Vibrio spp. disinfection and immunization of cobia (*Rachycentron canadum*) for the prevention of disease in aquaculture facilities.

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ABSTRACT

Cobia (*Rachycentron canadum*) is a tropical marine fish, with increasing commercial aquaculture importance worldwide. One of the major limitations to intensive aquaculture is disease. Diseases spread rapidly in an aquatic environment and pose a major threat to the development and introduction of new species, such as cobia, in aquaculture. This is due to the necessity to use wild caught broodstock, which pose a greater threat to introducing disease to a facility. Bacteria of the genus *Vibrio* play a major role in the diseases of cultured cobia and other species of marine fish. The goal of this study is to reduce the incidence of disease in a population, by either eliminating the potential pathogen or increasing the resistance of the host. To reach that goal, a disinfection assay to evaluate the effectiveness of nine common aquaculture chemical disinfecting compounds was evaluated against two bacterial pathogens (*Vibrio anguillarum* and *V. ordalii*). Both bacterial species were susceptible to a variety of common disinfecting compounds including Chloramine-T®, chlorine, ethanol, iodine, Lysol®, Roccal®-D Plus, and Virkon-S®. In addition, both species showed a resistance to disinfection with formalin and tap water. The humoral immune response of cobia to vaccination with a commercially-available vaccine for *Vibrio spp.* was evaluated by an ELISA. There was a significant difference between control and vaccinated groups ($P<0.0001$), showing significant antibody production resulting from vaccination.

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Chapter 1

Review of Cobia Life cycle, Culture, and Disease

1.1 Life cycle

Cobia (*Rachycentron canadum*) is a tropical marine fish that has been primarily considered a sport fish. Cobia is the only member of the family Rachycentridae, and is know as "ling," "lemon fish," or "crab-eater." There are no large commercial cobia fisheries, as the fish are not abundant. However, they are found globally in warm marine waters and have good aquaculture potential. The species is widely distributed in the Pacific Ocean as well as the southern Atlantic Ocean (Liao *et al*., 2004). Cobia is found in areas with salinities ranging from 22-44 ppt and temperatures varying from 16-32ºC.

Cobia reproduce in the wild from April to September, with males generally being 1-2 years of age and females being 2-3 years of age at sexual maturity. They typically spawn in small groups and can reproduce multiple times in a given spawning season. The female releases 400,000 to 5 million eggs after spawning (Kaiser and Holt, 2005).

Cobia has current aquaculture importance worldwide. In Taiwan, artificial cobia culture is well defined. Broodstock, either wild caught or captive raised, must be at least 2 years of age and 10 kg before captive spawning takes place. Fish are spawned in land-based ponds with flow-through water at 100 fish per pond in a 1:1 (male: female) sex ratio. Spawning takes place spontaneously year round with peaking in the spring and fall when water temperatures are between 23-27ºC. The fertilized eggs are collected by net and incubated in tanks for 21-37 hours. Larvae are reared in ponds for 20 days. The

ponds are maintained as "green water" (*Chlorella sp.*) with minimal water exchange and abundant copepods for food. Feeding begins 3 days post-hatch, and larvae are fed rotifers and copepod nauplii. During this stage, larval survival is 5-10%. The first phase of the nursery starts with larvae at 0.2 g and grows them to 2-5 g in outdoor ponds in 25 days. At this time, the fry are weaned to floating pellet feed. The fish are size graded every 4-7 days and fed manually 5-6 times daily to satiation. The second phase of the nursery continues for another 30 days, increasing the size of the fish to 30 g, with size grading conducted once during this stage. The third phase is continued in outdoor ponds or near shore cages and increases the size of the fish to 600-1000 g in 75-105 days. During the third nursery phase, size grading is conducted at least once to reduce cannibalism. Fish stocked to cages less than 30 g are more susceptible to strong water currents, increased cannibalism, bacterial infections, and parasitic infections. Final grow out is completed in open ocean cages until the fish reach 6-10 kg, which typically occurs in 6-8 months (Liao *et al*., 2004.) Recently, recirculating systems have been used in a nursery setup raising cobia fry (4-8 g) under intense conditions (370 fish/m^3) to produce juveniles (100-150 g) with survival rates above 95% for stocking into grow out cages (Liao *et al*., 2004). Fish are selectively harvested when over 6 kg for local market and export.

1.2 Market

The current market for cobia is restricted to very limited wild fishery and a few production facilities. Cobia is a valuable food fish and in the US market can sell for

$5.50/kg for whole gutted 6-7 kg fish shipped to wholesale markets in Miami, FL from culture production in Puerto Rico (Kaiser and Holt, 2005). Taiwan cobia culture was devastated by disease in 2002, reducing production from nearly 3000 metric tons in 2001, to only 1000 metric tons in 2002. Due to this, most of the cobia produced in Taiwan during this time was used for their domestic market. In Taiwan, the price for domestically farmed cobia is similar at $5.45 US/kg for 8 kg and larger fish, $5.15 US/kg for fish 7-8 kg, and $4.84 US/kg for fish 6-7 kg (Liao *et al*., 2004).

1.3 Aquaculture

The cobia aquaculture industry in the US is in its infancy, and much of the effort is presently focused on research to determine methods to maximize growth rates and reduce mortalities. There are two main cobia culture techniques. The first technique is intensive tank culture, including flow through and recirculating systems, which focuses on all life stages of cobia. The second technique is extensive culture, which includes open ocean cages and ponds, which primarily focuses on grow out. Marine cages have been used for over a decade in Taiwan (Lin *et al*., 2006), and have recently been gaining popularity in the US commercially for cobia.

The high fecundity and fast growth rate of cobia contributes to its large production potential in commercial aquaculture. Broodstock is generally collected during the warm summer months and transferred to tanks or ponds for captive spawning. Spawning is controlled by manipulation of photoperiod and temperature (27-32ºC) to simulate spring and fall, the natural spawning season (Kaiser and Holt, 2005). Cobia

larvae have a low survival of 5-10% for the first 20 days. The preferred culture temperature for grow out is 20-32ºC. When reared in culture systems, salinity can be maintained as low as 5 ppt (Kaiser, 2005), however greatest survival rates are associated with a salinity of 15-30 ppt (90-92% survival) (Resley *et al*., 2005). Harvestable adults can be obtained in 6-8 months at a weight of 6-10 kg (Kaiser and Holt, 2006).

Cobia require a high protein diet for best growth in captivity (Schwarz, *et al*., 2007). Typical feed consists of 40% protein from fishmeal. However, research has been conducted to evaluate non-animal protein sources, and cobia do well with up to 25% yeast–based protein (Lunger *et al*., 2006), which will have a future impact on the sustainability of this species in aquaculture.

1.4 Diseases

One of the biggest threats to the intensive aquaculture industry is infectious disease. Potential pathogens can be grouped in to three main categories: viral, parasitic, and bacterial. Cobia is susceptible to all three groups of pathogens. The reported bacterial diseases of cobia include mycobacteriosis, vibriosis, pasteurellosis, and streptococcosis, which are caused by pathogens including *Mycobacterium marinum, Vibrio anguillarum* and *V. ordalii, Pasteurella piscicida* and *Streptococcus spp.* (Liao *et al*., 2004, Lowery and Smith, 2006). The viral disease lymphocystis, and the parasitic diseases myxosporidosis, *Trichodina spp.*, *Neobenedenia spp.,* and *Amylodinium spp.* also can affect cobia (Kaiser and Holt, 2005).

The genus *Vibrio,* belonging to the family Vibrionaceae, is a group of Gram-negative, facultatively anaerobic, curved, rod-shaped bacteria that are oxidase positive and non-spore forming (Kaysner and Angelo, 1998). This genus includes the human pathogens *V. cholerae, V. mimicus, V. parahaemolyticus,* and *V. vulnificus,* as well as fish pathogens *Listonella anguillarum* (formerly *V. anguillarum*), *V. ordalii, V. damsela, V. carchariae, V. vulnificus, V. alginolyticus,* and *V. salmonicida* (Reed and Francis-Floyd, 2002). *Vibrio spp.* pathogens also affect other species of marine fish, penaeid shrimp, as well as abalone (Liu *et al*., 2004). In addition, *Vibrio spp*. bacteria account for a significant portion of the food-borne infections from eating raw or undercooked shellfish (Thompson *et al*., 2004).

Vibrio (*Listonella*) *anguillarum* is a halophilic Gram-negative, curved rod with polar flagella. *Vibrio ordalii* was formerly known as *V. anguillarum* biotype 2, with reclassification based on both phenotypic characteristics as well as DNA relatedness (Schiewe *et al*., 1981). The common non-specific clinical signs of *Vibrio spp.* infection in cobia include lethargy, darkened skin, abdominal distension, damaged eyes, and ulcerations of the skin. High mortalities are often observed in cultured fish, with 100% morbidity (Reed and Francis-Floyd, 2002) and mortality commonly over 80% (Liu *et al*., 2004). Fish less than 4 months old, <500 g, appear to be the most susceptible with the highest mortalities to these bacterial pathogens (Lin *et al*., 2005).

Vibrio (*Listonella*) *anguillarum*, one of the causative agents of vibriosis, is a primary pathogen of marine fish in salt and brackish waters. Disease outbreaks often occur in late summer in shallow near shore waters when water temperatures increase. Vibriosis affects close to 50 species of salt and freshwater fish, and is a major obstacle

for marine salmonid culture (Woo and Bruno, 1999). *Vibrio* (*L.*) *anguillarum* has been isolated from moribund striped bass (*Morone saxatilis*) from the Chesapeake Bay, exhibiting symptoms consistent with vibriosis, including hemorrhaging of the fins, eyes, and ventral surfaces, as well as internal petechiae (Toranzo *et al*., 2003). *Vibrio* (*L.) anguillarum* was first reported in North America in chum salmon (*Oncorhynchus keta*). The first reported case of vibriosis (*Vibrio alginolyticus)* in cobia occurred in 2001 in Taiwan (Liu *et al*., 2004).

While the precise route of transmission is unclear, it is suspected that *Vibrio spp.* infections are initiated through the oral route (Reed and Francis-Floyd, 2002). *Vibrio spp.* can be found in normal gut flora, but under certain conditions can cross the intestinal wall resulting in a systemic infection. Infected fish shed bacteria into the water, allowing the pathogen to infect other fish. Most of the pathogenic *Vibrio spp.* increase in number during warmer summer temperatures and spread well in warm water recirculating systems.

While a presumptive diagnosis can often be obtained by looking at the case history and clinical signs, a proper diagnosis of *Vibrio* infection is only accomplished by isolation, culture, and specific identification. Isolation of *Vibrio spp.* from mixed culture can be accomplished by utilizing a salt enriched selective media such as citrate bile salt media; further identification can be completed by use of bench top tests as well as molecular techniques (Thompson *et al*., 2004).

Vibrio anginolyticus has been reported as another major pathogen for cultured cobia in Taiwan (Liu *et al*., 2004), however it is not closely related to *V.* (*L.*) *anguillarum* and *V. ordalii* (Schiewe *et al*., 1981). Diseased cobia infected with *V. anginolyticus*

presented with lethargy, darkened skin, acites in the peritoneal cavity, and some fish exhibited damaged eyes. The disease can be present in cobia of varying sizes. In the study by Liu *et al*. (2004), *V. anginolyticus* was seen in cobia 100-120 g, as well as in cobia 8-12 g, from two different outbreaks in Taiwan during the summer season. Lin *et al*. (2006) also observed outbreaks associated with *Vibrio spp.* in early grow out of cobia, under 4 months of age and below 500 g.

Vibrio vulnificus has also been identified as a fish pathogen, as seen in ovate pompano (*Trachinotus ovatus*) in cage culture (Li *et al*., 2006). In fish infected with *V. vulnificus,* pathology is typically associated with external hemorrhages of the gill, head, ventral body, and base of the fins, especially the pectoral fins, along with hemorrhage in the liver and intestine. Symptoms may occasionally include ulceration of the dorsal and ventral body, deep muscle necrosis in the head region, as well as petechiae of the gills and fins (Li *et al*., 2006).

1.5 Immunity

Fish possess both innate and acquired immune systems. Innate immunity is non-specific and has no immune memory, while acquired immunity is specific and has memory. The innate immune system includes leukocytes, while acquired immunity includes B-cells and T-cells. B-cells produce antibodies for the humoral immune response, along with T-cells, which are involved with cell-mediated immunity.

Cell-mediated immunity is important for protection against intracellular pathogens, such as viruses and intracellular bacteria like *Mycobacterium spp*. Though the

development of cell-mediated immunity in cobia is not documented, the cell-mediated immune system in trout and carp develops by 4 weeks of age (Ellis, 1988).

