova and Steiner (1997) were able to detect the rearrangement activity of the heavy chain as early as day four and detected transcripts for membrane expression of Igµ on day seven of development from whole fish. Interestingly, the detection of RAG1 and Igµ in the pancreas on day four and 10 suggested that B cell development occurs in the pancreas, instead of the previously presumed pronephros.
In the Atlantic cod (*Gadus morhua*), Stenvik et al. (2001) showed the expression of the Ig heavy chain in the spleen and the head kidney, and demonstrated that the expression of IgD evenly in tissues, further suggesting that the IgD is a B-cell receptor in fish, much like the mammalian IgD. In contrast to the previous study, Petrie-Hanson and Ainsworth (2001) detected thymocytes in catfish for the first time at day 10, not at day four post-hatch.

**Cell Mediated Immunity – T-cells.** Cells involved in cellular immunity consist primarily of macrophages, T-cells, nonspecific cytotoxic cells, and neutrophils (Ellis 1982). The adaptive immune response cellular components are macrophages and T-cells. The α/β T cell receptor has been cloned in rainbow trout (Partula et al. 1996) and in channel catfish (Wilson et al. 1998). The cDNA sequences were found to belong to four different variable α (Vα) and six Vβ families that share characteristics with mammalian TCR Vα and β chains. These sequences show distinct immunoglobulin, connecting peptide, transmembrane, and cytoplasmic domains (Wilson et al. 1998). A clonal line was established encoding a TCR α/β expressing cell, designated 28S.1, that has T-cell function and constitutively produces supernatant factors with growth promoting activity (Wilson et al. 1998). Nam et al. (2003) identified all four TCR α, β, γ, and δ genes from a bacterial artificial chromosome created from a cDNA library from a Japanese flounder. Analysis of TCR transcripts identified hypervariability in the complement determining region 3 loops (CDR3), indicating that fish are capable of gene recombination to accommodate many antigenic peptides (Nam et al. 2003). Definitive classification or identification of two subsets of T helper cells, T-helper 1 or 2, as exists in the mammalian system has proven elusive in catfish T-cell populations, because the cytokine dichotomy that defines the helper sets has not been elucidated in fish. The CD4 molecule has not been identified in teleosts. Catfish T-cell culture supernatants failed to stimulate IL-2 or IL-4 dependent mammalian cell lines and murine
recombinant IL-2 or IL-4 did not stimulate catfish lines. Therefore a functional lack of cross-reactivity exists between catfish and mammalian T-lymphocyte cultures and makes identification and description of potential helper subsets difficult (Miller et al. 1998).

More recently, however, an IL-2-like cytokine has been identified in rainbow trout surface IgM- leukocyte culture after mitogenic stimulation (Blohm et al. 2003). IL-2 in mammalian systems functions as an autocrine growth factor for T-cells, wherein a T-cell stimulated by IL-2 can produce IL-2 and stimulate its own receptor and other T-cell receptors to proliferate further (Kuby 1997). A monoclonal antibody specific for mouse IL-2 cross reacted with supernatant from a mitogen stimulated leukocyte culture and neutralized growth factor-like stimulation of other cultured leukocytes, indicating its possible homology to mammalian IL-2 (Blohm et al. 2003). Production of IL-2 and proliferation of T-cells in mammals functions by an intracellular signaling pathway involving an NFAT protein. A protein homologous to mammalian NFAT has been identified in catfish and has been shown to bind to a murine IL-2 promoter sequence (Park et al. 2002). This finding indicates that catfish T-cell signaling to produce cytokines operates along a similar pathway to the mammalian NFAT pathway (Park et al. 2002).

Indications that fish utilize a system of antigen presentation by MHC expressing cells to T-cell receptors (TCRs) was provided by identification and immunoprecipitation of proteins of a similar size to mammalian MHCIα and class IIα, β, and invariant chains (Vallejo et al. 1992). Evidence that fish possess antigen specific cytotoxic T lymphocytes has come from both in vitro and in vivo studies testing graft vs. host rejections, suggesting that fish are capable of mounting antigen specific cytotoxic responses against nonself tissues (Nakanishi et al. 1999). The function and definitive identity of teleost cytotoxic T cells remains unclear due to lack of immunological
reagents (Shen et al. 2002). The establishment of channel catfish cytotoxic T cell lines had been a difficult undertaking, considering the inability to define these cells due to a lack of immunological reagents. Putative T-cytotoxic cell lines were established as a clonal, allogen-dependent cytotoxic leukocyte line from mixed leucocyte reaction studies (Stuge et al. 1997). This line was tested for target cell cytotoxicity by $^{51}$Cr release assay, and analyzed for transcripts of TCR $\alpha$ and $\beta$. Five types of cells were identified. The first type was postulated to be a cytotoxic T cell (CTL) because it was a TCR$\alpha$/\$\beta$ positive cell with allospecific cytotoxicity. The second type was postulated to be a T-helper type cell because it was TCR$\alpha$/\$\beta$ positive and allospecific in recognition, but non-cytotoxic. The third type was postulated to be an NK equivalent because it was TCR$\alpha$/\$\beta$ negative and nonspecifically cytotoxic. A fourth type was harder to categorize because it was TCR$\alpha$/\$\beta$ negative and allospecific in target cytotoxicity. This type of cell might be another type of cytotoxic T cell, possibly $\gamma$/\$\delta$ T cell, or another kind of NK type cell. And the last type of cell was not postulated to have mammalian equivalent, as it did not exhibit typical characteristics of any known mammalian cell. This type of cell was TCR$\alpha$/\$\beta$ positive and nonspecifically cytotoxic. None of these cells reacted with the NCC defining mab 5C6 (Stuge et al. 2000). Fisher et al. (2003) identified surface IgM- (sIgM-) leukocytes in the rainbow trout that have exhibited cell mediated cytotoxicity against allogeneic cells (RTG-2 cell line). Rainbow trout have recently been shown to have lymphocytes expressing CD8, a known indicator of cytotoxic T-cells in mammals (Hansen and Strassburger 2000). Further correlations between fish cellular immunity and mammalian were drawn by Nakanishi et al. (2002) who showed that in vitro killing of cells transfected with IHNV antigens by leukocytes was dependent upon MHC class I compatibility, indicating a T-like cell compatibility with the MHCI, as in mammals. The $\alpha$/\$\beta$ T-cell receptor was used by Boudinot et al. (2001) to investigate the T cell
response to viral infections. This group used the immunoscope methodology, which involved spectratyping the CDR3 region of the α/β TCR. In this study, the naïve T-cell population was shown to be polyclonal and highly diverse. Upon infection with VHSV, a skewed CDR3 size profile was identified for several Vβ/Jβ combinations. This indicates that recombination and clonal expansion has occurred during a primary and secondary response to VHSV. Boudinot et al. (2001) also showed a strong boost of the T-cell repertoire during the secondary response of the rainbow trout to VHSV.

**T-cell Interaction with MHC.** MHC class I molecules are expressed on every cell in mammals, and normally express a self antigen unless infected or tumorigenic. If expressing a nonself antigen, these molecules interact with T-cells expressing a CD8 molecule, which stimulates a primarily cytotoxic response. MHC class I molecules are made up of an α chain, a β chain and an invariant β2 microglobulin chain. MHC class II molecules are expressed only on antigen presenting cells (APC) and B-cells. This molecule interacts with T cells that express a CD4 molecule and stimulates the T-helper cellular and humoral arm of the immune system (Kuby 1997). MHC class I and II genes have been cloned from channel catfish (MHC Iα, IIα and β, β2 microglobulin), zebrafish (MHC I and II), the rainbow trout (MHC I and II), and the Japanese pufferfish (*Fugu rubripes*) (MHC I) (Godwin et al. 1997; Criscitiello et al. 1998; Antao et al. 1999; Takeuchi et al. 1995; Hansen et al. 1999; and Clark et al. 2001). The MHC class genes, unlike mammals, are unlinked and on separate chromosomes (Flajnik et al. 1999). The zebrafish MHC class II genes are separated from the MHC I class genes, which is postulated by Kuroda et al (2002) to have occurred by translocation, rather than chromosomal duplication. Other immune related genes were identified in rainbow trout by cDNA cloning including TAP1, TAP2, TAP2B, proteosome δ, and low molecular weight polypeptide (LMP)2 (Hansen et al.
TAP proteins function in loading of complementary proteins into MHC molecules (Kuby 1997). Genes found to cosegregate with the MHC class I locus were LMP2, LMP2δ, TAP1A, and TAP2B. The MHC class II locus was not found to not be linked to the MHC class I locus, suggesting that rainbow trout, zebrafish, and perhaps other teleost species function in this way (Hansen et al. 1999).

**Cellular Immune Response – Mode of Action.** A cellular immune response is primarily activated by intracellular bacteria and viruses. Macrophages find foreign material and ingest it by phagocytosis. The macrophage, or antigen presenting cell (APC), destroys the bacterium (unless it is resistant to the destructive forces of the cell) and presents epitopes of the bacterium on MHC type II receptors. The epitopes are recognized by T-helper like cells and the cellular immune response is activated. Viral antigens are produced by the host cell machinery and are presented by the endogenous pathway. Viral antigens are presented via MHC I type receptors as an altered-self protein (the immune system will recognize this epitope as non-self, and target it for destruction). If a virus gains entry into an APC as well as a bacterium, the bacterial antigens will be presented via MHC II and the viral antigens will be presented via MHC I. Thus the appropriate immune response will be activated for the appropriate pathogen (Kuby 1997).

**Factors Influencing Immune Responses in Fishes.** Factors influencing the immune response of a fish are multi-fold. Because ambient temperature is a critical factor of development of both specific and nonspecific immunity, low temperatures can be immunosuppressive in a fish host or can induce tolerance (Wishkovsky and Avtalion 1982). Nonpermissive temperatures have been shown to affect channel catfish lymphocytes *in vitro*, especially the T-dependent antigen response (Bly and Clem 1994). Bly and Clem (1988) studied the cell membrane fluidity in channel catfish and found that for short periods of time, catfish
thrombocytes, B-cells, and T-cells were able to acclimate to differences in ambient temperature over three weeks, with minimal membrane fluidity changes. However, after 3-5 weeks of acclimation, the membrane fluidity of all three cell types decreased and resulted in immunosuppression. These researchers therefore postulate that a combination of temperature and other unknown factors contribute to immunosuppressive function of B and T-cells at non-permissive temperatures. Nonpermissive temperatures can inhibit membrane Ig capping, and therefore inhibit intracellular signaling and activation/maturation of B-cells (Bly et al. 1987). Immunosuppression of fish immune cells can be rescued in vitro when suppressed cells are incubated with T-cell supernatants, showing that the suppression is due to a lack of the necessary autocrine stimulatory molecules or other cytokines (Bly and Clem 1994). The sensitivity of catfish T-cells to temperature is considered to be due mostly to the inability of catfish T-cells to desaturate membrane lipids at suboptimal temperatures. Because unsaturated fatty acid uptake is only able to occur at permissive temperatures, the function of the T-cell is compromised (Bly and Clem 1994). Looking at antibody response, Avtalion (1969) studied the primary and secondary response of carp to a model antigen. In the primary immune response to bovine serum albumin (BSA), it has been shown that the primary antibody immune response is suppressed at low temperatures. However, if fish are immunized at high temperatures, a secondary immune response can be elicited at low temperatures (Avtalion et al. 1972). In contrast, some studies have shown that lower temperatures induce a delay in the peak of the primary response but do not effect the magnitude of the response (Rijkers et al. 1980). Rijkers and collaborators (1980) therefore postulated the existence of one or more thermosensitive steps in the formation of the humoral response. Lymphoproliferative responses have been shown to be reduced in response to concavalin A (conA) at low temperatures in catfish (Cuchens and Clem 1997). Similar effects
were observed in carp and salmon. Therefore “immunologically permissive” and “non-
permissive” temperatures were established for salmonids (4°C), for carp (14°C) and for catfish
(22°C) (Bly and Clem 1992). After extensive testing, it was discovered that the generation of T-
helper functions, not memory T nor B-cell functions, was the component compromised by low
temperatures (Miller and Clem 1984). Channel catfish phagocytic cells were observed to be
more resistant to low temperatures than lymphocytes with respect to function (Scott et al. 1985).
Interestingly, adaptation to low temperature increased respiratory burst activity, implying an
increased bactericidal function (Dexiang and Ainsworth 1991). As phagocytosis is one of the
first lines of innate immune responses and is not downregulated by low temperatures,
nonspecific cytotoxic cells (NCCs) were studied (the equivalent of mammalian NK cells) to
further investigate the effect of low temperatures on innate immunity. In carp, lytic activity
mediated by NCCs was markedly increased at low temperatures (Le Morvan et al. 1995). This
response was detected as soon as 24 hours post-exposure to low temperatures and lasted eight
weeks, peaking at four weeks. This observation, coupled with the increased respiratory burst
properties and the predominance of complement alternative and classical pathways in low
temperature situations observed by LeMorvan and collaborators (1995), indicate that
immunosuppression due to low temperatures mainly effects the specific adaptive immune
response. The innate response appears even more effective, emphasizing the importance of
nonspecific defenses in low temperature situations (Hayman et al. 1992; Collazos et al. 1994).

Types of Vaccines

**Killed and Subunit Vaccines.** Vaccines against infectious diseases come in many
shapes and sizes. Popularly used vaccines include killed bacteria or viruses, protein/subunit
vaccines, live attenuated vaccines, and more recently, DNA vaccines. Subunit and killed
vaccines must be injected or otherwise artificially delivered to the animal because they are not able to invade. Because they do not replicate or persist, additional injections are often required to stimulate an adequate immune response. Killed vaccines consist of a pathogen that was killed by heat, pressure, or chemical treatment. Heat killed vaccines have not been proven to be the best type of vaccines because heat denaturation of epitopes on proteins prevents the elicitation of conformation dependent antibody responses (Kuby 1997). Killed vaccines are, however, relatively safe, as the pathogen is dead and can no longer invade. It also has no danger of recombination with other pathogens in the environment. Vaccines that require artificial delivery and do not invade or replicate in a host produce predominantly humoral responses and are poor stimulators of cell mediated immune responses or secretory IgA (Kuby 1997). These types of vaccines are most effective against pathogens that can be controlled with neutralizing antibody or other antibody responses. Subunit vaccines consist of a part of the pathogen than has been isolated and purified from the rest of the pathogen, i.e. lipopolysaccharide, capsular polysaccharides, inactivated exotoxins, recombinant surface antigens, or outer membrane proteins (Kuby 1997). This type of vaccine also must be delivered in an artificial manner, as it is not the whole pathogen and is incapable of invasion. Inactivated toxins or toxoids stimulate anti-toxin antibodies that neutralize toxins. A draw back of toxoid and other types of subunit vaccines is the need to produce mass quantities of pure toxin, a difficulty unless produced by recombinant DNA technology. Lipopolysaccharides or capsular polysaccharides stimulate an antibody response that works in opsonization and neutralization of pathogens. This type of vaccine works in a T-cell independent B-cell stimulatory manner and does not stimulate a T-helper response. Because it does not stimulate a T-helper response, B-cells have no way of interacting with T-cells and initiating class switching, maturation, and the formation of memory
B and T cells. These types of vaccines work most efficiently against pathogens than can be combated with antibody (Kuby 1997). Subunit vaccines are safe as well, with no chance of invasion, reversion, or recombination. There are a few reports of killed vaccines and subunit vaccines tested against CCV disease. A heat-killed vaccine against CCV did not have adequate immune stimulation (Plumb 1973b), probably due to heat lability of CCV antigens. This indicates that humoral protection against CCV is mostly due to conformationally dependent antibodies. A subunit vaccine was made from the harvested soluble envelope of the CCV virion (Awad et al. 1989). Three to four day old eggs and 1 week old fry were vaccinated by immersion with 250 µg/ml of the envelope suspension for 15 minutes. A booster was given to subgroups after two weeks. Eight weeks post-vaccination, a 1 X 10^{8.5} TCID_{50} CCV/ml challenge dose was administered and survival of vaccinated eggs was 31%, survival of fry was 82%, and survival of negative controls were 0%. Of the boosted groups, vaccinated eggs demonstrated a survival of 81%, survival of fry was 89%, and survival of negative controls were 44% (Awad et al. 1989). Although this vaccine had high survival rates, it is not an economically feasible vaccine to delivery by immersion to a large commercial pond.

**Live Attenuated Vaccines.** For bacteria or viruses that do not generate protective immunity with killed or subunit vaccines, live attenuated vaccines have been the vaccine of choice. Live, attenuated vaccines that retain the ability to invade a host in the specific way that the wild-type disease invades, and can briefly replicate before the death of the vaccine strain organism, stimulate a host immune response that is efficacious and long lasting against the virulent strain. Because of the prolonged exposure to the vaccine strain, memory cells are stimulated at a high level with a single vaccination and no boosting is required (Kuby 1997). Due to the fact that live attenuated vaccines proliferate and briefly utilize pathogen specific
virulence mechanisms, efficacious cellular and humoral immunity is elicited. However, live attenuated bacteria can have associated problems. First, there is a possibility that live attenuated vaccines could revert back to virulence or recombine with another strain to evolve new virulence factors. Live attenuated vaccines are also less stable than killed or subunit vaccines. However, compared to killed or subunit vaccines, live attenuated vaccines give the best, longest lasting protection, particularly when both cellular and humoral immunity are required because they mimic disease (Kuby 1997).

Considerable research has been conducted to develop live-attenuated vaccines against CCV. A live-attenuated CCV viral strain was produced by multiple passage in cultured cells from the walking catfish, *Clarius batrachus* (Noga and Hartman 1981). The attenuated strain was named V60 and was deficient in ORF 50, which encoded the protein V60. Efficacy of over 90% was reported when administered parenterally or as a waterborne vaccine. Sequencing and restriction mapping revealed that the mutation is a large deletion as well as many point mutations in ORF 50 (Vanderheijden et al. 1996). More recently, a live-attenuated vaccine strain was developed that had a deletion in the thymidine kinase gene (ORF 5). Thymidine kinase is an enzyme that phosphorylates thymidine to make dTMP, which is a precursor to dTTP, a nucleotide used in DNA synthesis. The thymidine kinase gene is especially useful to the virus in cells that are not divisional, such as neurons. Although this seems to be a good way to halt DNA synthesis, an organism can circumvent this block by transforming dUMP to dTMP by methylation (*de novo* synthesis) (Kancharla and Hanson 1996; Hanson and Thune 1993). The thymidine kinase gene of CCV is unique and can be distinguished biochemically from the catfish cellular thymidine kinase in that it cannot use dCTP as a phosphate donor (Hanson and Thune 1993). The interruption of ORF 5 confers resistance to Ara-T, a toxic thymidine analogue.
antiherpetic agent used to halt viral DNA synthesis (Hanson et al. 1994). In 1995, Zhang and Hanson showed that the deletion in the ORF 5 gene attenuates CCV. They tested the CCVTK-mutant in a vaccination experiment, immersing 5 cm channel catfish in $3 \times 10^5$, $1 \times 10^5$, $3 \times 10^4$, $1 \times 10^4$ PFU CCVTK-ml tank water for 30 minutes. Three weeks post-vaccination, fish were challenged with $3 \times 10^7$ PFU/ml wild-type CCV at 30°C. At the highest dose, CCVTK-vaccination resulted in 4.9% mortality. Average survival percentages following challenge were 100%, 73.7%, 60%, 31.7% from highest to lowest dose, respectively (Zhang and Hanson 1995). Production and shedding of CCVTK- versus CCV were investigated by Kancharla and Hanson (1996). Fish were vaccinated with 250 PFU/ml CCVTK- and CCV. Production and shedding were measured by quantifying infectious virus by plaque assay and viral DNA by quantitative PCR in various organs. Skin scrapings, gills, posterior kidney, liver, spleen, intestine, and water from tanks were analyzed. After analysis, it was determined that infectious CCV was isolated from skin, gills, posterior kidney, and intestine at day 1 post exposure, and from all tissues by day two. For CCVTK- exposed fish, infectious virus was isolated at day two from all organs. Virus reached peak levels in tissues at day three for CCV and day four for CCVTK-. Maximal virus was isolated from posterior kidney, followed by skin, gills, spleen, liver, and intestine. This was confirmed by quantitative PCR. In water samples, CCV was detected at a higher level than CCVTK- at all time points. This study shows that the kinetics of CCV and CCVTK-infections are similar, but CCVTK- infections persist for a shorter period and have reduced shedding ability (Kancharla and Hanson 1996). Mortalities, interestingly, were much higher in this study than in the previous study at 16% and 41% for CCVTK-.

