THESIS

EVALUATION OF AN ADJUVANTED HAPTEN-PROTEIN VACCINE APPROACH TO PREVENTING SEXUAL MATURATION OF FARMED ATLANTIC SALMON

Submitted by

Darcy Sonya Orahood

Department of Clinical Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2013

Master's Committee:

Advisor: M.D. Salman

Douglas C. Eckery
Lowell A. Miller
Christopher A. Myrick
Jack C. Rhyan

ABSTRACT

EVALUATION OF AN ADJUVANTED HAPTEN-PROTEIN VACCINE APPROACH TO PREVENTING SEXUAL MATURATION OF FARMED ATLANTIC SALMON

Aquaculture is a rapidly growing industry that significantly contributes to the world food supply. Sustainable practices in aquaculture are of increasing importance as an increasing proportion of fish in the global market come from aquaculture instead of wild catch. Maximizing aquaculture yields and minimizing the ecological impacts of these operations are two important goals towards sustainability. One approach to addressing these objectives is immunocontraception of fish which would increase the fish meat quality and yield in aquaculture production and prevent escaped farmed fish from undesirably altering wild fish population genetics through breeding.

The research presented here was conducted with the aim of proof of concept for contraceptive vaccine use in Atlantic salmon. Nine vaccine formulations, including a negative control vaccine, were formulated at the National Wildlife Research Center in Fort Collins, Colorado and injected into farmed Atlantic salmon in Sunndalsøra, Norway. Production of antibodies against three immunogenic components in each vaccine formulation was evaluated over the course of the 12-week study. Weight and length of each fish were also tracked over time to determine whether growth was affected by vaccination.

The study results indicate that Atlantic salmon will produce antibodies against BSA and KLH used as carrier proteins but that KLH is a stronger immunogen. Importantly, it was also determined that Atlantic salmon will produce antibodies against a small endogenous peptide (hapten) conjugated to the carrier protein, but to a lesser extent than the levels of antibody production against the carrier itself. Approximately 96% of samples from fish vaccinated against

KLH, 76% of samples from fish vaccinated against BSA, and 36% of samples from fish vaccinated against the hapten were identified as positive. Response rates for all three antigens were highest 12 weeks post-vaccination. Significant differences in antibody levels were also detected between groups vaccinated with different immunostimulants. Collectively, the results provide proof of concept and provide a building block for further research on immunocontraception of Atlantic salmon for application in aquaculture.

ACKNOWLEDGEMENTS

This research was funded by Prophylaxia AS (Norway) and was supported by the National Wildlife Research Center (Fort Collins, Colorado, USA).

Thank you to my mentors, co-workers, family, and friends for your support.

TABLE OF CONTENTS

INTRODUCTION

Aquaculture is a rapidly growing industry that significantly contributes to the world food supply. As wild fish stocks have diminished, aquaculture has taken over as the fastest growing food-animal producing sector (1) and provides 47% of fish for human consumption (2). Accordingly, sustainable fish farming practices are of increasing importance and are being heavily scrutinized by governmental and environmental agencies. The United Nations Food and Agriculture Association (FAO) predicts that the global demand for seafood will increase 25% between the years 2006 and 2030. More than 14% of fish produced globally are salmonids (2). Aquaculture production of salmonids continues to increase and has grown from 310,000 tons in 1990 to over 2.1 million tons in 2010, an average annual increase of more than 9.5% (2). Norway produces the majority of salmonids entering the global market and is the leading producer and exporter of Atlantic salmon (*Salmo salar*). The United States, Chile, Australia, Japan, Canada, the United Kingdom, and Russia also produce salmon (aquaculture and wild capture) for local consumption and export (1, 2).

With the continued expansion of aquaculture, an issue receiving increasing attention is escape of farmed salmon into bodies of water inhabited by wild fish populations (3). The ocean-rearing phase of salmon farming occurs in large sea pens made from nets that are suspended from floating docks and fastened to the seabed (4). The accidental release or escape of large numbers of Atlantic salmon every year can be caused by deterioration of nets, human error, and natural events such as flooding. It is estimated that farmed salmon account for 14-36% of the spawning population in Norwegian rivers (5). In Norway in particular, the unintentional release of farmed salmon is a problem because each fjord has its own genetically-unique wild Atlantic salmon population. Farmed salmon all originate from the same lineage and have severely limited genetic diversity due to founder effects, and other selection-related factors (6). The

Norwegian wild salmon population is estimated to be around 500,000 compared to the more than 200 million farmed salmon. While farmed salmon have reduced breeding performance compared to wild populations, they are still able to breed with the wild salmon, thereby altering the genetics of those unique populations (6, 7). As genetic variability decreases within a population, the survivability of that population is negatively affected (5, 6, 8).