Acquired, or humoral, immunity is affected by the age at which the fish is immunized. In rainbow trout fry for example, an immune response is present at 4 weeks of age, but in order to obtain a memory effect, the fish must be immunized after 8 weeks of age (Ellis, 1998). It is also important not to immunize too early, as a tolerance to the antigen may be induced. In rainbow trout, tolerance can be induced when injected with antigen at 21 days of age (Ellis, 1998). The type of antigen also plays a role in the time for first immunization. Bacterial lipopolysaccharide, or T-independent antigens, as well as soluble proteins, or T-dependent antigens, each have a different potential vaccination minimum age. The T-independent antigens, like a bacterin, are less prone to inducing tolerance at a young age than T-dependent antigens (Ellis, 1988).

Teleost fish have three major lymphoid organs: the thymus, kidney, and spleen. The lymphocytes develop in the thymus and are transported to peripheral blood and other lymphoid tissues. Antibody production does not occur in the thymus. The kidney is the major location of antibody production in the fish, as well as filtration by macrophages. The spleen also contains macrophages and works to trap immune-complexes. Once the lymphoid tissues in the kidney have matured, intake of antigen across the gill becomes an effective route of vaccine administration. This occurs by 8 weeks of age in carp and trout (Ellis, 1988).

The age at vaccination also affects the length of protection, as seen in vaccination against enteric red mouth (*Yersinia ruckeri*) and vibriosis (*Vibrio spp.*) in salmonids. At 1 g, fish are protected for 180 days; while at 4 g, fish are protected for over 1 year (Ellis,

1988). The length of protection is an important economic consideration for an aquaculture producer. The goal of vaccination is to provide protection through the most susceptible ages. In addition, if fish are vaccinated too early, then they will need to be revaccinated in order to maintain protective immunity throughout the production cycle, thus increasing the cost to the producer.

The goal of vaccination is to stimulate the acquired immune system to form B-cells and memory B-cells. The memory cells produce additional antibodies if the animal is exposed to the antigen again, for continuation of protective immunity over time. Similarly, T-cells will also form a memory towards antigens presented in cell-mediated immunity. However, no antibodies are produced.

1.6 Disinfection

Disinfection is a process where an antimicrobial agent is applied to non-living objects to reduce or eliminate microorganisms. Disinfection procedures include ozonation, ultraviolet (UV) irradiation, physical disinfection, and chemical disinfection. Ozone and UV are often used to disinfect raw seawater to prevent the introduction of pathogens into the fish culture systems.

Physical disinfection includes heat, radiation, and filtration. Salmon farms often use steam disinfection on large marine net pens where chemical disinfection is not suitable, due to cost and discharge of disinfectants. Net pens are removed from the water, and steam disinfected in sections. This is effective against the enveloped virus causing infectious salmon anemia, and was used after a disease outbreak in 2001 (Danner and

Merrill, 2006). Pasteurization, as well as autoclave sterilization, also fit in this category. Pasteurization reduces or destroys specific pathogens without altering the properties of the liquid. Autoclave sterilization destroys all organisms and spores by raising pressure one atmosphere and thus the boiling point of water to 121ºC. Dry heat is a less effective form of disinfection, as many organisms are resistant, so a higher temperature must be reached. Baking, flaming, and incineration are effective ways to sterilize objects that can withstand the treatment. Composting also will destroy bacteria, as long as an appropriate temperature is reached.

Natural sunlight is another physical disinfectant, and is often used on large aquaculture nets between uses. Artificial ultraviolet (UV) light disinfection is also effective in some situations and acts by damaging DNA. Similarly, beta and gamma radiation breaks strands of DNA and is more effective at penetrating through materials. Ionizing radiation is used to destroy anthrax spores in mail, and can disinfect soil, concrete, steel, lead, and water.

Filtration, another type of physical disinfection, can be used to remove pathogens from fluids and gases. Filtration to 0.25µ will remove bacteria, but not viruses. High efficiency particulate filtration is used to remove pathogens from air.

There are many chemical disinfectants currently utilized in aquaculture. Common ones include halogens such as chlorine and iodine, quaternary ammonium compounds, alcohols such as isopropanol and ethanol, phenolic compounds such as cresol (used in Lysol ®), oxidizing agents such as peroxide and peracetic acid, and alkylating agents such as formalin, glutaraldehyde, and ethylene oxide.

Halogens are good disinfectants only when all organic material and biofilm are removed prior to use. Household bleach, 5.25% aqueous sodium hypochlorite, contains 52,500 ppm available chlorine. Other, more concentrated, liquid or powder forms are better suited for general disinfection use due to reduced shipping costs. Other chlorine halogens include Chloramine-T® and chlorine dioxide. Iodophores are compounds that combine iodine with a carrier, such as with povidone iodine. Halogens also have an advantage as they also can be easily removed from water prior to discharge or use for fish by addition of sodium thiosulfate.

Quaternary ammonium compounds are considered surfactants, with a hydrophilic and hydrophobic region. They have a limited antimicrobial spectrum, and are most useful for cleaning non-critical areas such as floors and walls (Danner and Merrill, 2006).

Alcohols such as isopropyl alcohol and ethanol have been useful when diluted with water. They have a broad spectrum of effectiveness, but do not destroy spores, and are best used on small areas to clean and disinfect hard surfaces.

Phenolic compounds are not widely used in aquaculture due to the toxicity to fish even at low concentrations and the residue left behind. However, they are effective against *Mycobacterium spp*. where other disinfectants have limited effectiveness, and are worth considering for specific applications.

Oxidizing agents, such as hydrogen peroxide, are useful as they destroy membrane lipids and DNA. The byproduct of the oxidation is water, so there is little concern for contamination and discharge. Ethylene oxide, a gas, is useful for disinfecting delicate instruments, as it is non-corrosive.

Acids are effective against most bacterial species and enveloped viruses, but not against non-enveloped viruses or *Mycobacterium spp.* (Danner and Merrill, 2006). Peracetic acid is both an oxidizing agent as well as an acid, as it is often supplied as a solution of acetic acid and hydrogen peroxide. Peracetic acid is an effective disinfectant and has FDA approval for use on reusable medical and dental devices. Virkon S® also acts both as an acid and an oxidizer, and is useful in aquaculture, as it remains effective at temperatures below 0ºC (Danner, 2006). Alkalis are useful for killing most bacteria as well as enveloped viruses. Acids and alkalis can damage surfaces, such as metal tabletops, as well as tissues, and so must be used only on surfaces that will not be damaged.

Aldehydes, including formaldehyde and glutaraldehyde, are useful in disinfection and preservation of tissues by coagulation of proteins. Formaldehyde gas, or as a 37% solution known as formalin, is useful for preservation of tissues, and at lower concentrations is used in fish to treat external parasites and fungi. Its use as a disinfectant is limited, as vapors pose a health risk to persons using it. Glutaraldehyde is useful as a disinfectant, as its effectiveness is not reduced by the presence of organic matter. However, glutaraldehyde is toxic to both humans and animals and is seldom used outside medical facilities for disinfection.

Disinfectants need to be selected based on their effectiveness against specific pathogens (Danner and Merrill, 2006). The list of possible disinfectants is further reduced by what is effective and appropriate for use in the aquaculture industry. Surface damage to equipment and tanks caused by disinfection is a concern. Disinfectant discharge is also an important consideration, and is regulated by the Food and Drug

Administration, as well as the Environmental Protection Agency (Danner and Merrill, 2006). Most disinfectants are toxic to animals, necessitating the removal of the animals from the facility prior to disinfection, as well as being dangerous to the people, requiring proper personal protection. However proper disinfection is much less expensive than treatment of an infected population, or loss of that population due to disease outbreak.

In salmon hatcheries, disinfection is important to remove pathogens from the surface of eggs. This helps break the vertical transmission of certain bacterial pathogens from broodstock to egg and thus fry. Iodophores have been used extensively to reduce or eliminate *Aeromonas salmonicida,* the causative agent for furunculosis, from the surface of eggs (Cipriano *et al*., 2001). The use of iodophores are also effective against *Yersinia ruckeri* (Danner and Merrill, 2006). Disinfection is commonly done in a two-step process, one treatment at the spawning facility and another at the nursery after transportation of the eggs. Disinfection of the eggs will not protect against pathogens passed within the egg, such as infectious pancreatic necrosis virus, bacterial kidney disease, and bacterial cold-water disease (Cipriano *et al*., 2001).

Surface cleaning is also important prior to disinfection. Soaps and detergents will aid in cleaning of a soiled surface, and removal of up to 99% of infectious material (Danner and Merrill, 2006). Soaps and detergents often are used to clean a surface prior to disinfection, in order to obtain the most effective treatment. Concentration and time of exposure play an important role in the efficacy of any given chemical disinfectant.

1.7 Vaccination

Another method used in the prevention of disease is vaccination to increase the immune response and resistance of the host to a particular pathogen. Vaccination has been used for more than two centuries in humans. The first fish vaccine developed was for enteric red mouth (*Yersinia ruckeri*) in salmonids during the late 1970's. The first vaccine for prevention of vibriosis in salmonids was available in 1988 by Norvax® Vibriose (Intervet, Bergen, Norway).

There are several types of vaccines, which include attenuated live vaccines, killed vaccines, subunit vaccines, live recombinant vaccines, and DNA vaccines. A killed bacterial vaccine (bacterin) is currently commercially available for *V. anguillarum* and *V. ordalii*, and has been demonstrated effective in prevention of vibriosis in juvenile Atlantic halibut (Bricknell *et al*., 2000), as well as salmon (Bravo and Midtlyng, 2007). Cobia have been shown to develop protective immunity from vaccination with inactivated *Vibrio* bacterins (Lin *et al*., 2005).

The United States Department of Agriculture (USDA) licenses fish vaccines for use in food fish. The USDA currently lists vaccines for salmonids to protect against *Aeromonas salmonicida, Vibrio anguillarum, V. ordalii, V. salmonicida,* and *Yersinia ruckeri.* There is also a vaccine approved for prevention of *Edwardsiella ictaluri* infection in catfish. Presently there are no approved vaccines for use in cobia.

Different routes can be used to administer vaccines. Immersion vaccination, or bath administration, is one of the most commonly used vaccination routes. Fish can be dipped in a defined concentration of vaccine for a short period of time. Alternatively,

bath vaccination is possible using a lower concentration and longer exposure time. This method could be used prior to transporting juvenile fish to grow out locations with minimal stress to the fish. Tank water level is reduced to a minimum safe level with aeration and the vaccine is added to the tank water. In Norway, pre-smolt salmon are vaccinated in a ratio of 1:500 vaccine to tank water for 1 hour prior to transfer to sea water (Ellis, 1988). Intraperitoneal (IP) injection is commonly used in commercial aquaculture production as well as research settings. This technique delivers a specific amount of vaccine to each individual fish and uses less vaccine overall than immersion vaccination. Intramuscular (IM) vaccination is another route, but is less commonly used due to potential damage to the muscle (fillet) as well as potential leakage of the vaccine from the site of injection prior to absorption.

1.8 Hypothesis and Goal of Research

- Hypothesis 1. Some disinfectants will be more effective than others in killing *V. anguillarum* and *V. ordalii*, exhibiting varying bactericidal effects at different concentrations or exposure times.

- Hypothesis 2. An immune response will develop in hybrid striped bass and cobia after vaccination by either IP and/or bath routes with a *V. anguillarum*/ *V. ordalii* vaccine.

The goal of this study was to develop recommendations for the most effective disinfectant(s) for an aquaculture setting, as well as to investigate the effectiveness of vaccination using a commercially available vaccine as a way to prevent disease and economic loss from vibriosis in cultured cobia.

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Chapter 2

Vibrio anguillarum and *V. ordalii* disinfection for the prevention of disease in aquaculture facilities

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2.1 Introduction

Vibriosis, a disease caused by numerous species of *Vibrio*, is a primary disease of marine fish in salt and brackish waters. Disease outbreaks often occur in late summer in shallow near shore waters when water temperatures increase. Vibriosis has been reported in over 50 species of salt and freshwater fish, and is a major obstacle for marine salmonid culture (Woo and Bruno, 1999).

Vibrio (*Listonella*) *anguillarum* is a halophilic Gram-negative, curved rod with polar flagella. Vibriosis caused by this bacterial species has been identified in many finfish species including turbot (*Scophthalmus maximus*), eels (*Angullla anguilla*), and salmonids (*Oncorhynchus nerka*) (Austin and Austin, 1987, Tiecco *et al*., 1988, Antipa *et al*., 1980). High mortalities are often observed, with 100% morbidity (Reed and Francis-Floyd, 2002) and mortality commonly over 80% in cultured cobia, *Rachycentron canadum* (Liu *et al*., 2004). Cobia (*Rachycentron canadum*) less than 4 months old, < 500 g, appear to be the most susceptible, with the highest mortalities to this bacterial pathogen (Lin *et al*., 2006). Clinical signs present as hemorrhagic septicemia, skin discoloration, red necrotic lesions in the abdominal muscle, erythema at the base of the fins, vent and in the mouth, abdominal distension, and exophthalmia may be present (Austin and Austin, 1987).