The CCVTK- vaccine was also investigated as a vaccine vector to express a foreign gene. The $\text{lacZ}$ gene was cloned into the start site of the TK gene on a plasmid and homologous
recombination from that plasmid resulted in a CCVTK-lacZ strain. The CCVTK-lacZ was found to be resistant to Ara-T (indicating the disruption of the TK gene) and to elicit anti-lacZ antibodies in the fish. Therefore, this strain has been shown to express foreign gene products and elicit fish immune responses against that product, making it a candidate to express an antigen from another catfish pathogen (Zhang and Hanson 1996). Due to the mortalities sometimes observed post-vaccination and the reliance on a cold chain to deliver the vaccine, commercial production has not yet been approved for CCVTK-.

**DNA Vaccines.** A new type of vaccine became available when it was discovered that the injection of pure DNA was as effective as liposome-encapsulated DNA at mediating transfection of skeletal muscle cells (Wolff et al. 1990). It was then discovered, during a study investigating gene therapy in mice, that the encoded growth hormone did not have an effect on the mice, but that antibody was elicited to the encoded protein (Tang et al. 1992). This was the first study to demonstrate an immune response elicited by plasmid encoded antigens. Later, DNA vaccination was shown to elicit cytotoxic T-cell responses (Ulmer et al. 1993), provide protection after intradermal (ID) or intramuscular injection (IM) (Fynan et al. 1993), and modulate the T-helper type of immune response (Feltquate et al. 1997). DNA vaccines consist of bacterial plasmids that encode antigens for infectious diseases under the control of a eukaryotic promoter (Dietrich et al. 1999). The promoters used are largely viral in origin and are constitutively expressed (most commonly used is the cytomegalovirus immediate early promoter).

An advantage of a DNA vaccine is that it provides its own adjuvant in the form of unmethylated CpG motifs that induces an innate immune response (Kowalczyk and Ertl 1999). The immunostimulatory properties of bacterial DNA were first reported during experimentation with the nucleic acid fraction in bacillus Calmette-Guerin (BCG) of *Mycobacterium bovis*. This
fraction was seen to activate natural killer cells and induce interferon in studies looking at tumor regression in mice, whereas vertebrate DNA had no such effect (Tokunaga et al. 1984; Yamomato et al. 1988; Yamomato et al. 1992). The structural difference between bacterial and vertebrate DNA was due to the methylation of particular base contexts called CpG motifs. Vertebrate genomes are highly methylated at these areas, while bacterial DNA is not. This lack of DNA methylation in a vertebrate animal signals that foreign DNA is present and initiates an innate immune response (A. P. Bird 1987; Krieg et al. 1995). Furthermore, this phenomenon is exaggerated in fish because teleost DNA is twice as methylated as mammalian genomes (Jabbari et al. 1997). Bacterial DNA and synthetic nucleic acid motifs can trigger the upregulation of costimulatory molecules on B-cells such as MHCII and B7, and induce secretion of immunoglobulin and cytokines (Yi et al. 1996; Davis et al. 1998; Redford et al. 1998). Kanellos et al. (1999) investigated the CpG motifs in the ampicillin resistance gene (ampR or bla) in mice and in goldfish and its ability to enhance the immune response to the β-galactosidase protein injected into the animals. They tested a “reactive” AACGTT CpG motif (from the ampR gene), a “nonreactive” GACGTC CpG motif from a pUC-based plasmid, and a synthetic oligonucleotide containing the GACGTT motif. The “reactive” motif increased the specific antibody titers in fish and in mice, the “nonreactive” motif did not elicit antibody responses in fish or in mice, but the synthetic oligonucleotide elicited antibody only in the mice. This data suggests that higher and lower vertebrates have different recognition sequences for CpG motifs.

The mode of action of vaccine stimulation of immune responses by DNA is not fully understood. Delivery of plasmid vaccines is accomplished mostly by intramuscular injection of the purified DNA to skeletal muscle and skin. After intramuscular injection (IM), intradermal injection (ID), or particle bombardment of DNA into tissues via gene gun techniques, myocytes,
keratinocytes, Langerhans cells, and dendritic cells become directly transfected with plasmids (Davis et al. 1993; Hengge et al. 1995). Langerhans cells, dendritic cells, and tissue-associated macrophages are thought to be the most important cells in stimulating the immune response against DNA vaccines (Kowalczyk and Ertl 1999). Transfected dendritic cells have been shown in draining lymph nodes after intradermal transfection (Condon et al. 1996; Fu et al. 1997; Porgador et al. 1998; Akbari et al. 1999). Tranfected cells express encoded protein using the cell’s machinery, the way viruses use the cell’s machinery to express and process viral proteins. These cells can be directly transfected or recruited to the area by inflammation. Subsequent to gene encoded expression by the transfected cell, proteosomes in the cell degrade the antigen into peptides, which are translocated into the ER. In the ER, complementary antigens will bind to MHC I, allowing surface expression of the peptide/MHC I complexes. The specific immune response is activated following translation of the antigen from the plasmid and its presentation in the MHC I of antigen presenting cells (Kowalczyk and Ertl 1999). At this point, cytotoxic responses are elicited when CD8+ cells recognize the foreign peptide in the MHC I receptor because these cells appear as virally infected or “altered self” cells. The cytotoxic T-cells then lyse the “infected” cell and release the remaining protein, which could then be taken up by antigen presenting cells (APC) to be presented as exogenously produced antigens in the MHC II receptors. At this point, helper T-cells are stimulated by the peptide in the MHC II receptors and signal for help or stimulate B-cells to produce antibody. In general, DNA induction of humoral immunity is less effective than the induction of cellular immunity because B-cells predominantly respond to extracellular antigens, antigens that are freely floating in the cellular milieu or are bound by surface Ig molecules to the B-cell. Because B-cell presentation of antigen to T-cells involves either interaction with bound antigens on the surface of the B-cell, or endocytosed and
degraded antigens in its MHC II, B-cells are not very efficiently stimulated by plasmid DNA (Sharon 1998). The failure of DNA vaccines to elicit strong humoral responses has been dealt with by modifying the encoded antigen with a signal sequence to promote secretion or cell surface display (where specific antibody has access to the protein) (Boyle et al. 1997).

Nevertheless, neutralizing and specific antibodies can be elicited by plasmid encoded antigens. As a general rule, however, titers elicited by DNA vaccines are well below those achieved by subunit vaccines or recombinant viruses (Kowalczyk and Ertl 1999). The isotypes of antibodies induced by DNA vaccines are predominantly IgG (IgG2a in mice) but IgM and IgA have also been detected (Deck et al. 1997). The detection of IgG2a antibodies after DNA immunization indicates a Th1 type of response, which reflects the ability of CpG motifs to induce IL-12 (a known activator of Th1 responses and suppressor of Th2 responses) (Raz et al. 1996).

Another way to deliver plasmid vaccines is to use attenuated bacterial vectors. An attenuated bacterium retains the ability to invade host tissues in a way specific to the bacterium in question (Dietrich et al. 1999). The attenuated intracellular bacterium invades the host, is phagocytosed by the host antigen presenting cells, and is lysed by the APC due to the specific attenuation and the destructive action of the macrophage. This action releases the plasmid into the phagosome or cytosol of the APC and allows the plasmid to be transcribed by the host cell machinery in the APC’s nucleus. The protein of interest will then be translated and presented to the immune system, resulting in long lasting cellular and humoral immune responses (Dietrich et al. 1999). *Salmonella spp.*, *Shigella spp.*, *Listeria spp.*, and *Vibrio spp.* are a few of the different attenuated intracellular bacteria that have been used to deliver plasmid vaccines against viruses, tumors, and bacteria (Dietrich et al. 1998; Jain and Mekalanos 2000; Pasetti et al. 2003). Dietrich et al. (2001) tested the ability of *Listeria monocytogenes* to invade EPC cells *in vitro*.
and deliver a green fluorescent protein (gfp) encoding plasmid, which should be expressed after the *Listeria* escape the phagosome and enters the cytosol of the cell. The authors reported that gfp was detected as early as three hours post-infection and reached a high level at 24 hours, showing that the *Listera act-A* promoter was functional in fish cell cytosol and that bacterial delivery of plasmid vaccines is possible for fish.

A major advantage of DNA vaccines is that a potentially dangerous live-attenuated vaccine can be reduced to an epitope, which poses little threat to a naive host. Some concerns have arisen dealing with DNA vaccines, however, including reports of possible autoimmune problems as a result of exposure to foreign DNA, which could result in an immune response against any DNA of any type, or nonspecific inflammation induced by methylation patterns in bacterial plasmid vectors (CpG motifs) (FDA Points to Consider 1996; Mor et al. 1997; McMahon et al. 1998; Alton et al. 1999; Smith and Klinman 2001). This could result in the host making an immune response against its own DNA. However, there have not been reports of this event taking place. Another concern with the use of DNA vaccines is the possibility of integration into the host or into the bacterial carrier (Smith and Klinman 2001). DNA recombination into the bacterial carrier would result in clearance of the plamids prior to host cell expression and failure to induce an endogenous immune response against the plasmid-encoded antigen. Integration of the gene into the host genome, however, would be a bigger problem because the antigen would persist, but may not produce protein if that portion of the genome was not translationally active (Mor et al. 1997). Alternatively, if that portion of the genome in cells was actively producing protein, the body could be in a constant state of immune response, build up a tolerance to the antigen, induce a hypersensitivity allergy, or these cells may be lysed by cytotoxic T-cells (Mor et al. 1997; Smith and Klinman 2001). If the cells were eliminated early
by cytotoxic lysis, any autoimmunity could be avoided. Kanellos et al. (1999b) investigated integration of a β-gal expressing plasmid into the goldfish genome, the transference of the plasmid to other sites in the fish, and autoimmunity to single-stranded (ssDNA) or double-stranded DNA (dsDNA). Goldfish injected with 50 µg of plasmid DNA were euthanized at 4, 7, 14, 21, 28, 35, and 70 days to count positive muscle cells, determine antibody titer by ELISA, identify individual cells producing antibody by the antibody forming cell assay, assess lymphocyte activity by the lymphocyte proliferation assay, and test for antibody against ssDNA and dsDNA. At day 70, muscle fibers were still highly positive. Antibody forming cell assay showed positive responses at day four in the kidney tissue, which disappeared at day 35. Serum anti-β-gal antibodies increased between days 7 and 14 and plateaued from day 21 to day 70. The lymphoproliferation assay showed responses at day 7 and 14 and peaked at day 21 but showed a lower response at day 70. Serum antibody against ssDNA and dsDNA was not positive. In addition, plasmid β-gal DNA was not detected in the fish genome by PCR, indicating it had not integrated (Kanellos et al. 1999b). Dijkstra et al. (2001), however, demonstrated that in a glass catfish (Kryptopterus bicirrhus), a luciferase encoding plasmid was still expressing luciferase two years after injection. Luciferase expression was predominantly at the site of injection from day two post-injection and was detected in an undiminished capacity two years post-injection. The authors site the instability of luciferase protein in mice to suggest that the luciferase protein was recently translated from the DNA vaccine, and not a remnant of protein expressed in the past (Wolff et al. 1990). Dijkstra et al. (2001) did not establish the form of the plasmid at the injection site (i.e. circular, linear, or integrated), however, Anderson et al. (1996) found that plasmids exist as a stable extra-chromosomal entity after two months without integration in rainbow trout. This study highlights the potential longevity of a vaccine to elicit immune
responses, however, also brings to light concerns for potentially negative effects of long-term expression (i.e. tolerance). Thus, although there are concerns regarding DNA vaccination, data to date fails to support those concerns.

**DNA Vaccines Against Fish Pathogens.** The field of fish disease encompasses many bacterial, viral, fungal, and protist pathogens (Noga 1996). DNA vaccines in the aquatic disease field have been tested primarily against viral pathogens, and have experienced successes within the rhabdoviral disease family (Anderson et al. 1996; Heppell et al. 1998; Lorenzen et al. 1999; Corbeil et al. 1999; LaPatra et al. 2000; Lorenzen et al. 2000; Corbeil et al. 2002b; McLauchlan et al. 2003).

Expression of Foreign DNA in Fish and DNA Vaccine Structure. DNA vaccination has been used widely in mammalian systems against many pathogens. The first report of expression of plasmid mediated gene expression in fish muscle was by Hansen et al. in 1991. The reporter genes chloramphenicol acetyltransferase (CAT) and β-galactosidase were promoted by the SV40 promoter. Common carp (*Cyprinus carpio*) were injected with a range of 12.5-100 µg of DNA in 100 µl phosphate buffered saline (PBS). In the muscles of young growing carp (about 10 cm), CAT activity was detected at more than 90% chloramphenicol conversion in injected muscle and at 0% in uninjected muscle. In older fish, the level of CAT activity was 41%. CAT expression peaked at 50 µg of DNA but as little as 12.5 µg was enough to elicit expression. Muscle fibers observed to be expressing the *lacZ* or β-galactosidase gene were distributed along the path of injection (Hansen et al. 1991). Heppell et al. (1998) injected zebra fish and rainbow trout with a range of doses from 0.01-1 µg with a plasmid using a CMV promoter to drive the luciferase reporter gene. Dose dependent expression was observed for the 0.01-1 µg doses, but greater amounts (10 µg or 50 µg) did not increase observed luciferase activity. Mice were injected in a
parallel experiment and it was found that rainbow trout and zebra fish had consistently higher luciferase activity than mice. This luciferase activity was detected at the muscle site and, surprisingly, in the gills, even at the lowest dose of plasmid (0.01 µg). The optimal dose of DNA in fish muscle for expression of luciferase was 1 µg. The delivery volume was also investigated and it was found that the smaller the volume of delivery (the more concentrated the dose) the higher the level of luciferase activity (measured in relative light units (RLU)/second) that could be detected. For volumes of 5, 10, 25, 50, and 100 µl, 5 µl was the volume in which the most luciferase activity was detected, with RLUs reaching over $4 \times 10^5$ RLU/sec in the 5 µl treatment and activity barely reaching $1 \times 10^5$ RLU/sec in the 100 µl treatment. Kinetics experiments indicated that detection of luciferase with trout and zebra fish muscle was still high after 100 days. The earliest time at which luciferase was detected was in the 10 µg dose in zebra fish at two hours post-injection. Tissue damage at the site of injection was minimal and determined to be due to the needle-stick injury, not the injection of DNA. In another study, direct injection of plasmid DNA utilizing the carp $\beta$-actin promoter in tilapia muscle successfully drove CAT expression (Rahman and Maclean, 1992). Maximal expression was observed 48 hours post-injection and was undetectable by 7 days post-injection. The carp $\beta$-actin promoter was compared to the CMV promoter and the mummichog Fundulus heteroclitus lactate dehydrogenase-B promoter in Atlantic salmon (Salamo salar) by Gomez-Chiarri et al. (1999) using the luciferase gene. Luciferase activity was the greatest in the CMV promoter treatments, followed by the $\beta$-actin promoter. The lactate dehydrogenase-B promoter induced the lowest luciferase expression.

Anderson et al. (1996) investigated the optimal promoter, optimal DNA amount, and optimal volume for delivery of a luciferase reporter plasmid in 50 g rainbow trout.
(Oncorhynchus mykiss). Fish were injected with 10, 25, or 50 µg of plasmids containing the glucocorticoid-responsive mouse mammary tumor virus promoter (pMMTV-Luc), the cytomegalovirus plus translational promoter (pCMV4EAL), the CMV promoter (pCMVL), and the carp β-actin promoter (pFV4EAL). Luciferase activity was measured at 36 hours post-injection. Maximum luciferase activity was observed at 25 µg for all promoters. The highest level of luciferase was elicited by the pCMVL plasmid (3.2 million RLU). The fish promoter elicited an intermediate amount of luciferase (124,000 RLU). Maximal expression of luciferase was detected at day 7 post-injection, with the pCMVL and pCMV4EAL promoting approximately 500-fold greater activity than the β-actin promoter, which was detectable out to 115 days post-injection. The effect of volume delivered was tested on 1 g and 50 g fish after injection of 25 µg plasmid DNA in 100 µl and 200 µl volumes. Variability was considerable with the 100 µl volume but was more reproducible using the 200 µl volume. The optimal concentration determined in this study was 25 µg of plasmid in a 200 µl volume for delivery of DNA to fish. After restriction enzyme digestion of DNA extracted from fish tissue and Southern blot hybridization, the injected DNA appeared to remain in the tissue as unreplicated, unintegrated DNA (Anderson et al. 1996). When the organs of injected fish were analyzed, luciferase activity was found in the kidney, heart, liver, spleen, and gill at lower levels than the muscle site. This phenomenon may be explained by leukocytes carrying the plasmid to distant sites following injection (Anderson et al. 1996).

Alonso et al. (2003) compared the rainbow trout Mx gene promoter, the interferon regulatory factor 1A (IFR1A) promoter from rainbow trout, and the cytomegalovirus immediate early promoter (CMV) for their ability to express the IHNV-G gene in mouse cells NIH-3T3, Chinook salmon embryo cells (CHSE-214), Epithelioma Papulosum Cyprini cells (EPC), and in
in vivo in 0.4 g rainbow trout muscle. In both fish cell lines, the CMV promoter elicited the highest level of gene expression in a luciferase reporter gene study, although all these promoters drove gene expression. When promoting the IHNV-G gene in fish cell culture, however, the CMV and IFR1A promoters elicited greater gene expression than the Mx promoter. In rainbow trout muscle, the CMV promoter elicited the highest luciferase expression, followed by IFR1A. However, when used as a DNA vaccine promoting the IHNV-G gene, the IRF1A promoter generated the greatest protection after challenge, followed closely by the CMV promoter. This study suggests that fish promoters are an avenue of investigation for teleost DNA vaccines because teleost promoters may be more efficacious in driving gene expression and eliciting a protective immune response in fish.

The delivery of plasmid DNA into fish tissue was further investigated by Gomez-Chiarri et al. (1996). Fish were vaccinated by intramuscular injection or by particle bombardment with gold particles coated with naked DNA encoding the luciferase and β-galactosidase genes. Fish were injected with 100 µl of plasmid DNA of concentrations from 10-100 µg in PBS. Luciferase activity was maximal using 50 µg DNA and could be detected as soon as two days after injection into the muscle and skin. This response increased significantly when tested at 60 days post-injection. Muscle cells expressing the construct were found primarily along the needle track. When injected into the gills, no luciferase expression was observed. Particle bombardment was performed using 5-25 µg of plasmid DNA in an area along the anterior margin of the dorsal fin and the lateral line. Results of the dosage optimization using particle bombardment were varied and much lower than the direct injection for the same delivery dose. Only 59% of bombarded fish were positive for luciferase whereas 100% were positive in the intramuscular injection group. This may be a result of the particles not penetrating the scales as it was observed that
some gold particles remained on the surface of the scales. Therefore, the bombardment was repeated using targets of the eye, gills, midsection, between the dorsal fin and the lateral line, and close to the tail (peduncle area). The results were still varied, although best results were obtained when the eye was targeted. Luciferase activity was not significantly different from that in the flank muscle and no activity was detected in the gill target area. These data indicate that injection is a superior method of DNA vaccine delivery than particle bombardment in fish (Gomez-Chiarri et al. 1996).

Corbeil et al. (2000) investigated intramuscular injection, scarification of the skin, intraperitoneal injection (IP), intrabuccal injection, cutaneous particle bombardment using a gene gun, and immersion in water containing vaccine-coated beads as delivery methods for DNA vaccines in 1.8 g rainbow trout. The DNA vaccine tested encoded the IHNV-G gene and success was tested in percent survival from a lethal challenge of IHNV and serum neutralizing antibody. For intramuscular and intraperitoneal injection, 100 ng was delivered in 50 µl PBS at the base of the dorsal fin or at the base of the ventral fins, respectively. For the intrabuccal treatment, a microtip containing 100 ng plasmid DNA in solution was delivered into the throat while fish were held vertically. The skin was scarified vertically from the dorsal fin to the abdomen using a needle that had one drop of 200 ng/µl of DNA vaccine solution on its bevel to ensure that at least a 100ng dose was delivered. For the gene gun delivery, one dose of gold particles covered with a total of 100 ng DNA was delivered into the left lateral side below the dorsal fin. For the immersion treatment, fish were held for 40 minutes in 200 ml of water containing 3.4 X 10⁶ DNA coated magnetic polystyrene beads/ml. At 27 days post-vaccination, no neutralizing antibodies were detected, regardless of method of delivery. After challenge, the intramuscular injection treatment had the highest relative percent survival (RPS) at 100%, followed by gene
gun delivery at 96.2%, and intaperitoneal injection at 50.3%. Scarification of skin, intrabuccal administration, and immersed treatments resulted in an RPS of 25.9%, 11.1%, and 28%, respectively. These results support the results of Gomez-Chiarri et al. (1996) who reported intramuscular injection as a superior method to gene gun delivery. Corbeil et al. (2000) reported that the gene gun had some effect, most likely due to the differences between the immune response against a reporter protein versus an antigen from a disease agent. The authors postulated that failure of the four methods are multi-fold. First, the IP injection may have failed because the dosage optimal for muscle site injection is not optimal for IP injection. A previous study in goldfish showed antibody responses following IP injection, but used an oil-based adjuvant that may have elicited an influx of antigen presenting cells or protected the naked DNA from nuclease (Kanellos et al. 1999c). The intrabuccal route may have failed due to regurgitation of the vaccine by the fish or destruction of the vaccine in the digestive tract. The scarification method most probably failed because of the wet nature of fish skin and the need to quickly return the fish to water, which may have caused the vaccine to wash off before it could be absorbed. Finally, the immersion method may have failed because the DNA vaccine bound to the polystyrene beads was not easily absorbed into the fish and was therefore not available to the fish cells for transcription/translation of the encoded gene. These authors also postulate that the lack of neutralizing antibody in the presence of survival of a lethal challenge suggests that either non-neutralizing antibodies and/or cellular immunity are the components crucial for survival to IHNV (Corbeil et al. 2000).

