MODIFICATION OF A DNA VACCINE FOR ORAL ADMINISTRATION IN FISH FOR AQUACULTURE BY USING NON-MICROBIAL NANOPARTICLES

By

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ABSTRACT

Utilization of DNA vaccines in aquaculture has been gaining interest and recent efforts have been focused on methods of delivering DNA vaccines to fish. In the present study, a methodology was sought that could protect DNA vaccines such that they could be orally administered. The main objective of the study was to determine if a DNA vaccine could be effectively compounded into an orally administrable formulation with chitosan or polylactide-co-glycolide (PLG). The immune response of hybrid striped bass *(Morone saxatilis x Morone chrysops)* following oral delivery of a DNA vaccine containing *Mycobacterium marinum* Ag85A plasmid in either chitosan or PLG nanoparticle encapsulation was evaluated. Hybrid striped bass were divided into four experimental groups: IM immunization of the DNA vaccine as a positive control, oral delivery of uncomplexed DNA vaccine, oral delivery of chitosan or PLG alone as a negative control, and oral delivery of complexed chitosan or complexed PLG DNA vaccine. Fish were bled at regular intervals and an ELISA was used to evaluate antibody levels in individual fish. While the chitosan /plasmid DNA complex containing the *Mycobacterium marinum* Ag85A gene failed to produce a significant antibody response, the PLG/plasmid DNA matrix stimulated humoral immune response in the fish.

Dedication

To my loving parents for their support, inspiration and encouragement in all of my academic and personal decisions.

To my loving aunt, Saswati Hazra, for her thoughtful and mature suggestion to me for my entire life of academic career.

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Chapter 1: Literature Review

a. Hybrid Striped Bass

i. Classification

Hybrid striped bass is the crossing of two parents' species, striped bass *Morone saxatilis* (Walbaum) of order Perciformes and white bass *M. chrysops* (Rafinesque) (Kerby & Harrell 1990; Harrell 1997). Their body is elongate and laterally compressed in cross-section. The body color of the fish changes from silver and black dorsally to white underneath (Hodson 1989). Spines are present in the dorsal and anal fins. The dorsal fin near the center of the body contains 8-9 spines, while the anal fin bears 3 spines and 9-13 soft rays.

ii. Geographical Distribution

Striped bass are abundant from New Brunswick to Florida along the Atlantic Coast and from Florida to Texas along the Gulf Coast. On the Pacific Coast, they are found from British Columbia to south of the border between United States and Mexico (Hodson 1989).

iii. Life History

Being pelagic, striped bass are mainly found in open water areas and show activity during periods of low light such as dawn and dusk. They are predacious in nature and their food includes crustacean zooplanktons such as cladocerans and copepods, insects, fish like threadfin, shad and gizzard shad during various stages of their life cycle. Water temperature in the range of 25-27° C is optimal for the growth of striped bass and they grow rapidly during their first two years of life. The usual life span of striped bass is 5 to

6 years. They are oviparous and produce eggs and sperm in the spring when the water temperature reaches 15-20° C. Males and females mature at 2 and 3 years of age, respectively, and females spawn only once a year producing 160,000 eggs per pound of body weight (Hodson 1989).

iv. Aquacultural and Commercial Importance

Hybrid striped bass culture started in the United States, particularly in the southeastern region in the 1890s, and has rapidly grown since then to become an important aquaculture industry. Commercial production is heavily concentrated in the southern states like Mississippi, North Carolina, Texas, Florida, Louisiana, and South Carolina. Production of hybrid striped bass consists of four distinct phases (Hodson 1995). The hatchery phase consists of rearing of larvae for 3 to 5 days. Next, the larvae or fry are stocked into fertilized, outdoor ponds where they feed primarily on natural zooplankton. After attaining a length of 50 mm after approximately 30–45 days after stocking, these Phase I fingerlings are restocked into ponds for further growth until late winter or early spring. After this stage, the fish are collected as Phase II fingerlings where they usually reach 90–225 g in body weight. Phase II fingerlings are collected, graded and restocked once again into ponds where they attain a weight of 0.6 kg. During this stage they are ready for harvest and are called Phase III hybrid striped bass (Garber and Sullivan 2006). Commercial production of these fish has gained in momentum where the annual production has increased from 400,000 pounds in 1987 to about 11 million pounds in 2005 (USDA Aquaculture Census 2005). Hybrid striped bass is also an important game fish and used for recreational fishing under the regulation of various fisheries agencies or commissions of different states.

v. Diseases of Hybrid Striped Bass

Hybrid striped bass have been used for aquaculture for a long time in the United States and the major etiological agents causing disease in hybrid striped bass are well known. Several of these agents pose major constraint for the commercial production of the fish. The important viral diseases occurring in striped bass are lymphocystis (Krantz 1970) and infectious pancreatic necrosis (Wechsler et al. 2006). Striped bass are also affected by viral hemorrhagic septicemia (Gagne et al. 2007) where the clinical signs include lethargy, exophthalmia, anemia, darkening of skin, hemorrhages of skin, gills and eyes etc. Bacterial diseases such as vibriosis (*Vibrio sp.*) and motile aeromonas septicemia *(Aeromonas hydrophilia)* (Bowser 2004) are important sources of concern. Mycobacteriosis caused by *Mycobacterium sp.* is another concern for hybrid striped bass. Being a chronic disease, mycobacteriosis is responsible for causing low to moderate level mortality in fish populations. Diseased fish are characterized by emaciation, inflammation of the skin, exophthalmia and open lesions or ulcerations. The internal organs of the fish such as the liver, kidney, spleen, heart and muscles are often characterized by the formation of gray-white granuloma (Francis-Floyd 1999). Another disease similar to piscine mycobacteriosis is a bacterial disease caused by *Nocardia sp.* (Shimahara et al. 2009) where the clinical signs include anorexia, emaciation, lethargy, skin discoloration and ulcerations in the fish. During times of stress or injury, striped bass can also be affected by fungi (usually *Saprolegnia or Aphanomyces*) (Yanong 2003). These fungal infections usually present as a cottony-mass of hyphae attached to the external surface of the fish. Common external protozoan infecting hybrid striped bass are *Amyloodinium ocellatum*, *Trichodina sp., Ichthyophthirius mutifillis,* and *Cryptocaryon irritans* (Plumb 1991). The

yellow grub, the larval stage of a digenetic trematode, can also poses a threat to the commercial culture of hybrid striped bass. This parasite burrows into the flesh of the fish, and can affect an entire population of fish (Daniels, UNC-SG-BP-05-01). Striped bass are often attacked by *Epistylis sp.,* a sessile, stalked ciliated protozoan that primarily results in skin lesions (Miller and Chapman 1976).

b. Mycobacteriosis in Fish

i. Causative Agent

Piscine mycobacteriosis is a chronic bacterial disease caused by various species of *Mycobacterium* including *M. marinum, M. fortuitum*, and *M. chelonae*. The etiological agents responsible for mycobacteriosis in fish are non-tuberculous mycobacteria and are facultative intracellular bacterial pathogens that are capable of causing severe, progressive, systemic disease. Virtually all species of freshwater and marine fish can be infected (Austin and Austin 1993; Smith 1997). Reports of the first mycobacterial infection in fish occurred in 1897 (Bataillon et al. 1897) where an acid-fast bacillus from a tuberculous lesion was isolated in a common carp (*Cyprinus carpio*). *Mycobacterium marinum* was first detected from marine fish at the Philadelphia Aquarium (Aronson 1926). *Mycobacterium fortuitum* was identified in the early 1950's from neon tetra fish (*Paracheirodon innesi*) and later the presence of *M. chelonae* was documented in Pacific salmon (*Oncorhynchus spp.*) (Heckert et al. 2001). Massive mortalities due to piscine mycobacteriosis have occurred in intensive aquaculture systems in species such as European sea bass (*Dicentrachus labrax*), red drum

(*Sciaenops ocellatus*), rabbitfish (*Siganus doliatus*), and cobia (*Rachycentron canadum*) (Diamant et al. 2000; Lowry and Smith 2006).

ii. Disease Transmission

Cannibalism and eating contaminated feed and thereby ingesting the infectious agent are the main routes for the transmission of mycobacteriosis in fish (Chinabut et al. 1990; Grady et al. 1992; Post 1987). Mycobacterial lesions present in the digestive tract, gill and skin are the main source for releasing infectious materials into the water column. In addition, once the fish dies and decomposes, release of infective bacteria from infected tissues increases (Decostere et al. 2004; Smith 1997). Vertical transmission is also reported to occur through egg or sperm products in the Mexican platyfish, *Xiphophorus maculatus* (Decostere et al.).

iii. Disease Pathology

One of the essential virulence attributes of mycobacteria is their ability to grow within host macrophages and avoid the immune system of fish. (Pasnik et al. 2003; Cosma et al. 2004). Very few lymphocytes are reported in *Mycobacterium marinum* induced fish granuloma (Bouley et al. 2001; Swaim et al. 2006). It is thought that the survival of *Mycobacterium marinum* occurs within a vacuole in macrophage that does not undergo fusion with lysosomes or acidification (El-Etr et al. 2001). Survival of *Mycobacterium marinum* within the host cell depends partially on arresting phagosome maturation prior to phagolysosome fusion (Barkar et al. 1997; Rohde et al. 2007). In fish monocytes, the live *Mycobacterium marinum* are concentrated in non-acidified phagosomes and secrete vaculoa proton ATPase (Barkar et al. 1997). This seclusion helps the mycobacteria avoid

phagolysome-mediated killing and also changes the immune response of the host by altering antigen presentation (Russell et al. 2002).

Parenchymal tissue of the kidney, spleen and liver can show microscopic grayish white miliary granuloma (Frerichs 1993). The visceral granuloma is comprised of a thick capsule of epitheloid cells and the granuloma surrounds a necrotic center containing large numbers of acid-fast bacilli. The gray or yellowish tubercular nodules are prominent in the internal organs like the liver, spleen and gonads. Langhan's giant cells are not prominent in piscine mycobacteriosis (Post 1987; Talaat et al. 1997; Astrofsky et al. 2000).

iv. Clinical signs

Mycobacteriosis is mainly a chronic disease and because of the slow progress of the disease, fish do not often show any external signs in the initial stage. With time, clinical signs may include lethargic behavior, emaciation, and inflammation of the skin, exophthalmia, skin ulcerations, spinal curvature and lack of development of secondary sexual characteristics. (Smith 1997; Decostere et al. 2004). Mycobacteriosis can lead to corneal opacity and eventually cause exophthalmia. Changes in body color are mainly associated with changes in actual pigment cell density due to the accumulation of melanin-pigmented cells around the inflammatory foci.

v. Diagnosis

Mycobacteria are Gram-positive, acid-alcohol fast, non-motile, non-spore forming, pleomorphic rods. Diagnosis of mycobacteriosis mainly depends on clinical and histological signs and identifying the strains of

bacteria responsible for causing the disease. Smears from organs such as the spleen, kidney and skin lesions can be stained with Kinyoun modification of the Ziehl-Neelsen stain (Mycobacteriosis 2006. Center for Food Security and Public Health). Presence of gross and histologic lesions of necrogranulomas having acid-fast bacilli indicates the presence of *Mycobacterium.* Tissue biopsy for histology and bacterial culture are important diagnostic assays for mycobacteriosis. Both polymerase chain reaction (Wongtavatchai et al. 2003) and enzyme-linked immunosorbent assays (ELISA) are newer tools used to detect mycobacteriosis in fish (Adams et al. 1995).

vi. Human Health Concerns

Piscine mycobacteriosis is responsible for "Fish Handler's Disease" or "Fish Tank Granuloma" in humans when the individuals become infected with the etiological agent. Granulomas develop in the skin resembling a rash and they are particularly prevalent on the hands, arms or legs. (Francis-Floyd, Publication # VM-96). For certain professions like pet shop owners, *Mycobacterium marinum* infection may be an occupational hazard. But sometimes infections also spread to fish fanciers who keep home aquariums, and hence the disease is also known as "Fish Fanciers' Finger Syndrome" (Wheeler and Graham 1989). The potential route for the spread of infection includes cleaning or changing of water as well as direct injury from the fish fins or bites (Bhatty et al. 2000). Cutaneous disease with *M. marinum* can appear as papulonodular, noduloulcerative, granulomatous, verrucous plaques and sporotrichoid lesions or deep tissue infections of the tendon and the bone (Kullavanijaya et al. 1993; Holmes et al. 1999). *Mycobacterium fortuitum* and *M. chelonae* are also capable of infecting humans but they are

less common than *M. marinum* (Zenone et al. 1999; Hazelton et al. 2000; Collina et al. 2002). Thus mycobacteriosis has important zoonotic significance for humans.

vii. Control Measures

Sanitation, disinfection and destruction of carrier fish seem to be the most effective steps for control measures against piscine mycobacteriosis. Treatment of the disease is challenging and the use of anti-tuberculin drugs like isoniazied, kanamycin or Baytril ® have recently been reported (Ichinotsubo 2000). However, none of these treatments is approved or legal for foodfish. Currently, there is no commercially available vaccine against piscine mycobacteriosis, but the ability of the fish to develop a significant cell-mediated immune response against the mycobacterial antigens (Bartos and Sommer 1981; Chen et al. 1998) promises the development of BCG-like vaccines (Frerichs 1993; Austin and Austin 1999c). Recently a DNA vaccine prepared from the *Mycobacterium marinum* Ag85A gene was shown to produce a protective immune response to a live *M. marinum* challenge 90 days post-inoculation in fish. (Pasnik et al. 2005).

c. Drugs vs. Vaccines in Aquaculture

Compared to the drugs approved for the treatment of diseases in cattle, swine and poultry, the number of drugs approved for aquaculture is relatively small. In addition, the use of drugs in a food fish species causes concern for potential drug residues for human consumption, so most of these drugs cannot be considered under Animal Medicinal Drug Use Clarification Act of 1994. For the approval of a new drug under the New Animal Drug Application, a huge amount of time and financial investment is required. The

metabolism and excretion of a given drug can be affected by the water temperature, which in turn can affect the drug residues in the tissues (Stehly et al. 2000). Changes in different water parameters such as temperature, pH, hardness, oxygen levels can also cause a change in drug toxicity even at the recommended doses (Burka et al. 1997). The environmental impact of a drug is another important issue to be considered. Incomplete feed consumption or the excretion of the biologically active metabolites associated with the medicated feed should be considered carefully.

In order to control some infectious diseases of fish in aquaculture, the use of fish vaccines have been shown to be a cost-effective and efficient method. Thus, fish vaccines can be a good alternative to the use of antibiotics in aquaculture making fish farming more economically feasible and more sustainable. Being made up of natural biological materials, fish vaccines are not associated with toxic residues in the tissues of the organism or in the environment. Vaccines also do not contribute to the growth of resistant strains of bacteria. Today, fish vaccines follow strict federal regulations like other veterinary biologicals. Therefore, more aquaculture producers and managers of farms are relying on fish vaccines for their protective and cost effect (Vinitnantharat et al. 1999).

d. Vaccination in Fish

Pathogens are one of the most important concerns for finfish culture. Many chemicals and antibiotics suggested for controlling pathogens do not seem to be too effective as evidenced by their contamination of the aquatic environment, accumulation in the flesh of animals and development of drug-resistant strains (Munn 1994; Lillehaug 1995). Classical vaccines are preparations of antigens derived from specific pathogenic organisms that are

rendered non-pathogenic. They stimulate the immune system and increase the resistance to disease from subsequent infections by the specific pathogen. Research in vaccine development for aquaculture has been progressing slowly and most of the commercially available vaccines are mainly against bacterial diseases. There are a few vaccines against viral diseases, and no vaccines exist against parasitic infections. Relatively small acceptance by the aquaculture industry along with the high price associated with the cost of production of a vaccine is mainly responsible for this scenario.

DNA vaccines have several advantages over classical antigen vaccines. Factors like DNA being a stable molecule, relatively inexpensive and easy to produce, and potential for preparing multivalent vaccines have contributed to DNA vaccines being attractive to fish manufacturers (Heppell 2000). Similar to classical vaccines, there are several routes of DNA vaccine immunization in fish. Vaccination by injection seems to be the most effective method for boosting the immune response where purified DNA in a small volume of saline or buffer is intramuscularly injected in the flank, below or close to the dorsal fin. The persistence of DNA after intramuscular (IM) injection is stable in fish and there is little degradation as determined by southern blotting. Plasmids were detected even 63 days after injection (Anderson et al. 1996). The peritoneal route (Kanellos et al. 1999) has also been tried, as well as other sites for injection but the results were not as effective as the IM route. The optimal dose of vaccine for the fish depends upon the size and species. The expression level of the gene is also affected by the physiological condition of the fish (Heppell 2000). Another route of vaccination is orally, where the vaccine is either mixed or coated on top of the feed or bioencapsulated. When vaccines are used as top-dressing in feed,

a coating agent needs to be applied either to prevent the leaching of antigens from the pellets or to help restore the integrity of the antigen to the acidic environment in the stomach of the fish. Bioencapsulated feed is particularly suitable for fish fry. Live feeds such as *Artemia* nauplii, copepods or rotifers are incubated in the vaccine suspension and fed to the fish. The bioencapsulated feed then releases the vaccine in the digestive tract of the fish. This appears to be the most appealing method for delivery of vaccines in fish as there is reduced handling of fish, which thereby reduces the stress experienced by the fish in addition to being convenient for mass immunization. For effective oral delivery, the antigen should not undergo digestive hydrolysis and should be absorbed efficiently for inducing a protective immune response (Vandenberg 2004). But oral delivery appears to be less effective compared to the other routes of administration, like injection or immersion techniques. (Ellis 1995). Bath vaccination or immersion vaccination seems to be the most widely employed mode for delivering vaccines by aquaculturists. With these techniques the fish are immersed or sprayed with vaccine solution. This mode of vaccination has historically been more effective than oral administration because there is a better absorption of antigens through the skin and gills as compared to the gut (Ellis 1995).

e. Nanoparticle Based Gene Delivery

Nanoparticles are submicron-sized polymeric colloidal particles that are mainly used for encapsulating a therapeutic agent for their application in medicine (Zhang et al. 2008). Several disease-related bioactive molecules have been successfully encapsulated with nanoparticles in order to enhance their bioavailability, bioactivity and controlled delivery. Factors like high

encapsulation efficiency, longer retention time, as well as increased bioavailability are the driving forces for choosing a suitable polymeric system for creation of efficient nanomedicine. The size of the nanoparticles, surface charge, and surface modifications are important in determining whether the nanoparticles can interact with the cell membrane and their ability to overcome the physiological drug barriers. Nanoparticles can be used for distant target sites within a body either by a catheter-based approach with minimal involvement of invasive procedures (Song et al. 1998) or the coupling of nanoparticles with biospecific ligands can be done for targeted site delivery (Moghimi et al. 2001). The circulation factor, tissue and target site of the body influences the nanoparticles for crossing different biological barriers (Brannon-Peppas and Blanchette 2004). Delivery of therapeutic genes to the diseased cells has been a great challenge in the development of gene therapy. For gene delivery, non-viral vectors are safer than those with viral vectors (Aigner 2007). Factors like toxicity, immunogenicity and carcinogenicity associated with viral gene therapy encourages the use of non-viral gene therapy.

Biodegradable polymeric nanoparticles are being increasingly used today in the field of nanomedicine. Some of the advantages of these type of nanoparticles includes the slow releasing property of the encapsulated gene or drug, subcellular size, biocompatibility with tissues and cells, non-toxic, stable in blood, non-immunogenic, non-inflammatory, biodegradable and avoiding retinonucleoid systems (Rieux 2006). For the formulations of different biodegradable polymers, many synthetic and natural polymers have been used. By modulating polymeric characteristics, the release of a therapeutic agent or vaccine from the polymer with an optimal time for enhanced therapeutic efficiency can be controlled. Synthetic polymers have

the advantage of slow release of the drug over an extended period of time as compared to natural polymers. Synthetic polymers that have been popular for gene delivery include polylactide and polylactide-co-glycolide copolymers, while the list of natural polymers includes albumin, alginate, gelatin, collagen and chitosan (Moghimi et al. 2001).

In aquaculture, the use of nanoparticles such as chitosan has been reported. An increase in survival rate was reported in black tiger shrimp (*Penaus monodon)* after oral administration of a DNA construct with chitosan nanoparticles against white spot syndrome virus (Rajeshkumar et al. 2009). The successful gene transfer through the oral route using a chitosan-DNA plasmid (pVAOMP38) and development of a significant antibody response in fish against the OMP38 protein of *Vibrio anguillarum* has also been reported (Rajeshkumar et al. 2008). Oral delivery of the construct by encapsulating DNA expressing β-galactosidase reporter gene within chitosan showed β-galactosidase in the stomach, spleen and gills of tilapia (Ramos et al. 2005). In rainbow trout fry, specific antibodies were detected in the serum after the fry were orally immunized with an antigen encapsulated in polylactide-co-glycolide (Lavelle et al. 1997).

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Chapter 2: Oral Administration of Chitosan-Ag85A Complex to Hybrid Striped Bass

(To be submitted to the Journal of Aquatic Animal Health)

a. Introduction

The advantages of non-invasive oral vaccines (e.g. generation of both mucosal and systemic immunity providing a strong first line of defense, ease of administration, and cost effectiveness) make them preferable to the injectable routes (Aziz et al. 2007). Thus, the application of DNA vaccines through oral administration is gaining attention. Having unique practical and immunological advantages over traditional antigen vaccines, DNA vaccines have attracted many aquaculturists to opt for DNA vaccines as a preferred mode of vaccination. From a practical point, DNA vaccines are relatively easy to produce and the process of production of various types of DNA vaccines are identical. Also, different plasmids can be mixed together for the production of multivalent vaccines. This can ultimately decrease the manufacturing cost of a vaccine. As a result, in today's age of aquaculture, more and more aquaculturists are attracted to the use of DNA vaccines as an effective tool to combat disease in fish farm facilities (Nerland et al. 2007, Lorenzen and LaPatra 2005).