Vibrio ordalii was formerly referred to as *Vibrio anguillarum* biotype 2, and has been reclassified as a distinct species (Schiewe *et al*., 1981). *Vibrio ordalii* is another causative agent for vibriosis in fish. It can be distinguished in culture by biochemical

characteristics as well as DNA sequence relatedness (Schiewe *et al*., 1981). The type strain (LMG 13544) of *V. ordalii* was isolated from coho salmon (*Oncorhynchus rhoddiurus*) from Washington in 1973 (Thompson *et al*., 2004). Clinical signs are similar to *V. anguillarum* with differences including microcolony formation on skeletal and heart muscle as well as gills and gastrointestinal tract, slower progression of bacteremia, and marked leucopenia (Austin and Austin, 1987).

Disinfection is the process where an antimicrobial agent is applied to a non-living object or surface to reduce or eliminate microorganisms. A variety of disinfection procedures are applicable to aquaculture situations including chemical disinfection, ultraviolet (UV) exposure, irradiation, and ozonation. Ozone and UV are commonly used to disinfect raw seawater to prevent the introduction of pathogens into fish culture systems. In addition, a variety of chemical disinfectants is currently utilized in aquaculture, with concentration and time of exposure playing an important role in the efficacy of the given disinfectant.

Common disinfectants used in aquaculture include halogens such as chlorine and iodine, quaternary ammonia compounds, alcohols such as isopropanol and ethanol, phenolic compounds such as cresol, benzyl-4-chlorophenol-phenylphenol (used in Lysol ®), and alkylating agents such as formalin, glutaraldehyde, and ethylene oxide (Ellis, 1988). Most disinfectants are toxic to animals as well as dangerous to the people using them. This may require removal of the animals from the facility prior to disinfection, as well as proper personal protection for all individuals during the disinfection process. Thus, the list of possible disinfectants is reduced by what is appropriate for use in the aquaculture industry and those that are relatively non-toxic to both animals and humans.

Sanitization is the process where a surface is disinfected after a clean up procedure has already taken place. The clean up could consist of a simple physical cleaning or scrubbing and removal of biofilms or use of a chemical descaling agent, from all wet contact surfaces of a given system. The sensitivity of the bacteria to a given disinfectant is examined in as planktonic cells.

The goal of this study was to examine the efficacy of common aquaculture compounds for disinfecting against two bacterial species causing vibriosis to provide a recommendation of the most effective compound(s) for the prevention of vibriosis in an aquaculture setting.

2.2 Materials and Methods

Cultures of *Vibrio* (*Listonella*) *anguillarum* and *Vibrio ordalii* were obtained from the National Fish Health Research Laboratory in Kernersville, WV. Cultures were inoculated on brain heart infusion agar (Fisher Chemicals, Fair Lawn, NJ) with 1% NaCl (Fisher Chemicals, Fair Lawn, NJ) (BHIA + 1% NaCl), and grown for 24 hours at 25ºC. Ten milliliters brain heart infusion broth with 1% NaCl (BHI + 1% NaCl) was inoculated from the plate and grown for 24 hours at 25ºC. Bacteria were harvested by centrifugation at 1900 x g for 10 minutes at room temperature (22°C). Bacteria were washed twice in 10 ml sterile phosphate buffered saline (PBS, Sigma, St. Louis, MO), and the final pellet resuspended in 5 ml sterile PBS (stock solution). One milliliter of stock solution was added to 6 ml sterile PBS, working solution. Then 100 µl of working solution was added to each of 3 labeled (A, B, C) sterile 1.5 ml microcentrifuge tubes. For the control, Tube

A, 900 µl sterile PBS was added. Next, 100 µl from Tube A was taken and added to 9.9 ml sterile PBS and 10 x serial dilutions were made to 10^{-5}. Serial dilutions were made with 100 µl of previous concentration, in 900 µl of sterile PBS. Dilutions were plated with a multi-channel pipette in 10µl drops, four dilutions, and five rows to a plate. To Tube B and Tube C, 900 µl of individual disinfectant was added. The disinfectants used were Chloramine-T ®, Clorox ®, ethanol, formalin, iodine, Lysol ®[1], Roccal®-D Plus [2], sterile autoclaved tap water, and Virkon S ®[3], with the concentrations used listed in Table 1. Samples were diluted and plated as with the control at 1, 5, 10, 20, 30, and 60 minutes exposure time. After the 60 minute samples were made, another dilution was taken of the control, Tube A, and plated. Colonies were counted after 24 and 48 hours incubation at 25ºC for separate trials of *V.* (*L.*) *anguillarum* and *V. ordalii*, respectively and the number of colony forming units (CFUs) per ml was calculated.

2.3 Results

The results of the disinfection assay (Table 1) illustrate that Chloramine-T®, Clorox®, ethanol, iodine, Lysol®, Roccal®-D Plus, and Virkon-S® eliminated all growth of both species of bacteria at exposure times of 1 minute and longer. Formalin reduced bacterial growth at only 60 minutes, and was not effective in elimination of either of the species of bacteria within 60 minutes. Tap water demonstrated bacterial growth only to 10 minutes with *V. anguillarum* and to 5 minutes for *V. ordalii*, with no growth of either bacteria after those times. Control plates (PBS only) showed no significant change in CFU count over 60 minutes in any of the trials.

[1] Lysol ®: Ethanol/SD alcohol, isopropyl alcohol, p-chloro-o-benzylphenol, potassium hydroxide
[2] Roccal® D-Plus: Alkyl dimethyl benzyl ammonium, didecyl dimethyl ammonium, tributyltin oxide
[3] Virkon-S®: Potassium peroxomonosulfate, sodium dodecylbenzenesulphonate, sulfamic acid

2.4 Discussion

Both *V. anguillarum* and *V. ordalii* were susceptible to a number of common aquaculture chemicals including disinfectants and chemotherapeutic tested in this study. Clorox®, ethanol, iodine, Lysol®, Roccal®-D Plus, Virkon-S® were all effective at killing both species of *Vibrio* at 1 minute. Formalin and Chloramine-T® were also tested, as they have been commonly utilized chemotherapeutics in aquaculture as a disease treatment. Formalin is used to treat external protozoan parasitic infections as well as prevention of fungal infection on fish eggs, while Chloramine-T® has been used to treat external bacterial infections. Formalin was not effective at elimination of *Vibrio spp*. as was to be expected, as it was being used at a concentration typical for treatment of living fish for external parasites, however Chloramine-T® was effective at elimination of *Vibrio spp*.

It was noted that *Vibrio spp.* were susceptible to autoclaved sterilized tap water (Blacksburg, VA municipal city). This was probably a result of osmotic imbalance, as *Vibrio spp*. used in this study were cultured in salt enriched media, and washed in sterile PBS. It was also noted that washing of the bacteria in sterile de-ionized water also caused killing of the bacteria.

Each pathogen needs to be taken into consideration for disinfection. *Vibrio spp.* act differently than other bacterial species which may have different levels of resistance to disinfection. For example, *Mycobacterium marinum* was resistant to many disinfectants and only susceptible to Lysol® and 50% ethanol (Mainous and Smith, 2005). In another study, *Edwardsiella spp.* was susceptible to most disinfectants, but not

to Chloramine-T® and formalin (Mainous and Smith, accepted). *Aeromonas salmonicida* has been documented to be susceptible to disinfection with the use of iodophor (povidone iodine) to reduce the incidence of disease from contaminated salmon eggs (Cipriano *et al*. 2001).

Due to its high susceptibility to disinfection, *V. anguillarum* and *V. ordalii* would be eliminated by standard disinfection practices at manufacturers' recommended dosages. The price of the disinfectant as well as discharge regulations would be the primary concerns for choosing a disinfectant for these species of *Vibrio*. Additional precautions would need to be taken if other bacterial pathogens were suspected to be present in order to properly disinfect the facility.

Sanitization is the process where a surface is disinfected after a clean up procedure has already taken place. The clean up, or removal of organic matter from all wet contact surfaces of a given system, is important prior to sanitization with a chemical disinfectant, as many are deactivated by excess organic matter. The sensitivity of the bacteria to a given chemical disinfectant was examined as planktonic cells, not as a biofilm. The removal of organic mater and biofilms prior to disinfection to allow the disinfectant to work properly. This does pose some difficulties, as tanks, filters, and plumbing must be cleaned thoroughly for maximum disinfectant effectiveness. This can be conducted by scrubbing with the use of a detergent, or use of descaling agent, which should be addressed in future studies.

Disinfection is an important part of biosecurity to prevent disease outbreaks.

Proper disinfection can be expected to be less expensive than economic cost due to antimicrobial treatment of an infected population, or loss of part or all of that population due to disease outbreak.

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Table 2.1: Results of disinfection assay using various aquaculture compounds for efficacy against *V. anguillarum*, and *V. ordalii*. The results indicate the last time sample with the presence of growth.

	Bacteria Species	
Disinfectant	***Vibrio* (*L.*) *anguillarum***	***Vibrio ordalii***
Chloramine-T® (0.0015g/100ml)	no growth *	no growth
Clorox® 50ppt	no growth	no growth
Clorox® 200ppm	no growth	no growth
Clorox® 100ppm	no growth	no growth
Clorox® 50ppm	no growth	no growth
Ethanol 70%	no growth	no growth
Ethanol 50%	no growth	no growth
Ethanol 30%	no growth	no growth
Formalin (250ppm)	Reduced growth (1 log) at 60min	reduced growth (1 log)at 60min
Iodine 50ppm	no growth	no growth
Lysol® 1%	no growth	no growth
Roccal®-D Plus 1:256 (3.9ppt)	no growth	no growth
Tap Water (autoclave sterilized)	growth at 10min	growth at 5min
Virkon-S® 1% (0.1g/10ml)	no growth	no growth

* no growth – indicates no colonies at any time periods and any concentrations.

Chapter 3

Immune response of hybrid striped bass (*Morone chrysops* x *M. saxtilis*) to vaccination with a commercial vaccine against *Vibrio anguillarum* and *V. ordalii*

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3.1 Introduction

One of the greatest threats to the intensive aquaculture industry is infectious fish disease. These can be grouped in to three main categories: viral, parasitic, and bacterial. Hybrid striped bass, like other species of fish, are susceptible to all three groups of pathogens. The reported major bacterial diseases of striped bass and their hybrids include mycobacteriosis, vibriosis, and streptococcosis, which are caused by pathogens including *Mycobacterium spp., Vibrio anguillarum* and *V. ordalii,* and *Streptococcus spp.* (Akhlaghi, 1999, Rodgers and Xu, 1992, Wolf and Smith, 1999).

The genus *Vibrio,* belonging to the family Vibrionaceae, is a group of Gram-negative, facultatively anaerobic, curved, rod-shaped bacteria that are oxidase positive and non-spore forming (Kaysner and DePaola, 1998). This genus includes the human pathogens *V. cholerae, V. mimicus, V. parahaemolyticus,* and *V. vulnificus,* as well as fish pathogens *V.* (*Listonella*) *anguillarum*, *V. ordalii, V. damsela, V. carchariae, V. vulnificus, V. alginolyticus,* and *V. salmonicida* (Reed and Francis-Floyd, 2002). *Vibrio spp.* pathogens also affect other species of marine fish, penaeid shrimp, as well as abalone (Liu *et al*., 2004). *Vibrio spp*. bacteria also account for a portion of the food-borne infections from eating raw or undercooked shellfish. (Thompson *et al*., 2004)

Vibrio (*Listonella*) *anguillarum* is a halophilic Gram-negative, curved rod with polar flagella. It has been isolated from moribund striped bass (*Morone saxatilis*) from the Chesapeake Bay, displaying symptoms consistent with vibriosis including hemorrhaging of the fins, eyes, and ventral surfaces, as well as internal petechiae (Toranzo *et al*., 2003).

Vibrio ordalii, formerly referred to as *Vibrio anguillarum* biotype 2, has been reclassified as a distinct species (Schiewe *et al*., 1981). *Vibrio ordalii* is another causative agent for vibriosis in fish. It can be distinguished in culture by biochemical characteristics as well as DNA sequence relatedness (Schiewe *et al*., 1981). The type strain (LMG 13544) of *V. ordalii* was isolated from coho salmon (*Oncorhynchus rhoddiurus*) from Washington in 1973 (Thompson *et al*., 2004).

While the precise route of transmission is unclear, it is suspected that *Vibrio spp.* infections are initiated through the oral route (Reed and Francis-Floyd, 2002). *Vibrio spp.* can be found in normal gut flora, and under certain conditions can cross the intestinal wall resulting in a systemic infection. This increases the numbers of bacteria in the water, which can then infect other fish.