Infectious Hematopoietic Necrosis Virus. Infectious hematopoietic necrosis virus (IHNV) is a serious and costly rhabdoviral disease of farmed rainbow trout. The first report of DNA vaccination in fish against a pathogen was by Anderson et al. (1996b) with the G gene of
IHNV. The G gene of IHNV is the glycoprotein gene, which was cloned into a plasmid and delivered IM to 1 g rainbow trout. 10 µg of the G gene encoding plasmid (pCMV4-G) elicited neutralizing antibody and gene specific antibody by ELISA to levels of 1000 relative antibody activity units (RU) in comparison to negative controls that elicited an RU of 10. Following challenge with wild-type IHNV, significant survival was observed post-vaccination with pCMV4-G, where 75% RPS was observed, and 35% RPS was observed in the negative control. A combination of the N (nucleoprotein gene) was tested and although protection was not observed in this treatment (25% RPS), a combination of the N construct with the pCMV4-G elicited 78% RPS.

DNA vaccination testing all five proteins of IHNV was undertaken by Corbeil et al. (1999). The nucleoprotein (N), phosphoprotein (P), nonvirion protein (NV), matrix protein (M), and the glycoprotein (G) each were cloned into pcDNA3.1 (Invitrogen). The N and G constructs were transfected into EPC cells and confirmed to make the expected protein using indirect immunofluorescence using monoclonal antibodies against the N and G gene protein products. Large sockeye salmon (Oncorhynchus nerka) were vaccinated with each of the constructs in a 25 µg in 200 µl dose. The sockeye salmon were bled and neutralizing antibody titers were assessed. Serum was also pooled and used to passively immunize a group of rainbow trout fry. A second group of rainbow trout fry were injected with 1, 5, or 10 µg of groups of each construct in 25 µl of Tris-EDTA buffer (TE) and challenged at four and six weeks post-vaccination. Mean cumulative percent mortality (MCP) was 0, 2, and 2% for the G construct was 1, 5, and 10 µg doses, respectively, irrespective of time to challenge. Mortality data was reported for one dose of DNA for the M (10 µg), P (5 µg), N (1 µg), and NV treatments (1 µg). The M construct treatment had a MCP of 50%, the P treatment had a 47% MCP, the N treatment had a 76% MCP,
and the NV treatment had a 67% MCP. At weeks 4, 8, and 11 post-vaccination, the G construct protected fry with a MCP of 2, 31, and 49%, whereas the negative control plasmid showed MCPs of 60, 98, and 95%, respectively. Looking at neutralizing ability, the only construct with an appreciable neutralization titer was the G construct in large sockeye salmon. All others were negative. Fry passively immunized with neutralizing sera from G-construct vaccinated fish were protected after challenge with IHNV to a 100% relative percent survival (RPS). Negative control sera injected treatments had a 58% RPS. However, fry vaccinated with the G construct were protected from challenge and following testing for neutralization activity were negative for neutralizing antibody. The authors suggest that, even though serum from the larger fish displayed neutralizing activity, that the protective element in the passive transfer of sera was non-neutralizing antibodies.

Traxler et al. (1999) investigated the mode of challenge as well as life stage of the fish in DNA vaccination against IHNV of smolt (acclimated to sea water) or pre-smolt (not acclimated to sea water) Atlantic salmon (average weight of 53-73 g) at eight weeks post-vaccination. The two modes of challenge were immersion (fish immersed in the challenge dose) or cohabitation (10 fish injected with $4.9 \times 10^3$ PFU of IHNV and put in 16 tanks with non-injected fish). For pre-smolts vaccinated with the G construct (pCMV4-G from Anderson et al. 1996b) and immersion challenged, a RPS of 96% was observed, compared to 26% RPS in negative control treatments. For pre-smolts vaccinated with the G construct and cohabitation challenged, the RPS was 100%. Negative control treatments had 37% RPS. For smolts vaccinated with the G construct and immersion challenged and cohabitation challenged, 90% RPS and 93% RPS was observed, respectively. Challenge mode and life stage does not appear to effect vaccine efficacy (Traxler et al. 1999).
In addition to method of delivery, challenge mode, life stage, and gene target, the vaccination dose has been extensively studied in the IHNV system. LaPatra et al. (2000) conducted a dose response study evaluating protection and humoral response in subyearling rainbow trout (120 g). The doses tested were 25, 10, or 1 µg of a G gene construct, pIHNw-G, in 100 µl phosphate buffered saline (PBS). A second study was conducted evaluating the protective capacity of 10, 1, and 0.1 µg of pIHNw-G in 100 µl PBS. After 42 days, fish were challenged by injecting 1 X 10^6 PFU of IHNV IP. Fish injected with doses of 25, 10, and 1 µg of pIHNw-G had 100% survival following challenge. At a dose of 0.1 µg, percent mortality was the same as the negative control, which implies no protection conferred. Neutralization activity was present in the pIHNw-G treatments, and was highest in the 25 µg doses (LaPatra et al. 2000). A minimal dose experiment was also conducted by Corbeil et al. (2000b) wherein 0.8-1.8 g rainbow trout were vaccinated with 5, 1, 0.1, and 0.01 µg of pIHNw-G in 50 µl PBS. Fish were challenged six weeks post-vaccination with IHNV and significant protection was observed even at the 0.01 µg dose (all at 100% RPS). Fish injected with a 0.001 µg dose still had significant protection with an RPS of 60%. Serum was tested for neutralizing antibody activity and had high titers at six weeks post-vaccination (unchallenged), even in the 0.01 µg treatment (titer of 107). The neutralizing activity of the 0.01 µg treatment was not detected at 10 weeks post-vaccination (unchallenged), but the 5, 1, and 0.1 µg doses still showed titers greater than 70. Fish vaccinated with a 1 µg dose and challenged with six heterologous strains representing the geographic range of IHNV (WRAC, 220-90, AD14, RB-1, Col-85, Shizuoka (Japan), and 32-87 (French)) showed significant cross protection, RPS of 60% and above, between heterologous strains. This study showed that not only were very low doses of DNA vaccine efficacious (1 ng), but a 1 µg dose could protect against heterologous strains of IHNV (Corbeil et al. 2000b).
LaPatra et al. (2001) injected 2 g rainbow trout and challenged at 1, 2, 4, 7, 14, and 28 days post-vaccination with a lethal dose of IHNV and found significant protection at all time points at day four and after. Mortalities at day 1 and 2 post-vaccination were not different from negative controls. DNA vaccination against IHNV has been successful and extensively investigated, with the G gene being the most efficacious. Neutralizing antibody has been shown to be elicited by DNA vaccination but is not considered to be as important as specific antibodies and cellular immune responses (LaPatra et al. 2001).

Viral Hemorrhagic Septicemia Virus. Viral hemorrhagic septicemia virus (VHSV) is a serious and costly rhabdoviral disease of farmed rainbow trout. Heppell et al. (1998) created two vaccines against VHSV by cloning the G gene (glycoprotein) and the N gene (nucleocapsid) into the pcDNA3 expression plasmid utilizing the CMV promoter. These vaccines were then tested using 0.5-2 g rainbow trout. Fish were injected IM with 5-50 µg of DNA and challenged at 28 days post-vaccination. Both the N and the G gene were detected by immunofluorescence using monoclonal antibodies against the N and the G gene, confirming correct expression and conformation of the products of each gene. Significant protection was observed with the G construct at 50 µg, 10 µg, and a mixture of the N and the G constructs at a dose of 5 µg each, with an mean cumulative percent mortality (MCP) under 10% for all three treatments. The N construct alone at a dose of 5 µg protected fish with a MCP of 20% (Lorenzen et al. 1998).

Lorenzen et al. (1999) tested a smaller dose of the G construct for its protective capacity against one strain from serogroup I and one strain of VHSV from serogroup III. Fish weighing 3.5 g were vaccinated with 10, 1, and 0.1 µg of G construct and N construct plasmids from the serogroup I strain and challenged on day 9 and 21 post-vaccination. Expressed G protein could be detected in the fish muscle by immunohistochemistry. For the homologous challenge, relative
percent survival (RPS) for the G construct at 10, 1, and 0.1 µg was 84, 96, and 88% at day 9 post-vaccination and 78, 90, and 77% at day 21 post-vaccination respectively. For the N construct, RPS at 10 and 1 µg was 7% at day 9 and 6 and 8% at day 21. One treatment of 1 µg of the G and N construct mixed was tested and had a RPS of 88% for day 9 and 75% for day 21. For the serologically distant strain, RPS for the G construct at 10, 1 and 0.1 µg was 95, 81, and 85% at day 9 and 78, 54, and 70% at day 21 respectively. For the N construct, RPS at 10 and 1 µg was 11 and 0% for day 9 and 0% for day 21. For the mixed vaccination, RPS was 84% at day 9 and 62% at day 21. This study shows that 0.1 µg of G construct is effective even against a serologically distinct strain and that the N construct is not as effective as the G construct in eliciting protection (Lorenzen et al. 1999). In further studies, the G construct is almost exclusively utilized.

Lorenzen et al. (2000) investigated the dose of DNA vaccine necessary to protect against VHSV and duration of protection. Rainbow trout weighing 3-4 g were injected IM with 0.001, 0.01, 0.1, and 1 µg of the G construct of VHSV in 25 µl of saline. Fish were challenged 51 days post-vaccination with VHSV and at 103 days post-vaccination with two strains of VHSV, a homologous strain and a heterologous strain that was not neutralized with sera from fish vaccinated with the G DNA vaccine. The relative percent survival (RPS) for the dose response study challenged at 51 days post-vaccination, was 14, 78, 97, and 100 for doses 0.001, 0.01, 0.1, and 1 µg respectively. The RPS for the dose response study challenged with the homologous VHSV at day 103 post-vaccination was 9, 58, 100, and 100 for doses 0.001, 0.01, 0.1, and 1 µg respectively. For the heterologous challenge, the RPS was 2, 32, 67, and 72% for doses 0.001, 0.01, 0.1, and 1 µg respectively. Greater protection was seen with the homologous versus the heterologous virus, but significant protection was observed against the heterologous strain at the
0.1 and 1 µg doses. In another study, fish were vaccinated with 1 µg of the G construct and the negative control (vector only) plasmid and then challenged at 8, 19, 28, 61, 112, and 168 days post-vaccination with the homologous VHSV strain. At day 8, 19, 28, 61, 112, 168 post-vaccination, RPS was 66, 88, 97, 97, 79, and 64%, respectively. These studies show that fish challenged with at little as 0.1 µg were protected against challenge and that fish vaccinated with 1 µg were protected as early as day 8 and as long as 168 days post-vaccination.

In contrast to previous studies, McLauchlan et al. (2003) found some variations in protection with DNA vaccines against VHSV. McLauchlan et al. (2003) investigated fish size, dose, route of injection, duration of protection, and indicators of early nonspecific protection against VHSV using the G gene construct DNA vaccine in rainbow trout. Using 10 g trout, protection at 1 week post-vaccination with 0.5-10 µg of DNA was high when vaccine was administered IM and low using an IP injection. At four weeks post-vaccination, mortality was low using IM vaccination at a dose of 0.5, 0.1, and 0.01 µg DNA but was high at all doses of DNA using the IP injection route, contrasting with studies done with IHNV (Gomez-Chiarri et al. 1996; Corbeil et al. 2000). Using 100 g fish vaccinated with 0.5 or 5 µg DNA, fish were very well protected against challenge, whereas 10 µg IM and 0.5 µg IP doses were not protective at 1 week post-vaccination. At four weeks post-vaccination, the only dose delivered to 100 g fish that was protective was 0.5 µg IM. This contrasts with the results of Lorenzen et al. (2000) and Lorenzen et al. (1999), who tested 3.5 g fish and found much lower doses of DNA vaccines effective using an IM route. Smaller 4.5 g trout were immunized with 0.5 µg DNA and challenged at four and eight weeks post-vaccination with low mortalities (<20% MCP) (McLauchlan et al. 2003). Neutralizing antibody elicited by vaccination of 4.5, 10, and 100 g fish with doses of vaccine of 0.01, 0.1, 0.1, 5, or 10 µg DNA was quite low in all groups and at
all time points except in 100 g fish vaccinated with 10 µg at five weeks post-vaccination. All fish in this group were positive for neutralizing antibody, in agreement with Corbeil et al. (1999) who found neutralizing titers only in large fish with the IHNV system. Mx gene expression was detected in fish livers vaccinated IM with 0.5, 5, and 10 µg DNA at 1 week post-vaccination, illustrating early protection. To investigate long term protection, 100 g fish were vaccinated IM with 0.5 µg DNA and challenged 9 months post-vaccination with a lethal dose of VHSV, resulting in a RPS of 88% and illustrating long term immunity (McLauchlan et al. 2003).

Lorenzen et al. (2001) investigated the protection conferred by DNA vaccination at an early life stage. Three months post-hatch, rainbow trout fry (approximately 0.5 g in weight) were injected IM with 1 µg of the G construct in 10 µl of saline. The fry were challenged at 9 days post-vaccination and 70 days post-vaccination. Immunohistochemistry showed that fish sampled at day 21 post-vaccination revealed G protein positive muscle cells surrounded by inflammatory cells at the site of injection, indicating that a cellular inflammatory response was elicited by the vaccine. RPS at day 9 post-vaccination of the G construct treatment was 98% and 85% at day 71. This study shows that early and late protection is evident in very young susceptible fish (Lorenzen et al. 2001).

Immune responses against VHSV DNA vaccination were investigated looking for upregulation of antiviral genes in rainbow trout and antibody response (Collet et al. 2003). DNA vaccination using the VHSV G construct elicited upregulation of interferon regulatory elements and Mx in rainbow trout. One week after DNA vaccination with a high dose of 300 µg of G plasmid DNA, upregulation of Mx protein and interferon regulatory factor 1 was detected in the muscle site (Collet et al. 2003). Rocha et al. (2002) tested the antibody response to a fragment of the G gene of VHSV in rainbow trout to identify the immunogenic fragment of the gene product.
Fragment 11 (frg#11) is a 104 amino acid fragment of the G gene that is recognized by about 40% of immunized trout and might be implicated in viral fusion (Fernandez-Alonso et al. 1999; Estepa et al. 2001; Fernandez-Alonso et al. 2001). This fragment also incorporates a large portion of the linear epitopes thought to be predominantly recognized by immunized trout. The lack of consistent neutralizing titers (conformational epitopes) in the presence of efficacious immunization implies that the identification of antibodies against linear epitopes is a better indicator of survival after immunization and challenge than neutralizing titers (Fernandez-Alonso et al. 1999; Estepa et al. 2001; Fernandez-Alonso et al. 2001). Fish vaccinated with concentrated VHSV and the whole G protein produced antibodies capable of interacting with frg#11 by ELISA to high OD levels. Fish DNA vaccinated with the G gene also produced antibodies capable of interacting with frg#11 to high levels. This study shows that linear epitope antibody tests are better predictors of fish survival after DNA vaccination than neutralizing antibody titers (Rocha et al. 2002). Lorenzen et al. (2000b) created a plasmid encoding a recombinant neutralizing single chain antibody against VHSV, delivered IM to fish, and later detected recombinant circulating neutralizing antibodies in the serum. When challenged, protective immunity was established.

In order to begin the investigation into fish cellular responses to DNA vaccines, Heppell et al. (1998) tested the persistence or reduction in reporter gene positive muscle cells after co-injection with a DNA vaccine. Heppell et al. (1998) co-injected a luciferase expression plasmid with the vector carrying the G gene and observed that luciferase expression peaked at day 2.5 post-injection. Muscle fibers expressing luciferase decreased at day 28 and day 84 post-injection, whereas the luciferase plasmid co-injected with the vector only plasmid did not decrease over time. The decrease in the muscle cells expressing luciferase in the G construct co-
injected group is postulated to be the result of the fish raising a cellular immune response against the transfected cells, illustrating the immunogenicity of the G gene as an antigen of VHSV. In another study utilizing the β-galactosidase reporter gene, Kanellos et al. (1999) co-injected the mouse GM-CSF (Granulocyte/Macrophage-Colony Stimulating Factor) with a β-gal expressing plasmid and observed a reduction in cells expressing β-gal as opposed to the β-gal injected treatment only. A cellular immune response was activated by the murine GM-CSF gene, which drew inflammatory cells to the site of transfection. This finding further supports the conclusion of Heppell et al. (1998) who stated that cellular immune response activation caused a reduction in the number of transfected cells.

Cross Protection. Early cross protection elicited by DNA vaccines against nonhomologous viruses has been shown with IHNV and VHSV. LaPatra et al. (2001) showed that vaccination with the G genes (glycoprotein) of IHNV, VHSV, and RV (rabies virus) at a dose of 1 µg, resulted in protection in 2 g rainbow trout at days 4, 7, and 14 with either the IHNV or the VHSV construct when challenged with IHNV. Vaccination with the RV construct did not provide early protection at any time point. The early cross protection was not detected at day 28, where only the IHNV construct protected against IHNV, indicating that specific adaptive immune responses had developed at this point. Kim et al. (2000) postulated that the early nonspecific response might be due to the expression of alpha/beta interferons and tested for expression of the interferon inducible Mx protein at various times after vaccination with the G genes of IHNV, snakehead rhabdovirus (SHRV), and spring viremia of carp virus (SVCV). Rainbow trout weighing 0.5 g were injected with a 10 µg/25 µl dose of each plasmid and challenged at 30 days and 70 days post-vaccination with wild type IHNV. Kidney and liver were tested by immunoblot for expression of Mx protein, an antiviral protein induced by alpha/beta
interferon. All three G genes were sequenced and analyzed for amino acid conservation, of which, only 11% of all three genes were conserved at the amino acid level. The G genes were also screened for CpG motifs. There were only two CpG motifs in the G gene sequences, whereas 25 occur in the backbone plasmid DNA. At day 30 post-vaccination, mean cumulative percent mortality (MCP) was 4, 1, and 3% in the IHNV, SHRV, and SVCV treatments when challenged with IHNV. At day 70 post-vaccination, MCP was 12%, 68%, and 76% for the IHNV, SHRV, and SVCV treatments, showing specific protection at day 70 that was not apparent at day 30. In the IHNV treatment, Mx protein was detected in 3/3 fish in both kidney and liver at day 0, 1, and 2 post-challenge. At day 5 post-challenge, 2/3 fish were positive for Mx and none were positive at day 7. For the SHRV treatment and the SVCV treatment, Mx protein was detected in 2/3 fish at day 0, 1, 2, 5, and 7. No Mx protein was detected in negative control plasmid only treatments and PBS only treatments on day 1, and 2. Mx protein was detected in the negative control treatments at day 5 and 7 post-challenge in 3/3 fish, most probably due to the challenge. This study shows that Mx gene expression was elicited by all three plasmid vaccines at early time points (days 1, 2, and 5 post challenge) but that expression was undetectable at day 7 in the IHNV construct while remaining high in fish given the heterologous vaccines and in the negative controls. The authors postulate that the downregulation of the Mx gene at 7 days post-challenge signifies the upregulation of a specific immune response and subsequent vaccine-specific protection. No neutralizing titers were detected in any fish pre-challenge in any treatment, indicating that they were naïve prior to vaccination and challenge (Kim et al. 2000).