However the mechanism for development of the immune response in fish after DNA vaccination is poorly understood. DNA vaccination by injection seems to be most promising regarding the development of a successful immune response in the fish. Alternative routes of administering a DNA vaccine, such as via the oral or immersion route, are ideal for large numbers of fish as well as for very small fish. This is because these routes reduce the amount of labor required, as well as minimize the stress to the fish that is associated with vaccination by injection. In addition, one of the advantages of oral vaccination in fish is that the fish generates a significant mucosal immune response (Heppell and Davis 2000).

Since oral vaccination delivers the antigen to the fish through its digestive system, the vaccine needs to be protected from the enzymatic degradation and acid hydrolysis of the gut. One way of protecting a DNA vaccine from the undesirable action of the stomach and intestine of the fish is by encapsulating the DNA vaccine in biodegradable nanoparticle microspheres. Nanoparticles are submicron-sized polymeric colloidal particles that are mainly used for encapsulating a therapeutic agent or gene for use in medicine (Gelperina et al. 2005). Some of the advantages of biodegradable polymeric nanoparticles include the slow release of the encapsulated gene or drug, subcellular size, biocompatibility with tissues and cells, low toxicity, stability in blood, lack of immunogenicity, lack of inflammatory response as well as biodegradability (Rieux 2006). Nanoparticles have the ability to be incorporated in both hydrophobic and hydrophilic substances. Also, nanoparticle encapsulated drugs can be easily presented to the host through various routes of administration such as oral administration and inhalation (Gelperina et al. 2005). Thus, nanoparticles formulated with chitosan, alginate or polylactide-co-glycolide have numerous potential applications for vaccine and drug delivery in the fields of veterinary and human medicine.

Chitosan is a cationic polyelectrolyte and is the second most abundant polysaccharide present in nature (Wang and Xu 2003). Distinct advantages of chitosan such as biocompatibility (Hirano et al. 1990) and increasing the membrane permeability, both *in vitro* (Aspeden et al. 1997; Lehr et al. 1992) and *in vivo* (Takeuchi et al. 1996) ranks chitosan as one of the most used biodegradable polymeric nanoparticles for drug or gene delivery. The mucoadhesiveness and permeability enhancing properties of chitosan makes this nanoparticle a good absorption enhancer across intestinal epithelium

(Janes et al. 2001). Unfortunately, chitosan is soluble in an acidic pH range, thus chitosan by itself does not act as a suitable oral delivery material. However, N-acetylation of chitosan with acetic anhydride makes chitosan acid-resistant (Kofuji et al. 2001). Therefore, N-acetylated chitosan complexed with DNA is presumed to protect the encapsulated material and enhance the transfection efficiency. Studies in humans suggest that N-acetylated chitosan can retain DNA for a longer time than a simple chitosan/DNA mixture (Roy et al. 1999).

In aquaculture, successful gene transfer through the oral route using a chitosan-DNA plasmid (pVAOMP38) was confirmed by the development of a significant antibody response in fish against the OMP38 protein of *Vibrio anguillarum* (Rajeshkumar et al. 2008). Oral delivery of a construct encapsulating DNA expressing the β-galactosidase reporter gene within chitosan demonstrated β-galactosidase in stomach, spleen and gills of tilapia (Ramos et al. 2005). In addition, an increase in survival rate was reported in black tiger shrimp (*Penaus monodon)* after oral administration of a DNA construct with chitosan nanoparticles against white spot syndrome virus (Rajeshkumar et al. 2009).

In the present study, a methodology was sought that could protect a DNA vaccine such that it could be orally administered to fish. The main objective of the study was to determine whether vaccination via oral delivery of the Ag85A plasmid complexed with N-acetylated chitosan would result in a detectable humoral immune response.

b. Materials and Methods

i. Fish

Juvenile hybrid striped bass were obtained from a local state hatchery (Vic Thomas Striped Bass Hatchery, Brookneal, VA) and kept in recirculation aquaculture systems with appropriate biological filtration and aeration. Water temperature was maintained in the range of 23-26^{0}C along with a fluorescent light photoperiod of 12 hr light/12 hr dark. Fish were fed daily with the commercial pelleted food (Zeigler Bros Inc, Gardners, PA) at 3-5% of their body weight per day.

ii. Chitosan - Ag85A Plasmid Complex Formation

Two thousand μg of DNA (recombinant plasmid expressing the *M. marinum* Ag85A antigen) was mixed with 36 ml pentabasic practical grade 90-95% of sodium triphosphate solution (TPP, Sigma Aldrich Inc. St. Louis, MO). Equal volumes of low molecular weight chitosan (Sigma Aldrich Inc.) and the previously mixed solution of DNA and TPP were heated in a water bath (ISO TEMP 210, Fisher Scientific, Atlanta, GA) to 55^{0}C. The two solutions were mixed and centrifuged for 15 minutes at 1000 x g and the supernatant was checked for uncomplexed DNA by measuring the concentration of DNA in a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). The pellet was resuspended in water and again centrifuged and this process repeated until the supernatant contained no free DNA. The pellet was frozen overnight at -20^{0}C and the next day was lyophilized using a Flexi-dry Lyophilizer (VirTis Wizard 2.0, Gardiner, NY). The resulting powder was resuspended in 3% acetic anhydride (Sigma Aldrich Inc.) in methanol (v/v, Sigma Aldrich Inc.) and incubated at room

temperature (21^0C) for 3 hrs. The material was then frozen at -80^0C overnight and the following day lyophilized again and stored at -80^0C until used.

iii. Tagging and Vaccination

Fish were anesthetized with sodium bicarbonate buffered MS-222 (100-150 µg/l, Sigma Aldrich Inc.) and weighed. Fish were individually tagged with a PIT tag (Biomark Inc., Boise, ID) inserted per manufacturers instructions into the ventral coelomic cavity. Fish were randomly assigned to one of four groups: (Group 1) an intramuscular (IM) immunization of the uncomplexed DNA vaccine as a positive control, (Group 2) an oral delivery of uncomplexed DNA vaccine, (Group 3) an oral delivery of a chitosan alone as a negative control, and (Group 4) an oral delivery of the complexed-chitosan DNA vaccine. In all experimental groups, a dose of 50 µg of DNA/fish was targeted. The first group was given an intramuscular injection of uncomplexed DNA by 1 ml syringe with a 25-gauge needle, while the remaining three groups of fish were administered the experimental material by oral administration of the vaccine directly into the stomach by a metal gavage feeding tube (Popper®, Cadence Science, Lake Success, NY).

iv. Blood Collection

Blood was collected from each fish in all four groups pre-vaccination and then repeated five times at two-week intervals between each blood collection post-vaccination. Prior to each collection, the fish were anesthetized as described previously and blood collected with a 1 ml syringe with a 23-gauge needle from the caudal tail vessels. Approximately 0.2 ml of blood was collected from each fish. The blood samples were placed in

separate serum separator tubes (BD Microtainer, Becton, Dickinson and Co., Franklin Lakes, NJ) and stored at 4^0C overnight. The next day the blood was centrifuged at 14,000 x g for five minutes, the serum extracted and placed in individual microcentrifuge tubes, and stored at -20^0C until future analysis. After the final blood collection, all fish were humanely euthanized by an overdose of buffered MS-222 (200 μg/l).

v. ELISA Assay

The ELISA assay was performed using a modification of a previously described whole-cell lysate ELISA (Laal et al. 1997). Briefly, a 50 ml culture of *Mycobacterium marinum* was grown in Middlebook 7H9 broth (DIFCO Laboratories, Detroit, MI) for 2-3 weeks at 25^0C. The culture was harvested at 1000 x g for 10 minutes. The pellet was resuspended in 25 ml phosphate buffered saline (PBS) containing 1mM PMSF, 1mM EDTA and 1mM DTT (Fisher Scientific, Pittsburg, PA). The mixture was frozen and thawed in liquid nitrogen several times, and then sonicated for 20 minutes followed by centrifugation at 150 x g. A protein assay (BCA assay, Pierce Chemical, Rockford, IL) was performed on the supernatant and the protein solution adjusted to 15 μg/ml in coating buffer (0.05M sodium carbonate buffer, pH 9.6). Fifty ul of the protein solution was added to all but two wells of a 96-well ELISA plate (Corning, Corning, NY). To the final two wells, 200 μl of 1% BSA (Sigma Aldrich Inc, USA) in coating buffer was added which acted as the uncoated negative control wells. The plate was covered with Parafilm (Pechiney Plastic Packaging, WI, USA) and incubated overnight at 4^0C. The next day, the plate was washed three times with phosphate buffered saline plus 0.05% Tween 20 (Sigma Aldrich Inc.) in dH2O (PBST). Then, 250 μl of 1% BSA in coating buffer was added to each

well. The plate was incubated for 45 minutes at 37°C, then washed three times with PBST. Following this, 100 µl of individual fish serum diluted 1:1000 in PBST was added to appropriate wells. Duplicate wells were used for each sample, including controls. The plate was incubated for 2 hr at room temperature after which the plate was again washed three times with PBST. The primary antibody used was an affinity purified rabbit anti-hybrid striped bass antibody produced in our laboratory (Smith et al. 1994). This was diluted 1:10,000 in PBST, and 100ul was added to each well. The plate was incubated for 1 hr at room temperature, then washed three times with PBST. Next, 100 µl of the secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) diluted 1:100 in PBST was placed in each well. The plate was incubated for 45 minutes at room temperature, and again washed three times with PBST. Finally, 100 µl of TMB substrate (Kirkegaard & Perry Lab., Inc.) was added to each well. The plate was incubated for 6 minutes at room temperature, then 100 µl of stop solution (1 N HCL) was added to each well. The plate was read using an ELISA plate reader at 450 nm.

vi. Scanning Electron Microscopy

Aluminum stubs (Electron Microscopy Sciences, Hatfield, PA) of 12.7 mm were selected for placement of chitosan and chitosan encapsulated DNA lyophilized powder on the appropriate stage for scanning electron microscope (SEM) examination. After putting double-sided tape on the top surface of the stub, the samples were placed on the exposed surface of the double-sided tape. Conducting paint of colloidal silver paste (Electron

Microscopy Sciences) was added around the periphery of the sample, and the samples sputter coater with gold (SPI Supplies, PA). After coating, the samples were viewed in a scanning electron microscope (Carl Zeiss, Evo 40, Germany) and images digitally recorded.

vii. Statistical analysis

ANOVA model with random effects was applied to evaluate the humoral immune response development in all of the groups of fish through all of the five bleeding periods after the pre-bleed. Time as a co-variant had been categorically distributed in these five bleeding periods. Multiple comparisons with Tukey's Adjustment was used to compare the mean differences of the ELISA values of the humoral immune response generated by the four experimental groups of fish with a significance level of $p \leq 0.05$.

c. Results

i. Immunological Assays

Antibody titers (e.g. ELISA values) demonstrated by the four groups of fish over the six sampling periods are shown in Figure 1. A relative comparison of immune response elicited by the four groups; (1) the positive control, i.e. fish which were given uncomplexed intramuscular (IM) injection, (2) the group of fish which were orally administered the uncomplexed DNA, (3) the negative control, i.e. group of fish which were given only chitosan and (4) the experimental group, i.e. group of fish to which oral administration of chitosan-encapsulated DNA vaccine was given over six sampling periods are represented in Figure 1. The control DNA IM immunization reached a peak titer at 8 weeks post-immunization, and then

declined. Neither the chitosan alone nor the DNA encapsulated in N-acetylated chitosan-administered orally elicited a humoral immune response in fish. However, the uncomplexed DNA oral vaccination elicited an immune response similar to the uncomplexed DNA IM vaccinated fish. Significant difference in development of the humoral immune response among the treatment groups throughout the bleeding periods was indicated in Table 1. The mean humoral immune response generated by four groups of fish through the bleeding periods is shown in Table 2, while Table 3 represents a relative comparison of the immune response of the four groups with respect to each other and whether the differences observed were significant ($p \leq 0.05$).

ii. SEM analysis results

Both chitosan and chitosan encapsulated DNA lyophilized powders were viewed by SEM and images recorded (Figures 2, 3, 4 and 5). The size range of the chitosan particles as observed from SEM imaging was 400-1000 μm, while the chitosan-encapsulated DNA particles were in the size range of 100-500 μm. The microparticles of both the chitosan and chitosan-encapsulated DNA lyophilized powder did not attain any regular specific shape. The microparticles looked like shells with bent edges, and the microstructures of the chitosan powder were not as distinctly visible as previously described by Picker-Freyer and Brink (2006).

d. Discussion

Oral administration of a vaccine is an effective way to deliver a vaccination non-invasively, and in aquaculture, fish can be vaccinated with minimal stress. For effective oral vaccination, the delivered antigen or gene

should not undergo digestive hydrolysis in the stomach and should be efficiently absorbed in the hindgut of the fish in order to induce a protective immune response (Vandenberg 2004).

Biodegradable polymeric nanoparticles are being increasingly used today in the field of gene or vaccine delivery to target organs. A number of polymers have been used in pharmaceutical research for drug delivery to the target site and have recognized the decreased number of side effects, thus increasing the therapeutic benefit (Kreuter 1994). The ability of biodegradable polymers in delivering protein, peptides and genes through an oral route, as carriers of DNA in gene therapy and their ability to reach target sites have made the use of these polymers as a delivery system in the field of pharmacology (Langer 2000).

Chitosan is a natural biodegradable polysaccharide that is extracted from the shells of crustaceans (Leong et al. 1997). The potential for application of chitosan for gene transfer through oral delivery has been investigated since 1990 (Sashiwa and Alba 2004). In comparison with other non-viral vectors, chitosan is relatively non-toxic for animals, shows high transfection efficiency (Richardson 1999) and has good bioadhesive properties (Pan et al. 2002). Chitosan contains OH and NH_2 functional groups that facilitate hydrogen bonding and therefore makes the polymer chain flexible to a certain extent. These factors make chitosan suitable for mucoadhesion. Evidence of *in vivo* gene transfection exists for chitosan-bound plasmid DNA and the successful use of N-acetylated chitosan was demonstrated by Ramos et al. (2005). Fish fed plasmid DNA encapsulated in chitosan were shown to express the reporter gene, β-galactosidase. N-acetylated chitosan has also been shown to deliver DNA to the gut-

associated lymphoid tissue of mammals (Roy et al. 1999; Bozkir and Saka 2004; Kai and Ochiya 2004; Li et al. 2005).

In this study, efforts were made to encapsulate the Ag85A plasmid of *M. marinum* with a mixture of N-acetylated chitosan and TPP. The ionic cross linking chitosan-TPP nanoparticle was chosen as an alternative rather than using chitosan alone as a delivery system for plasmid DNA since chitosan-TPP nanoparticle complex shows appropriate encapsulation and controlled release of DNA and also acts as a biocompatible non-viral gene delivery system (Csaba 2009). Since the chitosan-TPP complex has shown negligible toxicity, this can act as a highly efficient gene delivery system.

The results of this study showed that the complexed DNA vaccine failed to elicit a specific immune response in hybrid striped bass. The immune response generated by our two experimental groups, i.e. fish orally administered the chitosan-encapsulated DNA vaccine and fish administered the uncomplexed DNA oral vaccine, were statistically different ($p \leq 0.05$) from each other. However, the uncomplexed DNA oral vaccination elicited an immune response similar to the uncomplexed DNA IM vaccinated fish that served as our positive control. The immune response generated by the positive control and the DNA oral vaccination groups were not statistically different from each other.

Failure of our orally administered microencapsulated DNA vaccine may be due to DNA inactivation within the gastrointestinal tract of hybrid striped bass. The acidic pH of the gastrointestinal tract promotes degradation of chitosan-polymer more than an alkaline pH; the protonation of the chitosan decreases rapidly at pH 6.5 or higher. The fish might not have developed a suitable immune response from the chitosan-encapsulated DNA particle as the alkaline pH of gastrointestinal tract of hybrid striped bass

might have prevented the release of DNA from the chitosan nanoparticles. This could result in preventing the encoded gene from precipitating out from the chitosan nanoparticle-matrix. Thus, the absorption-promoting properties of the chitosan are lost. One of the most important challenges in using chitosan as a suitable encapsulating agent is to deliver the chitosan polymer and gene at the same time in an absorbable soluble form.

SEM imaging of both chitosan and chitosan-encapsulated DNA lyophilized powder was undertaken in order to get a rough estimate of the distribution of particle size. Our imaging results showed that the commercial chitosan powder had a size in the range of 400-1000 μm. Nanoparticle size typically ranges from 0.1 to 100 nm but for the purpose of drug or gene delivery, nanoparticles greater than 100 nm can serve as drug-carrier (Jong and Borm 2008). Observations from SEM imaging revealed that the chitosan powder that was used for encapsulation of DNA was many times larger than the accepted nanoparticle size. This finding revealed that the chitosan powder was in the microparticulate form rather than being in the size range of nanoparticles. When the chitosan was used for encapsulation of DNA, the size range of the chitosan-DNA particles were in the range of 100-500 μm. Though this was a decrease in size from the chitosan alone, this indicated that even the chitosan-encapsulated DNA was also in the size range of microparticles. In the field of drug delivery, both nanoparticles and microparticles have been used as a suitable carrier system. Due to the large size of microparticles, they are often unable to cross the biological barriers and so the drugs coated with microparticles must be delivered directly to the target sites. However the sub-micron size of nanoparticles can allow the particles to cross and overcome most of the biological barriers. Like other colloidal drug carriers, nanoparticles can pass through blood capillaries after

injection and get adsorbed in blood stream (Kreuter 1994). On the other hand, the large size of microparticles can cause the particles to get mechanically trapped and subsequently undergo filtration by the capillary bed. Nanoparticles can be absorped by the macrophages of reticuloendothelial system from the blood stream while a similar mechanism of absorption does not generally occur in the case of microparticles (Kreuter et al. 1995). The cells, through the process of phagocytosis and pinocytosis, generally take up extracellular materials with subsequent processing. Phagocytic mechanisms can enable the cell to take up material up to 10 μm in diameter. All cell types employ pinocytic mechanisms that enable the cells to engulf any material of sub-micron size. As a result, microparticles can only be delivered to the phagocytic cells while nanoparticles can be used for all cell types (Kohane 2007). However, the large size of chitosan-encapsulated DNA (100-500 μm) that had been administered to the fish may have prevented the cells from employing the process of phagocytosis or pinocytosis. As a result, the lack of uptake of chitosan-encapsulated DNA resulted in no significant immune response in our experimental groups of fish.

The generation of an immune response in fish orally administered the uncomplexed DNA vaccine was unexpected. Interestingly, the immune response developed by this group of fish was not statistically different from the immune response developed by the positive control group administered the vaccine by the IM route. This was surprising since the experimental group orally vaccinated (e.g. N–acetylated–chitosan DNA plasmid complex) failed to produce a significant humoral immune response in the fish. Thus, neither complex appeared to be able to stimulate a detectable immune response in hybrid striped bass.

Figure 1: Comparison of the ELISA values elicited in hybrid striped bass *(Morone saxatilis x Morone chrysops)* vaccinated orally with chitosan alone as a negative control, vaccinated orally with the complexed-chitosan DNA vaccine, vaccinated intramuscularly with the uncomplexed DNA as a positive control, and vaccinated orally with the uncomplexed DNA, respectively.

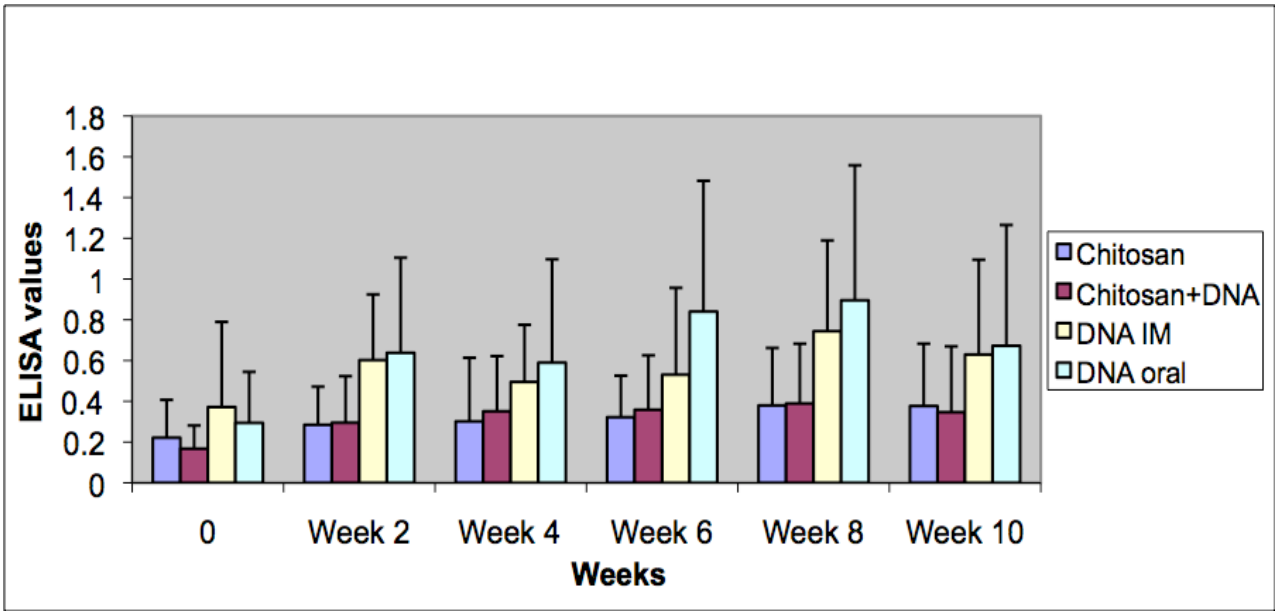

Figure 2: Scanning electron micrograph (SEM) image of chitosan (small particle size, approximately 500+ µm).