As *Vibrio spp.* are naturally present, preventing exposure may not be possible. Vaccination is one method used to protect fish from disease outbreaks due a naive immune system or higher than normal bacterial concentrations. Vaccination is used to increase the immune response and increase the resistance of the host. A killed bacterial vaccine (bacterin) is currently commercially available for *V. anguillarum* and *V. ordalii*, and has been demonstrated effective in prevention of vibriosis in juvenile Atlantic halibut (Bricknell *et al*., 2000), salmon (Bravo and Midtlyng, 2007), Testing of an autogenous bacterin has been shown to give protection to cobia after challenge (Lin *et al*., 2005.)

Different routes can be used to administer vaccines. Immersion (bath) administration is a commonly used route in aquaculture. Fish can be dipped in a defined concentration of vaccine for a short period of time. Alternatively, longer bath administration is also possible, using a lower concentration and longer exposure time.

Intraperitoneal (IP) injection is also used in commercial aquaculture production and research settings. For IP injection a small volume of vaccine is injected into the coelomic cavity, sometimes referred to as intracoelomic or intraabdominal. Intramuscular (IM) injection is another option, but it is less commonly used due to damage to the muscle (i.e. fillet) as well as potential leakage of vaccine from the injection site prior to absorption of the vaccine.

The goal of this study was to determine when hybrid striped bass develop an antibody titer to *Vibrio spp.* after vaccination by two different administration routes with a commercial vaccine in an effort to examine vaccination as a way to prevent disease. The goal of this study was to examine vaccination of hybrid striped bass in an aquaculture setting as a way to prevent disease and economic loss from vibriosis in cultured hybrid striped bass.

3.2 Materials and Methods

Fish and vaccination groups

Hybrid stripped bass (HSB, *Morone saxatilis* x *M. chrysops*) were obtained from a local fish hatchery and transported to the Aquatic Medicine Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine in Blacksburg, VA. Fish were acclimated and grown for 7 months prior to vaccination to a suitable size for vaccination and blood collection. Fish weighed 82.6+/- 15.6g (mean +/- standard deviation) at the time of vaccination. A total of 234 fish were used in the study and these were arbitrarily divided into three groups of 78 fish per group. Control fish were injected

intraperitoneally (IP) with 0.1 ml sterile PBS (Sigma, St. Louis, MO, USA), and then placed into two 350 liter fiberglass tanks. A second group of fish were injected IP with 0.1 ml (Vibrogen-2, Aqua Health LTD.) per manufacturer's directions, then placed in a separate multi-tank recirculating system, three fish per 75 liter tank, with a total volume of 1800 liters. A final group of bath vaccinated fish (designated as Bath)were placed in the prepared vaccine (Vibrogen-2) diluted per manufacturer's directions (500 ml vaccine, 4500 ml clean hatchery water) for 30 seconds, then placed in a system identical to the IP group. Water temperature was maintained between 19-26°C (due to seasonal variation) and the fluorescent light photoperiod consisted of 12 hours light/12 hours dark. Fish were fed a commercial pellet (3 mm) feed (Zeigler, Gardners, PA, USA) daily at 3% body weight. Water quality parameters (ammonia, nitrite, nitrate, pH) were monitored daily using a commercial water analysis kit (HACH, Loveland, CO, USA).

Six fish were arbitrarily selected from each group, sedated with sodium bicarbonate buffered MS-222 (100 mg/l, Sigma-Aldrich, St. Louis, MO, USA) and bled (23 gauge needle, 1 ml syringe) from the caudal vessels pre-vaccination day 0, and post-vaccination at 4, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77 days. Fish were then humanely euthanized after bleeding by anesthetic overdose using sodium bicarbonate buffered MS-222. Collected blood was allowed to clot in serum separation tubes (Becton Dickinson Microtainer®, Franklin Lakes, NJ, USA) for 1 hour at room temperature, and then refrigerated overnight at 4ºC. The next day, clotted blood was centrifuged at 10,000 x g for 6 minutes, and the serum separated and placed in 1.5 ml microcentrifuge tubes (USA Scientific, Ocala, FL, USA). These were frozen at -20ºC until analyses after all bleeding times were completed.

Vibrio ELISA

Part A: Antigen preparation

A culture of *Vibrio (Listonella) anguillarum,* obtained from the National Fish Health Research Laboratory in Kernersville, WV was grown overnight in 50 ml of TSB + 2% NaCl. Bacteria were killed by addition of 37% formalin (Fisher, Fair Lawn, NJ, USA) directly to the culture to a final concentration of 2%. The solution was stirred overnight at room temperature (22ºC). The determination of viability of the bacteria was tested by streaking one loop full onto TSA + 2% NaCl plates and observing for growth over 48 hours at 24ºC. The formalin-killed bacteria were harvested by centrifugation at 1900 x g for 10 min. The pellet was washed by resuspending in 50 ml Coating Buffer (0.05M sodium carbonate, (Sigma-Aldrich, St. Louis, MO, USA) in diH_2O, pH adjusted to 9.6) then centrifuged at 1900 x g for 10 min. The final pellet was resuspended in 50 ml Coating Buffer and the concentration of bacteria was adjusted to an optical density (OD) at 600nm of 0.132 for approximately $1.5x10^8$ cells per ml (Vervarcke *et al.*, 2004). Coating Buffer was used to dilute or resuspend cells as necessary (if original concentration was insufficient, cells were re-centrifuged and resuspended in a smaller volume). Then 1.0 ml of that solution was added to 49 ml of Coating Buffer for a final working concentration of $3x10^6$ cells per ml.

Part B: ELISA technique

A 96-well ELISA plate (Corning, New York, NY, USA) was coated by adding 200 µl of prepared antigen to all but two wells ($6x10^5$ cells per well). To the final two

wells, 200 µl of 1% BSA in Coating Buffer only was added (no *Vibrio* – uncoated well control). The plate was covered with Parafilm® and refrigerated overnight at 4^0 C. The next day the plate was washed 3x with PBS plus 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) in diH_2O (PBST). Next, 250 µl 1% BSA in Coating Buffer was added to each well, and incubated 45 minutes at 37^0C. The plate was washed 3x with PBST. Then 100 µl of hybrid stripped bass serum (primary antibody) to be tested, diluted 1:1000 in PBST, was added to the appropriate wells. Only PBST was added to the primary antigen control wells. Duplicate wells were used for each sample, including controls. Serum was incubated for 2 hours at room temperature. Then the plate was washed 3x with PBST.

Rabbit anti-hybrid striped bass Ig (secondary antibody, Hrubec *et al*., 1996, Smith, 1992) was diluted 1:10,000 in PBST, and 100 µl was added to appropriate wells. Only PBST was added to secondary antibody control wells. The plate was incubated 1 hour at room temperature. The plate was then washed 3x with PBST. Affinity purified horseradish peroxidase-conjugated goat anti-rabbit (tertiary antibody, KPL, Gaithersburg, MD, USA) diluted 1:100 in PBST 100 µl was added to each well. The plate was incubated 45 minutes at room temperature, and then the plate was washed 3x with PBST. Tetramethylbenzidine (TMB) microwell peroxidase substrate (KPL, Gaithersburg, MD, USA) was added at 100 µl per well, and incubated 6 minutes at room temperature. Then 100 µl of Stop Solution (1 N HCl, Fisher Chemicals, Fair Lawn, NJ, USA) was added to each well. The absorbance (optical density, OD) was then measured on an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

Statistical analysis was conducted using SAS® for box and whisker plots, analysis of covariance (ANCOVA) and Tukey's post-hoc analysis, using Proc GLM (Appendix 3).

3.3 Results

The immune response, as measured by the specific anti-*Vibrio* antibody level, of hybrid striped bass to the commercial vaccine (Vibrogen-2) is depicted in Figure 1. There was a significant difference between groups and between days ($P<0.0001$), and there was no interaction between group and day ($P=0.4059$). Tukey's pair wise comparison showed that there was a significant difference detected between groups: Bath and IP, Bath and HSB negative control, IP and PBS, IP and HSB negative control, PBS and HSB negative control ($\alpha<0.05$), but not between the Bath and PBS groups. Box and whisker plot, showing median, first and third quartile, +/- standard error bars, and outliers, is depicted in Figure 2.

3.4 Discussion

Vaccination is one method used in the prevention of disease, to increase the immune response and increase the resistance of the host to a particular pathogen. The measurement of antibody level is a direct way to evaluate the immune response of the host to vaccination.

In this study, the antibody levels of the vaccinated hybrid striped bass increased over time post-vaccination, and plateaued between 28 and 70 days. The IP vaccinated group had higher antibody levels than the bath vaccinated group, especially at 63 and 70 days post-vaccination. The results illustrate that both the bath and IP groups exhibited an immune response with specific antibody levels greater than the PBS control group.

These results agree with previous studies in hybrid striped bass and other species measuring the protective immunity based on a challenge with the pathogen and resultant lower mortality in vaccinated versus control fish. The correlation with past studies has been well documented previously in many species including Atlantic halibut (Bricknel *et al*., 2000), salmon (Bravo and Midtlyng, 2007; Midtylng *et al*., 1996), cobia (Lin *et al*., 2005), hybrid striped bass (Hrubec *et al*., 1996), and yellowtail (Shimahara *et al*., 2005).

The greatest difficulty in analysis of the data for this study was the variation between individual samples. A variation between fish would be expected, as each sample collection point was made up of six individual fish per group. This is apparent when observing the increase in OD value of the PBS controls, as well as the positive (previously vaccinated fish) and negative (normal hybrid striped bass) controls (Appendix 4). This could also be due in part to variation between materials, i.e. ELISA plates (separate packages of the same lot), as well as variation in coating buffer and dilution of antibody and batches of wash buffer.

To minimize variation, tank conditions were maintained as close to the same for each of the three groups (IP, Bath, and PBS) as was possible. Previous exposure, though undocumented, was possible. Hybrid striped bass, while commonly raised in freshwater, come from wild collected broodstock from a marine environment where exposure to

Vibrios is more likely. The temperature of the holding takes also varied seasonally as the facility only allows for heating, not cooling of the fish systems. Challenge studies have already been conducted in hybrid striped bass as well as other species with autogenous bacterins that demonstrate that an immune response to *Vibrio spp.* can be protective (Rogers *et al*, 1992, Lin *et al*, 2006). Autogenous bacterins, while useful for research purposes, are not the same as a commercially produced vaccine. A future challenge study could be conducted using the commercially produced vaccine should be conducted in order to examine the efficacy of a given commercial vaccine.

There are only two routes currently approved for *Vibrio spp.* vaccination in salmonids, Bath and IP, and thus these were the only two routes examined. To protect fish against vibriosis, producers can consider utilizing Bath or IP route to vaccinate their fish against vibriosis. Bath is much less labor intensive, and might be a better option economically. Though IP exhibited a slightly higher antibody level, these results were not significant ($P>0.05$), so it may not justify the additional labor and cost.

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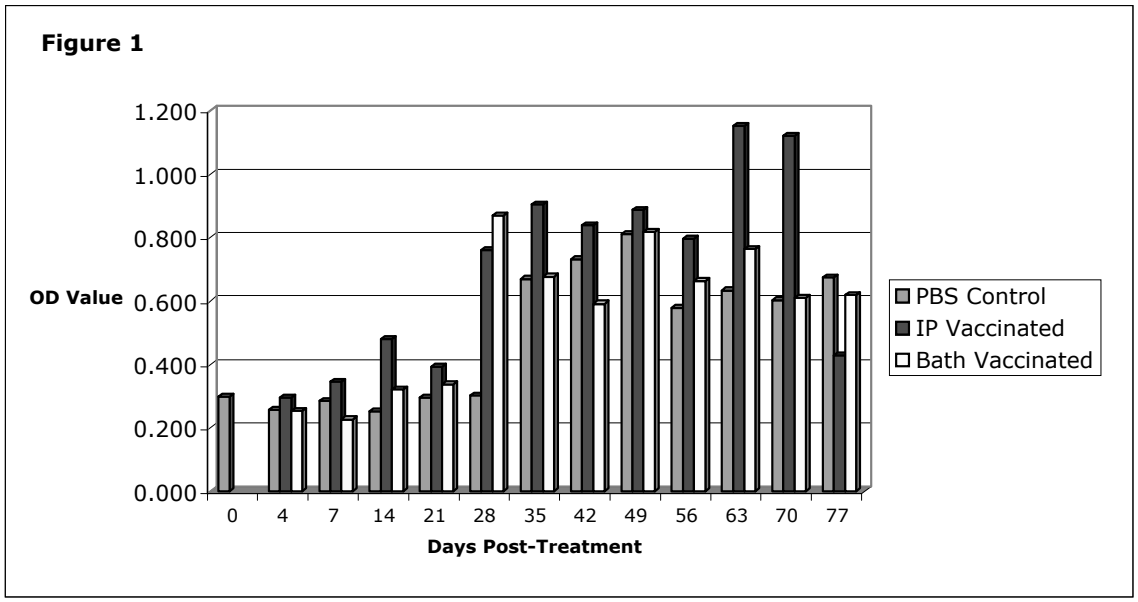

Figure 3.1: The immune response of hybrid striped bass (*Morone chrysops* x *M. saxtilis*) to *Vibrio spp.* after single immunization with a commercial *V. anguillarum/V. ordalii* vaccine. An increase in immune response depicted by increased OD value can be seen from day 7 onward in the IP vaccinated group, and day 14 onward in the Bath vaccinated group.