Considering the surprising finding that DNA vaccination is cross protective at early stages with IHNV and VHSV using the G gene, it is not surprising that Boudinot et al. (1998)
investigated dual DNA vaccination. Boudinot et al. (1998) investigated the efficacy of combined immunization with IHNV and VHSV G gene plasmids in rainbow trout. 150-200 g rainbow trout were immunized by multipoint IM injection with 30 µg of either the VHSV construct (gVHS), the IHNV construct (gIHN), or a mixture of the two. The fish were boosted twice on days 23 and 38 with an equal amount of DNA. Muscle samples were taken on day 45 to assay for plasmid persistence and transfection of muscle cells. After utilizing *in situ* PCR on muscle RNA and immunofluorescent antibody (IFA) tests on tissue sections, the VHS G gene was detected inside the cytoplasm of muscle cells and at the periphery of muscle fibrils. Inflammatory cells infiltrating this area were also positive for the G gene. The PCR method confirmed that the G gene was being transcribed from the plasmid in the muscle tissue. Neutralizing antibodies were detected in the single vaccinations against their specific viruses, and the combined vaccine produced a similar level of neutralizing antibody against both VHSV and IHNV as if the fish were vaccinated against only one virus. Therefore, double-specific neutralizing antibodies were detected. The double-specific antibodies were pooled and used in a passive transfer to protect juvenile fish to 100% against both viruses. As assayed by RT-PCR, both Mx genes and MHC class II beta transcripts were detected in fish vaccinated with the gVHS construct and not negative controls. This suggests that not only is the innate antiviral arm of the immune system activated (as shown by Mx expression) but immune cells have most likely infiltrated the site of injection (as phagocytic cells and B cells are the only cells that express MHC class II receptors) (Boudinot et al. 1998).

Further investigation into the early cross-protective phenomenon was undertaken by Lorenzen et al. (2002), who vaccinated rainbow trout with the G construct of VHSV and IHNV and challenged with the rhabdoviruses as well as *Yersinia ruckeri* and *Aeromonas salmonicida,*
two bacterial diseases of trout. When challenged with VHSV at days four and seven post-vaccination, there was protection with fish vaccinated with either VHSV or IHNV. Only specific protection was only seen at days 60 and 84 post-vaccination. When vaccinated with the VHSV construct and challenged 8 days post-vaccination with VHSV and *Y. ruckeri*, protection was seen against VHSV, but not against the bacterium. At 30 days post-vaccination, there was still protection against VHSV and not against the bacterium. This experiment was repeated with *A. salmonicida* and fish were challenged 18 days post-vaccination. Protection was seen against VHSV but not against the bacterium. Although DNA vaccination with rhabdoviruses provides early cross protection against heterologous viruses, it does not provide cross protection with bacterial pathogens.

The reason rhabdoviral genes work so well and so early after vaccination in a nonspecific fashion has not been definitively been explained. Two theories have been suggested by Lorenzen et al. (2002b) to explain the nonspecific early response, involving the role of CpG motifs and the expression of the viral G gene by transfected cells. CpG motifs are recognized by Toll-like receptors that are well conserved throughout the animal kingdom, and were recently suggested to be present in fish (Bayne et al. 2001). Recognition of CpG motifs induces the innate immune response, and after stimulation with CpG motifs, salmonid cells secreted antiviral compounds (Jorgensen et al. 2001). Only two CpG motifs are present in the rhabdoviral G genes, however, while many more are present on the plasmid backbone, suggesting that the early nonspecific response is not due to the CpG motifs in the G genes (Kim et al. 2000; Lorenzen et al. 2002b). The other theory presented by Lorenzen et al. (2002b) deals with the potentially intrinsic immunogenicity of the rhabdoviral G genes. Viral glycoproteins are known inducers of interferon in mammals (Ito 1994) and the detection of interferon-inducible proteins and/or gene
expression in fish during the nonspecific protection period suggests interferon-mediated protection. Type I interferon is known to be important in both innate and specific immune responses in mice because mice deficient in interferon type I receptors are unable to mount protective immune responses to the mammalian rhabdovirus VSV (Vesicular Stomatitis Virus) (Steinhoff et al. 1995) and are unable to respond to a DNA vaccine encoding the pseudorabies virus glycoprotein (Tudor et al. 2001). The recognition of the endogenously expressed G genes by the fish myocytes, phagocytes, or other immune cells might mimic natural infection well enough to stimulate a strong adaptive immune response and an effective innate immune response (Lorenzen et al. 2002b).

Channel Catfish Virus. DNA vaccination against CCV has been attempted by Nusbaum et al. (2002) with some success. Seven genes, ORFs 6, 7, 8, 10, 51, 53, and 59, were cloned into pCR2.1 following reverse transcription of CCV infected CCO cells. The genes were subcloned by restriction enzyme digestion into pcDNA3.1 (Invitrogen, Carlsbad, CA) and injected in a 50 μg dose into 4-8 g channel catfish. Four to six weeks post-vaccination, the fish were challenged with CCV at 20ºC and the temperature was raised to 26ºC over two days. After six independent experiments, the percent survival of fish injected with ORF59 was 60-100%, ORF6 was 52-100%, and the negative control PBS or vector only treatments was 34-56%. A combination of two constructs, ORF59 and ORF6, was also injected at a 50μg dose of each and challenged similarly. When analyzed using a Kaplan-Meyer survival plot, ORF 6 had a 39% survival, PBS had a 34% survival, ORF 59 had a 74% survival, and the combination of ORF 59 and ORF 6 (ORF 59+6) had a 78% survival. Nusbaum et al. (2002) also tested neutralizing titers elicited by each construct at 4-6 weeks post-vaccination and two weeks post-challenge. Expressed as geometric mean titer (GMT), titers for control, vector only, ORF 10, and ORF 6 barely reached a
mean titer of 5 when measured at four and six weeks post-vaccination. ORF 59 was barely detectable at four weeks and only reached a GMT of 10 at six weeks post-vaccination. When tested two weeks post-challenge, control, vector only, and ORF 10 had titers just above 5. ORF 6 and ORF 59 had titers that increased to 15 and 20, respectively. When tested at five weeks post-vaccination, control, vector only, ORF 8, ORF 6, ORF 59, and ORF 59+6 titers were low and the GMT was around 20. When tested two weeks post-challenge, ORF59+6 and ORF 59 reached a GMT of about 100 and ORF 6 reached a GMT of about 80. ORF 8 had a GMT of about 50 and the vector had a GMT of about 30.

In summary, vaccination against CCV has been attempted with killed, subunit, and live-attenuated vaccines with some success. The characterization of the genes in the literature has not been extensively investigated, with the only information available resulting from mass spectrometry and temporal gene regulation studies. Therefore, much more research is needed into the function of the viral genes in order to elucidate virulence factors for potential knock-out, and further investigation into vaccination is needed because a economically viable and safe vaccine is not currently available. Therefore, DNA vaccination, a successful strategy in combating IHNV and VHSV in aquaculture, was considered a potential boon to fish vaccinology, resulting in a proposal to develop a DNA vaccine against CCV. Using knowledge gained from the distantly related HSV viruses, where glycoprotein genes result in some protection when used as DNA vaccines, and success in the IHNV and VHSV systems, DNA vaccination is expected to reduce mortality and disease pathogenesis in CCV disease and provide a stepping stone to safer and more economically viable vaccines for the commercial catfish industry.
CHAPTER 2. DNA VACCINATION AGAINST CHANNEL CATFISH VIRUS (CCV) IS NOT EFFICACIOUS REGARDLESS OF DOSE OF DNA, GENE TARGET, OR NUMBER OF GENES COEXPRESSED, ALTHOUGH IMMUNE RESPONSES ARE ELICITED

Introduction

Commercial catfish production accounts for 85-90% of the total finfish aquaculture production in the USA, valued at $3 billion dollars in gross national product annually in 2002 (USDA Aquaculture Outlook Report 2003). Channel catfish virus (CCV) is the most devastating viral disease of channel catfish resulting in losses exceeding 90% in fry and fingerling channel catfish ponds (Plumb 1978; Noga 1996). Channel catfish virus (*Ictalurid herpesvirus* 1) is a hemorrhagic viremia wherein clinical signs include reddening of the base of fins and gills, abdominal distension, and necrosis of all major organ systems (Fijan et al. 1970; Plumb 1971; Noga 1996). CCV is an enveloped double stranded DNA virus comprised of a 134 kb genome (Davison 1992). Vaccination against CCV has achieved a moderate level of protection with a subunit vaccine, a heat killed vaccine, live-attenuated vaccines, and DNA vaccines (Plumb 1973b; Noga and Hartman 1981; Awad et al. 1989; Kancharla and Hanson 1996; Zhang and Hanson 1995; Zhang and Hanson 1996; Vanderheijden et al. 1996; Nusbaum et al. 2002). Due to various difficulties with the efficacy and the economic feasibility of delivering these vaccines, further research is necessary to elucidate an efficacious CCV vaccine.

The development of genetic immunization provides a powerful tool for evaluating protective proteins from CCV by delivering only the antigenic protein to the host, instead of the whole virus. Vaccination with DNA has been extensively studied in mammalian systems, in both therapeutic and prophylactic capacities since 1990 (Wolff et al. 1990; Tang et al. 1992; Fynan et al. 1993; Ulmer et al. 1993), including substantial work with herpesviruses. Most
research in DNA vaccination against HSV-1 utilizes the gB protein, the major envelope protein, and significant protection against lethal challenge and neutralizing antibody has been demonstrated after DNA vaccination with plasmids encoding the gB gene (Kuklin et al. 1997; Nass et al. 1998; Yu et al. 1998; Suter et al. 1999; Nass et al. 2001; Talaat et al. 2001; Baghian et al. 2002; Inoue et al. 2002). Similar success in DNA vaccination has been achieved against HSV-2 using the membrane protein gD, where significant protection was reported and neutralizing antibody titers were elicited (Sin et al. 1998; Gebhard et al. 2000; Strasser et al. 2000; Flo et al. 2001; Higgins et al. 2001; Lee et al. 2002; Meseda et al. 2002; Lasaro et al. 2003). In experiments utilizing more than one gene or epitope against a pathogen in the HSV-2 system, the combination of the protective gD vaccine with the less protective gB vaccine was more efficacious than either singly (Lee et al. 2002). In various animal systems and other herpes viruses, different types of genes have been tested for efficacy by DNA vaccination, including immediate early genes and tegument genes. A hallmark of herpesviruses is the temporal regulation of gene products, and immediate early genes often upregulate or stimulate the expression of later genes. In the Varicella-zoster virus (VZV) system in guinea pigs, the immediate early (transcriptional regulatory protein) and major tegument protein IE62 (ORF 62) was tested as an immunogen expressed in a vaccinia virus vector and resulted in cell mediated immune responses (Sabella et al. 1993). In the pseudorabies virus system, moderate protection is elicited by DNA vaccination with the immediate early IE180 gene (Bu et al. 2003).

In fishes, DNA vaccinations encoding the glycoprotein gene of Infectious Hematopoietic Necrosis Virus (IHNV) and Viral Hemorrhagic Septicemia Virus (VHSV) are efficacious with nanograms of DNA in rainbow trout and salmon (Anderson et al. 1996; Heppell et al. 1998; Lorenzen et al. 1999; Corbeil et al. 1999; LaPatra et al. 2000; Lorenzen et al. 2000; Corbeil et al.
In channel catfish, Nusbaum et al. (2002) tested ORFs 6, 7, 8, 10, 51, 53, and 59 of the CCV genome with some success. When 4-8 g catfish were intramuscularly vaccinated with these ORFs in a 50 µg dose, significant protection and neutralizing antibody titers were elicited with vaccines encoding ORF 59, ORF 6, and a combination of the two.

In order to study immune responses to DNA vaccination, Mx gene expression was investigated by Kim et al. (2000), who proposed that the expression of a known interferon inducible protein (Mx) would indicate the production of an innate, nonspecific immune response to DNA vaccination in a fish host. In studies investigating a cross reactive phenomenon observed after DNA vaccination with IHNV or VHSV, the Mx gene expression profile of fish was examined to possibly explain the cross protection of glycoprotein encoding DNA vaccines against heterologous viruses early in the immune response as an innate, nonspecific, interferon-mediated phenomenon (Lorenzen et al. 2000; LaPatra et al. 2001; Collet et al. 2003; McLauchlan et al. 2003). Serum neutralizing titers are often used as an indicator of a specific immune response in DNA vaccination. In other aquatic disease studies, DNA vaccination of large fishes (juvenile or adult) results in the elicitation of neutralizing antibody, whereas fry and fingerlings do not generally produce neutralizing titers following DNA vaccination (Corbeil et al. 1999; LaPatra et al. 2000; McLauchlan et al. 2003).

In light of the success achieved with these two aquatic rhabdoviruses in the aquaculture industry and the work demonstrating efficacy in other herpesviruses, studies were initiated to evaluate DNA vaccination for CCV. In order to evaluate antigens appropriate for CCV DNA vaccines, genes were selected based on their predicted function and temporal expression profile (ORF59 [putative major envelope glycoprotein gene], ORF46, ORF6, ORF19 [three membrane
genes], ORF1, and ORF3 [immediate early genes]) as types of genes that may be immunogenic for CCV (Davison 1992; Davison and Davison 1995; Kucktas et al. 1998; Silverstein et al. 1998; Stingley and Gray 2000). As indicated above, the DNA vaccines encoding the membrane genes of HSV-1, HSV-2, and the aquatic rhabdoviruses (IHNV and VHSV) are the most protective. In addition, indications that immediate early genes may have some protective capacity early in the viral infection were reported by Sabella et al. (1993) and Bu et al. (2003). Therefore, membrane genes (ORFs 6, 7, 8, 10, 19, 46, 51, and 59) and immediate early genes (ORFs 1 and 3) of CCV were chosen for testing in this study. In order to evaluate innate and specific immune responses to DNA vaccination, Mx gene expression was investigated to indicate an interferon-mediated response, and serum neutralizing antibodies and DNA vaccine specific antibodies were investigated to indicate an adaptive immune response.

Materials and Methods

Plasmid Construction. CCV genes were amplified by polymerase chain reaction (PCR) from an existing cosmid library (Hanson, 1990) with a GeneAmp® PCR System 9700 (Applied Biosystems). Each gene was amplified using unique primers that incorporated a Kozak sequence, ACCATGG, around the start codon at the 5’ end of the gene and 3’ modified to eliminate the stop codon (Kozak 1986). The elimination of the stop codon allowed read-through and translation of a V5 fusion protein on the 3’ end of the protein for subsequent analysis. Reaction mixtures consisted of 0.5 µM primer concentration, 200 mM dNTPs, 1 X Amplitaq Buffer I (containing Mg++,), and Amplitaq DNA polymerase (PerkinElmer, Boston, MA). Reaction conditions for amplification were 30 seconds at 94°C, 30 seconds at 53°C, and 1 minute at 72°C, cycled 30 times with a final 7 minute hold at 72°C. Five genes were amplified utilizing the primers listed in Table 2.1. ORFs 1 and 6 were amplified from cosmid 389 and
ORFs 3 and 19 were amplified from cosmid 395. ORF59 was amplified from cosmid 388. ORFs 1, 6, and 19 were amplified with one PCR reaction, whereas ORFs 3 and 59 were amplified with nested PCR due to difficulties with primer self binding and nonspecific amplification of some primer pairs. The resultant PCR product was then cloned into the plasmid pcDNA3.1/V5/His-TOPO (Invitrogen, Carlsbad, CA). Forward or reverse orientation of the gene was confirmed with differential restriction enzyme digestion and sequence analysis (Table 2.2). Clones in the forward, protein-encoding orientation were called pORF1, pORF3, pORF6, pORF19, and pORF59. Clones in reverse, non-protein encoding orientation were called pORF1rev, pORF3rev, pORF6rev, pORF19rev, and pORF59rev. Each plasmid was chemically transformed into *Escherichia coli* Top 10 and stored at -70°C. All plasmids were sequenced by the LSU Gene Lab using the Big Dye Cycle Terminator sequencing kit and read on an ABI Prism 327 Sequence analyzer. Sequence results were paired against template plasmids created with the published CCV sequence (accession # M75136) in GCG (Wisconsin) in order to determine orientation of the inserted gene and fidelity to the published sequence. Plasmids were isolated with Qiagen mini, midi, maxi, or gigaprep kits (Qiagen Inc., Valencia, CA). DNA was resuspended in concentrations of 1 µg/µl (50 µg/50 µl), 0.5 µg/µl (25 µg/50 µl), and 0.1 µg/µl (5 µg/50 µl) of TE for vaccination of fingerlings. Plasmids pORF46, pORF7, pORF8, pORF10, pORF39, and pORF51 were constructed and confirmed in the same manner as the above plasmids by Karen Plant and given as a gift for use in some experiments.

**Recombinant Protein Expression.** DNA concentration was determined on a Beckman DU-600 spectrophotometer. Each plasmid was then transfected into the COS-7 cell line and the Epithelioma Papulosum Cyprini (EPC) cell line with cationic liposome transfection
Table 2.1. Primer names and sequences used to amplify CCV genes from cosmids. Underlined sequences represent the Kozak sequence.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Primer</th>
<th>Size of Product</th>
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<tr>
<td>ORF1</td>
<td>5'ORF1</td>
<td>5'CGATCTGACCATGGACGG3</td>
<td></td>
</tr>
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<td>5'TACAACGACCCGAATAAG3’</td>
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<tr>
<td></td>
<td>3'ORF3nest</td>
<td>5'CCCAGAAAAATAGAGTCAAGAG3’</td>
<td>1789</td>
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<tr>
<td></td>
<td>5'ORF3</td>
<td>5'CTCGATCACCATGGCATTCT3’</td>
<td></td>
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<td></td>
<td>3'ORF3</td>
<td>5'GTTGAGGAGCGACGCAGTCG3’</td>
<td>908bp</td>
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<tr>
<td>ORF6</td>
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<td>GCACCGGACCATGGACTCTC3’</td>
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<tr>
<td></td>
<td>3'ORF6-P2</td>
<td>5'GACCCGGATCTCCGTTCTAG3’</td>
<td>417bp</td>
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<tr>
<td>ORF19</td>
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<td>5'ACCATGACCATGGAAACAAATG3’</td>
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<tr>
<td></td>
<td>3'ORF19-P2</td>
<td>5'CTCGTTCGCGATAGTGTAC3’</td>
<td>614bp</td>
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<tr>
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<td>forw388</td>
<td>5'TATTTGACACCCGTCGACC3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rev388</td>
<td>5'CGAGACGTGTATAACGAGAAG3’</td>
<td>1138bp</td>
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<tr>
<td></td>
<td>394NheI</td>
<td>5'ACTTGAGAGCTAGCAGACCATGGTCGGAAAAGGGTCTCCCC3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>59nostop</td>
<td>5'CGCCCGGCGAGGTGTGAGTTCCACTTCTCATACCG3’</td>
<td>1038bp</td>
</tr>
</tbody>
</table>
(Lipofectamine™ 2000, Invitrogen, Carlsbad, CA) according to the manufacturers recommendations. Briefly, three tubes of each plasmid was adjusted to a concentration of 1 µg DNA/50 µl with Dulbecco’s Minimal Essential Media supplemented with 15 mM HEPES and NaHCO₃ (DMEM SF). In a separate microfuge tube, 1.5 µl Lipofectamine was added to 50 µl DMEM SF and left at room temperature for no more than 5 minutes, after which each tube containing the DNA complex was added to one of the three tubes of Lipofectamine and allowed to incubate at room temperature for 20 minutes. After the 20 minute incubation, the 100 µl DNA/Lipofectamine complex was added to one well of a 24-well tissue culture plate containing confluent monolayers of either EPC or COS-7 cells that had been washed once with DMEM-SF. Each plasmid was allowed to express recombinant protein for 24 hours in COS-7 cells and 48 hours in EPC cells in a 37°C or 28°C 0.05% CO₂ incubator. Transfected cell lysate was harvested according to a protocol by Sambrook and Russell (2001). Briefly, the DNA/Lipofectamine complex was removed from each well and washed three times with 1 ml cold 0.9% phosphate buffered saline pH 7.4 without calcium or magnesium (PBS-CMF). The cells were then mechanically scraped off of the well surface and pooled into a microfuge tube (one set of triplicate wells were pooled in the COS-7 line and three sets of triplicate wells were pooled in the EPC line), and stored briefly on ice. Cells were then pelleted at 12,000 X g in a tabletop microcentrifuge (Eppendorf 5415D) for 30 seconds, washed with 1 ml PBS-CMF, and pelleted a second time. The supernatant was removed and cell pellets were stored at -20°C until use. Prior to use, cell pellets were resuspended in 100 µl 0.25 M Tris-HCl pH 7.8, and subjected to 3 freeze thaw cycles at 37°C and -70°C to lyse the cells (Sambrook and Russell 2001). The cell lysate was then centrifuged at 12,000 X g for 5 minutes to pellet particulates and the supernatant was stored at -20°C until use.
Separation and Immunodetection of Recombinant Proteins. Lysed cell supernatants were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Gels were run on a Mini-PROTEAN® II Dual Slab Cell according to the manufacturer’s protocol (Bio-Rad, Hercules, CA). Proteins were separated by discontinuous 0.75 mm SDS-PAGE made up of a 4% acrylimide, 0.125 M tris pH 6.8 stacking gel and a 12% acrylimide, 0.375 M tris pH 8.8 separating gel. Proteins were separated with a constant voltage of 200 V for 45 minutes. Following separation, gels were equilibrated in 25 mM tris, 192 mM glycine, 20% methanol pH 8.3 for 15 minutes prior to transfer. After separation, proteins were transferred to an Optitran 0.45 µm nitrocellulose membrane (Schleicher and Shuell, Keene, NH) with a Mini Trans-Blot® Electrophoretic Transfer Cell according to the manufacturer’s protocol (Bio-Rad, Hercules, CA). Following the 1 hour transfer at 100 V, blots were dried overnight in the dark and stored at room temperature until use. For some gels, molecular weight marker lanes (broad range marker, Bio-Rad) were removed prior to immunodetection and stained with colloidal gold total protein stain according to the manufacturer’s protocol (Bio-Rad). For blots in which the prestained molecular weight marker (Bio-Rad) was used, the marker lane was not removed. Blots were immunodetected according to a protocol provided with the pcDNA3.1/V5/His-TOPO instruction manual (Invitrogen, Carlsbad, CA). Briefly, each blot was blocked for 1 hour in 5% nonfat milk (w/v) in 100 ml PBS-0.5% Tween (PBST) and rinsed 3 times for 10 minutes in PBST. Blots were immunodetected with anti-V5 mouse monoclonal antibody (Invitrogen, Carlsbad, CA) conjugated to horse-radish peroxidase (anti-V5-HRPO) in PBST at a 1/5000 dilution in blocker for one hour. Blots were then washed 5 times for 10 minutes in PBST. Blots were visualized by autoradiography with the Pierce SuperSignal chemiluminescent detection kit (Pierce, Rockford, IL) as per the manufacturer’s instructions.
Expected molecular weight proteins were visualized as compared to a broad range SDS-PAGE prestained ladder (Bio-Rad, Hercules, CA).