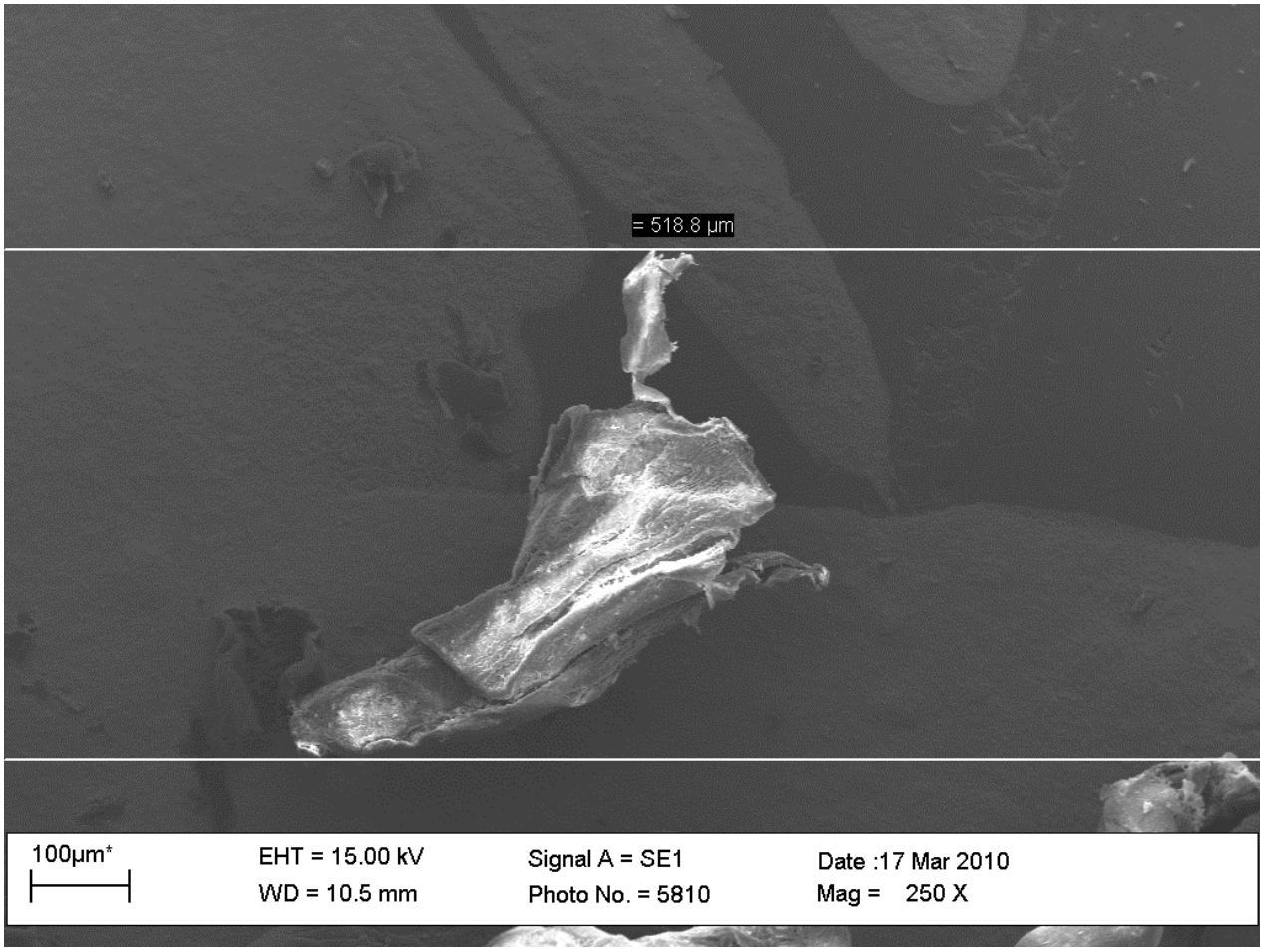

Figure 3: Scanning electron micrograph (SEM) image of chitosan (large particle size, approximately 1000+ µm).

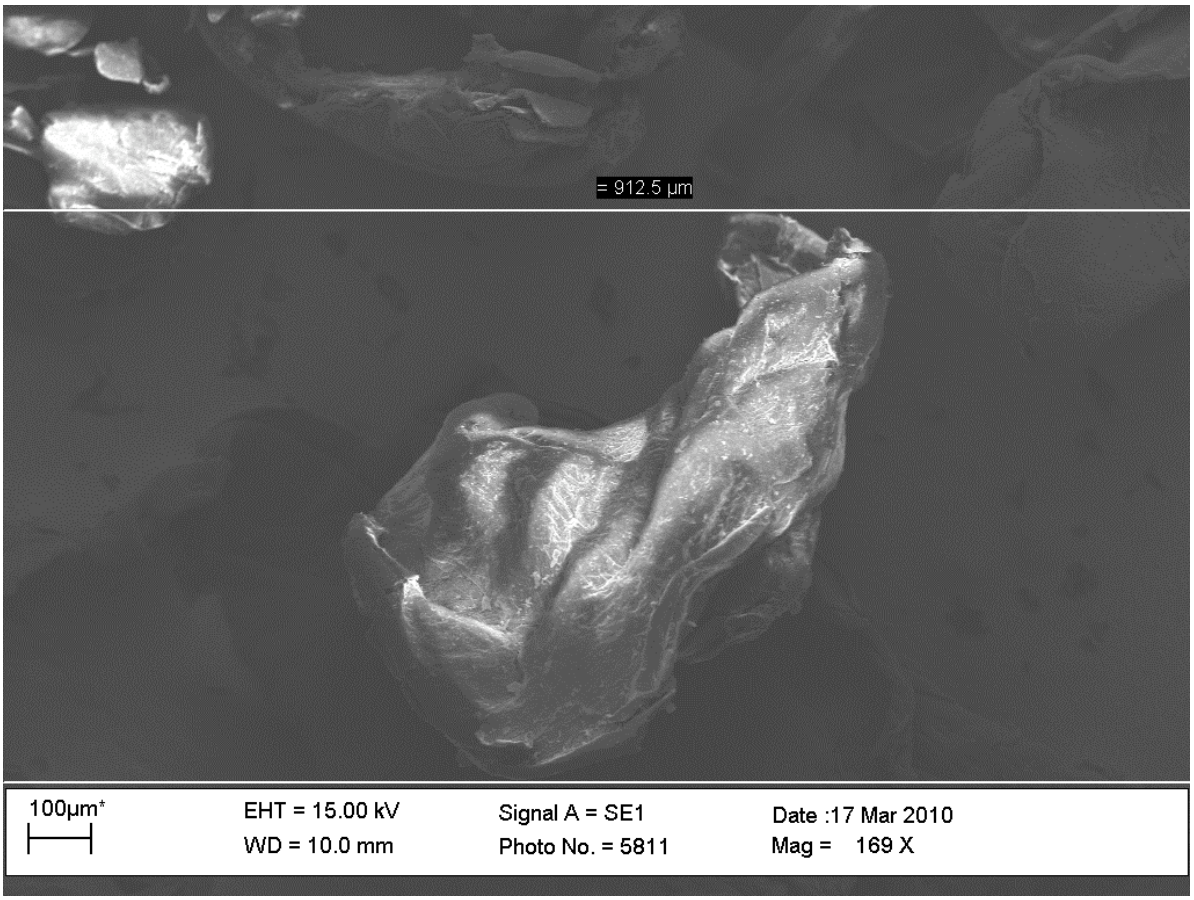

Figure 4: Scanning electron micrograph (SEM) image of chitosan-encapsulated DNA (small size, approximately 100+ µm).

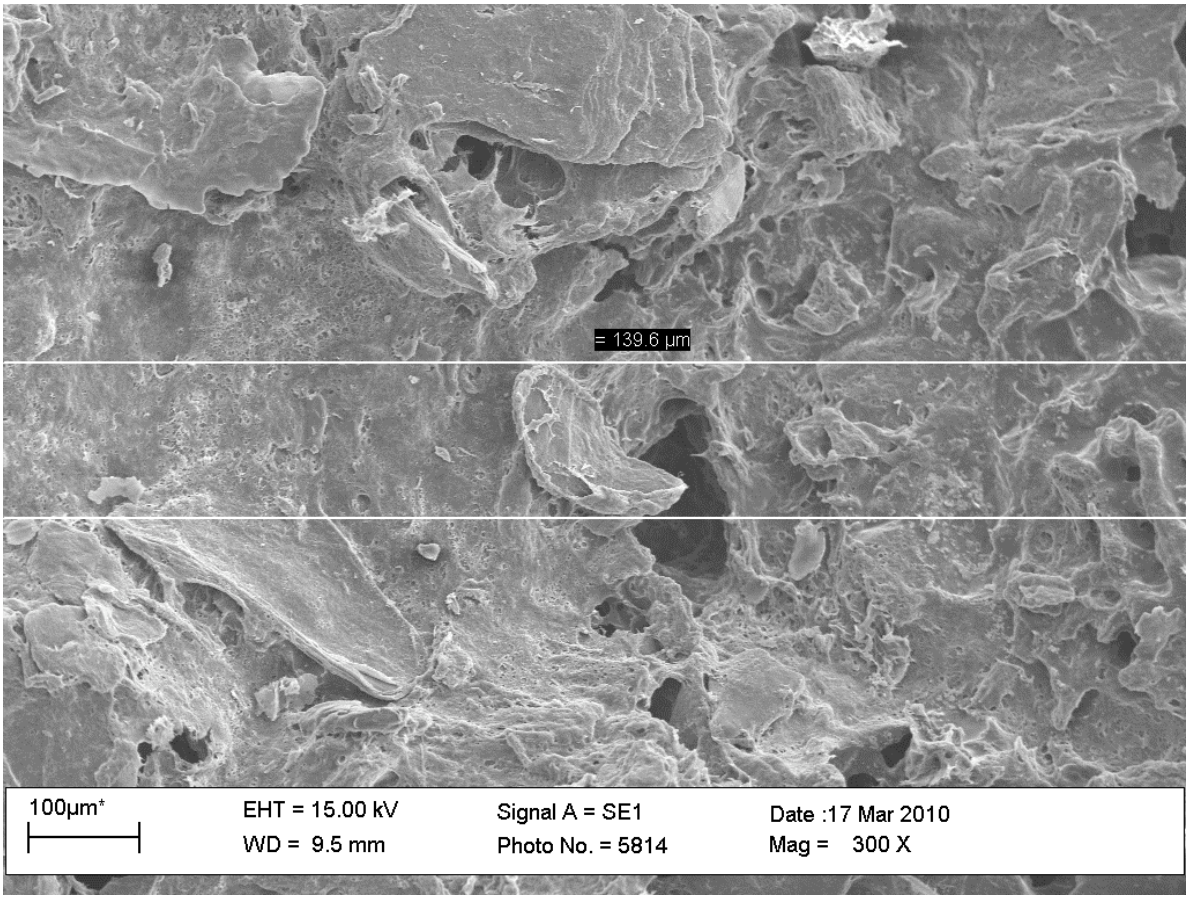

Figure 5: Scanning electron micrograph (SEM) image of chitosan-encapsulated DNA (large size, 400+ µm).

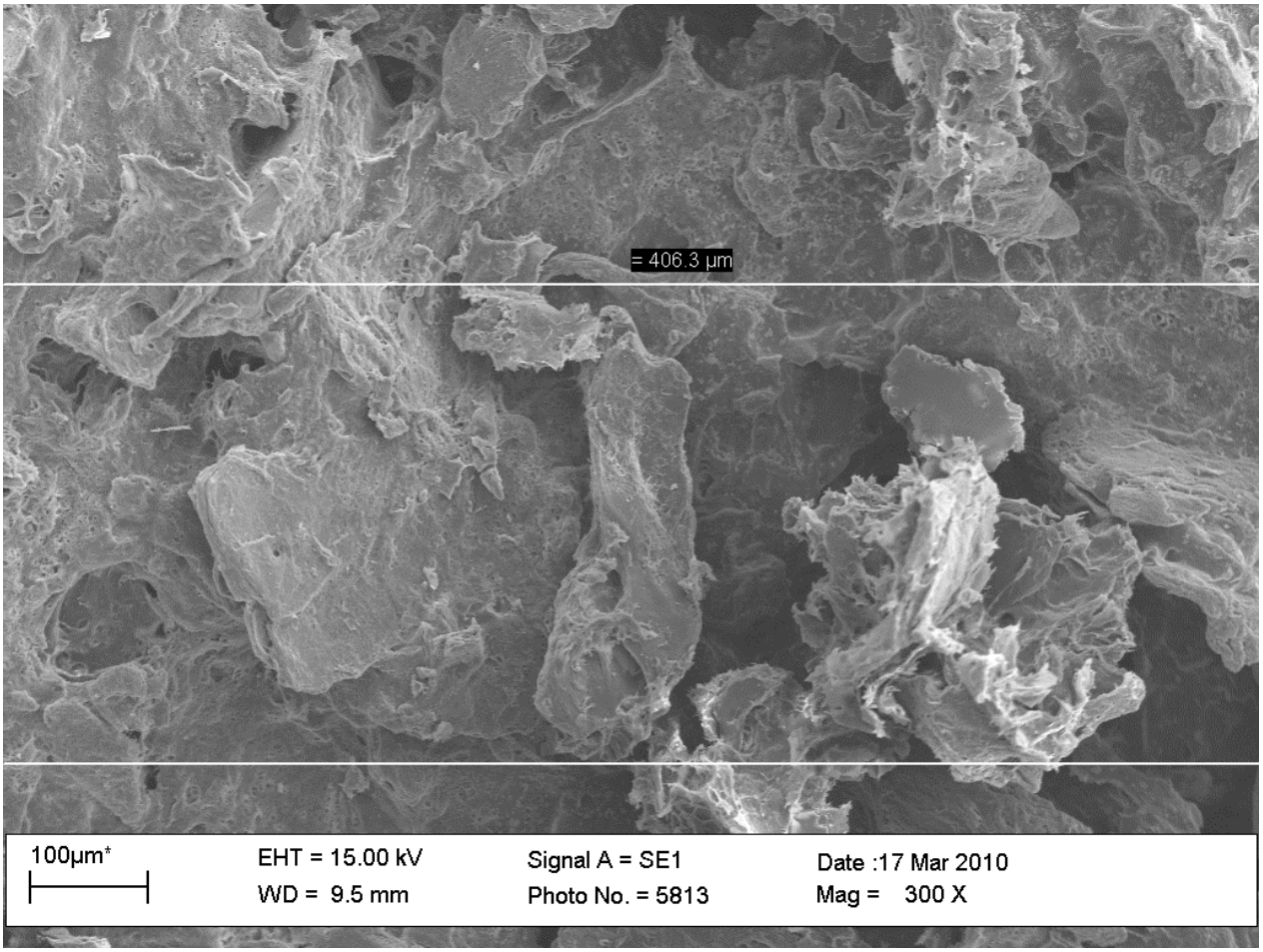

Table 1: ANOVA showing the humoral immune response generation in all treatment groups of chitosan experiment where time was used as co-variant and the significance level was $p \leq 0.05$.

Effect	F value	Pr>F
Experimental Groups	13.38	<0.0001
Time	2.35	0.0544

Table 2: Mean ELISA value (i.e. immune response) generated by the four groups of fish in the chitosan experiment for each individual bleeding period.

Week 0	
Treatment Group Response	**Mean ELISA Value**
Uncomplexed Chitosan	0.2213
Chitosan-encapsulated in DNA	0.1675
DNA intramuscular	0.3716
DNA oral	0.2941
Week 2	
Uncomplexed Chitosan	0.2846
Chitosan-encapsulated in DNA	0.2949
DNA intramuscular	0.6022
DNA oral	0.6375
Week 4	
Uncomplexed Chitosan	0.3013
Chitosan-encapsulated in DNA	0.3508
DNA intramuscular	0.4951
DNA oral	0.5892
Week 6	
Uncomplexed Chitosan	0.3217
Chitosan-encapsulated in DNA	0.3585
DNA intramuscular	0.5304
DNA oral	0.8402
Week 8	
Uncomplexed Chitosan	0.3799
Chitosan-encapsulated in DNA	0.3893
DNA intramuscular	0.7441
DNA oral	0.8957
Week 10	
Uncomplexed Chitosan	0.3769
Chitosan-encapsulated in DNA	0.3463
DNA intramuscular	0.6296
DNA oral	0.6726

Table 3-A: Comparison of the significance of the difference of mean ELISA values generated by the four groups of fish in the chitosan experiment with respect to each other at Week 2.

Experimental Group	Experimental Group	p-value*
Uncomplexed Chitosan	Chitosan-encapsulated in DNA	0.9997
Uncomplexed Chitosan	DNA Intramuscular	0.0227
Uncomplexed Chitosan	DNA Oral	0.008
Chitosan-encapsulated in DNA	DNA Intramuscular	0.0213
Chitosan-encapsulated in DNA	DNA Oral	0.0071
DNA Intramuscular	DNA Oral	0.9862

* $p \leq 0.05$ was considered significant

Table 3-B: Comparison of the significance of the difference of mean ELISA values generated by the four groups of fish in the chitosan experiment with respect to each other at Week 4.

Experimental Group	Experimental Group	p-value *
Uncomplexed Chitosan	Chitosan-encapsulated in DNA	0.9773
Uncomplexed Chitosan	DNA Intramuscular	0.4073
Uncomplexed Chitosan	DNA Oral	0.0907
Chitosan-encapsulated in DNA	DNA Intramuscular	0.6208
Chitosan-encapsulated in DNA	DNA Oral	0.176
DNA Intramuscular	DNA Oral	0.8519

* $p \leq 0.05$ was considered significant

Table 3-C: Comparison of the significance of the difference of mean ELISA values generated by the four groups of fish in the chitosan experiment with respect to each other at Week 6.

Experimental Group	Experimental Group	p-value *
Uncomplexed Chitosan	Chitosan-encapsulated in DNA	0.0064
Uncomplexed Chitosan	DNA Intramuscular	0.1445
Uncomplexed Chitosan	DNA Oral	0.995
Chitosan-encapsulated in DNA	DNA Intramuscular	0.1964
Chitosan-encapsulated in DNA	DNA Oral	0.0088
DNA Intramuscular	DNA Oral	0.6236

* $p \leq 0.05$ was considered significant

Table 3-D: Comparison of the significance of the difference of mean ELISA values generated by the four groups of fish in the chitosan experiment with respect to each other at Week 8.

Experimental Group	Experimental Group	p-value *
Uncomplexed Chitosan	Chitosan-encapsulated in DNA	0.9999
Uncomplexed Chitosan	DNA Intramuscular	0.051
Uncomplexed Chitosan	DNA Oral	0.0058
Chitosan-encapsulated in DNA	DNA Intramuscular	0.0459
Chitosan-encapsulated in DNA	DNA Oral	0.0046
DNA Intramuscular	DNA Oral	0.8541

* $p \leq 0.05$ was considered significant

Table 3-E: Comparison of the significance of the difference of mean ELISA values generated by the four groups of fish in the chitosan experiment with respect to each other at Week 10.

Experimental Group	Experimental Group	p-value *
Uncomplexed Chitosan	Chitosan-encapsulated in DNA	0.9966
Uncomplexed Chitosan	DNA Intramuscular	0.3305
Uncomplexed Chitosan	DNA Oral	0.1915
Chitosan-encapsulated in DNA	DNA Intramuscular	0.2031
Chitosan-encapsulated in DNA	DNA Oral	0.1033
DNA Intramuscular	DNA Oral	0.9907

* $p \leq 0.05$ was considered significant

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Chapter 3: Oral Administration of Polylactide-co-glycolide-Ag85A Complex to Hybrid Striped Bass

(To be submitted to the Journal of Aquatic Animal Health)

a. Introduction

The advantages of non-invasive oral vaccines (e.g. generation of both mucosal and systemic immunity providing a strong first line of defense, ease of administration, and cost effectiveness) make them preferable to the injectable routes (Aziz et al. 2007). Thus, the application of DNA vaccines through oral administration is gaining attention. Having unique practical and immunological advantages over traditional antigen vaccines, DNA vaccines have attracted many aquaculturists to opt for DNA vaccines as a preferred mode of vaccination. From a practical point, DNA vaccines are relatively easy to produce and the process of production of various types of DNA vaccines are identical. Also, different plasmids can be mixed together for the production of multivalent vaccines. This can ultimately decrease the manufacturing cost of a vaccine. As a result in today's age of aquaculture, more and more aquaculturists are attracted to the use of DNA vaccines as an effective tool to combat disease in fish farm facilities (Nerland et al. 2007, Lorenzen and LaPatra 2005).

However, due to a lack of knowledge of the fish immune system, the mechanism for development of the immune response in fish after DNA vaccination has been poorly understood. DNA vaccination by injection seems to be most promising regarding the development of a successful immune response in the fish. Alternative routes of administering a DNA vaccine, such as via the oral or immersion route, are ideal for large numbers of fish, as well as for very small fish. This is because these routes reduce the amount of labor required, as well as minimize the stress to the fish that is associated with vaccination by injection. In addition, one of the unique

advantages of oral vaccination in fish is that the fish generates a significant mucosal immune response (Heppell and Davis 2000).