A significant amount of energy is required for sexual maturation of salmon, particularly in the development of gonads and for gametogenesis (9). Alternatively, in the absence of sexual maturation, this energy can be used towards increasing fat and muscle. In bioenergetic energy budget models, energy in excess of basic maintenance requirements is dedicated to growth which is defined as the sum of somatic and reproductive growth. Under fixed food consumption, increased energy allocation towards reproductive growth must shift energy away from somatic growth (10). On average, approximately 74% of a female sockeye salmon's (*Oncorhyncus nerka*) stored energy, and 55-68% of a male's stored energy is invested in gonadal development and spawning (11). Sexual maturation of salmon reduces fat and protein content, increases water content, and diminishes the characteristic red color of the meat. These changes ultimately produce a fillet that has decreased flavor, paler color, and a tough, watery texture (12), all of which are traits consumers find less desirable. Behavioral changes are common in sexually mature salmon and may present as heightened aggression and diminished health. From a commercial standpoint, sexual maturation of salmon that will not become broodstock is unfavorable and, thus, modulation of the reproductive system is desirable.

Gonadotropin releasing hormone (GnRH) is an important regulator of the reproductive system. It is the first molecule to act in a signaling pathway that leads to production of sex hormones: estrogen and progesterone in females, and testosterone in males. The presence of these hormones, along with other molecules in the signaling pathway initiated by GnRH, leads

to development of gonads, expression of secondary sex characteristics, and production and maturation of germ cells (13, 14).

Salmon produce two forms of GnRH, salmon GnRH and chicken GnRH-II, which vary slightly in their amino acid sequences but are thought to perform the same function (13, 15). GnRH is synthesized in the hypothalamus and transported, via neurons, to the pituitary (13, 15, 16). In the pituitary, GnRH stimulates secretion of two protein hormones, gonadotropin I (GTH-I) and gonadotropin II (GTH-II), homologs of mammalian follicle stimulating hormone (FSH) and lutenizing hormone (LH), which are carried to gonads in the bloodstream (15). The primary role of FSH in fish is to stimulate gonad development, while both FSH and LH regulate functions of the mature reproductive system, such as ovulation and sperm release. GTH-I and GTH-II have similar structures and both consist of an alpha subunit and a beta subunit. The alpha subunit is necessary for structural integrity and the beta subunit is considered to be the biologically active portion of the molecule (16). Circulating gonadotropin concentrations are tightly regulated by negative feedback signals originating from receptors on dopamine neurons (13, 14).

Scientific research continues to provide greater insight into the way fish function as individual organisms and as part of the ecosystem around them. Despite this, the aquaculture industry in certain regions, Norway in particular, has grown at such a rapid rate that, in some cases, regulations related to fish farming practices have not kept pace with the recognition of environmental problems. The world population is also growing at a rapid rate and reliance on aquaculture for food production is increasing (1, 2). As food security becomes more of an issue, greater emphasis is being put on implementation of sustainable practices in agriculture and aquaculture. Thus, it will be of increasing importance that approaches are explored to optimize quality and yield of aquaculture products while maintaining an ecologically-conscious approach to fish production.

One approach to optimize production of farmed salmon is genetic modification. Aqua Bounty Technologies developed a process for production of triploid, all-female, transgenic Atlantic salmon that grow at an increased rate (17). However, significant barriers to approval of transgenic animals still exist in most of the world; regulatory and environmental issues pose a large obstacle and the public tends to look upon genetically modified organisms with suspicion (18). Troutlodge, Inc. also produces triploid, all-female eggs for commercial aquaculture but rainbow trout (*Oncorhynchus mykiss*) is the only species currently available (19).

An immunocontraceptive approach would provide a non-transgenic means to maintain high fish growth rates and prevent escaped salmon from cross-breeding with wild salmon. If administered at the proper stage in a fish's development, an immunocontraceptive could prevent fish from sexually maturing. A precedent for immunological intervention in culture salmon has already been established through the use of injectable oil-based vaccines against bacterial pathogens. Such vaccines are typically considered superior to immersion and oral vaccines (20). Thus, the ideal immunocontraceptive scheme would be easily incorporated into existing vaccination regimens and should be effective as a single-shot rather than requiring multiple vaccinations.

Under such an immunocontraceptive approach, induced auto-immunity to a reproductive molecule or its receptor causes temporary (months to years; species, animal, dose, and adjuvant-dependent) infertility in many species (21, 22). An immunocontraceptive may be administered like a conventional injectable vaccine against a pathogen and is designed to stimulate antibody production in a similar fashion. However, the antigen in the vaccine is not a virus or bacterium, but a molecule important to functioning of the reproductive system of the target species. Since the antigen is also endogenously produced by the salmon and will be recognized as self, it must be modified to appear foreign to a salmon's immune system.