**Channel Catfish.** Channel catfish egg masses were obtained from a commercial producer with no history of CCV outbreaks. The eggs were disinfected with 100 ppm free iodine and hatched in closed recirculating systems in the SPF laboratory at the School of Veterinary Medicine. Fish were reared on commercial catfish diets fed at 3% of their body weight per day until used for the challenge experiments. Prior to vaccination, fry were tested and certified to be free of CCV with the method outlined by Baek and Boyle (1996).

**Vaccination of Fingerlings –Experiment 1 Dose Response.** Preliminary studies using 5 µg and 1 µg doses of pORF59 failed to protect channel catfish fingerlings from wild-type CCV challenge (data not shown). Consequently experiments were conducted on a range of doses for all of the DNA vaccine constructs. Because of the number of plasmids that were constructed, however, it was not possible to do a dose titration in a single experiment with the number of tanks available, so two experiments were conducted consecutively. The first experiment dealt with treatments of each plasmid vaccine pORF6, pORF46, and pORF59 in 3 doses each. The second dealt with treatments of pORF1, pORF3, and pORF19 given in the same 3 doses.

Briefly, for each experiment, triplicate tanks of 25 average weight 1 g fingerlings were anesthetized with 100 mg/l tricane methyl sulfonate (MS222) and individually injected in the dorsal epaxial muscle with 50 µl of each vaccine concentration at 30°C. Doses consisted of 50 µg (1 µg/µl), 25 µg (0.5 µg/µl), and 5 µg (0.1 µg/µl) of plasmid DNA delivered in a volume of 50 µl TE of each treatment. One set of triplicate tanks was injected with 50 µl TE and another set was vaccinated with 3 doses of pcDNA (50 µg, 25 µg, and 5 µg) as negative controls in both experiments. In parallel to the fingerling vaccination, ten juvenile average weight 13 g channel
catfish were injected in the dorsal epaxial muscle with the same vaccines as the fingerlings in order to provide enough serum for antibody studies.

**Challenge of Vaccinated Fingerlings.** Five weeks post-vaccination fish were challenged with $1 \times 10^6$ TCID$_{50}$/ml of wild-type CCV for 8 hours at 30°C. Morbid and moribund fish were collected twice daily and 10% of the dead fish were necropsied. Liver, viscera, and/or head kidney were removed from dead fish, homogenized in sterile Hank’s balanced salt solution (HBSS) with 2% FBS, centrifuged for 3 minutes at 2300 X g, and the supernatants were removed. Fifty microliters of the supernatant were added in duplicate to 24 well tissue culture plates with confluent monolayers of CCO cells to confirm the viral diagnosis by the presence of CPE. Mortalities were normalized by arcsine transformation and significant difference was determined at a level of 0.05% with the analysis of variance test (ANOVA).

**Mx Gene Expression - RNA Extraction.** For both dose response experiments testing pORFs 6, 46, and 59, and pORFs 1, 3, and 19, samples were taken to measure Mx gene expression in the livers of vaccinated fish. On the day of vaccination (day 0) five fish were euthanized with an overdose of MS222. These fish were necropsied, the livers were removed, snap frozen in liquid nitrogen, and stored at -70°C. At days 1, 3, and 35 post vaccination, one fish from each tank per treatment (three fish per treatment) was similarly euthanized and the livers removed, frozen, and stored at -70°C for later RNA extraction. Frozen livers were thawed, weighed, adjusted to 50 mg, and RNA was extracted with Tri-reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s recommendations. Briefly, one milliliter of Tri-reagent was added to each 50 mg of catfish liver and homogenized with sterile, RNase-free pestles. The homogenates were pelleted in an Eppendorf 5415 R microcentrifuge at 12000 X gravity ($g$) for 10 minutes at 4°C, and supernatants were removed to new RNase-free
microcentrifuge tubes and incubated at room temperature for 5 minutes. Two hundred microliters of chloroform were added to the supernatants, which were then shaken for 15 seconds, incubated at room temperature for 10 minutes, and centrifuged at 12000 X g for 15 minutes at 4ºC. The clear upper aqueous phase containing RNA was transferred to new RNase-free microcentrifuge tubes, precipitated with 50 µl of isopropanol, washed once with 500 µl of isopropanol, washed once with 75% RNase-free ethanol, and then dried at room temperature for 10 minutes 4ºC. Pellets were resuspended in 50 µl of RNase-free molecular biology grade water and incubated at 60ºC to aid dissolution. The concentration of RNA recovered was determined by spectrophotometer and RNA was stored at -70ºC.

**Mx Gene Expression - RT-PCR.** Reverse transcription of Mx RNA into cDNA was performed by the method described by Plant et al. (2003). Briefly, 10 µg of RNA was mixed with 38 µl RNase-free molecular biology grade water with 0.5 µM primer MxcDNA2 (5’TCAGCCAGGTTGGGAATGGTG3’) and incubated for 5 minutes at 65ºC. A master mix containing 25 mM each dNTP (Promega, Madison, WI), reverse transcriptase buffer (Stratagene, LaJolla, CA), 1 unit RNase block and 1 unit reverse transcriptase (Stratagene, LaJolla, CA) was added to each tube at room temperature and then heated at 42ºC for one hour, at 90ºC for 5 minutes, and cooled to 4ºC to create Mx-specific cDNA. The cDNA was stored at -20ºC and Mx PCR was performed in a reaction mixture containing 1 µl of cDNA, 200 mM dNTPs, 0.2 µM Mx-specific forward and reverse primers, 0.2 units Taq polymerase, and 5 µl 1X Taq buffer I (PerkinElmer, Boston, MA). The primers used were Mx-P1 (5’GAGATCCAAAATCCAGCAGA3’) and Mx-P2 (5’ATGATCTCGTTATGGATGATG3’). Reaction mixtures were held at 94ºC for 2 minutes, and then cycled 40 times at 94ºC for 30
seconds, 52°C for 30 seconds, and 72°C for 30 seconds, followed by a 10 minute extension period at 72°C. The 400 bp product was visualized on a 1.5% agarose gel by electrophoresis.

**Serum Neutralization.** For the first experiment, with pORF6, pORF46, and pORF59, a parallel experiment was conducted in which ten juvenile average weight 13 g channel catfish were vaccinated with each treatment of vaccine to allow collection of serum for neutralization and ELISA antibody studies. Five weeks post-vaccination, fish were euthanized with an overdose of MS222 and serum was collected and stored at -70°C until assayed. For the second experiment testing pORF1, pORF3, and pORF19, fish were collected directly from the treatment tanks and serum was collected five weeks post-vaccination.

Serum neutralization titers for both experiments were determined with the constant virus-varying serum method of Plumb and Bowser (1983). Briefly, sera were thawed and diluted 1/5 in duplicate tubes in MEM with 25mM HEPES, 2%FBS, and pen/strep (MEM2%p/s). The diluted serum was heat-inactivated at 45°C for 30 minutes and a 1:2 serial dilution was made in a 96-well tissue culture plate using 100 µl volumes. One hundred microliters of 100 TCID<sub>50</sub>/ml CCV was added to all wells and incubated at room temperature for 1 hour, after which the serum/virus mixtures were transferred to 96-well flat-bottomed microtiter plates with confluent monolayers of CCO cells. Titers were read after seven days as the inverse of the dilution at which neither of the duplicate wells had detectable CPE.

**ELISA.** Enzyme-linked immunosorbant assays (ELISA) were performed with Dynex Immulon 1 96-well ELISA plates. Plates were coated with 100 µl per well of 0.1% poly-L-lysine (Sigma-Aldrich, St. Louis, MO). Plates were washed twice with 1 X PBS pH7.4 with 0.05% Tween (wash buffer), and were then coated overnight at 4°C with 5 µg viral pellet resuspension in carbonate coating buffer (0.05 M Na<sub>2</sub>CO<sub>3</sub> and 0.05 M NaHCO<sub>3</sub> pH 9.6).
Preparation of Coating Antigen. Antigen for coating ELISA plates was prepared from infected CCO cells after infection related lysis of the cell sheets. Cellular debris was removed by centrifugation in a Marathon 21/BK centrifuge at 3500 X g for 5 minutes and then pelleted by centrifugation for 3 hours at 48,000 X g at 4°C in a Beckman J2-21 ultracentrifuge in a JA-20 rotor. Viral pellets were dried for 10 minutes at room temperature and then resuspended in 100 µl sterile 1 X PBS pH 7.4 overnight at 4°C. Resuspended antigen was pooled and stored at -20°C. Antigen concentration of the resuspended pellet was determined by the Bradford method using the Biorad Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol and by viral titration methods as described previously. This antigen suspension resuspended to a concentration of approximately 5 µg viral pellet/100 µl in carbonate buffer and used at that concentration per well as a coating antigen in all ELISAs.

ELISA Protocol. The ELISA plates were coated overnight at 4°C with 5 µg of the antigen suspension per well in 100 µl carbonate coating buffer. Plates were washed 3 times with wash buffer, blocked for 1 hour at room temperature with 150 µl per well of a 10% dilution of goat serum in wash buffer, and washed 3 more times with wash buffer. Individual fish sera were applied to duplicate wells of the plate in a 1:2 dilution in wash buffer, serially diluted 1:2 down the plate, incubated for 2 hours at room temperature, and then washed 3 times with wash buffer. A positive control consisting of CCV antiserum collected from survivors of an infection was used as a positive control on each plate to ensure consistency between plates. A negative control of serum from catfish injected with saline only was also used to ensure consistency. The secondary antibody, a monoclonal mouse anti-catfish IgM antibody (mab9E1), was applied in a 1:4.5 dilution, incubated for 1 hour at room temperature, and washed 3 times with wash buffer. The tertiary antibody was a goat-anti-mouse antibody conjugated to horseradish-peroxidase.
(Sigma-Aldrich, St. Louis, MO), applied in a 1/16,000 dilution in wash buffer and incubated for 1 hour at room temperature. The plate was then washed 5 times with wash buffer and ABTS (2, 2'-azino-di(3-ethylbenzthiazoline-6-sulfonate)) Microwell Peroxidase Substrate System 2-C substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added according to the manufacturer’s instructions. The plates were incubated 10 minutes at room temperature and read at 405nm on a Dynex MRXTC Revelation ELISA plate with Revelation 4.02 software. Average ELISA titers were statistically analyzed by ANOVA using the mean of titers for each group of 10 fish per treatment.

**Vaccination of Fingerlings with Multiple Groups of Plasmids – Experiment 2.** An experiment was conducted to evaluate the possible efficacy of vaccinating with multiple plasmids. Treatment groups consisted of a combination of all 11 plasmids (pORF1, pORF3, pORF6, pORF7, pORF8, pORF10, pORF19, pORF39, pORF46, pORF51, and pORF59), the plasmids donated by Karen Plant, called KP6, (pORF7, pORF8, pORF10, pORF39, pORF46, and pORF51), the plasmids created by Heather Harbottle, called HH5, (pORF1, pORF3, pORF6, pORF19, and pORF59), three plasmids (pORF6, pORF46, and pORF59), the vector-only control pcDNA, and the negative control TE. Each plasmid was adjusted to a concentration of 1 µg in a final volume of 100 µl, which means that the all 11 treatment received a total of 11 µg/100 µl, the KP6 treatment received 6 µg/100 µl, the HH5 treatment received 5 µg/100 µl, the 3 plasmid treatment received 3 µg/100 µl, the pcDNA treatment received 1 µg/100 µl, and the TE treatment received only 100µl of TE. Detection of Mx gene expression and the serum neutralization assay were conducted as previously described. Fish were challenged after 5 weeks with 1 X 10⁶ TCID₅₀/ml of wild-type virus.
Results

Plasmid Construction. Five genes were successfully amplified via PCR from an existing cosmid library (Hanson 1990). To confirm ligation and determine orientation of insertion, whole plasmids were digested with various restriction enzymes and visualized on a 0.6% agarose gel. Plasmid size and restriction digest fragments are presented in Table 2.2 and Figure 2.1 and in all cases confirmed proper size and orientation. Sequencing data confirmed amplification of the appropriate gene and its orientation in the plasmid as either forward, protein-encoding or reverse, non-protein encoding.

Recombinant Protein Expression. Transfected COS-7 and EPC cell lysates were separated by discontinuous SDS-PAGE as described previously, transferred to nitrocellulose, and immunodetected with anti-V5-HRPO and visualized by autoradiography using West-Fempto SuperSignal Chemiluminescent substrate (PierceBiotechnology, Rockford, IL). Protein of the appropriate molecular weight was detected for each ORF in the forward orientation and no proteins were detected for any of the ORFs in the reverse orientation (Table 2.2 and Figure 2.2).

DNA Vaccination Results – Experiment 1 Dose Response. In both experiments testing ORFs 1, 3, 6, 19, 46, and 59 at three different vaccination doses, none of the mortalities of any of the treatments were significantly different from the negative control (Table 2.3, Figures 2.3 and 2.4). Fish vaccinated with pORF46 at a dose of 25 µg experienced the lowest mortality at 40 +/- 10.4%, but was not significantly different from the negative control.

Reverse transcription and Mx PCR were performed on liver samples taken at days 1, 3, and 35 post-vaccination. A positive result was visualized as a 400 bp product. For the first experiment with treatments of pORF6, pORF46, and pORF59, in each of the triplicate samples taken at day 1, 3, and 35 of all doses, no Mx transcription was detected (data not shown). For the
Table 2.2. Differential restriction enzyme digest of cloned CCV genes to distinguish between forward and reverse orientation of insert. Predicted molecular weight proteins based on mass spectrometry (Davison and Davison 1995).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Restriction enzyme</th>
<th>Plasmid size (bp)</th>
<th>Forward orientation</th>
<th>Reverse orientation</th>
<th>Predicted protein (kDal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1</td>
<td>EcoRI, HindIII</td>
<td>8088</td>
<td>978bp, 7110bp</td>
<td>1700bp, 6388bp</td>
<td>94 kDal</td>
</tr>
<tr>
<td>ORF3</td>
<td>SmaI</td>
<td>6431</td>
<td>1290bp, 5141bp</td>
<td>1930bp, 4501bp</td>
<td>33.3 kDal</td>
</tr>
<tr>
<td>ORF6</td>
<td>KpnI, BamHI</td>
<td>5948</td>
<td>442bp, 5506bp</td>
<td>369bp, 5579bp</td>
<td>20 kDal</td>
</tr>
<tr>
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<td>SmaI</td>
<td>6144</td>
<td>1245bp, 4899bp</td>
<td>1806bp, 4338bp</td>
<td>22.5 kDal</td>
</tr>
<tr>
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<td>SmaI</td>
<td>6720</td>
<td>1220bp, 5500bp</td>
<td>2217bp, 4503bp</td>
<td>38 kDal</td>
</tr>
</tbody>
</table>
Figure 2.1. Agarose gel separation of PCR isolated CCV genes and differential restriction enzyme digest to confirm forward or reverse orientation of the cloned gene. (A) Agarose gel of the nested PCR products of ORF3 and the PCR product of ORF1. Lane 1 is a 1 kb size marker. Lane 2 is the first part of the nest amplifying ORF3 at 1789 bp. Lane 3 is the second part of the nest of ORF3 at 908 bp. Lane 4 is the PCR amplified ORF1 at 2588 bp. (B) Agarose gel of the differential digestion of pORF3 and pORF3rev with SmaI. Lane 1 is the 1Kb size marker. Lane 2 is pORF3rev with fragments at 1930 bp and 4501 bp. Lane 3 is pORF3 with fragments at 1290 bp and 5141 bp. (C) Agarose gel of restriction digest of pORF1 and pORF1rev with EcoRI and HindIII. Lane 1 is the 1 kb size marker. Lane 2 represents pORF1 as evidenced by a fragment at 978 bp and 7110 bp. Lane 3 represents pORF1rev as evidenced by a fragment at 1700 bp and 6388 bp. (D) Agarose gel of PCR amplified ORF6. Lane 1 is a PCR size marker. Lane 3 represents the PCR product of ORF6 at 417 bp. (E) Agarose gel of restriction digest of pORF6 and pORF6rev with KpnI and BamHI. Lane 1 is the PCR size marker. Lane 2 represents pORF6 as indicated by a fragment at 442 bp and 5506 bp. Lane 3 represents pORF6rev as indicated by a fragment at 369 bp and 5579 bp. (F) Agarose gel of ORF19 PCR product. Lane 1 is the PCR size marker. Lane 3 is the PCR product of ORF19 at 614 bp. (G) Agarose gel of the restriction digestion of pORF19 and pORF19rev with Smal. Lane 1 is the 1 kb size marker. Lane 2 represents pORF19 as evidenced by a fragment at 1245 bp and 4899 bp. Lane 5 represents pORF19 as evidenced by a fragment at 1806 bp and 4338 bp. (H) Agarose gel of nested PCR amplification of ORF59. Lane 1. 1 kb ladder. Lane 2. First part of the nest from cosmid 394. Lane 3. Unique amplification of ORF59 from the nested product at 1038 bp. (I) Agarose gel of ORF59 inserted into pcDNA3.1/V5/His-TOPO as evidenced by a 1 kb shift and differential Smal digest to determine orientation of insertion. Lane 1. 1 kb ladder. Lane 2. pcDNA3.1/V5/His-TOPO. Lane 3. pcDNA3.1/V5/His-TOPO with ORF59 inserted in the T/A cloning site. Lane 4. pcDNA3.1/V5/His-TOPO. Lane 5. pcDNA3.1/V5/His-TOPO linearized with Smal. Lane 6. pORF59 cut with Smal showing a band at 1220 bp, identifying the forward orientation. Lane 7. pORF59rev cut with Smal showing a band at 2217 bp, identifying the reverse orientation.
Figure 2.2. Autoradiographs of cell lysates confirming the correct predicted molecular weight protein being made by each DNA vaccine. A) Autoradiograph of pORF59, pcDNA3.1/V5/His-TOPO/LacZ, and pORF59rev in COS-7 cell lysate. Lane 1 and 2 are immunodetections of pORF59 showing the predicted molecular weight protein of 43 kDal. Lanes 3 and 4 are of pcDNA3.1/V5/His-TOPO/LacZ showing the predicted molecular weight protein of 121 kDal. B) Autoradiograph of pORF1, pORF1rev, pORF6, and pORF6rev in COS-7 cell lysate. Lane 1 is an immunodetection of pORF1, showing the predicted molecular weight protein of 99 kDal. Lanes 2 and 3 are the immunodetections of pORF1rev and pORF6rev, showing no protein expression as expected. Lanes 4 and 5 are immunodetections of pORF6, showing a very dark band at the predicted molecular weight of 20 kDal. Lane 6 is a prestained broad range molecular weight marker. C) Autoradiograph of immunodetection of pORF3 from COS-7 cell lysate. Lane 1 is the immunodetection of pORF3 showing the predicted molecular weight protein at 38.3 kDal. Lane 2 is a total protein stained broad range SDS-PAGE molecular weight marker. D) Autoradiograph of immunodetection of pORF19 from COS-7 cell lysate. Lane 1 is the immunodetection of pORF19 showing the predicted molecular weight protein at 27.5 kDal. Lane 2 is the prestained broad range molecular weight marker. E) Immunodetection of EPC transfected cell lysates of pORF59 and pORF6. Lane 1 represents pORF6 protein product at approximately 20 kDal. Lane 2 represents pORF59 protein product at approximately 43 kDal. Lane 3 is the prestained broad range ladder. F) Immunodetection of EPC transfected cell lysates of pORF46 and pORF19. Lane 1 represents the pORF46 protein product at approximately 133 kDal. Lane 2 represents the pORF19 protein product at 27.5 kDal. G) Immunodetection of EPC lysate of pORF3 and pORF1 transfected cell lysate. pORF1 predicted molecular weight protein is at approximately 99 kDal (Lane 1). pORF3 predicted molecular weight protein is at approximately 33 kDal (Lane 2). Lane 3 is the broad range ladder.
Table 2.3. Experiment 1. Mean triplicate percent mortalities after challenge with 1 X $10^6$TCID$_{50}$/ml of wild-type CCV 5 weeks post vaccination with 3 doses (50 µg, 25 µg, and 5 µg) of plasmids pORF59, pORF46, pORF6, and pcDNA and pORF1, pORF3, pORF19, and pcDNA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
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<tr>
<td>pORF59 50 µg</td>
<td>63.33 +/- 11.6</td>
</tr>
<tr>
<td>pORF59 25 µg</td>
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<tr>
<td>pORF59 5 µg</td>
<td>88.33 +/- 15.8</td>
</tr>
<tr>
<td>pORF46 50 µg</td>
<td>53.33 +/- 10.9</td>
</tr>
<tr>
<td>pORF46 25 µg</td>
<td>40 +/- 10.4</td>
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<tr>
<td>pORF46 5 µg</td>
<td>66.67 +/- 3.3</td>
</tr>
<tr>
<td>pORF6 50 µg</td>
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</tr>
<tr>
<td>pORF6 25 µg</td>
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</tr>
<tr>
<td>pORF6 5 µg</td>
<td>50 +/- 8.6</td>
</tr>
<tr>
<td>pcDNA 50 µg</td>
<td>81.67 +/- 4.4</td>
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<tr>
<td>pcDNA 25 µg</td>
<td>60 +/- 7.6</td>
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<td>pcDNA 5 µg</td>
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<td>Experiment 2</td>
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<tr>
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<td>pORF3 5 µg</td>
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<tr>
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<tr>
<td>pORF19 5 µg</td>
<td>78.79 +/- 5.46</td>
</tr>
<tr>
<td>pcDNA 50 µg</td>
<td>80.32 +/- 8.28</td>
</tr>
<tr>
<td>pcDNA 25 µg</td>
<td>84.85 +/- 6.6</td>
</tr>
<tr>
<td>pcDNA 5 µg</td>
<td>78.79 +/- 4</td>
</tr>
<tr>
<td>TE</td>
<td>71.21 +/- 6.6</td>
</tr>
</tbody>
</table>
Figure 2.3. Experiment 1. Mean triplicate percent mortalities for treatments pORF6, pORF46, pORF59, and pcDNA vaccinated in 3 doses and challenged with $1 \times 10^6$ TCID$_{50}$ CCV/ml tank water. There was no significant difference between any of the treatment means.
Figure 2.4. Experiment 1. Mean triplicate percent mortalities from fish vaccinated with pORF1, pORF3, pORF19, and pcDNA in 3 doses and challenged with $1 \times 10^6$ TCID$_{50}$ CCV/ml tank water five weeks post vaccination. There were no significant differences between treatment means.
% Mortality