As oral vaccination delivers the antigen to the fish through its digestive system, the vaccine needs to be protected from the enzymatic degradation and acid hydrolysis of the gut. One way of protecting a DNA vaccine from the undesirable action of the stomach and intestine of the fish is by encapsulating the DNA vaccine in biodegradable nanoparticle microspheres. Nanoparticles are submicron-sized polymeric colloidal particles, which are mainly used for encapsulating a therapeutic agent or gene for use in medicine (Gelperina et al. 2005). Some of the advantages of biodegradable polymeric nanoparticles include the slow release of the encapsulated gene or drug, subcellular size, biocompatibility with tissues and cells, low toxicity, stability in blood, lack of immunogenicity, lack of inflammatory response as well as biodegradability (Rieux 2006). Nanoparticles have the ability to be incorporated in both hydrophobic and hydrophilic substances. Also, nanoparticles encapsulated drugs can be easily presented to the host through various routes of administration such as oral administration and inhalation (Gelperina et al. 2005). Thus, nanoparticles formulated with chitosan, alginate or polylactide-co-glycolide have numerous potential applications for vaccine and drug delivery in the field of veterinary and human medicine.

Owing to the nature of biocompatibility and biodegradability, polymers like poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers like polylactide-co-glycolide (PLG) have been explored vigorously for the preparation of nanoparticles. One of the distinct advantages of PLG nanoparticles is that this polymer is made from glycolic acid and lactic acid polymers that are approved by FDA. As a result after the

drug or gene is released, these polymers do not need to be surgically removal from the body (Kwon et al. 2001). These nanoparticles can successfully microencapsulate a therapeutic agent or gene and prevent the material from enzymatic degradation of the body. Nanoparticles can also release the drug in a controlled manner that maintains the concentration of the drug within a suitable therapeutic level for a prolonged period of time. In addition, the sub-micron size of PLG nanoparticles can enable the particles to cross highly permeable vasculature, can efficiently flow through the blood capillaries and can enter the tumor cells through the mechanism of endocytosis (Betancourt et al. 2005). Several studies have reported the use of PLG nanoparticles in aquaculture. PLG particles have been injected intraperitoneally to Atlantic Salmon (*Salmo sadar*) in order to see the expression of pro-inflammatory cytokines where the spleen and liver of the fish showed an increased level of TNF-α1, IL-6 and IL-8 (Myren 2007). Rohu carp (*Labeo rohita)* parenterally immunized with PLG microencapsulated antigen showed an increased response in both innate and adaptive immunity parameters at both 21 and 42 days post-immunization (Behera et al. 2010). Pre-smolt Atlantic salmon (*Salmo salar L.*) orally administered PLG-encapsulated antigen human gamma globulin (HGG) showed the presence of free antigen in serum, posterior epithelium and the kidney suggesting that PLG microparticles had been successful in protecting the test antigen from the enzymatic degradation of the body (O'Donnell et al. 1996).

In the present study, a methodology was sought that would protect a DNA vaccine such that it could be orally administered to fish. The main objective of the study was to determine whether vaccination via oral delivery

of the Ag85A plasmid complexed with PLG would result in a detectable humoral immune response.

b. Materials and Methods

i. Fish

Juvenile hybrid striped bass were obtained from a local state hatchery (Vic Thomas Striped Bass Hatchery, Brookneal, VA) and kept in recirculation aquaculture systems with appropriate biological filtration and aeration. Water temperature was maintained in the range of 23-26^{0}C along with a fluorescent light photoperiod of 12 hr light/12 hr dark. Fish were fed daily with the commercial pelleted food (Zeigler Bros Inc, Gardners, PA) at 3-5% of their body weight per day.

ii. Polylactide-co-glycolide - Ag85A Plasmid Complex Formation

Twenty ml of methylene chloride (Sigma-Aldrich, Inc., St. Louis, MO) was added to 2.0 g of polylactide-co-glycolide (PLG) (Sigma-Aldrich, Inc.) and the resulting solution was vortexed for 2 minutes. Then, 2.0 ml of phosphate buffered saline (PBS, pH 7.4, Sigma-Aldrich, Inc.) was added and emulsified using a homogenizer on high speed for 3 minutes. The resulting solution was divided equally into two beakers and 50 ml of hexadecyltrimethylammonium bromide (CTAB, Sigma-Aldrich, Inc.) solution was added to each of the two beakers and stirred overnight at medium-high speed at room temperature. The next day, the mixtures were centrifuged at 2200 × g for 30 minutes. The pellets were resuspended in 25 ml of dH2O and the mixtures were centrifuged at 2200 × g for 20 minutes.

The samples were frozen and then followed by lyophilization using a Flexi-dry Lyophilizer (VirTis Wizard 2.0, Gardiner, NY) for 48 hrs. Then 100 mg of the lyophilized PLG/CTAB mixture was added to 4.0 ml of plasmid DNA (approximately 2300 μg recombinant plasmid expressing the *Mycobacterium marinum* Ag85A antigen) in TE (Tris-EDTA buffer). In a separate container, another 100 mg of the lyophilized PLG/CTAB mixture was added to 4.0 ml of sterile PBS that would serve as a negative control. The two mixtures were incubated in the refrigerator (4^0C) overnight. Then 4.0 ml of each mixture (PLG/CTAB + Plasmid, or PLG/CTAB) was centrifuged at 20,000 × g for 10 minutes. The supernatant was removed and saved to test for the presence of DNA. The pellets were resuspended in 2.0 ml of dH2O and centrifuged again at 20,000 × g for 10 minutes. The supernatant was again removed and the four supernatant samples tested with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) for the presence of DNA.

iii. Tagging and Vaccination

Fish were anesthetized with sodium bicarbonate buffered MS-222 (100-150 μg/l, Sigma Aldrich, Inc.) and weighed. Fish were individually tagged with a PIT tag (Biomark Inc., Boise, ID, USA) inserted per manufacturers instructions into the ventral coelomic cavity. Fish were randomly assigned to one of four groups: (Group 1) an intramuscular (IM) immunization of the uncomplexed DNA vaccine as a positive control, (Group 2) an oral delivery of uncomplexed DNA vaccine, (Group 3) an oral delivery of a PLG alone as a negative control, and (Group 4) an oral delivery of the complexed-PLG DNA vaccine. In all experimental groups, a dose of

50 μg of DNA/fish was targeted. The first group was given an intramuscular injection of uncomplexed DNA by 1 ml syringe with a 25-gauge needle, while the remaining three groups of fish were administered the experimental material by oral administration of the vaccine directly into the stomach by a metal gavage feeding tube (Popper®, Cadence Science, Lake Success, NY).

iv. Blood Collection

Blood was collected from each fish in all four groups pre-vaccination and then repeated five times at two-week intervals between each blood collection post-vaccination. Prior to each collection, the fish were anesthetized as described previously and blood collected with a 1 ml syringe with a 23-gauge needle from the caudal tail vessels. Approximately 0.2 ml of blood was collected from each fish. The blood samples were placed in separate serum separator tubes (BD Microtainer, Becton, Dickinson and Co., Franklin Lakes, NJ) and stored at 4^0C overnight. The next day the blood was centrifuged at 14,000 x g for five minutes, the serum extracted and placed in individual microcentrifuge tubes, and stored at -20^0C until future analysis. After the final blood collection, all fish were humanely euthanized by an overdose of buffered MS-222 (200 μg/l, Sigma Aldrich, Inc).

v. ELISA Assay

The ELISA assay was performed using a modification of a previously described whole-cell lysate ELISA (Laal et al. 1997). Briefly, a 50 ml culture of *Mycobacterium marinum* was grown in Middlebook broth (Bacto Middlebrook 7H9 broth, DIFCO Laboratories, Detroit, MI) for 2-3 weeks at 25^0C. The culture was harvested at 1000 x g for 10 minutes. The pellet was resuspended in 25 ml phosphate buffered saline (PBS) containing 1mM

PMSF, 1mM EDTA and 1mM DTT (Fisher Scientific, Pittsburg, PA). The mixture was frozen and thawed in liquid nitrogen several times, and the mixture sonicated for 20 minutes followed by centrifugation at 150 x g. A protein assay (BCA assay, Pierce Chemical, Rockford, IL) was performed on the supernatant and the protein solution adjusted to 15 μg/ml in coating buffer (0.05M sodium carbonate buffer, pH 9.6). Then 50 ul of the protein solution was added to all but two wells of a 96-well ELISA plate (Corning, Corning, NY). To the final two wells, 200 μl of 1% BSA (Sigma Aldrich Inc) in coating buffer was added which acted as the uncoated negative control wells. The plate was covered with Parafilm (Pechiney Plastic Packaging, WI) and incubated overnight at 4^0C. The next day, the plate was washed three times with phosphate buffered saline plus 0.05% Tween 20 (Sigma Aldrich Inc) in dH2O (PBST). Then, 250 μl of 1% BSA in coating buffer was added to each well. The plate was incubated for 45 minutes at 37°C, then washed three times with PBST. Following this, 100 μl of fish serum diluted 1:1000 in PBST was added to appropriate wells. Duplicate wells were used for each sample, including controls. The plate was incubated for 2 hr at room temperature after which the plate was again washed three times with PBST. The primary antibody used was an affinity purified rabbit anti-hybrid striped bass antibody produced in our laboratory (Smith et al. 1994). This was diluted 1:10,000 in PBST, and 100ul was added to each well. The plate was incubated for 1 hr at room temperature, then washed three times with PBST. Next, 100 μl of the secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) diluted 1:100 in PBST was placed in each well. The plate was incubated for 45 minutes at

room temperature, and again washed three times with PBST. Finally, 100 µl of TMB substrate (Kirkegaard & Perry Lab., Inc.) was added to each well. The plate was incubated for 6 minutes at room temperature, then 100 µl of stop solution (1 N HCL) was added to each well. The plate was read using an ELISA plate reader at 450 nm.

vi. Scanning Electron Microscopy

Aluminum stubs (Electron Microscopy Sciences, Hatfield, PA) of 12.7 mm were selected for placement of PLG and PLG-encapsulated DNA lyophilized powder on the appropriate stage for scanning electron microscope (SEM) examination. After putting double-sided tape on the top surface of the stub, the samples were placed on the exposed surface of the double-sided tape. Conducting paint of colloidal silver paste (Electron Microscopy Sciences) was added around the periphery of the sample, and the samples sputter coater with gold (SPI Supplies, PA). After coating, the samples were viewed in a scanning electron microscope (Carl Zeiss, Evo 40 Germany) and images digitally recorded.

vii. Statistical analysis

ANOVA model with random effects was applied to see the humoral immune response development in all of the groups of fish through all of the five bleeding periods after the pre-bleed. Time as co-variant had been categorically distributed in these five bleeding periods. Multiple comparisons with Tukey's Adjustment was used to compare the mean differences of the ELISA values of the humoral immune response generated by the four experimental groups of fish with a significance level of $p \leq 0.05$.

c. Results

i. Immunological Assays

Antibody titers (e.g. ELISA values) demonstrated by the four groups of fish over the six sampling periods are shown in Figure 6. A relative comparison of immune response elicited by the four groups; (1) the positive control, i.e. fish which were given uncomplexed intramuscular (IM) injection, (2) the group of fish which were orally administered the uncomplexed DNA, (3) the negative control, i.e. group of fish which were given only PLG, and (4) the experimental group, i.e. group of fish to which oral administration of PLG-encapsulated DNA vaccine was given over six sampling periods are represented. The control DNA IM immunization reached a peak titer at 8 weeks post-immunization, and then declined. Both PLG and DNA encapsulated in PLG administered orally elicited a significant humoral immune response in fish. However, the uncomplexed DNA oral vaccination also elicited an immune response similar to our positive control. i.e. the uncomplexed DNA IM vaccinated fish. There was no significant difference in development of the humoral immune response among the treatment groups throughout the sampling periods. The mean humoral immune response generated by each of the four groups of fish through the sampling periods is shown in Table 5, while Table 6 represents a relative comparison of the immune response of the four groups with respect to each other and whether the differences observed were significant ($p \leq 0.05$).

ii. SEM analysis results

Both PLG and PLG-encapsulated DNA lyophilized powders were viewed by SEM and images recorded (Figures 7, 8, 9 and 10). The size range of the PLG particles was 1-40 µm, while the PLG-encapsulated DNA particles were in the size range of 700nm-20µm. The particulate size of both the PLG and PLG-encapsulated DNA lyophilized attained rigid spherical shaped structures with smooth surfaces (Stern et al. 2003).

d. Discussion

Oral incorporation of a vaccine is an effective way to administer a vaccination non-invasively, and in aquaculture, fish can be vaccinated with minimal stress. For effective oral vaccination, the delivered antigen or gene should not undergo digestive hydrolysis in the stomach and should be efficiently absorbed in the hindgut of the fish in order to induce a protective immune response (Vandenberg 2004).

Biodegradable polymeric nanoparticles are being increasingly used today in the field of gene or vaccine delivery to target organs. A number of polymers have been used in pharmaceutical research for drug delivery to the target site and have recognized the decreased number of side effects, thus increasing the therapeutic benefit (Kreuter 1994). The ability of biodegradable polymers in delivering protein, peptides and genes through an oral route, as carriers of DNA in gene therapy and their ability to reach target sites have made the use of these polymers as a delivery system a viable route of administration (Langer 2000).

Due to the biocompatibility and biodegradability PLG polymer has been widely used as a novel carrier system for gene and drug delivery (Andersen and Shive 1997). PLG protects the microencapsulated antigens

against enzymatic degradation until the time of release of antigen from the polymer. The adjuvant effect of PLG promotes more efficient antigen presentations and thereby incorporation of low doses of orally-delivered antigen e.g. 10 µg can allow for the induction of an antibody response (Eldridge et al. 1991, Jones et al. 1996). It has been observed that PLG encapsulating the plasmids for luciferase has a long retention time within the body and releases the plasmid slowly for 2 weeks (Baoum et al. 2009). PLG has also successfully been used for encapsulating potent antituberculosis drugs (ATDs). The PLG matrix exhibited gradual release of the ATDs through a sustained time period with the presence of ATDs demonstrated in plasma, lungs and spleen of mice and guinea pigs (Pandey et al. 2003, Sharma et al. 2004).

There have also been a few reports of successful use of PLG encapsulation and use of PLG-matrix as a carrier system in the field of aquaculture. An immune response was observed in Japanese flounder, *Paralichthys olivaceus*, after the fish were administered PLG microcapsules loaded with plasmid DNA against lymphocystis disease virus (Tian et al. 2008). Rainbow trout (*Oncorhynchus mykiss*), when administered human gamma globulin microencapsulated in PLG microparticles, showed the presence of intact antigens in the posterior intestine, intestinal mucus and bloodstream of the fish. The presence of antigens indicated that the PLG had successfully protected the antigens from digestive degradation in the fish (Lavelle et al. 1997). Therefore, polymeric particles like PLG could be a potential carrier system for gene or vaccine delivery in the field of aquaculture owing to the protection of the microencapsulated antigens against enzymatic degradation as well as the ability of delivery of microencapsulated antigens intact to lymphoid tissues in fish (Ellis 1998).

The results of this study showed that the complexed DNA vaccine elicited a significant humoral immune response in hybrid striped bass. The immune response generated by our two experimental groups, i.e. fish orally administered the PLG-encapsulated DNA vaccine and fish administered the uncomplexed DNA oral vaccine, were not statistically different ($p \leq 0.05$) from each other. In addition, the uncomplexed DNA oral vaccination elicited an immune response similar to the uncomplexed DNA IM vaccinated fish that served as our positive control. The immune response generated by the positive control and the DNA oral vaccination groups were also not statistically different from each other.

The humoral immune response generated by our negative control group was not statistically different from the immune response generated by our positive control, i.e. the group of fish that were given intramuscular injection of uncomplexed DNA vaccine. The fish of our negative control group as well as our experimental group were housed in the same experimental tank. Now, it had already been evident from SEM imaging that PLG encapsulated DNA vaccine were in the size of both nanoparticle and microparticle during the time of the encapsulated vaccine incorporation to the fish. It was hypothesized that the microencapsulated vaccine that was not taken up by the fish cells successfully might have been exposed to the water from the body of the fish through normal excretory process. As a result, there might have been an increase in the vaccine exposure in the water. Now, since the fish of our negative control group as well as our experimental group were housed in the same experimental tank, so it was hypothesized that there might be additional vaccine uptake by the fish of our negative control which might have resulted in high value of titer of negative control.

Scanning electron microscopic imaging of both PLG and PLG-encapsulated DNA lyophilized powder was undertaken in order to estimate the distribution of particle size. The PLG lyophilized powder had a size in the range of 1-40 μm. Nanoparticle size typically ranges from 0.1 to 100 nm but for the purpose of drug or gene delivery, nanoparticles greater than 100 nm are suitable as a drug-carrier (Jong and Borm 2008). Observations from our SEM imaging revealed that the PLG powder that has been used for encapsulation of DNA was larger than the accepted nanoparticle size. This finding revealed that the PLG powder was in the microparticulate size range rather than being in the size range of nanoparticles. After the PLG was used for encapsulation of DNA, the size ranges of the PLG-DNA complex particles were in the range of 700 nm-20 μm. This was a decrease in size from the PLG alone, and indicated that the PLG-encapsulated DNA complex was in the size range of both nanoparticles and microparticles.

In the field of drug delivery, both nanoparticles and microparticles have been used as a suitable carrier system. Due to the large size of microparticles, they are often unable to cross biological barriers and so the drugs coated with microparticles must be delivered directly to the target sites. However, the sub-micron size of nanoparticles allows the particles to cross membranes and overcome most biological barriers. Like other colloidal drug carriers, nanoparticles can pass through blood capillaries after injection and get adsorbed into the blood stream (Kreuter 1994). Nanoparticles can also be adsorbed by the macrophages of reticuloendothelial system from the blood stream (Kreuter et al. 1995). Cells, through the process of phagocytosis or pinocytosis, generally accomplish taking up of extracellular materials and subsequent processing. Phagocytic mechanisms can enable the cell to take material up to 10 μm in diameter. All

cell types employ pinocytic mechanisms that enable the cells to engulf any material of sub-micron size. As a result microparticles can only be delivered to the phagocytic cells while nanoparticles can be used for all cell types (Kohane 2007).

Our results from SEM imaging revealed that some of the PLG-encapsulated DNA particles were in the size range of nanometers, which may have allowed the cells to employ both phagocytosis and pinocytosis for successful uptake of PLG-encapsulated DNA resulting in developing humoral immune response in our experimental groups of fish. Therefore PLG might be recommended for use in DNA vaccines for in the field of aquaculture.

Figure 6: Comparison of the ELISA values elicited in hybrid striped bass *(Morone saxatilis x Morone chrysops)* vaccinated orally with polylactide-co-glycolide (PLG) alone as a negative control, vaccinated orally with the complexed-polylactide-co-glycolide DNA vaccine, vaccinated intramuscularly with the uncomplexed DNA as a positive control, and vaccinated orally with the uncomplexed DNA.

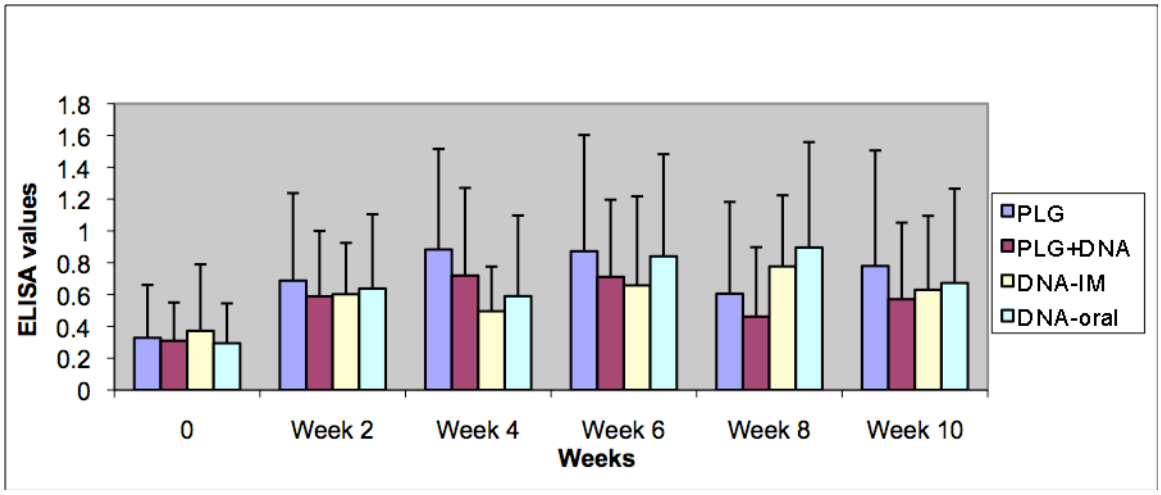

Figure 7: Scanning electron micrograph (SEM) image of polylactide-co-glycolide (PLG, small particle size, approximately 1+ µm).