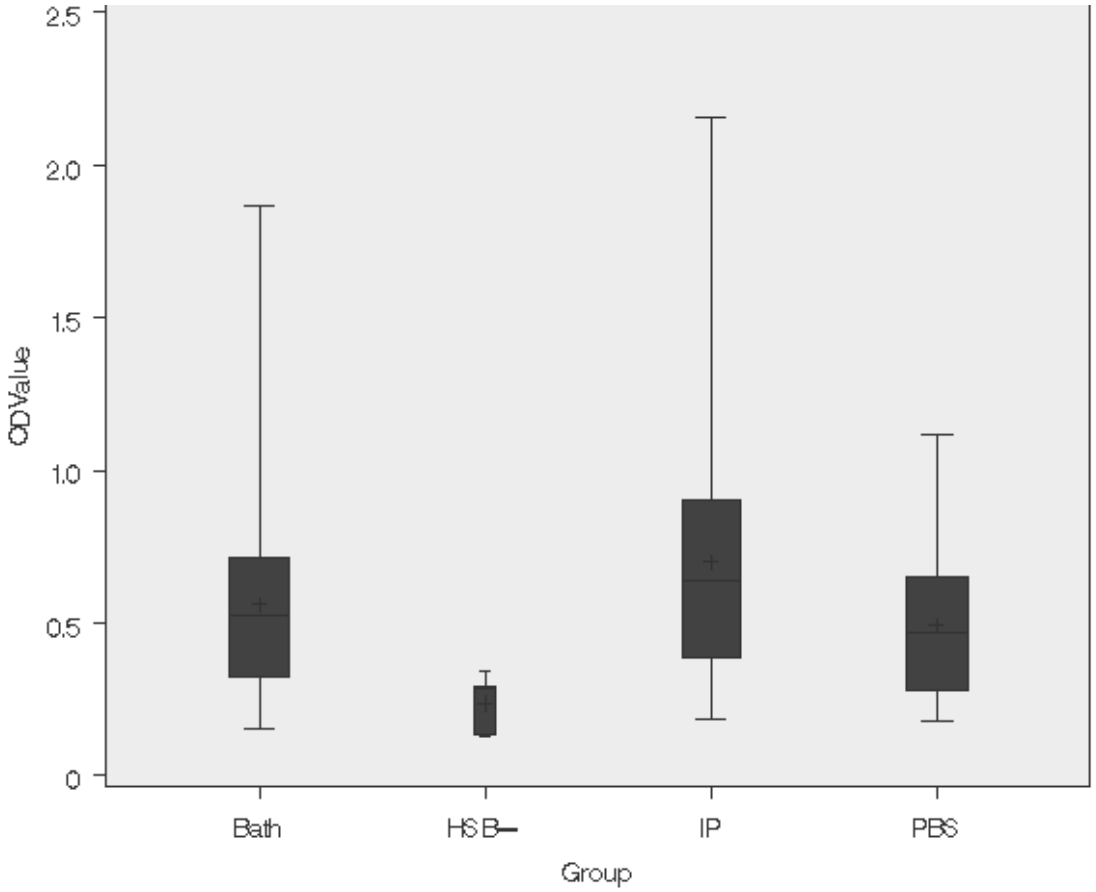

Figure 3.2: The immune response of hybrid striped bass (*Morone chrysops* x *M. saxtilis*) to *Vibrio spp.* after immunization with a commercial *V. anguillarum/V. ordalii* vaccine summarized by box and whisker plot for ELISA results (Optical Density, OD values) depicting median 1st and 3rd quartiles, standard error bars and outliers. Groups include Bath vaccinated, IP vaccinated, PBS control, and HSB- non-exposed normal serum control.

Chapter 4

Immune response of cobia (*Rachycentron canadum*) to vaccination with a commercial vaccine against *Vibrio anguillarum* and *V. ordalii*

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4.1 Introduction

One of the greatest threats to the intensive aquaculture industry is infectious fish disease. These can be grouped in to three main categories: viral, parasitic, and bacterial. Cobia is susceptible to all three groups of pathogens. The reported bacterial diseases of cobia include vibriosis, pasteurellosis and streptococcosis, and include *Mycobacterium marinum, Vibrio anguillarum* and *V. ordalii, Pasteurella spp.,* and *Streptococcus spp.* (Liao *et al*., 2004, Lowry and Smith, 2006).

The genus *Vibrio,* belonging to the family Vibrionaceae, is a group of Gram-negative, facultatively anaerobic, curved, rod-shaped bacteria that are oxidase positive and non-spore forming (Kaysner and DePaola, 1998). This genus includes the human pathogens *V. cholerae, V. mimicus, V. parahaemolyticus,* and *V. vulnificus,* as well as the fish pathogens *Listonella anguillarum* (formerly *V. anguillarum*), *V. ordalii, V. damsela, V. carchariae, V. vulnificus, V. alginolyticus,* and *V. salmonicida* (Reed and Francis-Floyd, 2002, Lhafia and Kühneb, 2007, Wang *et al.,* 2008). *Vibrio spp.* pathogens also affect other species of marine fish, penaeid shrimp, as well as abalone (Liu *et al*., 2004). *Vibrio spp*. bacteria also account for a significant portion of the food-borne infections from eating raw or undercooked shellfish (Lhafia and Kühneb, 2007, Wolf and Smith, 1999).

Vibrio (*Listonella*) *anguillarum* is a halophilic Gram-negative, curved rod with polar flagella. Vibriosis caused by this bacterial species has been identified in many finfish species including turbot (*Scophthalmus maximus*), eels (*Angullla anguilla*) and salmonids (*Oncorhynchus nerka*) (Austin and Austin, 1987, Tiecco *et al*., 1988, Antipa *et*

al., 1980). Significant outbreaks have been observed, with 100% morbidity (Reed and Francis-Floyd, 2002) and mortality commonly over 80% in cobia (Liu *et al*., 2004). Fish less than 4 months old, <500 g, appear to be the most susceptible, with the highest mortalities to this bacterial pathogen (Lin *et al*., 2006).

Vibrio ordalii was formerly referred to as *Vibrio anguillarum* biotype 2, and has been reclassified as a distinct species (Schiewe *et al*., 1981). *Vibrio ordalii* is another causative agent for vibriosis in fish. It can be distinguished in culture from other *Vibrios* by biochemical characteristics as well as DNA sequence relatedness (Schiewe *et al*., 1981). The type strain (LMG 13544) of *V. ordalii* was isolated from coho salmon (*Oncorhynchus rhoddiurus*) in Washington in 1973 (Thompson *et al*., 2004). Clinical signs are similar to *V. anguillarum* with differences including microcolony formation on skeletal and heart muscle as well as in the gills and gastrointestinal tract, slower progression of bacteremia, and marked leucopenia (Austin and Austin, 1987).

While the precise route of transmission in fish is unclear, it is suspected that *Vibrio spp.* infections are initiated through the oral route (Reed and Francis-Floyd, 2002). *Vibrio spp.* can be found in normal gut flora, and under certain conditions can cross the intestinal wall, resulting in a systemic infection. This increases the numbers of bacteria in the water, which can then infect other fish.

One method used in the prevention of disease is vaccination, to the increase immune response of the host. A killed bacterial vaccine is currently available for *V. anguillarum* and *V. ordalii*, and has been demonstrated effective in prevention of vibriosis in juvenile Atlantic halibut (Bricknell *et al*., 2000), as well as in salmon (Bravo

and Midtlyng, 2007). Cobia have also been demonstrated to develop protective immunity from vaccination with inactivated *Vibrio* bacterins (Lin *et al*., 2005.)

Different routes can be used to administer vaccines to fish. Immersion administration is a commonly used route in aquaculture. With this method, fish are immersed in a specific concentration vaccine for a pre-determined period of time. Intraperitoneal (IP) injection is also commonly used in commercial aquaculture as well as research settings. With this technique, the vaccine is delivered into the coelomic cavity of the fish. Intramuscular injection is another option, but it is less commonly used for aquaculture species due to potential damage to the muscle (fillet) of the fish as well as potential leakage of vaccine from the injection site prior to absorption.

The goal of this study was to develop recommendations for the most effective route of vaccination for cobia in an aquaculture setting, using a commercially available vaccine, as a way to prevent disease and economic loss from vibriosis in cultured cobia.

4.2 Materials and Methods

Fish

Cobia were obtained from a commercial producer (VA Cobia Farms, Saltville, VA) and housed at the Aquatic Medicine Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine of Virginia Tech in Blacksburg, VA. Fish weighed 111.0+/- 19.7g (mean +/- standard deviation) at the time of vaccination. A total of 162 fish were used in the study and arbitrarily divided into 3 equal groups of 54 fish per group. One group of fish were injected Intraperitoneally (IP) with 0.1 ml (Vibrogen-2,

Aqua Health LTD.) then placed three fish per 75 liter tank, in a multi-tank recirculating system with a total volume 1800 liters. A second group of fish was bath vaccinated (described as bath) by placing the fish in the prepared vaccine per manufacturer's directions (400 ml vaccine, 3600 ml salt water, 25 ppt) for 30 seconds, then the fish were placed in a system identical to the IP group. A third group of control fish were injected IP with 0.1ml sterile PBS (Sigma), then placed into a single 1300 liter fiberglass tank system.

Water temperature for all groups of fish was maintained between 24-31°C (due to seasonal variation), salinity was maintained at 25-28 ppt (due to evaporation), and the fluorescent light photoperiod consisted of 12 hours light/12 hours dark. Fish were fed a commercial pellet (5mm) feed (Corey Aquafeeds, Fredericton, NB, Canada) daily at 3% body weight, and water quality parameters (ammonia, nitrite, nitrate, salinity, pH) were monitored using a commercial water analysis kit (HACH, Loveland, CO, USA).

Six fish were arbitrarily selected from each group and sedated with sodium bicarbonate buffered MS-222 (100mg/l, Sigma-Aldrich, St. Louis, MO, USA) and bled (23 gauge needle, 1 ml syringe) from the caudal vessels pre-vaccination day 0, and post-vaccination at 7, 14, 21, 42, 56, 84, 112, 140 days. Blood was allowed to clot in serum separation tubes (Becton Dickinson Microtainer®, Franklin Lakes, NJ, USA) for 1 hour at room temperature, and then refrigerated overnight at 4ºC. The next day the clotted blood was centrifuged at 10,000 x g for 6 minutes, and the serum separated and placed in 1.5 ml microcentrifuge tubes (USA Scientific, Ocala, FL). These were frozen at -20ºC until analyses after all bleeding times were completed.

Isolation and purification of cobia Ig

A separate cobia (581g, Proaquatix, Maritech, Vero Beach, FL, USA) was immunized IP with 100µl prepared antigen (150µg goat IgG fraction to mouse IgG HC, ICN Biomedicals, Costa Mesa, CA, USA). The cobia was boosted on day 28 and day 35 post-immunization, and 1 ml of blood was taken on day 35 to check for antibody production. The cobia was bled on day 45 and day 56 post-immunization, and the serum pooled, for affinity purification (Smith, 1992; Smith *et al.*, 1993).

Rabbit anti-cobia antibody

A New Zealand white laboratory rabbit was immunized with purified cobia Ig emulsified with Freund's complete adjuvant by subcutaneous vaccination (0.2 ml each in two locations along the dorsal midline) and IM vaccination (0.2 ml, right rear hock). The rabbit was boosted at day 14 with purified cobia Ig emulsified in Freund's incomplete adjuvant by subcutaneous vaccination (0.2 ml in two locations along the dorsal midline) and IM vaccination (0.2 ml, left rear hock).

The rabbit was sedated with Acepromazine (10mg/ml) at a rate of 0.5mg/kg body weight, 0.15ml IV (marginal ear vein). The rabbit was bled (23 gauge needle, 1 ml syringe) from the central ear vein day 14 and 30 post-immunization. For terminal bleeding the rabbit was sedated with Acepromazine as before, then anesthetized with Ketamine (100 mg/ml) at 60mg/kg body weight, 1.4 ml IM. Final exsanguination was by cardiac puncture on day 45, obtaining approximately 85 ml blood. The blood was allowed to clot in serum separation tubes for 1 hour at room temperature, and refrigerated

overnight at 4ºC. The next day the clotted blood was centrifuged at 10,000 x g for 6 minutes, and the serum separated and frozen.