Treatment

pORF1 50ug
pORF1 25ug
pORF1 5ug
pORF3 50ug
pORF3 25ug
pORF3 5ug
pORF3 19 50ug
pORF3 19 25ug
pcDNA 50ug
pcDNA 25ug
pcDNA 5ug
TE
second experiment with treatments of pORF1, pORF3, and pORF19, Mx gene transcription was variable, but was identified in almost all of the treatments with the most numerous fish positive at day three post-vaccination (Table 2.4).

Serum neutralizing titers observed of all the treatments and doses were very low to negative. Table 2.5 shows the titers of neutralization detected for 5 fish per treatment as the reciprocal of the dilution at which no CPE was visible in either of the duplicate wells tested. All of the pcDNA treatment doses were negative. Very low titers (below 10) were detected in every treatment but pORF46 at a 25 µg dose, where an average titer of 10 was observed. This titer is not different from negative controls and cannot be considered significant. In the second experiment, mostly negative titers were observed (Table 2.6). The only treatment with a non-negative neutralization titer was pORF19 at the 5 µg dose, where the average titer was 3.3. However, these mean titer values were not significantly different from the negative control values.

**ELISA Results.** Semi-purified CCV was used as the coating antigen in an indirect sandwich ELISA to test the sera from 10 individual fish per dose per treatment for antibody elicited by DNA vaccination for Experiment 1, the dose response study. An ANOVA was performed comparing treatment versus dose (10 fish sampled per dose were averaged) and treatments versus treatments (all 30 fish sampled within a treatment were averaged). After analysis, no significant differences were found between doses within a treatment. However, significant differences were found when treatments irrespective of dose were compared (Figure 2.5). Significant differences were found between average optical densities (OD) of sera from pORF46 vaccinated fish and pORF3 vaccinated fish from the negative control, pcDNA. pORF46 treatment at an average OD of 0.0656 +/- 0.005 was significantly different from
Table 2.4. Mx gene expression at days 1, 3, and 35 post vaccination of three fish per treatment in the second experiment with treatments of pORF1, pORF3, pORF19, and pcDNA. Five prevaccinate fish were sampled at day 0. Fish of an average weight of 1 g were vaccinated with treatments pORF1, pORF3, pORF19, and pcDNA in doses of 50 µg, 25 µg, and 5 µg. Three fish per treatment were sampled at days 1, 3, and 35 and total RNA was extracted and subjected to Mx-specific RT-PCR. Results reflect positive fish out of three tested by the presence of a 400 bp band after Mx PCR.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevaccinate</td>
<td>0/5</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>TE</td>
<td>0/3</td>
<td>0/3</td>
<td>2/3</td>
</tr>
<tr>
<td>pcDNA 50 µg</td>
<td>1/3</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>pcDNA 25 µg</td>
<td>0/3</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>pcDNA 5 µg</td>
<td>0/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>pORF1 50 µg</td>
<td>1/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>pORF1 25 µg</td>
<td>0/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>pORF1 5 µg</td>
<td>2/3</td>
<td>1/3</td>
<td>2/3</td>
</tr>
<tr>
<td>pORF3 50 µg</td>
<td>2/3</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>pORF3 25 µg</td>
<td>0/3</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>pORF3 5 µg</td>
<td>0/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>pORF19 50 µg</td>
<td>0/3</td>
<td>1/3</td>
<td>3/3</td>
</tr>
<tr>
<td>pORF19 25 µg</td>
<td>0/3</td>
<td>3/3</td>
<td>1/3</td>
</tr>
<tr>
<td>pORF19 5 µg</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
</tr>
</tbody>
</table>
Table 2.5. Serum neutralizing titers of five fish tested per treatment. Five fish were vaccinated with three doses (50 µg, 25 µg, and 5 µg) of treatments pORF59 (p59), pORF46 (p46), pORF6 (p6), and pcDNA. At 5 weeks post vaccination, vaccinated fish were terminally bled and subjected to serum neutralization tests according to the constant virus/varying serum method. All treatments but the positive control CCV antiserum showed very low to negative neutralizing activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fish 1</th>
<th>Fish 2</th>
<th>Fish 3</th>
<th>Fish 4</th>
<th>Fish 5</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA 50 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pcDNA 25 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pcDNA 5 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>p59 50 µg</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p59 25 µg</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>p59 5 µg</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>p46 50 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p46 25 µg</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>p46 5 µg</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>0</td>
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<td>p6 25 µg</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>p6 5 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCV antiserum</td>
<td>160</td>
<td>320</td>
<td>80</td>
<td>640</td>
<td>160</td>
<td>272</td>
</tr>
<tr>
<td>TE</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2.6. Serum neutralizing titers of three fish tested per treatment. Three fish were vaccinated with three doses (50 µg, 25 µg, and 5 µg) of treatments pORF1, pORF3, pORF19, and pcDNA. At five weeks post vaccination, vaccinated fish were terminally bled and subjected to serum neutralization tests according to the constant virus/varying serum method. Neutralizing titers were defined as the reciprocal of the dilution at which no CPE was present in either of the duplicate test wells. All treatments but the positive control CCV antiserum showed very low to negative neutralizing activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fish 1</th>
<th>Fish 2</th>
<th>Fish 3</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA 50 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pcDNA 25 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pcDNA 5 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pORF1 50 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pORF1 25 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pORF1 5 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pORF3 50 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pORF3 25 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pORF3 5 µg</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>pORF19 50 µg</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>pORF19 25 µg</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pORF19 5 µg</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>3.3</td>
</tr>
<tr>
<td>TE</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 2.5. ELISA results of pORF59, pORF46, pORF6, and pcDNA comparing mean OD per treatment, regardless of dose. The OD at 405 nm of the lowest dilution of each fish in duplicate was averaged as well as all fish within a treatment (n=30). As analyzed by ANOVA, pORF46 was significantly different from the negative control pcDNA. None of the other treatments, although a low level of response is evident, were significantly different from the pcDNA treatment. All treatments showed higher OD readings than the TE and saline treatments. The positive control CCV antiserum was used as a positive test control.
negative control pcDNA treatment at an average OD of 0.026 +/-0.0029. The pORF3 treatment had an average OD of 0.0494 +/- 0.008, which was significantly different from negative control pcDNA treatment at an average OD of 0.0167 +/- 0.004 (Figure 2.6).

**DNA vaccination – Multiple groups.** For the third experiment multiple group treatment, mortalities were over 50% in all treatments at any dose (Figure 2.7 and Table 2.7). Mean mortalities were not significantly different from negative control treatments, pcDNA and TE.

Mx gene transcription was identified in almost all of the treatments (Table 2.8). No Mx gene expression was detected at day 0. In contrast to single DNA vaccination experiments, Mx gene expression was detected at all sampling points and in all treatments, even the vector only control, indicating an innate immune response to DNA vaccination. None of the treatments were significantly different from one another, as Mx gene expression was elicited by the negative control pcDNA as well as the DNA vaccinated treatments. However, more fish were positive for Mx gene expression in each treatment and at day 35 than in previous experiments.

**Serum Neutralizing Titers Results.** In the third experiment testing multiple groups of plasmids, low neutralizing titers were observed (Table 2.9). The all 11 treatment showed an average neutralizing titer of 10. The KP6 treatment had an average neutralization treatment had an average neutralization titer of 13.3. The TE treatment and vector only treatment had average neutralization titers of 0. The HH5 treatment had an average titer of 0. The pORF59, pORF46, and pORF6 treatment (3) had a titer of 10. The all 11, KP6, and 3 group treatments all had titers of 10 or greater, which might be considered weakly reactive, but are not significantly positive over negative controls.
Figure 2.6. Mean ELISA OD of 10 fish tested per treatment per dose (pORF1, pORF3, pORF19, and pcDNA at doses 50 µg, 25 µg, and 5 µg), averaged as a whole treatment. Ten fish were individually bled and serum assayed in duplicate in an indirect sandwich ELISA using semi-purified CCV antigen as the coating antigen. The OD value of the lowest dilution was averaged for each fish within a dose (n=10) and all doses in one treatment (n=30). Taken as a whole treatment (n=30), pORF3 is significantly different from pcDNA negative controls (p=0.05). The remaining treatments were not significantly different from pcDNA negative controls. CCV antiserum positive control was used to ensure consistency between tests.
Table 2.7. Mean percent mortalities after challenge with $1 \times 10^6$TCID$_{50}$/ml of wild-type CCV 5 weeks post vaccination with 3 doses (50 µg, 25 µg, and 5 µg) of treatments All 11, KP6, HH5, 3 (59, 46, 6), pcDNA, and TE.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 plasmids</td>
<td>84.8 +/- 3.03</td>
</tr>
<tr>
<td>HH5</td>
<td>87.8 +/- 9.2</td>
</tr>
<tr>
<td>KP6</td>
<td>71.2 +/- 8.02</td>
</tr>
<tr>
<td>59, 46, 6</td>
<td>86.4 +/- 7.87</td>
</tr>
<tr>
<td>pcDNA</td>
<td>89.4 +/- 8.43</td>
</tr>
<tr>
<td>TE</td>
<td>87.8 +/- 3.03</td>
</tr>
</tbody>
</table>
Figure 2.7. Mean triplicate percent mortalities of treatments from multiple plasmid vaccination. Fish were vaccinated with 1µg per plasmid and challenged five weeks post vaccination. None of the group means were significantly different from each other.
% Mortality

Treatments

11 plasmids  HH5  KP6  59, 46, 6  pcDNA  TE
Table 2.8. Mx gene expression in each treatment at days 1, 3, and 35. Three fish of an average weight of 1 g were vaccinated with All 11 plasmids, the treatment KP6, the treatment HH5, a group of 3 plasmids (pORF59, pORF46, pORF6), pcDNA, and TE. Five prevaccinate fish were sampled at day 0. Fish were sampled at days 1, 3, and 35, and total RNA was extracted. Mx specific RT-PCR was performed on each sample. Results reflect positive fish as detected by a positive band at 400 bp on agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevaccinate</td>
<td>0/5</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>TE</td>
<td>0/3</td>
<td>1/3</td>
<td>2/3</td>
</tr>
<tr>
<td>pcDNA</td>
<td>2/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>All 11</td>
<td>2/3</td>
<td>2/3</td>
<td>1/3</td>
</tr>
<tr>
<td>KP6</td>
<td>2/3</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>HH5</td>
<td>3/3</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>3</td>
<td>2/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
</tbody>
</table>
Table 2.9. Serum neutralizing titers of three fish tested per treatment. Three fish were vaccinated with multiple plasmid treatments All 11, KP6, HH5, 3, pcDNA, and TE. At five weeks post vaccination, vaccinated fish were terminally bled and subjected to serum neutralization tests according to the constant virus/varying serum method. Neutralizing titers were defined as the reciprocal of the dilution at which no CPE was present in either of the duplicate test wells. All treatments but the positive control CCV antiserum showed very low to negative neutralizing activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fish 1</th>
<th>Fish 2</th>
<th>Fish 3</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>All 11</td>
<td>10</td>
<td>0</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>KP6</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>13.3</td>
</tr>
<tr>
<td>HH5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>59, 46, 6</td>
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<td>10</td>
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<tr>
<td>pcDNA</td>
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</tr>
<tr>
<td>TE</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>
Discussion

The potential utility of DNA vaccination in fishes was first demonstrated against aquatic rhabdoviruses, where significant protection was demonstrated with the glycoprotein genes of these viruses, after delivery of even nanogram doses of the DNA vaccine. Vaccination with the G gene of IHNV and VHSV elicits 100% protection after vaccination with 1 µg of DNA (Anderson et al. 1996; Heppell et al. 1998; Lorenzen et al. 1999; Corbeil et al. 1999; LaPatra et al. 2000; Lorenzen et al. 2000; Corbeil et al. 2002; McLauchlan et al. 2003). The only report of DNA vaccination against CCV is by Nusbaum et al. (2002). Using ORF59 and ORF6 of the CCV genome to vaccinate via IM injection, survival after vaccination with ORF59 was 60-100%, with ORF6 was 52-100%, and survival after injection with the negative control PBS or vector only treatments was 34-56%. A combination of the two constructs, injected at a 50 µg dose of each and challenged similarly, was more effective than either singly.

In light of the reported efficacy extensive testing of DNA vaccines against other commercially important fish viruses, DNA vaccination was investigated to protect channel catfish fingerlings from CCV infections and evaluate immune responses to vaccination. All plasmids were cloned with the gene of choice, sequenced, and confirmed for protein expression in COS-7 (mammalian) and EPC (teleost) cell lines. The visualization of predicted molecular weight proteins for each gene supports the functionality of these DNA vaccines in eukaryotic cell culture. Differences in expression levels and multiple forms of expressed proteins were detected for each gene. This may indicate that lower levels of expression of some genes may be responsible for the lack of protection conferred by some DNA vaccines.

In vivo preliminary studies testing pORF59 in a 5 µg dose and pORFs 1, 3, and 59 in a 1 µg dose resulted in no protection, indicating that 1 µg and 5 µg doses of DNA vaccine may not
be sufficient. Consequently to investigate the protective capacity of these DNA vaccines, a dose response study was conducted wherein 50 μg, 25 μg, and 5 μg doses of DNA vaccines pORF1, 3, 6, 19, 46, and 59 were tested. Protection was not observed at any dose using any gene, even at a 50 μg dose. The only treatment that showed lower mortalities was pORF46 at a 25 μg dose, which had a cumulative mortality level of 40%. However, this was still not significantly different from the negative vector only control. In addition to the dose of DNA delivered, the volume of delivery has been studied in rainbow trout IHNV system and has been determined to be most effective at 50-200 μl per fish (Anderson et al. 1996). The delivery volume employed in the dose response study was 50 μl, well within this established range of efficacy.

Time to challenge was not a factor in this dose response study, as fish challenged at 5 weeks post vaccination, and in preliminary studies 7 and 10 weeks post vaccination, were not protective time frames. In the rainbow trout system, significant specific protection against IHNV has been elicited after 28 days post-vaccination. Early non-specific protection occurred within four days post-vaccination in this system, but specific protection was not seen sooner than 28 days (LaPatra et al. 2001).

Possible explanations for the differences in the efficacy of the reported DNA vaccines by Nusbaum et al. (2002) and the efficacies reported here are multifold. Familial strains of channel catfish have been observed to have genetic differences in their responsiveness to vaccination or susceptibility to disease (Wolters and Johnson 1994). Plumb et al. (1975) tested eight strains of 1-3 month old CCV-free channel catfish fingerlings (Falcon, Kentucky, Marion, Tennessee, Warrior, Yazoo, Warrior-Yazoo, and Yazoo-Tennessee) for susceptibility to wild-type CCV. Following lethal challenge with CCV, significant differences were observed between strains of fish in mortality. The Falcon strain suffered 71% mortality, which was significantly higher than
the rest. The least susceptible strains were the Yazoo and the Tennessee, experiencing 12% mortality, and the hybrids of these strains (Warrior-Yazoo and Yazoo-Tennessee) experienced 10% mortality. Plumb et al. (1975) attribute the wide range of susceptibility of strains to CCV as familial genetic resistance to disease, supported by the inability they experienced in isolating virus from experimentally challenged fish. Therefore, the channel catfish strains used in these two studies could have had genetic differences that resulted in increased survival of fish after vaccination in the study by Nusbaum et al. (2002). Additionally, differences in the virulence of the CCV strains used in these two studies could result in differences in mortalities, resulting in differences in responses to vaccination. Fish used in the study by Nusbaum et al. (2002) were 6-10 months old, 4-8 cm long, and maintained at a temperature of 20°C until challenge, as opposed to fish used in this study, which were 1 month old, weighed an average of 1 g, and were maintained at a temperature of 27°C. The increased age and size of the fish, in addition to the decreased temperature of maintenance used by Nusbaum et al. (2002) could have produced an environment where a low level of immunity stimulated by the DNA vaccines would have been enough to be moderately protective. In addition the 26°C challenge temperature used by Nusbaum et al. (2002), is just inside optimal temperature for infection with CCV (25-30°C (Plumb 1978)), which could have slowed the kinetics of CCV infection and allowed that moderate level of protective immunity to result in the observed efficacy of the vaccines.

In addition to biological differences, Nusbaum et al. (2002) conducted six experiments replicated in time. It is unclear what replicates were employed, however, the use of the Chi-square to test for significance implies that the replication of each group was limited (Norman and Streiner 2000). In the current study, triplicate tanks of each treatment and each dose of each
treatment were employed to normalize variations introduced by tank-to-tank differences, and the inherent variations involved when testing an outbred and genetically heterogenous population.