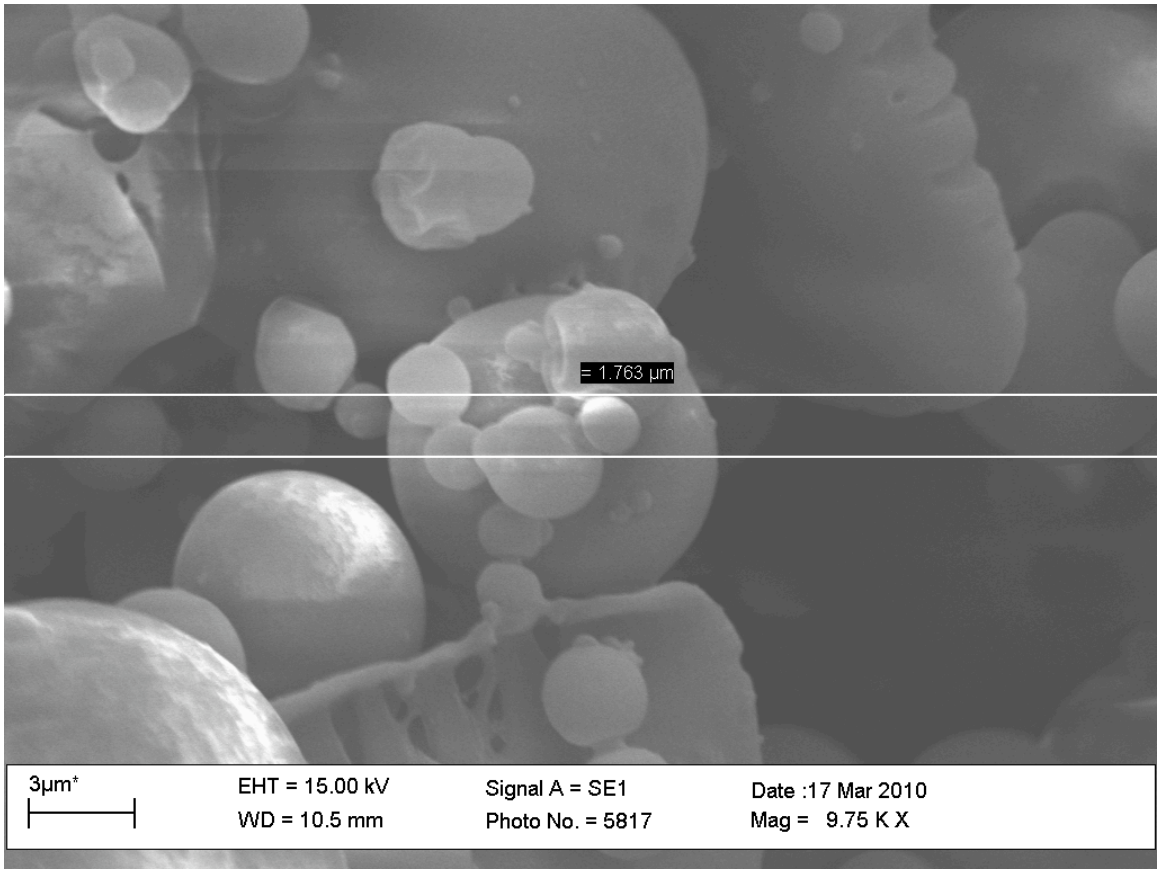

Figure 8: Scanning electron micrograph (SEM) image of polylactide-co-glycolide (PLG, large particle size, approximately 36+ µm).

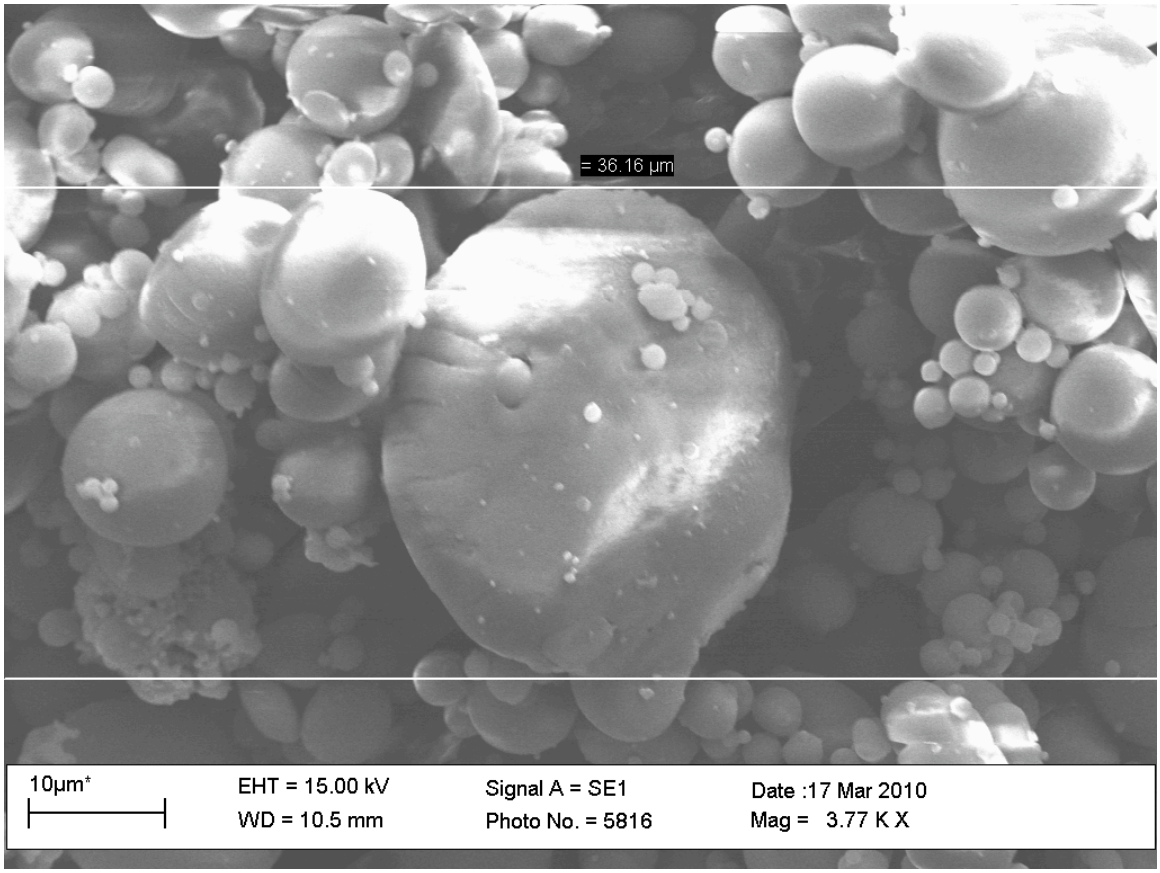

Figure 9: Scanning electron micrograph (SEM) image of polylactide-co-glycolide encapsulated DNA (PLG, small particle size, approximately 730+ nm).

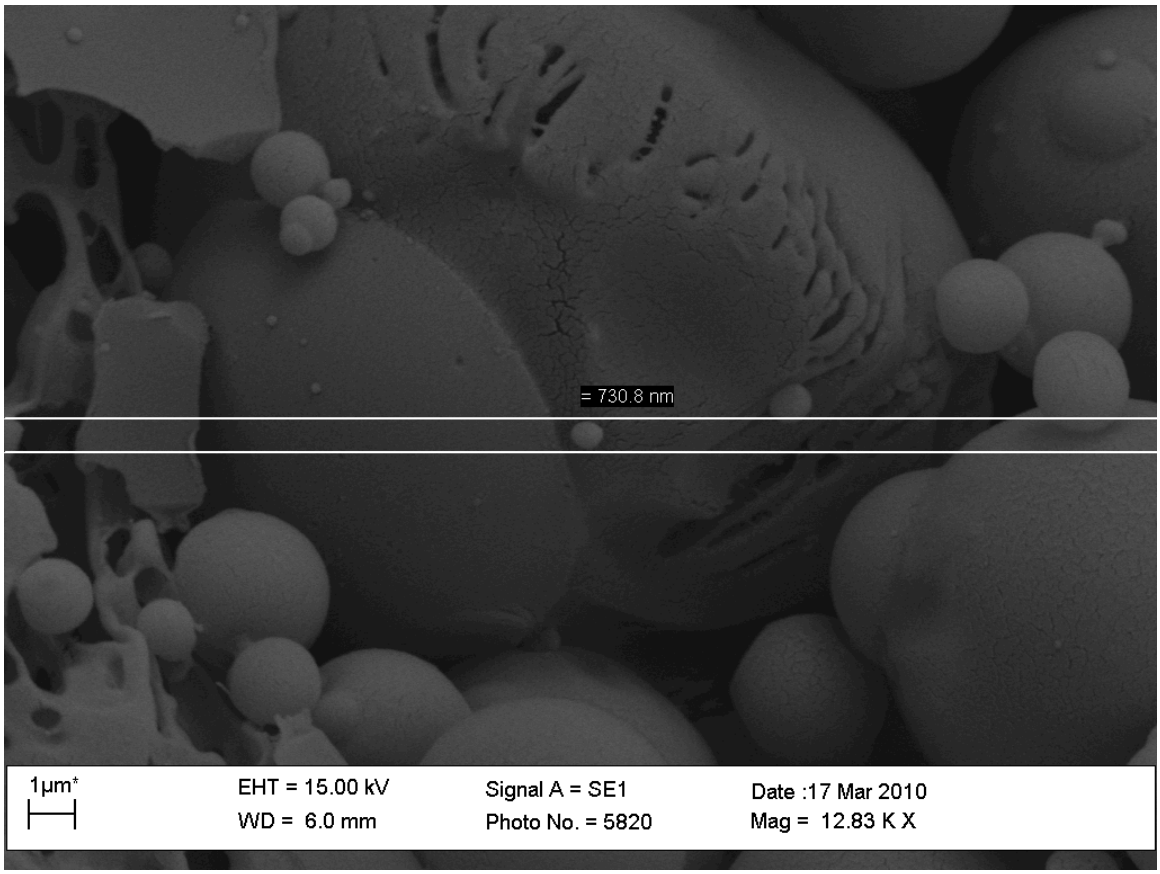

Figure 10: Scanning electron micrograph (SEM) image of polylactide-co-glycolide encapsulated DNA (PLG, large particle size, approximately 16+ µm).

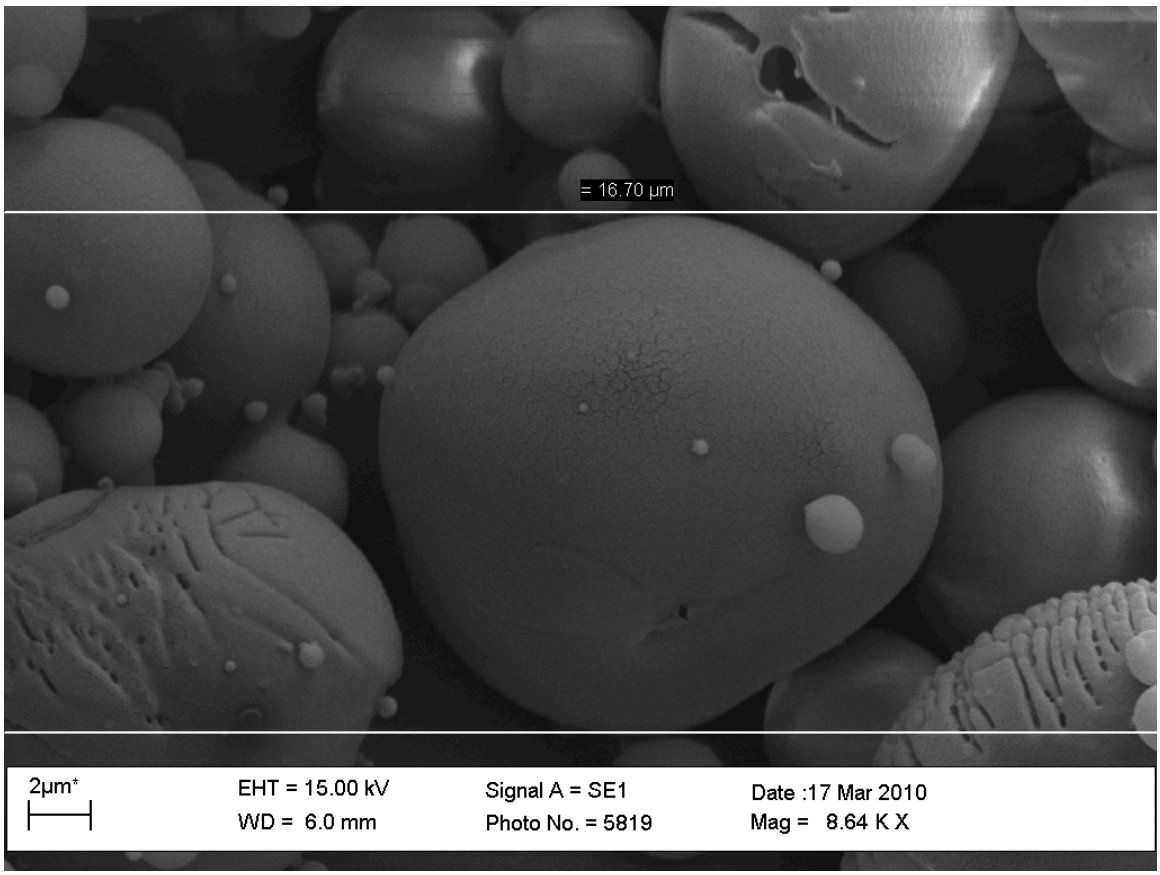

Table 4: ANOVA showing the humoral immune response generation in all treatment groups of polylactide-co-glycolide (PLG) experiment where time was used as co-variant and the significance level was p≤ 0.05.

Effect	F value	Pr>F
Experimental Groups	2.64	0.0492
Time	3.13	0.0778

Table 5: Mean ELISA value (i.e. immune response) generated by the four groups of fish in the polylactide-co-glycolide (PLG) experiment for each individual bleeding period.

Week 0	
Treatment Group Response	**Mean ELISA Value**
Uncomplexed PLG	0.3278
PLG-encapsulated in DNA	0.3094
DNA intramuscular	0.3716
DNA oral	0.2941
Week 2	
Uncomplexed PLG	0.6874
PLG-encapsulated in DNA	0.5884
DNA intramuscular	0.6022
DNA oral	0.6375
Week 4	
Uncomplexed PLG	0.8832
PLG-encapsulated in DNA	0.7193
DNA intramuscular	0.4951
DNA oral	0.5892
Week 6	
Uncomplexed PLG	0.8721
PLG-encapsulated in DNA	0.7107
DNA intramuscular	0.5304
DNA oral	0.8402
Week 8	
Uncomplexed PLG	0.6057
PLG-encapsulated in DNA	0.4612
DNA intramuscular	0.7441
DNA oral	0.8957
Week 10	
Uncomplexed PLG	0.7796
PLG-encapsulated in DNA	0.5708
DNA intramuscular	0.6296
DNA oral	0.6726

Table 6-A: Comparison of the significance of the difference of mean ELISA values generated by the four groups of fish in the polylactide-co-glycolide (PLG) experiment with respect to each other at Week 2.

Experimental Group	Experimental Group	p-value *
Uncomplexed PLG	PLG-encapsulated in DNA	0.7983
Uncomplexed PLG	DNA Intramuscular	0.912
Uncomplexed PLG	DNA Oral	0.9951
PLG-encapsulated in DNA	DNA Intramuscular	0.9929
PLG-encapsulated in DNA	DNA Oral	0.8999
DNA Intramuscular	DNA Oral	0.9734

* $p \leq 0.05$ was considered significant

Table 6-B: Comparison of the significance of the difference of mean ELISA values generated by the four groups of fish in the polylactide-co-glycolide (PLG) experiment with respect to each other at Week 4.

Experimental Group	Experimental Group	p-value *
Uncomplexed PLG	PLG-encapsulated in DNA	0.7638
Uncomplexed PLG	DNA Intramuscular	0.1187
Uncomplexed PLG	DNA Oral	0.3067
PLG-encapsulated in DNA	DNA Intramuscular	0.5305
PLG-encapsulated in DNA	DNA Oral	0.8511
DNA Intramuscular	DNA Oral	0.9402

* $p \leq 0.05$ was considered significant

Table 6-C: Comparison of the significance of the difference of mean ELISA values generated by the four groups of fish in the polylactide-co-glycolide (PLG) experiment with respect to each other at Week 6.

Experimental Group	Experimental Group	p-value *
Uncomplexed PLG	PLG-encapsulated in DNA	0.85420
Uncomplexed PLG	DNA Intramuscular	0.6775
Uncomplexed PLG	DNA Oral	0.9986
PLG-encapsulated in DNA	DNA Intramuscular	0.9874
PLG-encapsulated in DNA	DNA Oral	0.9118
DNA Intramuscular	DNA Oral	0.7552

* $p \leq 0.05$ was considered significant

Table 6-D: Comparison of the significance of the difference of mean ELISA values generated by the four groups of fish in the polylactide-co-glycolide (PLG) experiment with respect to each other at Week 8.

Experimental Group	Experimental Group	p-value *
Uncomplexed PLG	PLG-encapsulated in DNA	0.8518
Uncomplexed PLG	DNA Intramuscular	0.7831
Uncomplexed PLG	DNA Oral	0.3874
PLG-encapsulated in DNA	DNA Intramuscular	0.2897
PLG-encapsulated in DNA	DNA Oral	0.0758
DNA Intramuscular	DNA Oral	0.9096

* $p \leq 0.05$ was considered significant

Table 6-E: Comparison of the significance of the difference of mean ELISA values generated by the four groups of fish in the polylactide-co-glycolide (PLG) experiment with respect to each other at Week 10.

Experimental Group	Experimental Group	p-value *
Uncomplexed PLG	PLG-encapsulated in DNA	0.6952
Uncomplexed PLG	DNA Intramuscular	0.8656
Uncomplexed PLG	DNA Oral	0.9435
PLG-encapsulated in DNA	DNA Intramuscular	0.9894
PLG-encapsulated in DNA	DNA Oral	0.9468
DNA Intramuscular	DNA Oral	0.9958

* $p \leq 0.05$ was considered significant

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Appendices

ELISA Data of Chapter 2

Chitosan administration in hybrid striped bass

Chitosan Oral FISH (TAG NO.)	Pre - Bleed	Pre - Bleed	Average Pre - Bleed	True Value	Plate
365011	0.7461	0.6373	0.6917	0.3769	1
366745	0.4621	0.4653	0.4637	0.021	6
375776	0.4805	0.4929	0.4867	0.1719	1
20978599	0.6378	0.5488	0.5933	0.2785	1
366246	0.4373	0.5254	0.48135	0.16655	1
371354	0.6058	0.5784	0.5921	0.2773	1
368985	0.3788	0.3788	0.3788	0.064	1
375435	0.5835	0.6902	0.63685	0.32205	1
375941	0.334	0.4354	0.3847	0.0699	1
358820	0.6122	0.6464	0.6293	0.3145	1
366624	0.4901	0.5393	0.5147	0.1999	1
374657	0.4951	0.4634	0.47925	0.16445	1
352965	0.4732	0.3805	0.42685	0.11205	1
352389	0.33	0.3656	0.3478	0.033	1
352969	1.3688	1.13	1.2494	0.8067	6
373700	0.528	0.5771	0.55255	0.10985	6
369161	0.5343	0.6461	0.5902	0.2754	1
366835	no blood	no blood	no blood	-	-

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	1st Bleed	1st Bleed	Average 1st Bleed	True Value	Plate
365011	0.73	0.8653	0.79765	0.35495	6
366745	0.7649	0.7616	0.76325	0.41975	2
375776	0.4337	0.4511	0.4424	0.0989	2
20978599	0.7445	0.8395	0.792	0.4485	2
366246	0.3924	0.4034	0.3979	0.0544	2
371354	0.7802	0.7371	0.75865	0.41515	2
368985	0.6819	0.5999	0.6409	0.2974	2
375435	0.8078	0.7884	0.7981	0.4546	2
375941	0.5247	0.5607	0.5427	0.1992	2
358820	0.5526	0.5318	0.5422	0.1987	2
366624	0.658	0.5414	0.5997	0.2562	2
374657	0.3548	0.4292	0.392	0.0485	2
352965	-	-	-	-	-
352389	0.4214	0.4411	0.43125	0.08775	2
352969	1.058	1.3722	1.2151	0.7724	6
373700	0.4663	0.5718	0.51905	0.17555	2
369161	0.4648	0.5953	0.53005	0.18655	2
366835	0.679	0.7462	0.7126	0.3691	2

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	2nd Bleed	2nd Bleed	Average 2nd Bleed	True Value	Plate
365011	1.4558	1.3024	1.3791	1.0356	2
366745	0.8737	0.9623	0.918	0.5745	2
375776	0.409	0.4737	0.44135	0.09785	2
20978599	0.9564	0.9088	0.9326	0.5891	2
366246	0.4491	0.4572	0.45315	0.10965	2
371354	0.6247	0.6636	0.64415	0.30065	2
368985	0.4611	0.4309	0.446	0.1025	2
375435	0.7404	0.7045	0.72245	0.3531	3
375941	0.4821	0.42	0.45105	0.0817	3
358820	no blood	no blood	-	-	-
366624	0.4668	0.4651	0.46595	0.0966	3
374657	0.3823	0.3638	0.37305	0.0037	3
352965	-	-	-	-	-
352389	0.3626	0.4256	0.3941	0.02475	3
352969	1.0894	1.2721	1.18075	0.8114	3
373700	0.4095	0.4646	0.43705	0.0677	3
369161	0.4857	0.4321	0.4589	0.08955	3
366835	0.886	0.8173	0.85165	0.4823	3