An Ouchterlony immuno-diffusion assay was conducted to test for the presence of rabbit anti-cobia antibodies. Nine ml of 1% immunodiffusion grade agarose (ICN Biomedicals, Costa Mesa CA, USA) was liquefied and poured onto a sheet of Gel-bond® film (FMC Bio Products, Philadelphia, PA, USA) and allowed to cool. Wells were punched in the agarose using a template of 6 holes in a circle equidistant from a single well in the center. Rabbit anti-cobia serum to be tested was placed in the center well. PBS, cobia serum, and serum of three other fish species (hybrid striped bass, channel catfish, and tilapia) were placed in the surrounding test wells. The gel was placed in a humidified chamber for 48 hours to allow adequate time for diffusion of the serum through the gel. The gel was the washed in PBS overnight. The next day the gel was stained with Coomassie® brilliant blue 1% (G-250, Bio-Rad Laboratories, Richmond, CA, USA) for 10 minutes, and de-stained in Coomassie de-stain (450 ml methanol (Fisher Chemicals, Fair Lawn, NJ, USA), 450 ml de-ionized water, 100 ml glacial acetic acid (Sigma-Aldrich, St. Louis MO, USA)). The presence of a band of precipitation between wells indicated a positive antigen antibody reaction between the rabbit anti-cobia serum and the serum in the particular test well.

Vibrio ELISA

A culture of *Vibrio* (*Listonella*) *anguillarum,* obtained from the National Fish Health Research Laboratory in Kernersville, WV, was grown overnight in 50 ml of TSB

+ 2% NaCl. Bacteria were killed by addition of 37% formalin (Fisher, Fair Lawn, NJ, USA) directly to the culture to a final concentration of 2%. The solution was stirred overnight at room temperature (22ºC). The termination of viability of the bacteria was tested by streaking one loop full onto TSA + 2% NaCl plates. The formalin-killed bacteria were harvested by centrifugation at 1900 x g for 10 min. The pellet was washed by resuspending in 50 ml Coating Buffer (0.05M sodium carbonate (Sigma-Aldrich, St. Louis, MO) in diH_2O, pH adjusted to 9.6) then centrifuged at 1900 x g for 10 min. The final pellet was resuspended in 50 ml Coating Buffer and the concentration of bacteria was adjusted to an optical density (OD) at 600nm of 0.132 for approximately $1.5x10^8$ cells per ml (Vervarcke *et al.*, 2004). Coating Buffer was used to dilute or resuspend cells as necessary (if original concentration was insufficient, cells were re-centrifuged and resuspended in a smaller volume). Then 1.0 ml of that solution was added to 49 ml of Coating Buffer for a final working concentration of $3x10^6$ cells per ml.

ELISA technique

A 96-well ELISA plate (Corning, New York, NY, USA) was coated by adding 200 µl of prepared antigen ($6x10^5$ cells per well) to all but two wells. To the final two wells, 200 µl of 1% BSA in Coating Buffer only was added (no *Vibrio* – uncoated well control). The plate was covered with Parafilm® and refrigerated overnight at 4º C. The next day the plate was washed 3x with PBS plus 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) in diH_2O (PBST). Next, 250 µl 1% BSA in Coating Buffer was added to each well, and incubated 45 minutes at 37º C. The plate was washed 3x with PBST. Then 100 µl of cobia serum (primary antibody) to be tested, diluted 1:1000 in PBST, was

added to the appropriate wells. Only PBST was added to the primary antigen control wells. Duplicate wells were used for each sample, including controls. Serum was incubated for 2 hours at room temperature (21ºC), then the plate was washed 3x with PBST.

Rabbit anti-cobia (secondary antibody), diluted 1:10,000 in PBST, 100 µl was added to appropriate wells. Only PBST was added to secondary antibody control wells. The plate was incubated 1hourat room temperature, then washed 3x with PBST. Affinity purified horseradish peroxidase-conjugated goat anti-rabbit (tertiary antibody (KPL, Gaithersburg, MD)) diluted 1:100 in PBST was added 100 µl of to each well. The plate was incubated 45 minutes at room temperature, and then the plate was washed 3x with PBST. Tetramethylbenzidine (TMB) microwell peroxidase substrate (KPL, Gaithersburg, MD, USA) was added 100 µl to each well, and incubated 6 minutes at room temperature. Then 100 µl of Stop Solution (1 N HCl, Fisher Chemicals, Fair Lawn, NJ, USA) was added to each well. Absorbance (Optical Density, OD) was then measured on an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

Statistical analysis was conducted using SAS® for box and whisker plots, analysis of covariance (ANCOVA) and Tukey's post-hoc analysis, using Proc GLM (Appendix 3).

4.3 Results

The immune response, as measured by the specific anti-*Vibrio* antibody level, of cobia to the commercial vaccine (Vibrogen-2) is depicted in Figure 1. There was a significant difference between groups ($P<0.0001$) and between days ($P=0.0003$), and there was no interaction between group and day ($P=0.2553$). Tukey's pair wise comparison of groups showed that there was a significant difference detected between all three groups: Bath, IP and PBS ($\alpha<0.05$). Tukey's pair wise comparison of days showed only a significant difference between day 112 and 21 and between day 112 and 28. This is indicative of an outlier value on day 112, one fish had a mean OD value of 3.2665, where median value for all Bath group OD values was 0.771. Box and whisker plot, showing median, first and third quartile, +/- standard error bars, and outliers, is depicted in Figure 2.

4.4 Discussion

Vaccination is one method used in the prevention of disease, to increase the immune response and increase the resistance of the host to a particular pathogen. The measurement of antibody level is a direct way to evaluate the immune response of the host to vaccination. In this study, the antibody levels of the vaccinated cobia increased over time post-vaccination, and then plateaued. The IP vaccinated group had higher antibody levels than the Bath vaccinated group post-vaccination. The results showed that

both the Bath and IP groups exhibited an immune response with specific antibody levels greater than the PBS control group.

These results agree with previous studies in cobia and other species measuring the protective immunity based on a challenge with the pathogen and resultant lower mortality in vaccinated versus control fish. The correlation with past studies has been well documented previously in many species including Atlantic halibut (Bricknel *et al*., 2000), salmon (Bravo and Midtlyng, 2007; Midtylng *et al*., 1996), cobia (Lin *et al*., 2005), hybrid striped bass (Hrubec *et al*., 1996), and yellowtail (Shimahara *et al*., 2005).

The greatest difficulty in analysis of the data for this study was the variation between individual samples. A variation between fish would be expected, as each sample collection point was made up of six individual fish per group. This is apparent when observing the increase in OD value of the PBS controls, as well as the positive (previously vaccinated fish) and negative (normal cobia) controls (Appendix 6). This could be due in part to varying ability of fish to respond to vaccination or variation between materials, i.e. ELISA plates (separate packages of the same lot), as well as variation in coating buffer and dilution of antibody and batches of wash buffer.

To minimize variation, tank conditions were maintained as close to the same for each of the three groups (IP, Bath, and PBS) as was possible. Previous exposure, though undocumented, was possible. Cobia, while raised in this study in artificial seawater with presumably no *Vibrio spp.* present, came from wild collected broodstock from a marine environment where exposure to *Vibrios* is more likely. The temperature of the holding takes also varied seasonally as the facility only allows for heating, not cooling of the fish systems. Challenge studies have already been conducted in cobia as well as other species

with autogenous bacterins and confirm the results of this vaccination study (Rogers *et al*, 1992, Lin *et al*, 2006). Autogenous bacterins, while useful for research purposes, are not the same as a commercially produced vaccine. A future challenge study could be conducted using the commercially produced vaccine should be conducted in order to examine the efficacy of a given commercial vaccine.

Presently the commercial vaccine evaluated in this study is only approved for salmonids. In addition, the only two routes of administration currently approved for this vaccine is by the bath and IP routes, and thus these were the ofnly two routes examined in this study. The IP route generally stimulates a better immune response than the Bath vaccination route. However, bath vaccination is much less labor intensive, and might be a better option economically in some aquaculture facilities. The IP route generally exhibits a significantly higher antibody level than the bath vaccinated group, which sometimes relates to greater protection and therefore may justify the additional labor and cost.

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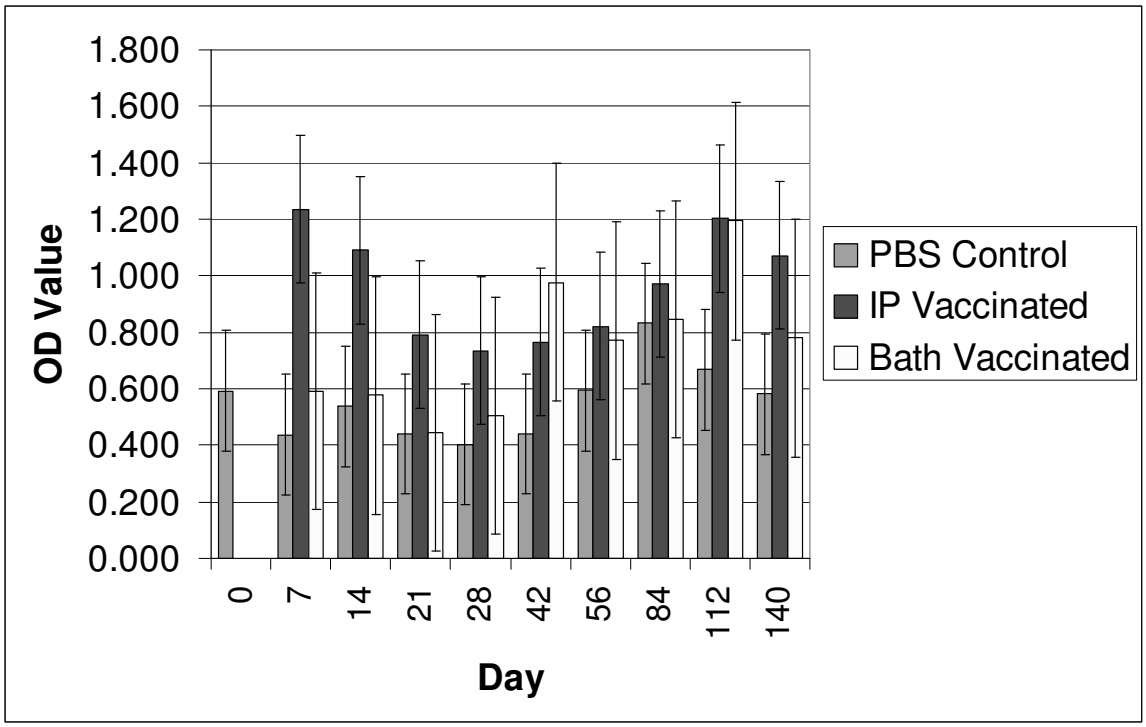

Figure 4.1: The immune response of cobia (*Rachycentron canadum*) to *Vibrio spp.* after single immunization with a commercial *V. anguillarum/V. ordalii* vaccine. An increase in immune response depicted by increased OD value can be seen from day 7 onward in the IP vaccinated group, and day 14 onward in the Bath vaccinated group. The mean OD for the PBS Controls of 0.558 was consistent with previous studies for normal background.