Even though these DNA vaccines did not provide protection against CCV, the DNA vaccines did elicit an innate immune response in the form of Mx gene expression. In the first study testing pORF6, 46, and 59, no Mx gene expression was detected. The reason for this anomaly is not fully understood. Although the RT-PCR was repeated several times, no Mx gene expression was detected in any dose at any time point. According to studies conducted on genetic resistance in fishes, certain families of fish are less immunologically responsive and susceptible to disease. Observed differences in catfish responses to antigens have been postulated to be due to differences in sensitivity of channel catfish strains and families to ESC (Wolters and Johnson 1994). It is possible that the inability to detect Mx gene expression in this group is due to genetic strain differences in the catfish population used in this part of the study. Other explanations as to why in this group of fish Mx gene expression was not detected involves the existence of secondary infections in the fish and the presence of a secondary immune response in progress. Kim et al. (2000) postulated that the failure of detection of Mx gene expression in challenged fish 7 days post challenge involved the downregulation of the innate immune response as a result of an upregulation of the secondary immune response, because this group was vaccinated with the glycoprotein gene of IHNV and protection was significant in this group only. In other groups, protection was not observed and Mx gene expression was detected throughout the experiment with no downregulation at any time. This suggests that the downregulation in the only group that was protected correlates with the upregulation of the specific response. It is then logical to assume that the presence of a secondary immune response to a ubiquitous pathogen in the environment, such as *Columnaris* spp. or *Aeromonas* spp. could
down regulate Mx gene expression in vaccinated fish. Fish in this study did not exhibit gross clinical signs of any *Columnaris* spp. or *Aeromonas* spp. disease, however a low level or chronic state of disease may have gone undetected.

In the second study testing pORF1, 3, and 19, Mx gene expression was variably detected in most treatments and most often at day 3. This indicates that the fish were responsive on an immediate and innate level to the injection and transcription of foreign DNA. An Mx gene response was even observed to the vector-only negative control. The detection of Mx gene expression is postulated to indicate that a nonspecific, interferon mediated response was elicited. The expression of Mx was detected in fish injected with DNA vaccines against other fish diseases as early as day 7 to as long as day 30 post-vaccination (Kim et al. 2000; Boudinot et al. 1998; McLauchlan et al. 2003).

In serum neutralizing studies, only very low to negative titers were detected. Interestingly, the only treatment with an individual titer of 10 or more was also the only treatment that experienced a lower mortality, pORF46 at a 25μg dose. This indicates that pORF46 may have some efficacy against CCV. It is interesting that pORF46 was not efficacious at 50 μg, but this might be due to tolerance.

Neutralizing antibody has been elicited by DNA vaccination in other fish systems and correlates to protection, but most often by older and larger fish (Corbeil et al. 1999; LaPatra et al. 2000; McLauchlan et al. 2003). Corbeil et al. (1999) DNA vaccinated large sockeye salmon (*Oncorhynchus nerka*) with the IHNV-G DNA vaccine and passively transferred neutralizing antibodies to smaller fingerling rainbow trout (*Oncorhynchus mykiss*), which subsequently survived lethal challenge with IHNV. Another group of small trout were concurrently DNA vaccinated with the IHNV-G construct and failed to produce neutralizing antibodies, but were
protected from challenge, indicating that the protective element in the passive transfer could have been specific antibody, not the neutralizing antibody. One contrasting study was conducted by Corbeil et al. (2000b), who detected high neutralizing titers resulting from DNA vaccination of 0.8-1.0 g rainbow trout of 0.01 µg with the IHNV-G vaccine up to 6-weeks post-vaccination. At doses of 5, 1, and 0.1 µg of vaccine, high neutralizing titers were detected out to week-10 post-vaccination. Nusbaum et al. (2002) tested neutralizing titers in 4-8cm and 8-10 month old channel catfish elicited by CCV ORFs 59, 6, and the combination (59+6) at 4-6 weeks post-vaccination and 2-weeks post-challenge. Expressed as geometric mean titer (GMT), neutralizing antibody in fish vaccinated with ORF6 barely reached a mean titer of 5 when measured at 4 and 6 weeks post-vaccination. ORF59 was barely detectable at 4 weeks and only reached a GMT of 10 at 6 weeks post-vaccination. When tested at 5-weeks post-vaccination, however, ORF6, ORF59, and ORF59+6 titers were low and the GMT was around 20. Our results confirm that very low or negative neutralizing antibody titers result from DNA vaccination. This indicates that the genes used in these two studies do not carry neutralizing epitopes or that they are not presented to the immune system in a way conducive to neutralizing antibody production. For example, if the proteins encoded by the genes were not being expressed in a correct conformational manner, conformational neutralizing antibodies would not be elicited. Neutralizing antibodies are most often produced against the available external sites on a virus, most often components of the outer membrane or envelope, and are generally conformational antibodies (Kuby 1997).

When analyzing the same DNA vaccinated sera that was used in the neutralization studies by ELISA using a semi-purified CCV coating antigen, significant differences were detected between treatments. Significant differences were detected between antibody responses
between pORF46 and pcDNA and the pORF3 treatment and pcDNA. The presence of significant antibody responses by ELISA and not in neutralization, supports the findings of Corbeil et al. (1999), who demonstrated that the detection of neutralizing antibodies, although neutralizing titers correlate with protection, is the most accurate predictor of protection following vaccination, and specific antibodies are additionally important components in DNA vaccination. Overall, our results demonstrate that the fish are responding to DNA vaccination against a single gene in an innate manner (Mx gene expression) and in a specific manner (significant ELISA titers), but are not protected.

DNA vaccination utilizing more than one gene or epitope has been investigated in the HSV and CCV system with some success. When given in combination, the gB and gD genes provided better protection together than singly in terms of reduction of viral shedding, increase in neutralization titers, and better cytotoxic lysis in the HSV system (Lee et al. 2002). Nusbaum et al. (2002) reported better protection when catfish were vaccinated with DNA vaccines encoding ORFs 59 and 6 together rather than singly. In the current study, however, DNA vaccination with multiple genes delivered concurrently was not more protective, as even 11 genes delivered at once were not protective. Expression of the Mx gene, however was upregulated in response to the multiple DNA injections as Mx gene expression was detected in all treatments and in all doses, even as long as day 35 post-vaccination. Serum neutralizing antibodies were detected in higher levels than in previous experiments but these levels were still low. Although not significantly different from each other, the treatments containing all 11 plasmids, the KP6 plasmids, and the pORF46, 6, and 59 plasmids resulted in an average titer that was over 10. The common denominator in these three treatments is pORF46, which again indicates that this gene is involved in antibody elicitation.
In light of the success that has been achieved with nanogram amounts of DNA in the IHNV and VHSV systems, it is surprising that these DNA vaccines were not protective even in large doses. These studies indicate that DNA vaccination for CCV, at any dose and with any of the gene targets are not protective, but do stimulate an immune response. Even multiple genes delivered at the same time did not provide protection, but did increase innate responses and indicated a trend of increasing neutralizing antibody titer. Possible future DNA vaccines for CCV could be coupled with alternative immune modulators or used in a prime/boost strategy, if an immunodominant antigen is identified. For example, coupling the plasmid encoding pro-inflammatory cytokines murine GM-CSF and the VHSV G gene elicits a more robust immune response than the viral genes alone (Kanellos et al. 1999). Expression of the cytokine increased the number of proinflammatory cells to the injection site and provided a means of initiating a cellular immune response. This approach, however requires an increase in the knowledge concerning channel catfish cytokines.
CHAPTER 3. A COMPARISON OF CURRENTLY AVAILABLE VACCINES AGAINST CCV: LIVE ATTENUATED CCVTK- IS MORE VIRULENT THAN REPORTED AND DNA VACCINES ARE NOT EFFICACIOUS

Introduction

Channel catfish virus (CCV) causes the most devastating viral disease of channel catfish (*Ictalurus punctatus*) with outbreaks resulting in losses exceeding 90% in fry and fingerling channel catfish ponds (Plumb 1978; Noga 1996). *Ictalurus herpesvirus* 1 (Channel catfish virus) presents as a hemorrhagic viremia exhibiting clinical signs including abdominal distension, reddening of the base of fins and gills, and necrosis of all major organ systems (Fijan et al. 1970; Plumb 1971; Noga 1996). Channel catfish virus is an enveloped, double-stranded DNA virus comprised of a 134 kb genome encoding 77 genes (Davison 1992). A live-attenuated mutant of CCV was created by Zhang and Hanson (1995) (Channel catfish virus deficient in thymidine kinase or CCVTK-) with a disruption of the thymidine kinase gene that reportedly attenuates the virus and provides low vaccination mortality but high levels of protection against CCV challenge. More recently, Nusbaum et al. (2002) created two DNA vaccines against CCV from ORF59, encoding the major glycoprotein, and ORF6, encoding a membrane gene, that provide significant protection against CCV singly, but together are even more protective, with reported efficacies reaching 100%. In previous studies in chapter 2, however, newly constructed DNA vaccines using pORF59 and pORF6 were not protective even at a dose of 50 µg per fish, even though they carry the identical genes tested by Nusbaum et al. (2002) (herein called KN59 and KN6). Because of this discrepancy, this study was initiated to compare the efficacies of the apparently identical vaccines, and further compare them to the reportedly very efficacious CCVTK- mutant. Immunological parameters of innate and adaptive immunity were investigated to establish the ability of the vaccines being tested to induce an immune response.
Materials and Methods

Dose Response for CCVTK- Vaccination. The live attenuated thymidine kinase deficient CCV mutant (CCVTK-) was a gift from Dr. Larry Hanson and was propagated in a manner consistent to published protocols (Zhang and Hanson 1995). Triplicate tanks of 20 average weight 1 g fingerlings were vaccinated by immersion with 4 doses of CCVTK- for 2 hours at 30°C. Tank volumes were adjusted to 2 L after which 2 mls, 0.2 mls, 0.02 mls, and 0.002 mls of CCVTK- was added to triplicate tanks, resulting in final doses of 1 X 10^{4.5}, 1 X 10^{3.5}, 1 X 10^{2.5}, 1 X 10^{1.5} TCID_{50}/ml CCVTK-. Mortalities were collected twice daily and 10% of dead fish were necropsied and diagnosed for CCVTK- by cell culture. After 3 weeks, surviving fish were challenged with 1 X 10^{6} TCID_{50}/ml CCV for 8 hours at 30°C. Mortalities were collected twice daily and 10% of dead fish were necropsied and diagnosed for CCV by cell culture. Following an arcsin transformation of the percent mortalities, the treatments were compared by Analysis of variance (ANOVA).

Plasmid Methods. Plasmids pORF59, pORF6, and pcDNA3.1/V5/His-TOPO (hereafter called pcDNA) were constructed as described previously in chapter 2. Plasmids pCR3ORF59 (KN59) and pCR3ORF6 (KN6) were donated by Dr. Ken Nusbaum for use in this experiment (Nusbaum et al. 2002). Each plasmid was isolated from the E. coli Top 10 strain with Qiagen gigaprep kits and resuspended to 50 μg DNA in 20 μl TE.

Channel Catfish. Channel catfish egg masses were obtained from a commercial producer with no history of CCV outbreaks. Eggs were disinfected with 100 ppm free iodine and hatched in closed recirculating systems in the SPF laboratory at the School of Veterinary Medicine. Fish were reared on commercial catfish diets fed at 3% of their body weight per day at a temperature ranging from 24-28°C until used for the challenge experiments. Prior to
vaccination, fry were tested and certified to be free of CCV by methods outlined by Baek and Boyle (1996).

**Vaccination of Fingerlings.** This experiment was conducted to replicate an experiment conducted by Nusbaum et al. (2002), using DNA vaccines pORF59, pORF6, KN59, and KN6. All plasmids were resuspended in a concentration of 2.5 µg/µl of TE, and triplicate groups of 23 average weight 1 g channel catfish fingerlings were vaccinated with 50 µg of DNA in 20 µl TE delivered individually on each side of the dorsal fin in the dorsal epaxial muscle. For the combination vaccination, fish received two injections, one on either side of the dorsal fin with the plasmids used in the Nusbaum et al. (2002) experiment (KN59+6) or pORF59+6. Triplicate groups of 23 fish were also vaccinated with the vector pcDNA and TE as negative controls. Similar groups of fish were also vaccinated with 1 X 10^{3.5} TCID_{50}/ml CCVTK- by immersion for 2 hours, after which, water flow was resumed. Fish were maintained at 30°C until challenge, a divergence from Nusbaum et al. (2002) who maintained 4-8 cm catfish at 20°C until challenge.

**Mx Gene Expression - RNA Extraction.** Samples were taken to measure Mx gene expression in the muscle site of injection. On the day of vaccination (day 0) five fish were euthanized with an overdose of MS222. These fish were necropsied, the muscle was removed, snap frozen in liquid nitrogen, and stored at -70°C. At days 1, 3, and 35 post vaccination, one fish from each tank per treatment (three fish per treatment) was similarly euthanized and the muscle removed, frozen, and stored at -70°C for later RNA extraction. Frozen muscle sections were thawed, weighed, adjusted to 50 mg, and RNA was extracted with Tri-reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s recommendations and briefly described in Chapter 2.
**Mx Gene Expression - RT-PCR.** Reverse transcription of Mx RNA into cDNA was performed by the method described by Plant et al. (2003) and briefly described in Chapter 2. For vaccine specific mRNA detection, reverse transcription and PCR conditions were identical to the conditions used for Mx detection. Two specific cDNA primers were used to specifically amplify cDNA from ORF59 and ORF6 in all treatments vaccinated with ORF59 or ORF6. The ORF59 cDNA primer was called 59cDNA (5’AGGTGCAGGTACAGTTCGTG3’) and the ORF6 primer was called 6cDNA (5’TTCAGACCGGATCTCCG3’). Specific ORF 59 PCR was performed on the amplified cDNA using primers 59RTB5’ (5’ACGGGAGGTGATACA3’) and 59RTB3’ (5’GTGCGGAGCTGGTG3’) for ORF59. A 500 bp product represents a positive product for the ORF59 gene. Amplification of ORF6 was performed from reverse transcription using the 6cDNA primer and PCR primers 6RTPCRfor (5’TCTCCTCTCCGCTCA3’) and 6RTPCRrev (5’CTCTGCTGCTGCGGCTC3’). A 360 bp product represents a positive product for the ORF6 gene.

**Challenge of Vaccinated Fingerlings.** Fish were challenged five weeks post-vaccination by immersion in 1 X 10^6 TCID_{50}/ml of wild-type CCV for 8 hours at 30°C. Morbid and moribund fish were collected twice daily and 10% of dead fish were necropsied. Liver, viscera, and/or head kidney was removed from dead fish and homogenized in sterile HBSS with 2% FBS. Homogenates were centrifuged for 3 minutes at 2300 X g and the supernatants were removed. Fifty microliters of the supernatant was added to duplicate wells of a 24 well tissue culture plate with a confluent monolayer of CCO cells for viral confirmation by the presence of CPE.
**Serum Neutralization.** Five weeks post-vaccination, fish were euthanized with an overdose of MS222 and serum was collected and stored at -70°C until assayed. Fish were collected directly from the treatment tanks and serum was collected 5 weeks post-vaccination. Serum neutralization titers for both experiments were determined using the constant virus-varying serum method of Plumb and Bowser (1983) and briefly described in Chapter 2.

**Results**

**CCVTK- Vaccination Results.** After vaccination, vaccination-associated mortalities occurred at higher doses (Figure 3.1). At the 1 X 10^{4.5} TCID_{50}/ml CCV dose, the average cumulative mortality was 42.6 +/- 6.66%. At the 1 X 10^{3.5} TCID_{50}/ml CCV dose, the average cumulative mortality was 26.6 +/- 5.2%. There were no mortalities from vaccination in the 1 X 10^{2.5} and 1 X 10^{1.5} TCID_{50}/ml CCVTK- dose. Statistical differences occurred between the highest two doses (1 X 10^{4.5} TCID_{50}/ml CCVTK- and 1 X 10^{3.5} TCID_{50}/ml CCVTK- dose) and the lowest two doses. Mortalities following challenge were high in the low doses and low in the high doses. In the 4.5, 3.5, 2.5, and 1.5 log doses, the average cumulative mortalities were 1.3 +/- 0.46%, 26.6 +/- 9.22%, 66.6 +/- 2%, and 66.6 +/- 0.46%, respectively (Figure 3.2). In the nonvaccinated/challenged fish, the average cumulative mortality was 54.6 +/- 0.46%. Survival was statistically higher in the high doses (1 X 10^{4.5} and 1 X 10^{3.5} TCID_{50}/ml CCVTK-) as compared to the low doses (1 X 10^{2.5} and 1 X 10^{1.5} TCID_{50}/ml CCVTK-), whereas, in the vaccination mortality study, survival was statistically lower in the high doses as compared to the low doses.
Figure 3.1. Cumulative percent mortalities resulting from vaccination of fingerlings with 4 doses of CCVTK-. Nonvaccinated fish that were subsequently challenged (n/c) served as a negative control. Asterisks denote significant difference from the negative control.
Log of CCVT- vaccination dose

% Mortality

Log of CCVT- vaccination dose

4.5 3.5 2.5 1.5 n/c

% Mortality

*
Figure 3.2. Cumulative mortalities resulting from vaccination of fingerlings with 4 log doses of CCVTK- that were challenged with $1 \times 10^6$ TCID$_{50}$/ml CCV 3 weeks post vaccination. Asterisks denote significant difference from the negative control.
**DNA Vaccination Results.** Prior to vaccination, KN59 and KN6 were sequenced, confirming their identity. Additionally, both plasmids were subjected to gene specific PCR used to isolate ORF59 and ORF6 used previously. The predicted 1038 bp and 417 bp products were identified for both pORF59 and KN59 and KN6 and pORF6, respectively (Figure 3.3). Following challenge with the wild-type virus, average mortalities in DNA vaccinated treatments were high and not significantly different from negative controls (Figure 3.4). For the comparison to the CCVTK- vaccine, considerable mortalities were again observed at this dose of CCVTK-following vaccination. The mortalities were highly variable, however, with one tank of fish experiencing 100% mortality. One tank experienced 30% mortality, and the other 0% vaccination related mortality. Therefore, the CCVTK- treatment only consisted of duplicate tanks for the challenge study and the average mortality was 50 +/- 0.14% (Figure 3.4).

**Mx and Gene Specific Gene Expression.** Mx gene expression resulted in the amplification of a 400 bp product from the catfish Mx gene. Mx gene transcription was identified in all treatments containing DNA including the negative control pcDNA, with the most numerous fish positive at day three post vaccination (Table 3.1). Vaccine specific PCR amplified a 500 bp product for ORF59 containing treatments, and a 350 bp product for ORF6 containing treatments (Table 3.2). Vaccine specific gene expression was identified in all treatments, at all 3 sampling times, indicating that the plasmid vaccines were transcriptionally active in the muscle.

**Serum Neutralization Results.** Of all the treatments and doses, only low neutralizing titers were observed (Figure 3.7). In the TE and pcDNA treatments, negative titers were observed, with an average titer of 0. In this experiment, the titers of some treatments (KN59+6 and CCVTK-) were higher than a titer of 10, and therefore might be considered reactive.
Figure 3.3. Agarose gel of PCR products from pORF6, pORF59, KN6, and KN59. Lane 1 is the 1kb ladder. Lanes 2 and 4 are the products of 417 bp ORF6 amplified from pORF6 and KN6. Lanes 3 and 5 are the 1038 bp products of ORF59 amplified from pORF59 and KN59 at 1038 bp.
Figure 3.4. Average percent mortalities resulting from fish vaccinated with 2 types of DNA vaccines and a live attenuated vaccine (CCVTK-). Triplicate tanks of fingerlings were vaccinated with 50 µg of DNA vaccines KN59, KN6, KN59+6, pORF59, pORF6, pORF59+6, pcDNA3.1/V5/His-TOPO, and vaccinated with CCVTK- and the negative control TE. Fish were challenged 5 weeks post vaccination and mortalities were counted for 7 days.
Table 3.1. Mx gene expression of fish vaccinated with DNA vaccines pORF59, pORF6, pORF59+6HH, KN59, KN6, KN59+6, TE, pcDNA, and CCVTK-. Treatments pORF59+6HH and KN59+6 consist of two muscle sites vaccinated with one of the combination vaccines and are designated with an ORF number after the combination treatment name to determine which construct is being tested for, ie., pORF59+6HH(59) is the muscle site from the combination treatment that was vaccinated with pORF59. Muscle was sampled from three fish per treatment on day 1, 3, and 35 post vaccination and total RNA extracted was subjected to Mx-specific RT-PCR. Results shown are positive fish out of 3 sampled.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>0/5</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>pORF59</td>
<td>2/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>pORF6</td>
<td>1/3</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>59+6H(59)</td>
<td>3/3</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>59+6H(6)</td>
<td>0/3</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>KN59</td>
<td>0/3</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>KN6</td>
<td>0/3</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>KN59+6(59)</td>
<td>2/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>KN59+6(6)</td>
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<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>TE</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>pcDNA</td>
<td>0/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>CCVTK-</td>
<td>2/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
</tbody>
</table>
Table 3.2. Gene specific gene expression of fish vaccinated with DNA vaccines pORF59, pORF6, pORF59+6HH, KN59, KN6, KN59+6, TE, pcDNA, and CCVTK-. Treatments pORF59+6HH and KN59+6 consist of two muscle sites vaccinated with one of the combination vaccines and are designated with an ORF number after the combination treatment name to determine which construct is being tested for, ie., pORF59+6HH(59) is the muscle site from the combination treatment that was vaccinated with pORF59. Muscle was sampled from three fish per treatment on day 1, 3, and 35 post vaccination and total RNA extracted was subjected to gene specific RT-PCR. Results shown are positive fish out of 3 sampled.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>pORF6</td>
<td>3/3</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>59+6H(59)</td>
<td>3/3</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>59+6H(6)</td>
<td>2/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>KN59</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>KN6</td>
<td>0/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>KN59+6(59)</td>
<td>3/3</td>
<td>0/3</td>
<td>3/3</td>
</tr>
<tr>
<td>KN59+6(6)</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
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<tr>
<td>TE</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>pcDNA</td>
<td>0/3</td>
<td>0/3</td>
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</table>
Figure 3.5. Serum neutralization titers. Fingerlings (average weight 1 g) were vaccinated with pORF59, pORF6, pORF59+6HH, KN59, KN6, KN59+6, pcDNA, and TE. Three fish were sampled at day 35 for neutralizing antibodies and subjected to the varying serum/constant virus serum neutralization test. Results shown are the average of the 3 fish where the titer represented is the reciprocal of the dilution at which no CPE was detected.
Average titer vs Treatment
However, due to fish to fish variations and low levels of cross reactivity in the negative controls, these titers cannot be considered significant.