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	3rd Bleed	3rd Bleed	Average 3rd Bleed	True Value	Plate
365011	0.791	0.8799	0.83545	0.4661	3
366745	0.6476	0.7529	0.70025	0.3309	3
375776	0.4341	0.3934	0.41375	0.0444	3
20978599	0.8334	0.7689	0.80115	0.4318	3
366246	0.3792	0.4311	0.40515	0.0358	3
371354	0.7834	0.7016	0.7425	0.37315	3
368985	0.974	1.0097	0.99185	0.6225	3
375435	0.8815	0.8768	0.87915	0.5098	3
375941	0.5719	0.6043	0.5881	0.21875	3
358820	0.6637	0.5368	0.60025	0.2309	3
366624	0.7113	0.8377	0.7745	0.40515	3
374657	0.5858	0.5435	0.56465	0.1953	3
352965	-	-	-	-	-
352389	0.4478	0.4236	0.4357	0.06635	3
352969	1.0045	1.3195	1.162	0.79265	3
373700	0.6002	0.5918	0.596	0.22665	3
369161	0.6988	0.588	0.6434	0.2409	4
366835	0.6987	0.6674	0.68305	0.28055	4

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	4th Bleed	4th Bleed	Average 4th Bleed	True Value	Plate
365011	1.1016	1.2634	1.1825	0.78	4
366745	1.0272	1.0425	1.03485	0.63235	4
375776	0.4757	0.4187	0.4472	0.0447	4
20978599	0.9096	0.9412	0.9254	0.5229	4
366246	0.386	0.4245	0.40525	0.00275	4
371354	0.9716	0.997	0.9843	0.5818	4
368985	0.7027	0.653	0.67785	0.27535	4
375435	0.8675	0.9732	0.92035	0.51785	4
375941	0.7567	0.7405	0.7486	0.3461	4
358820	0.5898	0.6487	0.61925	0.21675	4
366624	0.8335	0.81	0.82175	0.41925	4
374657	0.5716	0.5983	0.58495	0.18245	4
352965	-	-	-	-	-
352389	0.5249	0.5262	0.52555	0.12305	4
352969	1.6014	1.3781	1.48975	1.08725	4
373700	0.6062	0.6465	0.62635	0.22385	4
369161	0.5457	0.625	0.58535	0.18285	4
366835	0.6543	0.7891	0.7217	0.3192	4

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	5th Bleed	5th Bleed	Average 5th Bleed	True Value	Plate
365011	1.247	1.173	1.21	0.8425	5
366745	0.865	0.82	0.8425	0.475	5
375776	0.37	0.412	0.391	0.0235	5
20978599	0.883	0.807	0.845	0.4775	5
366246	0.503	0.489	0.496	0.1285	5
371354	0.729	0.635	0.682	0.3145	5
368985	0.522	0.427	0.4745	0.107	5
375435	0.878	0.871	0.8745	0.507	5
375941	0.6529	0.6153	0.6341	0.1914	6
358820	0.845	0.852	0.8485	0.481	5
366624	0.763	0.755	0.759	0.3915	5
374657	0.515	0.586	0.5505	0.183	5
352965	-	-	-	-	-
352389	0.648	0.548	0.598	0.2305	5
352969	1.657	1.573	1.615	1.2475	5
373700	0.51	0.433	0.4715	0.104	5
369161	0.541	0.614	0.5775	0.21	5
366835	1.0159	0.8586	0.93725	0.49455	6

Chitosan administration in hybrid striped bass

Chitosan and DNA FISH (TAG NO.)	Pre - Bleed	Pre - Bleed	Average Pre - Bleed	True Value	Plate
368838	0.6859	0.6371	0.6615	0.3467	1
370927	0.3666	0.3425	0.35455	0.03975	1
361462	0.3621	0.3274	0.34475	0.02995	1
374581	0.4603	0.5693	0.5148	0.2	1
372263	0.3062	0.3672	0.3367	0.0219	1
372418	0.4937	0.5773	0.5355	0.2207	1
357581	0.4955	0.5295	0.5125	0.1977	1
361492	0.3953	0.3461	0.3707	0.0559	1
374497	0.4007	0.3499	0.3753	0.0605	1
356916	0.3987	0.397	0.39785	0.08305	1
366651	0.3367	0.403	0.36985	0.05505	1
1006353	0.6428	0.5319	0.58735	0.27255	1
357675	0.5369	0.5766	0.55675	0.24195	1
364962	0.543	0.5443	0.54365	0.22885	1
363453	0.4645	0.4563	0.4604	0.1456	1
357225	0.4397	0.3906	0.41515	0.10035	1
372211	0.5304	0.5048	0.5176	0.2028	1
369701	0.5604	0.6131	0.58675	0.27195	1
367027	0.933	0.7663	0.84965	0.40695	6
372868	-	-	-	-	-

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	1st Bleed	1st Bleed	Average 1st Bleed	True Value	Plate
368838	0.6252	0.6567	0.64905	0.30555	2
370927	0.5519	0.6635	0.6077	0.2642	2
361462	0.5525	0.5205	0.5365	0.193	2
374581	1.6067	1.469	1.53785	1.09515	6
372263	0.7833	0.6654	0.72435	0.38085	2
372418	0.6117	0.7616	0.68665	0.34315	2
357581	0.4758	0.5215	0.49865	0.15515	2
361492	0.5284	0.5794	0.5539	0.2104	2
374497	0.5893	0.4889	0.5391	0.1956	2
356916	0.6782	0.5785	0.62835	0.28485	2
366651	0.55	0.4211	0.48555	0.14205	2
1006353	0.4509	0.4903	0.4706	0.1271	2
357675	0.7822	0.63	0.7061	0.2634	6
364962	0.7514	0.8978	0.8246	0.4811	2
363453	0.6055	0.5431	0.5743	0.1316	6
357225	0.3996	0.3827	0.39115	0.04765	2
372211	0.426	0.527	0.4765	0.133	2
369701	0.8204	0.7887	0.80455	0.46105	2
367027	0.9337	0.983	0.95835	0.51565	6
372868	0.4615	0.5638	0.51265	0.16915	2

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	2nd Bleed	2nd Bleed	Average 2nd Bleed	True Value	Plate
368838	0.4702	0.4754	0.4728	0.10345	3
370927	0.6145	0.664	0.63925	0.2699	3
361462	0.6606	0.5794	0.62	0.25065	3
374581	1.5971	1.6331	1.6151	1.24575	3
372263	0.732	0.6565	0.69425	0.3249	3
372418	0.68	0.6923	0.68615	0.3168	3
357581	0.5691	0.5683	0.5687	0.19935	3
361492	0.6318	0.6074	0.6196	0.25025	3
374497	0.6784	0.5459	0.61215	0.2428	3
356916	0.858	0.8818	0.8699	0.50055	3
366651	0.5967	0.5295	0.5631	0.19375	3
1006353	no blood	no blood	-	-	-
357675	0.7539	0.6485	0.7012	0.33185	3
364962	1.0026	1.0422	1.0224	0.65305	3
363453	0.5943	0.7034	0.64885	0.2795	6
357225	0.4915	0.5017	0.4966	0.12725	3
372211	0.4983	0.5027	0.5005	0.13115	3
369701	0.9732	0.9228	0.948	0.57865	3
367027	0.9125	0.9386	0.92555	0.5562	3
372868	0.4421	0.5216	0.48185	0.1125	3

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	3rd Bleed	3rd Bleed	Average 3rd Bleed	True Value	Plate
368838	0.577	0.5195	0.54825	0.14575	4
370927	0.8376	0.7761	0.80685	0.40435	4
361462	0.7168	0.6542	0.6855	0.283	4
374581	1.5506	1.6133	1.58195	1.17945	4
372263	0.745	0.7403	0.74265	0.34015	4
372418	0.7219	0.7136	0.71775	0.31525	4
357581	0.5015	0.5059	0.5037	0.1012	4
361492	0.5969	0.5252	0.56105	0.15855	4
374497	0.7483	0.7289	0.7386	0.3361	4
356916	0.7649	0.6544	0.70965	0.30715	4
366651	0.5633	0.5464	0.55485	0.15235	4
1006353	0.5415	0.5264	0.53395	0.09125	6
357675	0.8628	0.7978	0.8303	0.4278	4
364962	0.9395	1.0107	0.9751	0.5726	4
363453	0.8877	0.9478	0.91775	0.51525	4
357225	0.56	0.5659	0.56295	0.16045	4
372211	0.5624	0.5268	0.5446	0.1421	4
369701	0.9646	0.9528	0.9587	0.5562	4
367027	1.1309	1.2352	1.18305	0.78055	4
372868	0.6254	0.579	0.6022	0.1997	4

Chitosan administration in hybrid striped bass

FISH TAG NO.)	4th Bleed	4th Bleed	Average 4th Bleed	True Value	Plate
368838	0.582	0.6019	0.59195	0.18945	4
370927	0.6505	0.616	0.63325	0.23075	4
361462	0.7939	0.6565	0.7252	0.3227	4
374581	1.8184	1.6424	1.7304	1.3279	4
372263	1	0.954	0.977	0.6095	5
372418	0.749	0.651	0.7	0.3325	5
357581	0.462	0.483	0.4725	0.105	5
361492	0.643	0.657	0.65	0.2825	5
374497	0.598	0.645	0.6215	0.254	5
356916	0.7847	0.7103	0.7475	0.3048	6
366651	0.876	0.874	0.875	0.5075	5
1006353	0.643	0.649	0.646	0.2785	5
357675	0.65	0.623	0.6365	0.269	5
364962	0.809	0.772	0.7905	0.423	5
363453	0.778	0.65	0.714	0.3465	5
357225	0.495	0.483	0.489	0.1215	5
372211	0.56	0.584	0.572	0.2045	5
369701	1.198	1.162	1.18	0.8125	5
367027	1.14	1.063	1.1015	0.734	5
372868	0.552	0.441	0.4965	0.129	5

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	5th Bleed	5th Bleed	Average 5th Bleed	True Value	Plate
368838	0.592	0.592	0.592	0.2245	5
370927	0.712	0.631	0.6715	0.304	5
361462	0.684	0.603	0.6435	0.276	5
374581	1.925	1.667	1.796	1.4285	5
372263	0.836	0.771	0.8035	0.436	5
372418	0.784	0.838	0.811	0.4435	5
357581	0.423	0.477	0.45	0.0825	5
361492	0.6113	0.5849	0.5981	0.1554	6
374497	0.719	0.718	0.7185	0.351	5
356916	0.472	0.485	0.4785	0.111	5
366651	0.5759	0.706	0.64905	0.20635	6
1006353	0.6253	0.5499	0.5876	0.1449	6
357675	0.5745	0.5549	0.5647	0.122	6
364962	1.0138	0.944	0.9789	0.5362	6
363453	0.6621	0.6325	0.6473	0.2046	6
357225	0.6106	0.6256	0.6181	0.1754	6
372211	0.7969	0.7671	0.782	0.3393	6
369701	1.0106	0.9225	0.96655	0.52385	6
367027	1.3491	1.259	1.30405	0.86135	6
372868	0.4523	0.4366	0.44445	0.00175	6

Chitosan administration in hybrid striped bass

Naked DNA intramuscular **FISH (TAG NO.)**	**Pre - Bleed**	**Pre - Bleed**	**Average Pre - Bleed**	**True Value**	**Plate**
363778	0.562	0.6952	0.6286	0.1859	6
356118	0.6707	0.5936	0.63215	0.18945	6
369127	0.524	0.611	0.5675	0.2315	12
363630	0.8383	0.7946	0.81645	0.37375	6
375610	0.6541	0.5203	0.5872	0.1445	6
368720	0.4797	0.4896	0.48465	0.04195	6
367715	0.6497	0.65	0.64985	0.20715	6
372298	1.2655	1.1554	1.21045	0.76775	6
353902	0.8963	1.1746	1.03545	0.59275	6
372827	-	-	-	-	-
365229	0.4043	0.4104	0.40735	0.09555	7
373979	0.4025	0.3296	0.36605	0.05425	7
359895	0.4228	0.4066	0.4147	0.1029	7
370753	1.061	0.927	0.994	0.658	12
356723	0.602	0.5115	0.55675	0.24495	7
370728	-	-	-	-	-
355464	-	-	-	-	-
364826	1.483	1.611	1.547	1.211	12
378405	1.996	1.81	1.903	1.567	12
364624	0.4801	0.4932	0.48665	0.17485	7
371990	0.3581	0.3537	0.3559	0.0441	7
367250	0.5108	0.4632	0.487	0.1752	7

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	1st Bleed	1st Bleed	Average 1st Bleed	True Value	Plate
363778	1.041	1.1898	1.1154	0.8173	8
356118	0.715	0.804	0.7595	0.4235	12
369127	0.7449	0.6766	0.71075	0.41265	8
363630	1.4324	1.299	1.3657	1.0676	8
375610	0.4502	0.5319	0.49105	0.19295	8
368720	0.6634	0.6001	0.63175	0.33365	8
367715	0.8705	0.7022	0.78635	0.48825	8
372298	0.617	0.654	0.6355	0.2995	12
353902	0.7159	0.855	0.78545	0.48735	8
372827	1.3416	1.4758	1.4087	1.1106	8
365229	0.8595	0.9305	0.895	0.5969	8
373979	1.0402	1.1125	1.07635	0.77825	8
359895	0.6607	0.7336	0.69715	0.39905	8
370753	1.2468	1.2979	1.27235	0.97425	8
356723	0.5	0.525	0.5125	0.1765	12
370728	-	-	-	-	-
355464	1.5738	1.3871	1.48045	1.18235	8
364826	-	-	-	-	-
378405	-	-	-	-	-
364624	0.5197	0.5109	0.5153	0.2172	8
371990	0.9838	1.2456	1.1147	0.8166	8
367250	0.9669	0.9623	0.9646	0.6665	8

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	2nd Bleed	2nd Bleed	Average 2nd Bleed	True Value	Plate
363778	1.0772	0.9238	1.0005	0.7024	8
356118	1.0076	0.9159	0.96175	0.66365	8
369127	0.4672	0.3898	0.4285	0.1304	8
363630	1.1944	1.2647	1.22955	0.93145	8
375610	0.4881	0.5166	0.50235	0.2304	9
368720	0.4942	0.4638	0.479	0.20705	9
367715	0.4548	0.5089	0.48185	0.2099	9
372298	0.646	0.665	0.6555	0.3195	12
353902	0.6511	0.5952	0.62315	0.3512	9
372827	1.3001	1.359	1.32955	1.0576	9
365229	0.8806	0.9666	0.9236	0.65165	9
373979	1.1798	1.0236	1.1017	0.82975	9
359895	0.7779	0.7235	0.7507	0.47875	9
370753	0.6286	0.6891	0.65885	0.3869	9
356723	0.6013	0.6126	0.60695	0.335	9
370728	-	-	-	-	-
355464	-	-	-	-	-
364826	-	-	-	-	-
378405	-	-	-	-	-
364624	0.4813	0.4625	0.4719	0.19995	9
371990	1.119	0.9717	1.04535	0.7734	9
367250	0.7325	0.7184	0.72545	0.4535	9

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	3rd Bleed	3rd Bleed	Average 3rd Bleed	True Value	Plate
363778	0.6445	0.6332	0.63885	0.3669	9
356118	0.9263	0.9723	0.9493	0.67735	9
369127	0.4615	0.5154	0.48845	0.2165	9
363630	1.2736	1.2798	1.2767	1.00475	9
375610	0.3673	0.4647	0.416	0.14405	9
368720	0.436	0.3073	0.37165	0.0997	9
367715	0.4892	0.3807	0.43495	0.163	9
372298	0.6103	0.59	0.60015	0.3282	9
353902	0.5236	0.6178	0.5707	0.29875	9
372827	1.621	1.583	1.602	1.2925	10
365229	0.877	0.988	0.9325	0.623	10
373979	1.841	1.599	1.72	1.4105	10
359895	0.616	0.627	0.6215	0.2855	12
370753	1.126	1.145	1.1355	0.826	10
356723	0.562	0.552	0.557	0.221	12
370728	-	-	-	-	-
355464	-	-	-	-	-
364826	-	-	-	-	-
378405	-	-	-	-	-
364624	0.741	0.815	0.778	0.4685	10
371990	2.224	2.603	2.4135	2.104	10
367250	1.556	1.675	1.6155	1.306	10

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	4th Bleed	4th Bleed	Average 4th Bleed	True Value	Plate
363778	0.658	0.741	0.6995	0.3635	12
356118	1.113	1.337	1.225	0.9155	10
369127	1.021	0.976	0.9985	0.689	10
363630	1.929	1.847	1.888	1.5785	10
375610	0.531	0.474	0.5025	0.1665	12
368720	0.753	0.682	0.7175	0.408	10
367715	0.872	0.795	0.8335	0.524	10
372298	0.881	0.993	0.937	0.6275	10
353902	0.959	0.881	0.92	0.6105	10
372827	1.525	1.372	1.4485	1.139	10
365229	1.0695	0.9828	1.02615	0.6955	11
373979	1.6753	1.6131	1.6442	1.31355	11
359895	0.7609	0.6328	0.69685	0.3662	11
370753	1.7093	1.9117	1.8105	1.47985	11
356723	0.583	0.658	0.6205	0.2845	12
370728	-	-	-	-	-
355464	-	-	-	-	-
364826	-	-	-	-	-
378405	-	-	-	-	-
364624	0.7723	0.667	0.71965	0.389	11
371990	1.6345	1.8525	1.7435	1.41285	11
367250	1.21	1.4787	1.34435	1.0137	11

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	5th Bleed	5th Bleed	Average 5th Bleed	True Value	Plate
363778	0.9001	0.9334	0.91675	0.5861	11
356118	1.0081	1.085	1.04655	0.7159	11
369127	0.7028	0.6972	0.7	0.36935	11
363630	2.0774	1.8428	1.9601	1.62945	11
375610	0.51	0.5074	0.5087	0.17805	11
368720	0.7985	0.7113	0.7549	0.42425	11
367715	0.5316	0.4813	0.50645	0.1758	11
372298	0.7911	0.7671	0.7791	0.44845	11
353902	0.7892	0.7865	0.78785	0.4572	11
372827	1.9084	1.9243	1.91635	1.5857	11
365229	0.8759	0.8969	0.8864	0.55575	11
373979	1.39	1.474	1.432	1.096	12
359895	0.504	0.484	0.494	0.158	12
370753	0.789	0.788	0.7885	0.4525	12
356723	0.535	0.525	0.53	0.194	12
370728	-	-	-	-	-
355464	-	-	-	-	-
364826	-	-	-	-	-
378405	-	-	-	-	-
364624	0.66	0.725	0.6925	0.3565	12
371990	1.68	1.564	1.622	1.286	12
367250	0.963	1.036	0.9995	0.6635	12

Chitosan administration in hybrid striped bass

Naked DNA oral FISH (TAG NO.)	Pre - Bleed	Pre - Bleed	Average Pre - Bleed	True Value	Plate
359888	1.6214	1.5164	1.5689	1.1262	6
356834	0.7866	0.7416	0.7641	0.3214	6
1124497	0.6263	0.6655	0.6459	0.2032	6
375240	1.0928	1.0083	1.05055	0.60785	6
374583	0.4951	0.4103	0.4527	0.01	6
353928	0.4939	0.5893	0.5416	0.0989	6
359200	0.5523	0.5397	0.546	0.1033	6
375384	0.9981	0.886	0.94205	0.49935	6
372412	0.5035	0.5751	0.5393	0.2275	7
364775	0.5622	0.5399	0.55105	0.23925	7
372385	-	-	-	-	-
369482	0.5563	0.5233	0.5398	0.228	7
352486	0.5756	0.4171	0.49635	0.18455	7
357112	0.756	0.725	0.7405	0.4045	12
372483	0.582	0.577	0.5795	0.2435	12
365359	0.5185	0.5782	0.54835	0.23655	7
1125649	0.6692	0.6357	0.65245	0.34065	7
1301904	0.4574	0.4364	0.4469	0.1351	7
358802	0.7906	0.8044	0.7975	0.4857	7
364691	0.4148	0.5133	0.46405	0.15225	7
368510	0.3367	0.3546	0.34565	0.03385	7

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	1st Bleed	1st Bleed	Average 1st Bleed	True Value	Plate
359888	1.965	2.021	1.993	1.657	12
356834	0.9825	0.9144	0.94845	0.65035	8
1124497	1.2114	1.2552	1.23333	0.93523	8
375240	0.7301	0.85	0.79005	0.49195	8
374583	0.3448	0.3662	0.3555	0.0574	8
353928	0.657	0.5135	0.58525	0.28715	8
359200	0.4947	0.5112	0.50295	0.20485	8
375384	1.5101	1.5287	1.5194	1.2213	8
372412	0.6177	0.6076	0.61265	0.31455	8
364775	-	-	-	-	-
372385	0.409	0.423	0.416	0.08	12
369482	0.7665	0.795	0.78075	0.48265	8
352486	2.353	1.2379	1.79545	1.49735	8
357112	1.2868	1.2379	1.26235	0.96425	8
372483	0.5743	0.5196	0.54695	0.24885	8
365359	0.6274	0.5218	0.5746	0.2765	8
1125649	1.0115	1.0516	1.03155	0.73345	8
1301904	0.7155	0.6869	0.7012	0.4031	8
358802	1.5028	1.4475	1.47515	1.17705	8
364691	0.9422	1.0768	1.0095	0.7114	8
368510	0.6259	0.6874	0.65665	0.35855	8

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	2nd Bleed	2nd Bleed	Average 2nd Bleed	True Value	Plate
359888	1.9107	2.1108	2.01075	1.7388	9
356834	0.7579	0.6426	0.70025	0.4283	9
1124497	1.4831	1.5241	1.5036	1.23165	9
375240	0.7407	0.8126	0.77665	0.5047	9
374583	0.4152	0.3204	0.3678	0.09585	9
353928	0.5323	0.5529	0.5426	0.27065	9
359200	0.5697	0.6139	0.5918	0.31985	9
375384	1.1969	1.2352	1.21605	0.9441	9
372412	0.4287	0.5525	0.4906	0.21865	9
364775	-	-	-	-	-
372385	0.5109	0.4358	0.47335	0.2014	9
369482	0.7945	0.8692	0.83185	0.5599	9
352486	2.0023	2.2218	2.11205	1.8401	9
357112	0.863	0.87	0.8665	0.5305	12
372483	0.5997	0.4507	0.5252	0.25325	9
365359	0.5291	0.4294	0.47925	0.2073	9
1125649	0.8532	0.7877	0.82045	0.5485	9
1301904	0.5172	0.4835	0.50035	0.2284	9
358802	1.3658	1.2108	1.2883	1.01635	9
364691	0.6189	0.7318	0.67535	0.4034	9
368510	0.5272	0.5187	0.52295	0.251	9