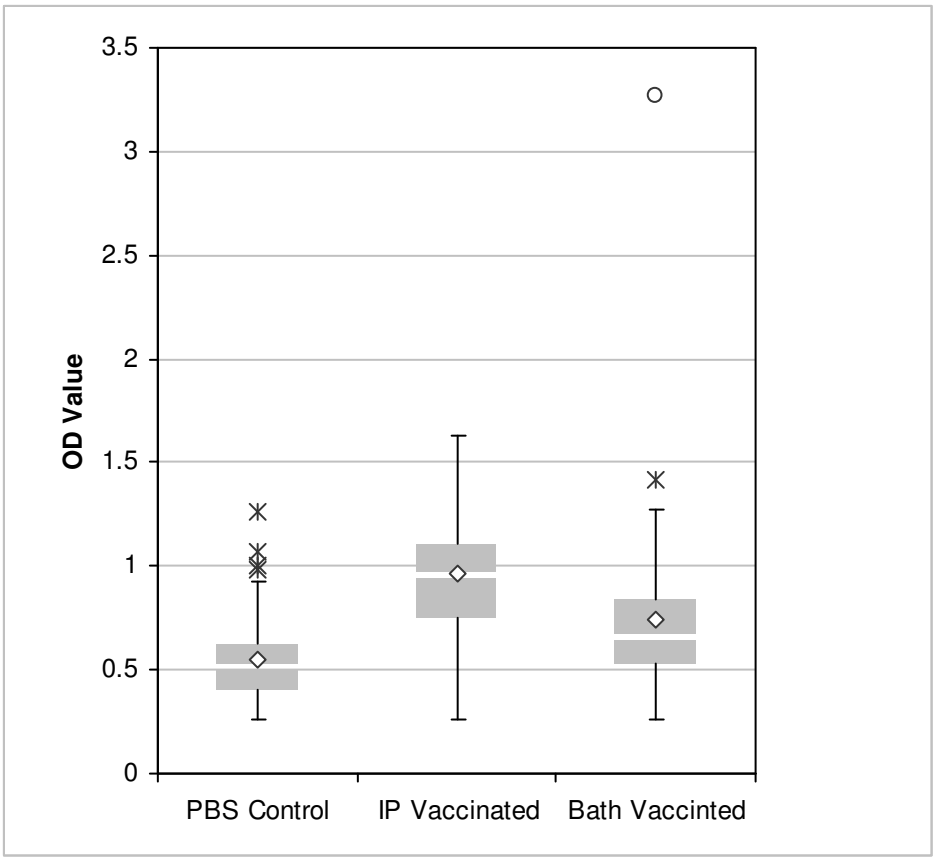

Figure 4.2: The immune response of cobia (*Rachycentron canadum*) to *Vibrio spp.* after immunization with a commercial *V. anguillarum/V. ordalii vaccine* summarized by box and whisker plot for ELISA results (Optical Density values) depicting median 1st and 3rd quartiles, standard error bars and outliers. Groups include Bath vaccinated, IP vaccinated, and PBS controls.

Appendix 1

Summary of disinfectants and their effectiveness against *V. anguillarium and V. ordalii.*

	Bacteria Species	
Disinfectant	***Vibrio* (*L.*) *anguillarum***	***Vibrio ordalli***
Chloramine T (0.0015g/100ml)	no growth	no growth
Clorox 50ppm	no growth	no growth
Ethanol 30%	no growth	no growth
Formalin (2.5ul of 37%/10ml)	reduced growth at 60min	reduced growth at 60min
Iodine 50ppm	no growth	no growth
Lysol 1%	no growth	no growth
Roccal 1:256 (3.9ppt)	no growth	no growth
Tap Water	growth at 10min	growth at 5min
Virkon 1% (0.1g/10ml)	no growth	no growth

Appendix 2

The table below displays raw data for *V. anguillarum* disinfection study. Plate counts at dilutions for A (control), B and C (replicates) disinfectant treatment. Number of colonies present out of five 10µl drops. Calculated number of colony forming units (CFU) per milliliter.

Disinfection *Vibrio* (*Listonella*) *angullarum*

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Chloramine T 15mg/l					
A begin	TNTC	76	13	1	1.50E+06
A end	TNTC	59	5	0	1.20E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Chloramine T 15mg/l Replicate					
A begin	TNTC	TNTC	46	6	9.20E+06
A end	TNTC	TNTC	33	4	6.60E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Clorox 50000ppm					
A begin	TNTC	TNTC	216	40	8.00E+07
A end	TNTC	TNTC	48	6	9.60E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Clorox 50000ppm Replicate					
A begin	TNTC	TNTC	39	2	7.80E+06
A end	TNTC	TNTC	33	4	6.60E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Clorox 200ppm					
A begin	TNTC	TNTC	42	8	8.40E+06
A end	TNTC	TNTC	34	8	6.80E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Clorox 200ppm Replicate					
A begin	TNTC	TNTC	46	2	9.20E+06
A end	TNTC	TNTC	51	6	1.00E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Clorox 100ppm					
A begin	TNTC	61	3	1	1.20E+06
A end	TNTC	TNTC	48	4	9.60E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Clorox 100ppm Replicate					
A begin	TNTC	TNTC	44	7	8.80E+06
A end	TNTC	TNTC	44	6	8.80E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Clorox 50ppm					
A begin	TNTC	TNTC	38	8	7.60E+06
A end	TNTC	TNTC	64	8	1.30E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Clorox 50ppm Replicate					
A begin	TNTC	TNTC	61	13	1.20E+07
A end	TNTC	TNTC	56	3	1.10E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	1.00E-03	1.00E-04	1.00E-05	cfu/ml
Ethanol 70%					
A begin	TNTC	TNTC	55	6	1.10E+07
A end	TNTC	TNTC	48	3	9.60E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Ethanol 70% Replicate					
A begin	TNTC	TNTC	56	1	1.10E+07
A end	TNTC	TNTC	52	6	1.00E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	1.00E-03	1.00E-04	1.00E-05	cfu/ml
Ethanol 50%					
A begin	TNTC	TNTC	36	8	7.20E+06
A end	TNTC	TNTC	29	1	5.80E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Ethanol 50% Replicate					
A begin	TNTC	TNTC	49	4	9.80E+06
A end	TNTC	TNTC	45	4	9.00E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Ethanol 30%					
A begin	TNTC	TNTC	52	6	1.00E+07
A end	TNTC	TNTC	34	3	6.80E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Ethanol 30% Replicate					
A begin	TNTC	TNTC	43	1	8.60E+06
A end	TNTC	TNTC	28	1	5.60E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Formalin 2.5ul/10ml (250ppm)					
A begin	TNTC	TNTC	38	5	7.60E+06
A end	TNTC	TNTC	56	4	1.10E+07
B 1 min	TNTC	TNTC	36	4	7.20E+06
B 5 min	TNTC	TNTC	54	4	1.10E+07
B 10 min	TNTC	TNTC	80	8	1.60E+07
B 20 min	TNTC	179	25	3	5.00E+06
B 30 min	TNTC	150	21	4	4.20E+06
B 60 min	TNTC	77	3	0	1.50E+06
C 1 min	TNTC	TNTC	55	6	1.10E+07
C 5 min	TNTC	TNTC	46	7	9.20E+06
C 10 min	TNTC	TNTC	36	3	7.20E+06
C 20 min	TNTC	TNTC	33	4	6.60E+06
C 30 min	TNTC	161	18	5	3.60E+06
C 60 min	TNTC	80	15	0	1.60E+06

Formalin 2.5ul/10ml replicate					
A begin	TNTC	TNTC	79	9	1.60E+07
A end	TNTC	TNTC	60	12	1.20E+07
B 1 min	TNTC	TNTC	80	7	1.60E+07
B 5 min	TNTC	TNTC	73	18	1.50E+07
B 10 min	TNTC	TNTC	76	6	1.50E+07
B 20 min	TNTC	TNTC	44	6	8.80E+06
B 30 min	TNTC	TNTC	30	6	6.00E+06
B 60 min	TNTC	109	24	1	2.20E+06
C 1 min	TNTC	TNTC	79	12	1.60E+07
C 5 min	TNTC	TNTC	87	7	1.70E+07
C 10 min	TNTC	TNTC	62	8	1.20E+07
C 20 min	TNTC	TNTC	46	4	9.20E+06
C 30 min	TNTC	TNTC	36	4	7.20E+06
C 60 min	TNTC	141	12	5	2.80E+06

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Iodine 50ppm					
A begin	TNTC	TNTC	64	5	1.30E+07
A end	TNTC	TNTC	53	5	1.10E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Iodine 50ppm Replicate					
A begin	TNTC	TNTC	76	5	1.50E+07
A end	TNTC	TNTC	88	9	1.80E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution 1.00E-02	1.00E-03	1.00E-04	1.00E-05	cfu/ml
Lysol 1%					
A begin	TNTC	TNTC	77	7	1.50E+07
A end	TNTC	TNTC	47	6	9.40E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Lysol 1% Replicate					
A begin	TNTC	TNTC	67	11	1.30E+07
A end	TNTC	TNTC	51	7	1.00E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution 1.00E-02	1.00E-03	1.00E-04	1.00E-05	cfu/ml
Roccal 1:256 (3.9ppt)					
A begin	TNTC	TNTC	52	8	1.00E+07
A end	TNTC	TNTC	55	5	1.10E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Roccal 1:256 Replicate					
A begin	TNTC	TNTC	59	8	1.20E+07
A end	TNTC	TNTC	57	8	1.10E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Tap Water					
A begin	TNTC	TNTC	89	5	1.80E+07
A end	TNTC	TNTC	64	9	1.30E+07
B 1 min	146	20	0	0	4.00E+05
B 5 min	79	9	0	0	1.80E+05
B 10 min	16	1	0	0	3.20E+04
B 20 min	0	0	0	0	No Growth
C 1 min	TNTC	64	6	2	1.30E+06
C 5 min	TNTC	37	8	0	7.40E+05
C 10 min	8	0	0	0	1.60E+04
C 20 min	0	0	0	0	No Growth
Tap Water Replicate					
A begin	TNTC	TNTC	44	11	8.80E+06
A end	TNTC	TNTC	48	7	9.60E+06
B 1 min	TNTC	77	13	2	1.50E+06
B 5 min	TNTC	59	8	0	1.20E+06
B 10 min	92	22	0	0	1.80E+05
B 20 min	0	0	0	0	No Growth
C 1 min	TNTC	90	10	2	1.80E+06
C 5 min	TNTC	48	10	2	9.60E+05
C 10 min	65	10	2	0	1.30E+05
C 20 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Virkon 1%					
A begin	TNTC	TNTC	89	8	1.80E+07
A end	TNTC	TNTC	84	9	1.70E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

Virkon 1% Replicate

A begin	TNTC	TNTC	48	11	9.60E+06
A end	TNTC	184	21	5	4.20E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

Appendix 3

The table below displays raw data for *V. ordalii* disinfection study. Plate counts at dilutions for A (control), B and C (replicates) disinfectant treatment. Number of colonies present out of five 10µl drops. Calculated number of colony forming units (CFU) per milliliter.

Disinfection *Vibrio ordalii*

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Chloramine T 15mg/l					
A begin	TNTC	TNTC	99	15	2.00E+07
A end	TNTC	TNTC	68	11	1.40E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Chloramine T 15mg/l Replicate					
A begin	TNTC	TNTC	69	8	1.40E+07
A end	TNTC	TNTC	93	12	1.90E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Clorox 50ppm					
A begin	TNTC	TNTC	108	17	1.00E+07
A end	TNTC	TNTC	92	7	6.80E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

Clorox 50ppm Replicate					
A begin	TNTC	TNTC	33	2	6.60E+06
A end	TNTC	TNTC	110	11	2.20E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Ethanol 30%					
A begin	TNTC	TNTC	87	5	1.70E+07
A end	TNTC	TNTC	74	9	1.50E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Ethanol 30% Replicate					
A begin	TNTC	TNTC	117	24	2.30E+07
A end	TNTC	TNTC	95	11	1.90E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	1.00E-03	1.00E-04	1.00E-05	cfu/ml
Formalin 2.5ul/10ml (250ppm)					
A begin	TNTC	TNTC	TNTC	23	4.60E+07
A end	TNTC	TNTC	118	11	2.40E+07
B 1 min	TNTC	TNTC	138	14	2.80E+07
B 5 min	TNTC	TNTC	90	11	1.80E+07
B 10 min	TNTC	TNTC	47	3	9.40E+06
B 20 min	TNTC	86	11	1	1.70E+06
B 30 min	TNTC	44	6	0	8.80E+05
B 60 min	84	11	1	0	1.70E+05
C 1 min	TNTC	TNTC	145	30	2.90E+07
C 5 min	TNTC	TNTC	98	12	2.00E+07
C 10 min	TNTC	TNTC	84	6	1.70E+07
C 20 min	TNTC	83	13	0	1.70E+06
C 30 min	TNTC	73	7	2	1.50E+06
C 60 min	TNTC	30	7	1	6.00E+05
Formalin 2.5ul/10ml replicate					
A begin	TNTC	TNTC	103	11	2.10E+07
A end	TNTC	TNTC	52	2	1.00E+07
B 1 min	TNTC	TNTC	95	11	1.90E+07
B 5 min	TNTC	TNTC	48	13	9.60E+06
B 10 min	TNTC	TNTC	24	1	4.80E+06
B 20 min	TNTC	50	9	0	1.00E+06
B 30 min	8	1	0	0	1.60E+04
B 60 min	0	0	0	0	No Growth
C 1 min	TNTC	TNTC	83	11	1.70E+07
C 5 min	TNTC	TNTC	43	6	8.60E+06
C 10 min	TNTC	TNTC	20	1	4.00E+06
C 20 min	33	1	0	0	6.60E+04
C 30 min	39	11	1	0	7.80E+04
C 60 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Iodine 50ppm					
A begin	TNTC	TNTC	97	12	1.00E+07
A end	TNTC	TNTC	119	19	6.80E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Iodine 50ppm Replicate					
A begin	TNTC	TNTC	105	12	2.10E+07
A end	TNTC	TNTC	111	14	2.20E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Lysol 1%					
A begin	TNTC	TNTC	124	12	1.00E+07
A end	TNTC	TNTC	100	13	6.80E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Lysol 1% Replicate					
A begin	TNTC	TNTC	98	14	2.00E+07
A end	TNTC	TNTC	105	12	2.10E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Roccal 1:256 (3.9ppt)					
A begin	TNTC	TNTC	75	13	1.00E+07
A end	TNTC	TNTC	89	15	6.80E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Roccal 1:256 Replicate					
A begin	TNTC	TNTC	118	19	2.40E+07
A end	TNTC	TNTC	104	9	2.10E+07
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Tap Water					
A begin	TNTC	TNTC	72	13	1.40E+07
A end	TNTC	TNTC	94	8	1.90E+07
B 1 min	TNTC	61	4	1	1.20E+06
B 5 min	0	0	0	0	No Growth
C 1 min	TNTC	55	8	0	1.10E+06
C 5 min	0	0	0	0	No Growth
Tap Water replicate					
A begin	TNTC	TNTC	95	21	1.90E+07
A end	TNTC	TNTC	60	8	1.20E+07
B 1 min	TNTC	TNTC	33	2	6.60E+06
B 5 min	1	0	0	0	2.00E+03
B 10 min	0	0	0	0	No Growth
C 1 min	TNTC	TNTC	20	3	4.00E+06
C 5 min	0		0	0	No Growth