**Discussion**

The potential utility of DNA vaccination in fish was first demonstrated against aquatic rhabdoviruses, where significant protection was demonstrated with glycoprotein genes of these viruses, after delivery of even nanogram doses of the DNA vaccine. Vaccination with the G gene of IHNV and VHSV elicits 100% protection after vaccination with 1 µg of DNA (Anderson et al. 1996; Heppell et al. 1998; Corbeil et al. 1999; Lorenzen et al. 1999; LaPatra et al. 2000; Lorenzen et al 2000; Corbeil et al. 2002b; McLauchlan et al. 2003). The only report of DNA vaccination against CCV is by Nusbaum et al. (2002). Using ORF59 and ORF6 of the CCV genome to vaccinate via IM injection, survival after vaccination with ORF59 was 60-100%, with ORF6 survival was 52-100%, compared to survival after injection with the negative control PBS or vector-only treatments at 34-56%. A combination of the two constructs, injected at a 50 µg dose of each and challenged similarly, was more effective than either singly.

In this study, the DNA vaccines constructed for ORF6 and ORF59 of the CCV genome and reported to be protective by Nusbaum et al. (2002) were tested alongside pORF59 and pORF6, DNA vaccines encoding the same ORFs that were reported in chapter 2. None of the vaccinated treatments were significantly different from the negative controls, with mortalities of over 50% in all treatments, with some treatments as high as 90%. From work previously conducted in chapter 2, this was expected, but directly conflicted with the results of Nusbaum et al. (2002) despite the fact that the experiment mirrored the conditions of that study. The current study indicates that, despite success previously reported by Nusbaum et al. (2002), DNA
vaccination in channel catfish derived from ORF59, ORF6, or a combination of the two, is not efficacious against CCV.

Possible explanations for the difference in the efficacy reported by Nusbaum et al. (2002) and the efficacy reported here are multifold. Possible differences reported in chapter 2 included variation in the strains of channel catfish (Plumb et al. 1975). Plumb et al. (1975) tested 8 strains of 1-3 month old CCV-free channel catfish fingerlings (Falcon, Kentucky, Marion, Tennessee, Warrior, Yazoo, Warrior-Yazoo, and Yazoo-Tennessee) for susceptibility to wild-type CCV. Following lethal challenge with CCV, significant differences were observed between strains of fish in mortality. The Falcon strain suffered 71% mortality, which was significantly higher than the rest. The least susceptible strains were the Yazoo and the Tennessee, experiencing 12% mortality, and the hybrids of these strains (Warrior-Yazoo and Yazoo-Tennessee) experienced 10% mortality. Plumb et al. (1975) attribute the wide range of susceptibility of strains to CCV as familial genetic resistance to disease, supported by the inability they experienced in isolating virus from experimentally challenged fish. The age and size of the fish, and the experimental temperature used by Nusbaum et al. (2002) could have produced an environment where a low level of immunity stimulated by the DNA vaccines was moderately protective. In the direct comparison reported here, however, all of the variables were controlled, leaving the lack of efficacy more difficult to explain compared to the previous study Nusbaum et al. (2002). One important difference that remains between the studies is CCV carrier status of the fish. Nusbaum et al. (2002) did not evaluate the experimental fish for CCV and latency. Vertical transmission of CCV leads to asymptomatic, latent infection in the offspring that would impact any vaccination regime (Wise et al. 1985; Wise et al. 1988; Boyle and Blackwell 1991; Baek and Boyle 1996; Gray et al. 1999). The fish used by Nusbaum et al. (2002) were not certified as
disease free with standard methods (Baek and Boyle 1996). Consequently, the DNA vaccines used may have been a boost of a previously exposed animal, with an unknown impact on the protection of that animal against subsequent disease.

In addition to biological differences, Nusbaum et al. (2002) conducted 6 experiments replicated in time. Nusbaum et al. (2002) does not report the numbers of replicates per experiment, but the use of Chi-square to test for statistical significance implies that replication was limited (Norman and Streiner 2000). In this study, triplicate tanks of each treatment were run concurrently to eliminate variations in tank allocation, temperature, and time, and allowing analysis by ANOVA which found no significant differences in efficacy between treatments and the negative controls.

In order to investigate innate and adaptive immunological parameters stimulated by DNA vaccination in channel catfish, Mx gene expression (an α/β interferon induced antiviral protein) and serum neutralizing antibodies were analyzed. Mx gene expression showed, in agreement with previous studies in chapter 2, that DNA vaccination elicits an Mx gene response in almost all treatments, with the highest number of fish positive for Mx gene expression at day 3 post vaccination. Although protection against disease was not evaluated in this study, these fish were responsive to DNA vaccination with an innate, interferon-mediated response regardless of gene or combination thereof.

Serum neutralizing titers, however low to negative, were also induced in the current experiment comparing Nusbaum et al. (2002) vaccines to pORF59 and pORF6. Nusbaum et al. (2002) reported that low neutralizing antibody titers were produced in response to ORF 6 and ORF 59 at 4-6 weeks post-vaccination. McLauchlan et al. (2003) examined 4.5, 10, and 100 g rainbow trout for serum neutralizing titers after DNA vaccination against VHSV. In agreement
with Corbeil et al. (1999), they found neutralizing titers were only at significant levels in larger (in her study 100 g) fish.

Lack of gene expression would be a possible explanation for the failure of the DNA vaccines to elicit protection. Specific expression of the DNA vaccines encoded genes, however, demonstrated that the plasmids were functional at a transcriptional level. In each treatment, specific expression of the encoded gene was detected in all treatments except the negative controls at most time points, even out to day 35. Disregarding a potential problem with folding or cellular compartmentalization, these DNA vaccines should be protective if the genes encoded are protective antigens.

The reason for the non-protective nature of DNA vaccines against CCV, in the face of success in other aquatic viral systems, is not clear. It is possible that a complex virus such as CCV, where the genome comprises 77 temporally regulated genes, may not be protected by vaccination with a single ORF. Researchers studying DNA vaccination against human HSV-1 and 2, other alphaherpesviruses, have yet to discover a completely protective DNA vaccine that elicits sterile and protective immunity against that herpesvirus (Nass et al. 1998; Mester et al. 2000; Baghian et al. 2002; Lee et al. 2002). Perhaps a prime-boost strategy, once an immunologically protective antigen is identified, would be a good vaccination strategy against CCV. In addition, this study demonstrates that the live attenuated thymidine kinase deficient CCV mutant is more virulent than previously reported and requires further analysis before any application in a prime/boost or commercial system.
SUMMARY

DNA vaccination against Channel Catfish Virus (Ictalurid herpesvirus 1) has been extensively studied in this work. Multiple genes of two different temporal classes, different doses of genes, different combinations of genes, and comparisons to currently existing and genetically identical genes have been investigated. All of the data indicates that DNA vaccines are not protective against lethal challenge with CCV despite the detection of vaccine specific gene expression, Mx gene expression in response to vaccination, and statistically reactive antibody titers measured by ELISA, indicating that the plasmids were functional.

Protection against CCV disease was not observed at any dose using any gene of the six tested, even at a 50 µg dose. The only treatment that showed lower mortalities was pORF46 at a 25 µg dose, which had a mean percent mortality of 40%. However, this was still not significantly different from the negative, vector-only control.

Even though these DNA vaccines did not provide protection against CCV, they did elicit an innate immune response in the form of Mx gene expression. Mx gene expression was variably detected in most treatments and most often at day 3. This indicates that the fish were responsive on an immediate and innate level to the injection and transcription of foreign DNA. An Mx gene response was even observed to the vector-only negative control. The detection of Mx gene expression is postulated to indicate that a nonspecific, interferon mediated response was elicited. The expression of Mx was detected in fish injected with DNA vaccines against other fish diseases as early as day 7 to as long as day 30 post-vaccination (Kim et al. 2000; Boudinot et al. 1998; McLauchlan et al. 2003).

In serum neutralizing studies, only very low to negative titers were detected. Interestingly, the only treatment with a mean titer of 10 or more was also the only treatment that
experienced a lower mortality, pORF46 at a 25μg dose. This indicates that pORF46 may have some efficacy against CCV. It is interesting that pORF46 was also not efficacious at 50 μg, which might be due to tolerance.

Neutralizing antibody has been elicited by DNA vaccination in other fish systems and correlate with protection, but most often by older and larger fish (Corbeil et al. 1999; McLauchlan et al. 2003; LaPatra et al. 2000). Nusbaum et al. (2002) tested neutralizing titers elicited by CCV ORFs 59, 6, and the combination (59+6) at 4-6 weeks post-vaccination and 2-weeks post-challenge. In agreement with this study, only low titers were detected prechallenge. This indicates that the genes used in these studies do not carry neutralizing epitopes or that they are not presented to the immune system in a way conducive to neutralizing antibody production. If the proteins encoded by the genes are not expressed in a correct conformational manner, conformational neutralizing antibodies would not be elicited (Kuby 1997). Some reports illustrate the ability to measure specific antibodies elicited by DNA vaccination against IHNV as an alternative method of prediction of the efficacy of DNA vaccines (Fernandez-Alonso et al. 1999; Estepa et al. 2001; Fernandez-Alonso et al. 2001). In the current study, ELISA of vaccinated fish sera indicated significant differences between some DNA vaccinated fish sera (pORF46 and pORF3) and the negative control sera, suggesting that a more specific, non-neutralizing humoral immune response to DNA vaccination had taken place. Overall, our results demonstrate that the fish are responding to DNA vaccination against a single gene in an innate manner (Mx gene expression) and in a specific manner (significant ELISA titers), but are not protected.

Multiple gene DNA vaccines have been investigated in the HSV and CCV system with some success. When given in combination, the gB and gD genes provided better protection
together than singly in terms of reduction of viral shedding, increase in neutralization titers, and better cytotoxic lysis in the HSV system (Lee et al. 2002). Nusbaum et al. (2002) reported better protection when catfish were vaccinated with DNA vaccines encoding ORFs 59 and 6 together rather than singly. In the current study, however, DNA vaccination with multiple genes delivered concurrently was not more protective, even when 11 genes were delivered. Expression of the Mx gene, however, was upregulated in response to the multiple DNA injections as Mx gene expression was detected in all treatments and in all doses, even as long as day 35 post-vaccination. Serum neutralizing antibodies were detected in higher levels than in previous experiments but these levels were still low. Although not significantly different from each other, the treatments containing all 11 plasmids, the KP6 plasmids, and the pORF46, 6, and 59 plasmids resulted in an average titer that was over 10. The common denominator in these three treatments is pORF46, which again indicates that this gene is involved in antibody elicitation.

In the last study, the DNA vaccines constructed for ORF6 and ORF59 of the CCV genome and reported to be protective by Nusbaum et al. (2002) were tested alongside pORF59 and pORF6 that were tested in chapter 2. Nusbaum et al. (2002) demonstrated significant protection post-vaccination with ORFs 59 and 6, and greater protection after combination vaccination. However, in this comparison study, mortalities reached over 50% in all treatments, with some treatments as high as 90%. None of the vaccinated treatments were significantly different from the negative controls. This corroborates the work reported in chapter 2, but directly conflicted with the results of Nusbaum et al. (2002), despite the fact that the experimental conditions were the same, excepting temperature of challenge and size of fish used. The current study indicates that, despite success previously reported by Nusbaum et al. (2002),
DNA vaccination in channel catfish using ORF59, ORF6, or a combination of the two, is not efficacious against CCV.

Possible explanations for the difference in the efficacy reported by Nusbaum et al. (2002) and the efficacy reported here are multifold. Possible differences reported in chapter 2 included variation in the strains of channel catfish (Plumb et al. 1975). Plumb et al. (1975) tested 8 strains of 1-3 month old CCV-free channel catfish fingerlings (Falcon, Kentucky, Marion, Tennessee, Warrior, Yazoo, Warrior-Yazoo, and Yazoo-Tennessee) for susceptibility to wild-type CCV and found drastically different mortalities, ranging from 12-71% mortality. Plumb et al. (1975) attribute the wide range of susceptibility of strains to CCV as familial genetic resistance to disease, supported by the inability they experienced in isolating virus from experimentally challenged fish. The age and size of the fish (8-10 months old and 4-8 cm), and the experimental temperature (26°C) used by Nusbaum et al. (2002) could have been a suboptimal challenge and produced an environment where a low level of immunity stimulated by the DNA vaccines was moderately protective. In the direct comparison reported here, however, all of the variables were controlled, leaving the lack of efficacy more difficult to explain compared to the previous study Nusbaum et al. (2002). One important difference that remains between the studies is CCV carrier status of the fish. Nusbaum et al. (2002) did not evaluate the experimental fish for CCV and latency. Vertical transmission of CCV leads to asymptomatic, latent infection in the offspring that would impact any vaccination regime (Wise et al. 1985; Gray et al. 1999; Boyle and Blackwell 1991; Wise et al. 1988; Baek and Boyle 1996). The fish used by Nusbaum et al. (2002) were not certified as disease free using standard methods (Baek and Boyle 1996). Consequently, the DNA vaccines used may have been a boost of a previously exposed animal, with an unknown impact on the protection of that animal against subsequent disease.
In addition to biological differences, Nusbaum et al. (2002) conducted six experiments replicated in time. Nusbaum et al. (2002) does not report the numbers of replicates per experiment, but the use of Chi-square to test for statistical significance implies that replication was limited (Norman and Streiner 2000). In this study, triplicate tanks of each treatment were run concurrently to eliminate variations in tank allocation, temperature, and time, and allowing analysis by ANOVA which found no significant differences in efficacy between treatments and the negative controls.

Possibilities explanations for the failure of the DNA vaccines created and tested in chapter 2 to provide protection include a potential non-functional set of plasmids. However, transfection of mammalian (COS-7) and teleost (EPC) cell culture with each of these vaccines produced a predicted molecular weight protein indentifiable by SDS-PAGE and immunodetection using a monoclonal antibody against a fusion protein, indicating that a protein of the proper size was being translated in cell culture. Because specific monoclonal or polyclonal antibodies were not available against any of these proteins, the three dimensional structure was not studied. The addition of the V5 fusion protein to the 3’ end of the genes may have caused a change in three dimensional folding of the encoded protein, exhibiting non-immunodominant antigens to the immune system that are normally hidden within the folding of the protein. However, the ORF 59 and ORF 6 vaccines created by Nusbaum et al. (2002) did not encode a fusion protein, and should not be conformationally distorted. Since the KN6 and KN59 plasmids were not efficacious in the comparison study, a folding problem due to the V5 epitope on pORF59 and pORF6 protein products is not a likely factor.

Future studies investigating DNA vaccination against Channel Catfish Virus should focus on the study of the function of the genes in order to elucidate what genes might be
immunodominant or protective. As none of the genes in this study were protective by DNA vaccination, further functional studies are needed to investigate their potential role in CCV immune response modulation. The ORF59 protein product was added to CCO cells by Kucktas et al. (1998) and found to prevent formation of CPE after addition of CCV. However, in what capacity the CPE formation was prevented was not demonstrated. This gene product is thought to be the major envelope glycoprotein (Davison and Davison 1995), and function in the prevention of CPE formation (Kucktas et al. 1998), but was not protective by DNA vaccination in our studies. Further investigation in to this gene product and the other genes of CCV will shed light on which genes, or possibly epitopes of genes, may be protective. Once this information is available, alternative types of vaccination can be tested. If a protective combination of antigens is available, a prime-boost strategy can be investigated for protection. In this scheme, a DNA vaccine is used in concert with another type of vaccine, such as live attenuated or subunit vaccine, to modulate the immune system and provide better protection than either alone. This strategy has been used in other systems with success. Meseda et al. (2002) used the HSV-2 plasmid encoding gD as a primary vaccination followed by a boost with a vaccinia virus that expresses the gD protein. Highest levels of antibody were produced when the vaccinia construct was used as the primary vaccine, followed by a boost with either the DNA vaccine or the vaccinia construct.

It has been postulated that DNA vaccines are not as effective as other types of vaccines because they do not self-replicate, and therefore do not spread to stimulate a stronger immune response. An interesting twist put on a DNA vaccine by Hung et al. (2001) involved creating a plasmid vaccine that had the potential to spread to other cells, thereby enhancing transfection and vaccine potency. This team created a novel fusion of VP22, a HSV-1 protein that is involved in
intracellular transport, with a model antigen and tested efficacy of the DNA vaccine. With the addition of VP22, the plasmid vaccine was able to amplify itself and spread the way HSV-1 might in a normal infection, and the way that live-attenuated, replication competent viral vaccines amplify themselves. This group showed that the addition of VP22 as a fusion protein on the model antigen, there was a dramatically increased the number of model antigen specific cytotoxic T-cell precursors and enhanced vaccine potency. In another approach, Suter et al. (1999) utilized a bacterial artificial choromosome (BAC) containing a replication-competent but packaging defective virus genome of HSV-1 (BAC-VAC) to induce protective immunity. BACs are large plasmids capable of carrying and expressing large inserts. With a mutated genome of HSV-1 encoded, the BAC can mimic a modified live virus vaccination. Protective immunity was elicited by gene gun vaccination of the BAC-VAC, opening the door for this type of molecularly defined vaccine. The use of increasingly engineered and defined methods to manipulate the immune response to protection against CCV is a possible direction for further investigation of DNA vaccines against CCV. Future studies should focus on the molecular manipulation of antigens to combat disease and the reduction of virulence of available live attenuated vaccines.
REFERENCES


Bowser, P. R., and J. A. Plumb. 1980. Growth rates of a new cell line from channel catfish ovary and channel catfish virus replication at different temperatures. Canadian Journal of Fisheries and Aquatic Sciences 37:871-873.


Kuroda, N., F. Figueroa, C. O’hUigin, and J. Klein. 2002. Evidence that the separation of MHC class II from class I loci in the zebrafish, Danio rerio, occurred by translocation. Immunogenetics 54:418-430.


Lorenzen, E., K. Einer-Jensen, T. Martinussen, S. E. LaPatra, and N. Lorenzen. 2000. DNA vaccination of rainbow trout against viral hemorrhagic septicemia virus: a


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


thymus-dependent and thymus-independent antigens. Journal of Immunology 133:2356-2359.


VITA

Heather Christina Harbottle was born on June 18, 1976, to Sharon and Philip Harbottle in Fairfax, Virginia. Soon after her birth, Heather and her family moved to Cleveland, Ohio where the family lived for 18 years. In 3 years, her sister Candice was born, making the family complete. Heather graduated from Mentor High School with honors in 1994, during which she enjoyed singing in the Top 25 show choir and acting in musical theater. Heather then attended Texas A&M University in Galveston, where she graduated with honors in 1998 in the field of marine biology. Keeping with her aquatic interests but delving into the world of diseases, Heather joined the graduate program at Louisiana State University under the direction of Ronald Thune, studying Channel Catfish Virus. Upon graduation, Heather has accepted a job with the FDA in Laurel, Maryland, studying genetic and phenotypic characteristics of food borne bacteria.