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	3rd Bleed	3rd Bleed	Average 3rd Bleed	True Value	Plate
359888	2.123	2.171	2.147	1.8375	10
356834	0.888	0.745	0.8165	0.507	10
1124497	1.769	1.845	1.807	1.4975	10
375240	0.875	0.944	0.9095	0.6	10
374583	0.356	0.469	0.4125	0.103	10
353928	0.604	0.727	0.6655	0.356	10
359200	0.576	0.463	0.5195	0.1835	12
375384	1.113	1.19	1.515	1.2055	10
372412	0.544	0.528	0.536	0.2265	10
364775	-	-	-	-	-
372385	0.557	0.498	0.5275	0.218	10
369482	1.133	0.961	1.047	0.7375	10
352486	2.842	2.731	2.7865	2.477	10
357112	1.114	1.079	1.0965	0.787	10
372483	0.949	0.904	0.9265	0.617	10
365359	0.902	0.883	0.8925	0.583	10
1125649	0.833	1.199	1.016	0.68	12
1301904	0.863	0.972	0.9175	0.608	10
358802	2.192	1.898	2.045	1.7355	10
364691	1.409	1.218	1.3135	1.004	10
368510	-	-	-	-	-

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	4th Bleed	4th Bleed	Average 4th Bleed	True Value	Plate
359888	2.17	2.128	2.149	1.8395	10
356834	1.049	1.157	1.103	0.7935	10
1124497	1.347	1.372	1.3595	1.05	10
375240	1.241	1.13	1.1855	0.876	10
374583	0.434	0.496	0.465	0.1555	10
353928	0.5694	0.5344	0.5519	0.22125	11
359200	0.8726	0.7791	0.82585	0.4952	11
375384	1.3713	1.4194	1.39535	1.0647	11
372412	0.6799	0.6181	0.649	0.31835	11
364775	-	-	-	-	-
372385	0.6263	0.6502	0.63825	0.3076	11
369482	1.1117	1.0737	1.0927	0.76205	11
352486	2.965	3.278	3.1215	2.7855	12
357112	1.5774	1.2931	1.43525	1.1046	11
372483	-	-	-	-	-
365359	0.7899	0.7626	0.77625	0.4456	11
1125649	1.0295	1.1208	1.07515	0.7445	11
1301904	0.9225	0.8137	0.8681	0.53745	11
358802	1.8334	2.2694	2.0514	1.72075	11
364691	1.1383	1.323	1.23065	0.9	11
368510	-	-	-	-	-

Chitosan administration in hybrid striped bass

FISH (TAG NO.)	5th Bleed	5th Bleed	Average 5th Bleed	True Value	Plate
359888	2.0192	2.1337	2.07645	1.7458	11
356834	1.4431	1.2891	1.3661	1.03545	11
1124497	0.8027	0.7831	0.7929	0.46225	11
375240	0.9981	1.1027	1.0504	0.71975	11
374583	0.3954	0.407	0.4012	0.07055	11
353928	0.6262	0.6103	0.61825	0.2876	11
359200	0.784	0.8666	0.8253	0.49465	11
375384	1.4989	1.1143	1.3066	0.97595	11
372412	0.6246	0.5786	0.6016	0.27095	11
364775	-	-	-	-	-
372385	0.5513	0.5292	0.54025	0.2096	11
369482	0.781	0.825	0.803	0.467	12
352486	2.801	2.804	2.8025	2.4665	12
357112	0.688	0.739	0.7135	0.3775	12
372483	0.698	0.797	0.7475	0.4115	12
365359	0.759	0.73	0.7445	0.4085	12
1125649	0.649	0.742	0.6955	0.3595	12
1301904	0.715	0.729	0.722	0.386	12
358802	1.443	1.606	1.5245	1.1885	12
364691	1.03	0.524	0.777	0.441	12
368510	-	-	-	-	-

Chitosan administration in hybrid striped bass

CONTROLS

	Plate 1	**Plate 1**
Rabbit Anti Fish	0.1235	0.1223
Primary Antibody	0.2472	0.2534
Normal	0.3181	0.3115
Positive	1.7942	1.7468
Uncoated	0.3315	0.424

	Plate 2	**Plate 2**
Rabbit Anti Fish	0.1062	0.093
Primary Antibody	0.257	0.207
Normal	0.384	0.303
Positive	2.375	2.292
Uncoated	0.224	0.191

	Plate 3	**Plate 3**
Rabbit Anti Fish	0.1599	0.1212
Primary Antibody	0.3298	0.2112
Normal	0.4063	0.3324
Positive	2.0488	2.2197
Uncoated	0.2984	0.2779

Chitosan administration in hybrid striped bass

	Plate 4	**Plate 4**
Rabbit Anti Fish	0.1355	0.131
Primary Antibody	0.249	0.2906
Normal	0.4557	0.3493
Positive	2.2784	1.9165
Uncoated	0.4183	0.4225

	Plate 5	**Plate 5**
Rabbit Anti Fish	0.109	0.158
Primary Antibody	0.244	0.274
Normal	0.356	0.379
Positive	1.719	1.863
Uncoated	0.364	0.269

	Plate 6	**Plate 6**
Rabbit Anti Fish	0.1415	0.2665
Primary Antibody	0.3055	0.3411
Normal	0.4684	0.417
Positive	2.1119	1.9717
Uncoated	0.422	0.2736

Chitosan administration in hybrid striped bass

	Plate 7	**Plate 7**
Rabbit Anti Fish	0.1265	0.1281
Primary Antibody	0.1894	0.2046
Normal	0.3235	0.3001
Positive	1.8133	1.6938
Uncoated	0.2847	0.28

	Plate 8	**Plate 8**
Rabbit Anti Fish	0.112	0.1256
Primary Antibody	0.2535	0.2164
Normal	0.3088	0.2874
Positive	1.7562	1.6539
Uncoated	0.2597	0.2787

	Plate 9	**Plate 9**
Rabbit Anti Fish	0.0999	0.1185
Primary Antibody	0.2187	0.2345
Normal	0.2978	0.2461
Positive	1.452	1.3469
Uncoated	0.2539	0.2038

Chitosan administration in hybrid striped bass

	Plate 10	**Plate 10**
Rabbit Anti Fish	0.143	0.159
Primary Antibody	0.236	0.215
Normal	0.299	0.32
Positive	1.643	1.558
Uncoated	0.344	0.442

	Plate 11	**Plate 11**
Rabbit Anti Fish	0.1279	0.1303
Primary Antibody	0.2306	0.2302
Normal	0.3735	0.2878
Positive	2.0621	1.7606
Uncoated	0.2858	0.2681

	Plate 12	**Plate 12**
Rabbit Anti Fish	0.13	0.112
Primary Antibody	0.206	0.19
Normal	0.293	0.379
Positive	1.932	1.644
Uncoated	0.348	0.232

ELISA Data of Chapter 3

PLG administration in hybrid striped bass

DNA
Intramuscular

FISH (TAG NO.)	Pre - Bleed	Pre - Bleed	Average Pre - Bleed.	True Value	Plate
363778	0.562	0.6952	0.6286	0.1859	6
356118	0.6707	0.5936	0.63215	0.18945	6
369127	0.524	0.611	0.5675	0.2315	12
363630	0.8383	0.7946	0.81645	0.37375	6
375610	0.6541	0.5203	0.5872	0.1445	6
368720	0.4797	0.4896	0.48465	0.04195	6
367715	0.6497	0.65	0.64985	0.20715	6
372298	1.2655	1.1554	1.21045	0.76775	6
353902	0.8963	1.1746	1.03545	0.59275	6
372827	-	-	-	-	-
365229	0.4043	0.4104	0.40735	0.09555	7
373979	0.4025	0.3296	0.36605	0.05425	7
359895	0.4228	0.4066	0.4147	0.1029	7
370753	1.061	0.927	0.994	0.658	12
356723	0.602	0.5115	0.55675	0.24495	7
370728	-	-	-	-	-
355464	-	-	-	-	-
364826	1.483	1.611	1.547	1.211	12
378405	1.996	1.81	1.903	1.567	12
364624	0.4801	0.4932	0.48665	0.17485	7
371990	0.3581	0.3537	0.3559	0.0441	7
367250	0.5108	0.4632	0.487	0.1752	7

PLG administration in hybrid striped bass

FISH (TAG NO.)	1st Bleed	1st Bleed	Average 1st Bleed	True Value	Plate
363778	1.041	1.1898	1.1154	0.8173	8
356118	0.715	0.804	0.7595	0.4235	12
369127	0.7449	0.6766	0.71075	0.41265	8
363630	1.4324	1.299	1.3657	1.0676	8
375610	0.4502	0.5319	0.49105	0.19295	8
368720	0.6634	0.6001	0.63175	0.33365	8
367715	0.8705	0.7022	0.78635	0.48825	8
372298	0.617	0.654	0.6355	0.2995	12
353902	0.7159	0.855	0.78545	0.48735	8
372827	1.3416	1.4758	1.4087	1.1106	8
365229	0.8595	0.9305	0.895	0.5969	8
373979	1.0402	1.1125	1.07635	0.77825	8
359895	0.6607	0.7336	0.69715	0.39905	8
370753	1.2468	1.2979	1.27235	0.97425	8
356723	0.5	0.525	0.5125	0.1765	12
370728	-	-	-	-	-
355464	1.5738	1.3871	1.48045	1.18235	8
364826	-	-	-	-	-
378405	-	-	-	-	-
364624	0.5197	0.5109	0.5153	0.2172	8
371990	0.9838	1.2456	1.1147	0.8166	8
367250	0.9669	0.9623	0.9646	0.6665	8

PLG administration in hybrid striped bass

FISH (TAG NO.)	2nd Bleed	2nd Bleed	Average 2nd Bleed	True Value	Plate
363778	1.0772	0.9238	1.0005	0.6257	8
356118	1.0076	0.9159	0.96175	0.6178	8
369127	0.4672	0.3898	0.4285	0.0917	8
363630	1.1944	1.2647	1.22955	0.9666	8
375610	0.4881	0.5166	0.50235	0.24465	9
368720	0.4942	0.4638	0.479	0.19185	9
367715	0.4548	0.5089	0.48185	0.23695	9
372298	0.646	0.665	0.6555	0.3195	12
353902	0.6511	0.5952	0.62315	0.32325	9
372827	1.3001	1.359	1.32955	1.08705	9
365229	0.8806	0.9666	0.9236	0.69465	9
373979	1.1798	1.0236	1.1017	0.75165	9
359895	0.7779	0.7235	0.7507	0.45155	9
370753	0.6286	0.6891	0.65885	0.41715	9
356723	0.6013	0.6126	0.60695	0.34065	9
370728	-	-	-	-	-
355464	-	-	-	-	-
364826	-	-	-	-	-
378405	-	-	-	-	-
364624	0.4813	0.4625	0.4719	0.19055	9
371990	1.119	0.9717	1.04535	0.69975	9
367250	0.7325	0.7184	0.72545	0.44645	9

PLG administration in hybrid striped bass

FISH (TAG NO.)	3rd Bleed	3rd Bleed	Average 3rd Bleed	True Value	Plate
363778	0.6445	0.6332	0.63885	0.3669	9
356118	0.9263	0.9723	0.9493	0.67735	9
369127	0.4615	0.5154	0.48845	0.2165	9
363630	1.2736	1.2798	1.2767	1.00475	9
375610	0.3673	0.4647	0.416	0.14405	9
368720	0.436	0.3073	0.37165	0.0997	9
367715	0.4892	0.3807	0.43495	0.163	9
372298	0.6103	0.59	0.60015	0.3282	9
353902	0.5236	0.6178	0.5707	0.29875	9
372827	1.621	1.583	1.602	1.2925	10
365229	0.877	0.988	0.9325	0.623	10
373979	1.841	1.599	1.72	1.4105	10
359895	0.616	0.627	0.6215	0.2855	12
370753	1.126	1.145	1.1355	0.826	10
356723	0.562	0.552	0.557	0.221	12
370728	-	-	-	-	-
355464	-	-	-	-	-
364826	-	-	-	-	-
378405	-	-	-	-	-
364624	0.741	0.815	0.778	0.4685	10
371990	2.224	2.603	2.4135	2.104	10
367250	1.556	1.675	1.6155	1.306	10

PLG administration in hybrid striped bass

FISH (TAG NO.)	4th Bleed	4th Bleed	Average 4th Bleed	True Value	Plate
363778	0.658	0.741	0.6995	0.3635	12
356118	1.113	1.337	1.225	0.9155	10
369127	1.021	0.976	0.9985	0.689	10
363630	1.929	1.847	1.888	1.5785	10
375610	0.531	0.474	0.5025	0.1665	12
368720	0.753	0.682	0.7175	0.408	10
367715	0.872	0.795	0.8335	0.524	10
372298	0.881	0.993	0.937	0.6275	10
353902	0.959	0.881	0.92	0.6105	10
372827	1.525	1.372	1.4485	1.139	10
365229	1.0695	0.9828	1.02615	0.6955	11
373979	1.6753	1.6131	1.6442	1.31355	11
359895	0.7609	0.6328	0.69685	0.3662	11
370753	1.7093	1.9117	1.8105	1.47985	11
356723	0.583	0.658	0.6205	0.2845	12
370728	-	-	-	-	-
355464	-	-	-	-	-
364826	-	-	-	-	-
378405	-	-	-	-	-
364624	0.7723	0.667	0.71965	0.389	11
371990	1.6345	1.8525	1.7435	1.41285	11
367250	1.21	1.4787	1.34435	1.0137	11

PLG administration in hybrid striped bass

FISH (TAG NO.)	5th Bleed	5th Bleed	5th Bleed	True Value	Plate
363778	0.9001	0.9334	0.91675	0.5861	11
356118	1.0081	1.085	1.04655	0.7159	11
369127	0.7028	0.6972	0.7	0.36935	11
363630	2.0774	1.8428	1.9601	1.62945	11
375610	0.51	0.5074	0.5087	0.17805	11
368720	0.7985	0.7113	0.7549	0.42425	11
367715	0.5316	0.4813	0.50645	0.1758	11
372298	0.7911	0.7671	0.7791	0.44845	11
353902	0.7892	0.7865	0.78785	0.4572	11
372827	1.9084	1.9243	1.91635	1.5857	11
365229	0.8759	0.8969	0.8864	0.55575	11
373979	1.39	1.474	1.432	1.096	12
359895	0.504	0.484	0.494	0.158	12
370753	0.789	0.788	0.7885	0.4525	12
356723	0.535	0.525	0.53	0.194	12
370728	-	-	-	-	-
355464	-	-	-	-	-
364826	-	-	-	-	-
378405	-	-	-	-	-
364624	0.66	0.725	0.6925	0.3565	12
371990	1.68	1.564	1.622	1.286	12
367250	0.963	1.036	0.9995	0.6635	12

PLG administration in hybrid striped bass

DNA Oral

FISH (TAG NO.)	Pre - Bleed	Pre - Bleed	Average Pre - Bleed	True Value	Plate
359888	1.6214	1.5164	1.5689	1.1262	6
356834	0.7866	0.7416	0.7641	0.3214	6
1124497	0.6263	0.6655	0.6459	0.2032	6
375240	1.0928	1.0083	1.05055	0.60785	6
374583	0.4951	0.4103	0.4527	0.01	6
353928	0.4939	0.5893	0.5416	0.0989	6
359200	0.5523	0.5397	0.546	0.1033	6
375384	0.9981	0.886	0.94205	0.49935	6
372412	0.5035	0.5751	0.5393	0.2275	7
364775	0.5622	0.5399	0.55105	0.23925	7
372385	-	-	-	-	-
369482	0.5563	0.5233	0.5398	0.228	7
352486	0.5756	0.4171	0.49635	0.18455	7
357112	0.756	0.725	0.7405	0.4045	12
372483	0.582	0.577	0.5795	0.2435	12
365359	0.5185	0.5782	0.54835	0.23655	7
1125649	0.6692	0.6357	0.65245	0.34065	7
1301904	0.4574	0.4364	0.4469	0.1351	7
358802	0.7906	0.8044	0.7975	0.4857	7
364691	0.4148	0.5133	0.46405	0.15225	7
368510	0.3367	0.3546	0.34565	0.03385	7

PLG administration in hybrid striped bass

FISH (TAG NO.)	1st Bleed	1st Bleed	Average 1st Bleed	True Value	Plate
359888	1.965	2.021	1.993	1.657	12
356834	0.9825	0.9144	0.94845	0.65035	8
1124497	1.2114	1.2552	1.23333	0.93523	8
375240	0.7301	0.85	0.79005	0.49195	8
374583	0.3448	0.3662	0.3555	0.0574	8
353928	0.657	0.5135	0.58525	0.28715	8
359200	0.4947	0.5112	0.50295	0.20485	8
375384	1.5101	1.5287	1.5194	1.2213	8
372412	0.6177	0.6076	0.61265	0.31455	8
364775	-	-	-	-	-
372385	0.409	0.423	0.416	0.08	12
369482	0.7665	0.795	0.78075	0.48265	8
352486	2.353	1.2379	1.79545	1.49735	8
357112	1.2868	1.2379	1.26235	0.96425	8
372483	0.5743	0.5196	0.54695	0.24885	8
365359	0.6274	0.5218	0.5746	0.2765	8
1125649	1.0115	1.0516	1.03155	0.73345	8
1301904	0.7155	0.6869	0.7012	0.4031	8
358802	1.5028	1.4475	1.47515	1.17705	8
364691	0.9422	1.0768	1.0095	0.7114	8
368510	0.6259	0.6874	0.65665	0.35855	8

PLG administration in hybrid striped bass

FISH (TAG NO.)	2nd Bleed	2nd Bleed	Average 2nd Bleed	True Value	Plate
359888	1.9107	2.1108	2.01075	1.7388	9
356834	0.7579	0.6426	0.70025	0.4283	9
1124497	1.4831	1.5241	1.5036	1.23165	9
375240	0.7407	0.8126	0.77665	0.5047	9
374583	0.4152	0.3204	0.3678	0.09585	9
353928	0.5323	0.5529	0.5426	0.27065	9
359200	0.5697	0.6139	0.5918	0.31985	9
375384	1.1969	1.2352	1.21605	0.9441	9
372412	0.4287	0.5525	0.4906	0.21865	9
364775	-	-	-	-	-
372385	0.5109	0.4358	0.47335	0.2014	9
369482	0.7945	0.8692	0.83185	0.5599	9
352486	2.0023	2.2218	2.11205	1.8401	9
357112	0.863	0.87	0.8665	0.5305	12
372483	0.5997	0.4507	0.5252	0.25325	9
365359	0.5291	0.4294	0.47925	0.2073	9
1125649	0.8532	0.7877	0.82045	0.5485	9
1301904	0.5172	0.4835	0.50035	0.2284	9
358802	1.3658	1.2108	1.2883	1.01635	9
364691	0.6189	0.7318	0.67535	0.4034	9
368510	0.5272	0.5187	0.52295	0.251	9

PLG administration in hybrid striped bass

FISH (TAG NO.)	3rd Bleed	3rd Bleed	Average 3rd Bleed	True Value	Plate
359888	2.123	2.171	2.147	1.8375	10
356834	0.888	0.745	0.8165	0.507	10
1124497	1.769	1.845	1.807	1.4975	10
375240	0.875	0.944	0.9095	0.6	10
374583	0.356	0.469	0.4125	0.103	10
353928	0.604	0.727	0.6655	0.356	10
359200	0.576	0.463	0.5195	0.1835	12
375384	1.113	1.19	1.515	1.2055	10
372412	0.544	0.528	0.536	0.2265	10
364775	-	-	-	-	-
372385	0.557	0.498	0.5275	0.218	10
369482	1.133	0.961	1.047	0.7375	10
352486	2.842	2.731	2.7865	2.477	10
357112	1.114	1.079	1.0965	0.787	10
372483	0.949	0.904	0.9265	0.617	10
365359	0.902	0.883	0.8925	0.583	10
1125649	0.833	1.199	1.016	0.68	12
1301904	0.863	0.972	0.9175	0.608	10
358802	2.192	1.898	2.045	1.7355	10
364691	1.409	1.218	1.3135	1.004	10
368510	-	-	-	-	-

PLG administration in hybrid striped bass

FISH (TAG NO.)	4th Bleed	4th Bleed	Average 4th Bleed	True Value	Plate
359888	2.17	2.128	2.149	1.8395	10
356834	1.049	1.157	1.103	0.7935	10
1124497	1.347	1.372	1.3595	1.05	10
375240	1.241	1.13	1.1855	0.876	10
374583	0.434	0.496	0.465	0.1555	10
353928	0.5694	0.5344	0.5519	0.22125	11
359200	0.8726	0.7791	0.82585	0.4952	11
375384	1.3713	1.4194	1.39535	1.0647	11
372412	0.6799	0.6181	0.649	0.31835	11
364775	-	-	-	-	-
372385	0.6263	0.6502	0.63825	0.3076	11
369482	1.1117	1.0737	1.0927	0.76205	11
352486	2.965	3.278	3.1215	2.7855	12
357112	1.5774	1.2931	1.43525	1.1046	11
372483	-	-	-	-	-
365359	0.7899	0.7626	0.77625	0.4456	11
1125649	1.0295	1.1208	1.07515	0.7445	11
1301904	0.9225	0.8137	0.8681	0.53745	11
358802	1.8334	2.2694	2.0514	1.72075	11
364691	1.1383	1.323	1.23065	0.9	11
368510	-	-	-	-	-