	Dilution				
	1.00E-02	**1.00E-03**	**1.00E-04**	**1.00E-05**	**cfu/ml**
Virkon 1%					
A begin	TNTC	TNTC	79	5	1.00E+07
A end	TNTC	TNTC	71	6	6.80E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth
Virkon 1% Replicate					
A begin	TNTC	TNTC	32	4	6.40E+06
A end	TNTC	TNTC	39	4	7.80E+06
B 1 min	0	0	0	0	No Growth
C 1 min	0	0	0	0	No Growth

Appendix 4

Raw data for hybrid striped bass (*Morone chrysops* x *M. saxatilis*) immune response to *Vibrio spp.* vaccination.

The table below displays optical density (OD) values, which are an average of duplicate wells, for each fish, group and time post vaccination. This is representative of the immune response in the form of specific antibodies to *V. anguillarum*.

	Average OD	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6
Control Day 0	0.299	0.232	0.275	0.284	0.393	0.271	0.339
Control Day 4	0.258	0.231	0.243	0.233	0.319	0.255	0.268
Control Day 7	0.285	0.385	0.278	0.234	0.176	0.429	0.212
Control Day 14	0.253	0.206	0.210	0.313	0.280	0.253	0.256
Control Day 21	0.296	0.305	0.335	0.256	0.270	0.282	0.331
Control Day 28	0.303	0.291	0.315	0.210	0.237	0.452	0.316
Control Day 35	0.669	0.504	0.480	0.564	0.817	0.680	0.972
Control Day 42	0.732	1.114	0.433	0.846	0.744	0.774	0.480
Control Day 49	0.811	1.107	0.739	0.809	0.676	0.907	0.628
Control Day 56	0.579	0.648	0.568	0.466	0.785	0.474	0.537
Control Day 63	0.633	0.653	0.872	0.535	0.669	0.490	0.580
Control Day 70	0.602	0.667	0.526	0.442	0.652	0.800	0.524
Control Day 77	0.674	0.490	0.891	0.514	0.619	0.889	0.643
IP Day 4	0.296	0.342	0.259	0.316	0.327	0.241	0.294
IP Day 7	0.347	0.482	0.465	0.338	0.186	0.331	0.279
IP Day 14	0.481	0.315	0.799	0.528	0.630	0.334	0.284
IP Day 21	0.394	0.388	0.466	0.359	0.536	0.231	0.385
IP Day 28	0.760	0.825	0.567	0.558	1.031	0.858	0.722
IP Day 35	0.904	0.840	0.485	1.115	0.939	0.713	1.335
IP Day 42	0.838	0.596	1.135	0.908	0.790	0.641	0.962
IP Day 49	0.887	0.994	1.076	0.831	0.772	0.966	0.683
IP Day 56	0.796	0.854	0.857	0.954	0.902	0.580	0.629
IP Day 63	1.153	0.763	1.009	1.903	1.436	0.748	1.058
IP Day 70	1.121	2.157	1.363	0.516	0.845	0.846	1.000
IP Day 77	0.429	0.345	0.421	0.492	0.426	0.545	0.343
Bath Day 4	0.255	0.233	0.272	0.272	0.367	0.170	0.215
Bath Day 7	0.227	0.239	0.242	0.225	0.239	0.154	0.266

Bath Day 14	0.322	0.384	0.294	0.242	0.279	0.413	0.323
Bath Day 21	0.338	0.382	0.277	0.349	0.457	0.314	0.252
Bath Day 28	0.870	0.628	1.868	0.704	0.727	0.604	0.689
Bath Day 35	0.676	0.718	0.521	0.670	0.536	1.113	0.500
Bath Day 42	0.592	0.429	0.839	0.630	0.664	0.526	0.463
Bath Day 49	0.818	1.162	0.824	0.807	0.714	0.871	0.532
Bath Day 56	0.662	0.500	0.568	0.712	0.978	0.417	0.801
Bath Day 63	0.764	0.538	0.885	1.235	0.418	0.597	0.911
Bath Day 70	0.610	0.433	0.577	0.452	0.943	0.559	0.695
Bath Day 77	0.619	0.494	0.627	0.327	1.078	0.727	0.459

Plate controls are included in the table below, for the respective groups of samples.

		No Fish	**No Rabbit**	**No Antigen**	**HSB -**	**HSB +**	**Blank**
Control 0-7	0-7	0.078	0.038	0.227	0.133	0.576	0.000
Control 14-28	14-28	0.078	0.044	0.222	0.131	0.660	0.000
Control 28-42	28-42	0.129	0.052	0.254	0.291	0.698	0.000
Control 42-56	42-56	0.102	0.055	0.281	0.341	0.778	0.000
Control 63-77	63-77	0.113	0.051	0.317	0.288	0.819	0.000
Control 77-extra	77	0.090	0.045	0.221	0.225	0.644	0.000

Appendix 5

Statistical analysis, from Statistical Analysis Software (SAS), of raw data for hybrid striped bass (*Morone saxatilis* x *M. chrysops*) immune response to *Vibrio spp.* vaccination. The data below displays analysis of optical density (OD) values, which are an average of duplicate wells, for each fish, group and time post vaccination. This is representative of the immune response in the form of specific antibodies to *V. anguillarum*.

SAS® Output results summary (Proc GLM for ANCOVA) for Hybrid Striped Bass immunization measured by ELISA.

The SAS System Proc glm Analysis

The GLM Procedure

Dependent Variable: ODValue

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	9.14105345	1.30586478	20.00	<.0001
Error	227	14.82468005	0.06530696		
Corrected Total	234	23.96573350			

R-Square	Coeff Var	Root MSE	ODValue Mean
0.381422	45.35123	0.255552	0.563496

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Group	3	3.13676029	1.04558676	16.01	<.0001
Day	1	5.81353826	5.81353826	89.02	<.0001
Day*Group	3	0.19075490	0.06358497	0.97	0.4059

Appendix 6

Raw data for Cobia (*Rachycentron canadum*) immune response to *Vibrio spp.* vaccination.

The table below displays optical density (OD) values, which are an average of duplicate wells, for each fish, group and time post vaccination. This is representative of the immune response in the form of specific antibodies to *V. anguillarum*.

	Average OD	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6
Control Day 0	0.593	0.361	0.595	0.755	0.607	0.608	0.635
Control Day 7	0.438	0.310	0.448	0.437	0.515	0.404	0.513
Control Day 14	0.538	0.420	0.583	0.980	0.475	0.427	0.343
Control Day 21	0.440	0.310	0.324	0.322	0.902	0.421	0.364
Control Day 28	0.403	0.338	0.522	0.659	0.261	0.287	0.353
Control Day 42	0.441	0.594	0.412	0.372	0.344	0.471	0.451
Control Day 56	0.594	0.730	0.471	0.768	0.543	0.538	0.513
Control Day 84	0.831	1.267	0.888	0.680	0.864	0.623	0.666
Control Day 112	0.668	0.444	0.926	0.491	0.592	1.068	0.487
Control Day 140	0.582	0.542	0.483	0.533	0.578	0.357	1.002
IP Day 7	1.236	1.018	1.291	1.337	1.010	1.594	1.167
IP Day 14	1.091	1.259	1.102	0.922	0.995	1.264	1.003
IP Day 21	0.792	0.883	1.004	0.984	0.626	0.730	0.525
IP Day 28	0.736	1.057	0.656	0.871	0.574	0.695	0.562
IP Day 42	0.765	0.825	0.685	0.848	0.794	0.640	0.799
IP Day 56	0.822	0.868	0.744	0.729	0.768	0.735	1.091
IP Day 84	0.971	1.110	0.808	1.324	0.735	1.167	0.686
IP Day 112	1.202	1.058	1.225	1.175	1.567	0.999	1.191
IP Day 140	1.071	0.895	1.041	0.881	0.929	1.630	1.053
Bath Day 7	0.592	0.499	0.471	0.904	0.481	0.781	0.417
Bath Day 14	0.577	0.746	0.574	0.585	0.477	0.518	0.561
Bath Day 21	0.444	0.626	0.339	0.526	0.333	0.407	0.434
Bath Day 28	0.505	0.526	0.385	0.565	0.530	0.470	0.553

Bath Day 42	0.976	0.811	0.777	1.274	0.837	0.744	1.417
Bath Day 56	0.771	0.804	0.763	0.689	0.863	0.965	0.542
Bath Day 84	0.846	0.658	1.016	1.077	0.664	0.807	0.855
Bath Day 112	1.194	0.744	3.267	0.639	0.845	0.597	1.072
Bath Day 140	0.780	0.739	0.505	1.010	0.931	0.534	0.963

Plate controls are included in the table below, for the respective groups of samples.

		No Fish	**No Rabbit**	**No Antigen**	**Negative Cobia -**	**Positive Cobia+**	**Blank**
Control 0-14	0.500	0.124	0.090	0.667	0.441	1.678	0.000
Control 14-28	0.487	0.085	0.110	0.669	0.383	1.673	0.000
Control 28-56	0.508	0.097	0.170	0.744	0.411	1.629	0.000
Control 56-112	0.478	0.0975	0.165	0.5295	0.539	1.5365	0.000
Control 140	0.520	0.094	0.217	0.651	0.410	1.751	0.000
Control extra	0.472	0.097	0.117	0.695	0.390	1.532	0.000

Appendix 7

Statistical analysis, from Statistical Analysis Software (SAS), of raw data for Cobia (*Rachycentron canadum*) immune response to *Vibrio spp.* vaccination. The data below displays analysis of optical density (OD) values, which are an average of duplicate wells, for each fish, group and time post vaccination. This is representative of the immune response in the form of specific antibodies to *V. anguillarum*.

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The ANOVA Procedure

Class Level Information

Class	Levels	Values
Group	4	BathVacc IPVaccin NewPBS oldPBS

Number of Observations Read	195
Number of Observations Used	186

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The ANOVA Procedure

Dependent Variable: ODValue

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	6.94332415	2.31444138	27.05	<.0001
Error	182	15.57462492	0.08557486		
Corrected Total	185	22.51794907			

R-Square	Coeff Var	Root MSE	ODValue Mean
0.308346	41.22148	0.292532	0.709659

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Group	3	6.94332415	2.31444138	27.05	<.0001

The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for ODValue

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	182
Error Mean Square	0.085575
Critical Value of Studentized Range	3.66697

Comparisons significant at the 0.05 level are indicated by ***.

Group Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
IPVaccin - BathVacc	0.23704	0.09106	0.38301	***
IPVaccin - NewPBS	0.46285	0.31688	0.60883	***
IPVaccin - oldPBS	0.46866	0.28257	0.65474	***
BathVacc - IPVaccin	-0.23704	-0.38301	-0.09106	***
BathVacc - NewPBS	0.22581	0.07984	0.37179	***
BathVacc - oldPBS	0.23162	0.04554	0.41770	***
NewPBS - IPVaccin	-0.46285	-0.60883	-0.31688	***
NewPBS - BathVacc	-0.22581	-0.37179	-0.07984	***
NewPBS - oldPBS	0.00581	-0.18028	0.19189	
oldPBS - IPVaccin	-0.46866	-0.65474	-0.28257	***
oldPBS - BathVacc	-0.23162	-0.41770	-0.04554	***
oldPBS - NewPBS	-0.00581	-0.19189	0.18028	