PLG administration in hybrid striped bass

FISH (TAG NO.)	5th Bleed	5th Bleed	Average 5th Bleed	True Value	Plate
359888	2.0192	2.1337	2.07645	1.7458	11
356834	1.4431	1.2891	1.3661	1.03545	11
1124497	0.8027	0.7831	0.7929	0.46225	11
375240	0.9981	1.1027	1.0504	0.71975	11
374583	0.3954	0.407	0.4012	0.07055	11
353928	0.6262	0.6103	0.61825	0.2876	11
359200	0.784	0.8666	0.8253	0.49465	11
375384	1.4989	1.1143	1.3066	0.97595	11
372412	0.6246	0.5786	0.6016	0.27095	11
364775	-	-	-	-	-
372385	0.5513	0.5292	0.54025	0.2096	11
369482	0.781	0.825	0.803	0.467	12
352486	2.801	2.804	2.8025	2.4665	12
357112	0.688	0.739	0.7135	0.3775	12
372483	0.698	0.797	0.7475	0.4115	12
365359	0.759	0.73	0.7445	0.4085	12
1125649	0.649	0.742	0.6955	0.3595	12
1301904	0.715	0.729	0.722	0.386	12
358802	1.443	1.606	1.5245	1.1885	12
364691	1.03	0.524	0.777	0.441	12
368510	-	-	-	-	-

PLG administration in hybrid striped bass

DNA & PLG Oral **FISH (TAG NO.)**	**Pre - Bleed**	**Pre - Bleed**	**Average Pre - Bleed**	**True Value**	**Plate**
352882	0.5296	0.4023	0.46595	0.15415	7
374648	0.8748	0.8038	0.8393	0.5275	7
365914	1.289	1.284	1.2865	0.9505	12
353965	0.624	0.7032	0.6636	0.3518	7
363050	0.3195	0.4426	0.38105	0.06925	7
1126707	0.7551	0.7145	0.7348	0.423	7
361121	0.3158	0.3331	0.32445	0.01265	7
366217	0.824	0.826	0.825	0.489	12
355200	0.3922	0.3471	0.36965	0.05785	7
364437	0.3845	0.4068	0.39565	0.08385	7
369704	-	-	-	-	-
370620	0.7057	0.679	0.7057	0.3807	13
363143	0.4729	0.4851	0.4729	0.1479	13
355245	0.4214	0.4187	0.4214	0.0964	13
368636	0.557	0.5209	0.557	0.232	13
366058	0.5311	0.4825	0.5311	0.2061	13
363365	0.6375	0.5968	0.6375	0.3125	13
366332	0.5459	0.5236	0.5459	0.2209	13
371820	0.9569	0.9711	0.9569	0.6319	13
369946	0.8548	0.7426	0.8548	0.5298	13

PLG administration in hybrid striped bass

FISH (TAG NO.)	1st Bleed	1st Bleed	Average 1st Bleed	True Value	Plate
352882	0.4592	0.3698	0.4145	0.0895	13
374648	1.706	1.765	1.7355	1.3575	17
365914	1.1	1.123	1.1115	0.7335	17
353965	1.044	0.901	0.9725	0.5945	17
363050	0.4904	0.4883	0.48935	0.16435	13
1126707	0.742	0.757	0.7495	0.467	18
361121	0.4644	0.4342	0.4493	0.1243	13
366217	0.986	0.89	0.938	0.6555	18
355200	0.5214	0.4803	0.50085	0.17585	13
364437	0.6986	0.699	0.6988	0.3738	13
369704	1.147	1.061	1.104	0.695	14
370620	1.831	1.746	1.7885	1.3795	14
363143	0.884	0.813	0.8485	0.4395	14
355245	1.348	1.367	1.3575	0.9485	14
368636	0.79	0.631	0.7105	0.3015	14
366058	0.812	0.741	0.7765	0.3675	14
363365	0.704	0.702	0.703	0.294	14
366332	0.705	0.74	0.7225	0.3135	14
371820	1.511	1.416	1.4635	1.0545	14
369946	1.598	1.702	1.65	1.241	14

PLG administration in hybrid striped bass

FISH (TAG NO.)	2nd Bleed	2nd Bleed	Average 2nd Bleed	True Value	Plate
352882	0.763	0.706	0.7345	0.3255	14
374648	2.685	2.746	2.7155	2.3065	14
365914	1.162	1.196	1.179	0.77	14
353965	1.061	0.988	1.0245	0.6155	14
363050	0.623	0.638	0.6305	0.2215	14
1126707	0.791	0.717	0.754	0.345	14
361121	0.62	0.648	0.634	0.225	14
366217	1.225	1.17	1.1975	0.7885	14
355200	0.637	0.651	0.644	0.235	14
364437	0.959	0.83	0.8945	0.4855	14
369704	1.098	1.124	1.111	0.702	14
370620	1.769	1.829	1.799	1.39	14
363143	0.765	0.785	0.775	0.366	14
355245	1.519	1.4721	1.49555	1.14155	15
368636	1.048	0.9312	0.9896	0.6356	15
366058	0.6825	0.6525	0.6675	0.3135	15
363365	0.698	0.626	0.662	0.3795	18
366332	0.611	0.602	0.6065	0.324	18
371820	1.7275	1.7136	1.72055	1.36655	15
369946	1.7505	1.8613	1.8059	1.4519	15

PLG administration in hybrid striped bass

FISH (TAG NO.)	3rd Bleed	3rd Bleed	Average 3rd Bleed	True Value	Plate
352882	0.5788	0.6757	0.62725	0.27325	15
374648	-	-	-	-	-
365914	1.1461	1.1796	1.16285	0.80885	15
353965	0.9419	0.9987	0.9703	0.6163	15
363050	0.853	0.7729	0.81295	0.45895	15
1126707	1.2106	0.9204	1.0655	0.7115	15
361121	0.6718	0.5781	0.62495	0.27095	15
366217	1.7028	1.6455	1.67415	1.32015	15
355200	0.6204	0.6631	0.64175	0.28775	15
364437	0.8779	0.8861	0.882	0.528	15
369704	1.1441	1.2307	1.1874	0.8334	15
370620	1.9577	2.0782	2.01795	1.66395	15
363143	0.7068	0.8137	0.76025	0.40625	15
355245	1.4188	1.676	1.5474	1.1934	15
368636	0.6872	0.6063	0.64675	0.29275	15
366058	0.7392	0.6322	0.6857	0.3317	15
363365	0.5954	0.6621	0.62875	0.27475	15
366332	0.54	0.53	0.535	0.2525	18
371820	1.8815	1.7585	1.82	1.466	15
369946	1.7875	1.9504	1.86895	1.51495	15

PLG administration in hybrid striped bass

FISH (TAG NO.)	4th Bleed	4th Bleed	Average 4th Bleed	True Value	Plate
352882	0.5521	0.6509	0.6015	0.1965	16
374648	-	-	-	-	-
365914	1.3102	1.3333	1.32175	0.91675	16
353965	0.9259	1.066	0.99595	0.59095	16
363050	0.5603	0.5702	0.56525	0.16025	16
1126707	0.6346	0.5303	0.58245	0.17745	16
361121	0.3853	0.4361	0.4107	0.0057	16
366217	1.1829	1.1973	1.1901	0.7851	16
355200	0.4789	0.5366	0.50775	0.10275	16
364437	0.6839	0.6297	0.6568	0.2518	16
369704	0.9655	0.9424	0.95395	0.54895	16
370620	1.2825	1.3503	1.3164	0.9114	16
363143	0.5232	0.4205	0.47185	0.06685	16
355245	1.4231	1.4015	1.4123	1.0073	16
368636	0.5757	0.4928	0.53425	0.12925	16
366058	0.5434	0.5287	0.53605	0.13105	16
363365	0.5358	0.5214	0.5286	0.1236	16
366332	0.6514	0.6072	0.6293	0.2243	16
371820	1.1941	1.3053	1.2497	0.8447	16
369946	1.9958	1.9949	1.99535	1.59035	16

PLG administration in hybrid striped bass

FISH (TAG NO.)	5th Bleed	5th Bleed	Average 5th Bleed	True Value	Plate
352882	0.564	0.616	0.59	0.212	17
374648	-	-	-	-	-
365914	1.049	1.085	1.067	0.689	17
353965	0.822	0.846	0.834	0.456	17
363050	0.47	0.418	0.444	0.066	17
1126707	0.62	0.665	0.6425	0.2645	17
361121	0.506	0.437	0.4715	0.0935	17
366217	1.648	1.743	1.6955	1.3175	17
355200	0.498	0.55	0.524	0.146	17
364437	0.57	0.593	0.5815	0.2035	17
369704	1.514	1.563	1.5385	1.1605	17
370620	1.138	1.337	1.2375	0.8595	17
363143	0.609	0.602	0.6055	0.2275	17
355245	1.92	1.973	1.9465	1.5685	17
368636	0.498	0.51	0.504	0.126	17
366058	0.764	0.643	0.7035	0.3255	17
363365	0.718	0.73	0.724	0.346	17
366332	0.848	0.885	0.8665	0.4885	17
371820	1.463	1.34	1.4015	1.0235	17
369946	1.604	1.695	1.6495	1.2715	17

PLG administration in hybrid striped bass

PLG oral FISH (TAG NO.)	Pre - Bleed	Pre - Bleed	Average Pre - Bleed	True Value	Plate
354787	1.411	1.361	1.386	1.05	12
358406	0.653	0.736	0.6945	0.3585	12
373843	0.884	0.876	0.88	0.544	12
361633	0.8273	0.784	0.80565	0.49385	7
368932	0.4863	0.4744	0.48305	0.17125	7
353275	0.5745	0.5257	0.5501	0.2383	7
357901	0.5128	0.4846	0.4987	0.1869	7
353973	0.6396	0.7633	0.70145	0.38965	7
357159	0.46	0.435	0.4475	0.1357	7
874565	0.553	0.4435	0.49825	0.18645	7
357240	-	-	-	-	-
357994	0.726	0.6984	0.7122	0.3872	13
368214	0.4285	0.3999	0.4142	0.0892	13
365106	0.4054	0.4457	0.42555	0.10055	13
355464	0.4629	0.4521	0.4575	0.1325	13
370188	0.3956	0.4191	0.40735	0.08235	13
367531	0.3967	0.372	0.38435	0.05935	13
353744	0.4681	0.4028	0.43545	0.11045	13
372827	0.8216	0.7505	0.78605	0.46105	13
357354	1.223	1.189	1.206	0.828	17
374810	0.3669	0.3963	0.3816	0.0566	13
357240	0.4139	0.4323	0.4231	0.0981	13
370728	1.543	1.551	1.547	1.169	17
368315	1.392	1.506	1.449	1.071	17
1125357	0.438	0.4133	0.42565	0.10065	13
372385	0.3906	0.4609	0.42575	0.10075	13
352875	0.4246	0.4812	0.4529	0.1279	13
369362	0.4488	0.4538	0.4513	0.1263	13

PLG administration in hybrid striped bass

FISH (TAG NO.)	1st Bleed	1st Bleed	Average 1st Bleed	True Value	Plate
354787	1.849	2.029	1.939	1.6565	18
358406	0.8699	0.9767	0.9233	0.5983	13
373843	0.988	0.9526	0.9703	0.6453	13
361633	0.9892	0.9487	0.96895	0.64395	13
368932	0.5082	0.6139	0.56105	0.23605	13
353275	-	-	-	-	-
357901	0.8297	0.7545	0.7921	0.4671	13
353973	0.933	0.822	0.8775	0.595	18
357159	-	-	-	-	-
874565	-	-	-	-	-
357240	0.817	0.735	0.776	0.367	14
357994	0.962	0.956	0.959	0.55	14
368214	0.726	0.811	0.7685	0.3595	14
365106	-	-	-	-	-
355464	-	-	-	-	-
370188	0.859	0.742	0.8005	0.3915	14
367531	-	-	-	-	-
353744	1.307	1.149	1.228	0.819	14
372827	-	-	-	-	-
357354	2.846	2.696	2.771	2.362	14
374810	0.78	0.737	0.7585	0.3495	14
357240	-	-	-	-	-
370728	-	-	-	-	-
368315	2.207	2.106	2.1565	-	18
1125357	0.653	0.73	0.6915	0.2825	14
372385	-	-	-	-	-
352875	0.738	0.79	0.764	0.355	14
369362	1.402	1.431	1.4165	1.0075	14

PLG administration in hybrid striped bass

FISH (TAG NO.)	2nd Bleed	2nd Bleed	Average 2nd Bleed	True Value	Plate
354787	2.112	2.078	2.095	1.686	14
358406	1.735	1.729	1.732	1.323	14
373843	1.244	1.418	1.331	0.922	14
361633	1.611	1.455	1.533	1.124	14
368932	0.878	0.937	0.9075	0.4985	14
353275	-	-	-	-	-
357901	1.767	1.98	1.8735	1.4645	14
353973	1.028	1.132	1.08	0.671	14
357159	-	-	-	-	-
874565	-	-	-	-	-
357240	0.973	0.939	0.956	0.547	14
357994	0.815	0.895	0.855	0.446	14
368214	0.794	0.8152	0.8046	0.4506	15
365106	-	-	-	-	-
355464	-	-	-	-	-
370188	0.636	0.587	0.6115	0.329	18
367531	-	-	-	-	-
353744	1.6627	1.6899	1.6763	1.3223	15
372827	-	-	-	-	-
357354	-	-	-	-	-
374810	0.8444	0.9527	0.89855	0.54455	15
357240	-	-	-	-	-
370728	-	-	-	-	-
368315	2.973	2.9062	2.9396	2.5856	15
1125357	0.449	0.457	0.453	0.1705	18
372385	-	-	-	-	-
352875	0.534	0.6492	0.5916	0.2376	15
369362	0.9372	1.1552	1.0462	0.6922	15

PLG administration in hybrid striped bass

FISH (TAG NO.)	3rd Bleed	3rd Bleed	Average 3rd Bleed	True Value	Plate
354787	2.3408	2.0372	2.189	1.835	15
358406	1.5834	1.5489	1.56615	1.21215	15
373843	1.1714	1.388	1.2797	0.9257	15
361633	1.4088	1.1476	1.2782	0.9242	15
368932	0.8267	0.7531	0.7899	0.4359	15
353275	-	-	-	-	-
357901	2.3798	2.1387	2.25925	1.90525	15
353973	0.9957	1.1479	1.0718	0.7178	15
357159	-	-	-	-	-
874565	0.8671	0.7561	0.8116	0.4576	15
357240	0.7627	0.8305	0.7966	0.4426	15
357994	-	-	-	-	-
368214	0.6829	0.6311	0.657	0.252	16
365106	-	-	-	-	-
355464	-	-	-	-	-
370188	0.4273	0.4375	0.4324	0.0274	16
367531	-	-	-	-	-
353744	1.7526	2.1418	1.9472	1.5422	16
372827	-	-	-	-	-
357354	-	-	-	-	-
374810	0.7397	0.6946	0.71715	0.31215	16
357240	-	-	-	-	-
370728	-	-	-	-	-
368315	3.0751	3.0704	3.07275	2.66775	16
1125357	0.7447	0.7752	0.75995	0.35495	16
372385	-	-	-	-	-
352875	0.6545	0.5892	0.62185	0.21685	16
369362	1.0174	0.9896	1.0035	0.5985	16

PLG administration in hybrid striped bass

FISH (TAG NO.)	4th Bleed	4th Bleed	Average 4th Bleed	True Value	Plate
354787	1.5732	1.434	1.5036	1.0986	16
358406	1.2638	1.3272	1.2955	0.8905	16
373843	1.1802	1.1465	1.16335	0.75835	16
361633	0.885	0.8881	0.88655	0.48155	16
368932	0.549	0.5027	0.52585	0.12085	16
353275	-	-	-	-	-
357901	1.2461	1.435	1.34055	0.93555	16
353973	0.768	0.77	0.769	0.4865	18
357159	-	-	-	-	-
874565	0.5149	0.5199	0.5174	0.1124	16
357240	0.7765	0.7995	0.788	0.383	16
357994	-	-	-	-	-
368214	0.6389	0.6272	0.63305	0.22805	16
365106	-	-	-	-	-
355464	-	-	-	-	-
370188	0.2927	0.2443	0.2685	-0.1365	16
367531	-	-	-	-	-
353744	1.2372	1.4123	1.32475	0.91975	16
372827	-	-	-	-	-
357354	-	-	-	-	-
374810	0.5712	0.572	0.5716	0.1666	16
357240	-	-	-	-	-
370728	-	-	-	-	-
368315	2.7427	2.8826	2.81265	2.40765	16
1125357	0.7621	0.7437	0.7529	0.3479	16
372385	-	-	-	-	-
352875	0.866	0.748	0.807	0.5245	18
369362	0.9655	0.9865	0.976	0.571	16

PLG administration in hybrid striped bass

FISH (TAG NO.)	5th Bleed	5th Bleed	Average 5th Bleed	True Value	Plate
354787	2.415	2.393	2.404	2.026	17
358406	1.043	1.189	1.116	0.738	17
373843	1.569	1.209	1.389	1.011	17
361633	0.963	0.888	0.9255	0.5475	17
368932	0.593	0.61	0.6015	0.2235	17
353275	-	-	-	-	-
357901	1.062	1.092	1.077	0.699	17
353973	0.817	0.773	0.795	0.417	17
357159	-	-	-	-	-
874565	0.553	0.54	0.5465	0.1685	17
357240	0.726	0.753	0.7395	0.457	18
357994	-	-	-	-	-
368214	0.706	0.662	0.684	0.306	17
365106	-	-	-	-	-
355464	-	-	-	-	-
370188	0.678	0.666	0.672	0.294	17
367531	-	-	-	-	-
353744	1.839	1.83	1.8345	1.4565	17
372827	-	-	-	-	-
357354	-	-	-	-	-
374810	1.059	0.972	1.0155	0.6375	17
357240	-	-	-	-	-
370728	-	-	-	-	-
368315	3.241	3.24	3.2405	2.8625	17
1125357	0.716	0.701	0.7085	0.3305	17
372385	-	-	-	-	-
352875	0.569	0.551	0.56	0.182	17
369362	1.392	1.156	1.274	0.896	17

PLG administration in hybrid striped bass

CONTROLS

	Plate 6	**Plate 6**
Rabbit Anti fish Primary Antibody	0.1415	0.2665
Normal	0.3055	0.3411
Positive	0.4684	0.417
Uncoated	2.119	1.9717
	0.422	0.2736

	Plate 7	**Plate 7**
Rabbit Anti fish Primary Antibody	0.1265	0.1281
Normal	0.1894	0.2046
Positive	0.3235	0.3001
Uncoated	1.8133	1.6938
	0.2847	0.28

	Plate 8	**Plate 8**
Rabbit Anti fish Primary Antibody	0.112	0.1256
Normal	0.2535	0.2164
Positive	0.3088	0.2874
Uncoated	1.7562	1.6539
	0.2597	0.2787

PLG administration in hybrid striped bass

	Plate 9	**Plate 9**
Rabbit Anti fish	0.0999	0.1185
Primary Antibody	0.2187	0.2345
Normal	0.2978	0.2461
Positive	1.452	1.3469
Uncoated	0.2539	0.2038

	Plate 10	**Plate 10**
Rabbit Anti fish	0.143	0.159
Primary Antibody	0.236	0.215
Normal	0.299	0.32
Positive	1.643	1.558
Uncoated	0.344	0.442

	Plate 11	**Plate 11**
Rabbit Anti fish	0.1279	0.1303
Primary Antibody	0.2306	0.2302
Normal	0.3735	0.2878
Positive	2.0621	1.7606
Uncoated	0.2858	0.2681

PLG administration in hybrid striped bass

	Plate 12	**Plate 12**
Rabbit Anti fish	0.13	0.112
Primary Antibody	0.206	0.19
Normal	0.293	0.379
Positive	1.932	1.644
Uncoated	0.348	0.232

	Plate 13	**Plate 13**
Rabbit Anti fish	0.096	0.106
Primary Antibody	0.185	0.198
Normal	0.376	0.274
Positive	1.39	1.456
Uncoated	0.28	0.196

	Plate 14	**Plate 14**
Rabbit Anti fish	0.17	0.148
Primary Antibody	0.298	0.327
Normal	0.369	0.449
Positive	1.942	1.746
Uncoated	0.391	0.471

PLG administration in hybrid striped bass

	Plate 15	**Plate 15**
Rabbit Anti fish	0.166	0.179
Primary Antibody	0.246	0.249
Normal	0.373	0.335
Positive	1.788	2.017
Uncoated	0.298	0.307

	Plate 16	**Plate 16**
Rabbit Anti fish	0.108	0.143
Primary Antibody	0.213	0.215
Normal	0.342	0.468
Positive	1.884	2.194
Uncoated	0.264	0.437

	Plate 17	**Plate 17**
Rabbit Anti fish	0.121	0.184
Primary Antibody	0.214	0.231
Normal	0.419	0.337
Positive	1.519	1.552
Uncoated	0.275	0.302

PLG administration in hybrid striped bass

	Plate 18	Plate 18
Rabbit Anti fish	0.204	0.203
Primary Antibody	0.208	0.222
Normal	0.253	0.312
Positive	1.826	1.81
Uncoated	0.349	0.25