Antibodies produced against the reproductive molecule will bind to it in the bloodstream, making the molecule unable to bind its receptors.

Limited research has been conducted on this type of approach to induced infertility of Atlantic salmon. A pioneering limited-scope study was conducted with the aim of proof of concept for this means of contraception in salmon. The purposes of this study were to: 1) assess the feasibility of stimulating Atlantic salmon to produce antibodies against an endogenous peptide, 2) compare immunogenicity of two different carrier proteins, 3) determine which immunostimulant is most efficient at exciting non-specific antibody production, and 4) evaluate the suitability of an oil adjuvant for use in Atlantic salmon.

REFERENCES

1. Sommerset I, B Krossøy, E Biering, and P Frost. Vaccines for fish in aquaculture. Expert Review of Vaccines 2005; 4(1):89-101.
2. The State of World Fisheries and Aquaculture 2012. Rome: Food and Agriculture Organization of the United Nations. Fisheries and Aquaculture Department, 2012.
3. Myrick CA. Ecological impacts of escaped organisms. *Aquaculture and the Environment in the United States*, editor JR Tomasso. Baton Rouge: United States Aquaculture Society/World Aquaculture Society, 2002. 225-245.
4. European Commission. Directorate-General for Maritime Affairs and Fisheries. *Fisheries and Aquaculture in Europe* [Salmon (*Salmo salar*)]. Brussels, 2012; 58.
5. Liu Y, JO Olaussen, A Skonhoft. Wild and farmed salmon in Norway – A review. Marine Policy 2011; 35:413-418.
6. McGinnity P, et al. Fitness reduction and potential extinction of wild populations of Atlantic salmon, *Salmo salar*, as a result of interactions with escaped farmed salmon. Proceedings of the Royal Society of London 2003; 270:2443-2450.
7. Jonsson B, N Jonsson. Farmed Atlantic salmon in nature. Ecology of Atlantic Salmon and Brown Trout (2011); 33:517-566.
8. Reed DH and R Frankham. Correlation between fitness and genetic diversity. Conservation Biology 2003; 17(1):230-237.
9. Fleming IA. Reproductive strategies of Atlantic salmon: ecology and evolution. Reviews in Fish Biology and Fisheries 1996; 6:379-416.
10. Busacker GP, IR Adleman, and EM Goolish. Growth. *Methods for Fish Biology*, eds CB Schreck and PB Moyle. Bethesda: American Fisheries Society, 1990. 363-388.
11. Hendry AP and OK Berg. Secondary sexual characters, energy use senescence, and the cost of reproduction in sockeye salmon. Canadian Journal of Zoology 1999; 77:1663-1675.

12. Aksnes A, B Gjerde, and SO Roald. Biological, chemical and organoleptic changes during maturation of farmed Atlantic salmon, *Salmo salar*. Aquaculture 1986; 53:7-20.
13. Trudeau VL. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*. Reviews of Reproduction 1997; 2:55-68.
14. Nozaki M, et al. Salmonid Pituitary Gonadotrophs: II. Ontogeny of GHT I and GTH II cells in the rainbow trout (*Salmo gairdneri irideus*). General and Comparative Endocrinology 1990; 77(3):358-367.
15. Melamed P and N Sherwood. Molecular Aspects of Fish & Marine Biology, Volume 4: Hormones and Their Receptors in Fish Reproduction. Singapore: World Scientific Publishing Co. Pte. Ltd., 2005.
16. Levavi-Sivan B, J Bogerd, EL Mañanós, A Gómez, JJ Lareyre. Perspectives on fish gonadotropins and their receptors. General and Comparative Endocrinology 2010; 165:412-437.
17. AquAdvantage® Fish, AquaBounty Technologies. 2012. Web. [4 Sept 2012].
18. Association of Fish and Wildlife Agencies. White paper on genetically modified salmon. Washington DC, 2012.
19. Benefits of Triploid Eggs, Troutlodge, Inc. 2013. Web. [4 Nov 2013].
20. Evensen Ø. Development in fish vaccinology with focus on delivery methodologies, adjuvants and formulations. Options Méditerranéennes, Series A 2009; 86:177-186.
21. Gionfriddo JP, AJ DeNicola, LA Miller, KA Fagerstone. Health effects of GnRH immunocontraception of wild white-tailed deer in New Jersey. Wildlife Society Bulletin 2011; 35(3):149-160.
22. Sambroni E, L Abdennebi-Najar, J-J Remy, F Le Gac. Delayed sexual maturation through gonadotropin receptor vaccination in the rainbow trout *Oncorhynchus mykiss*. General and Comparative Endocrinology 2009; 164**:**107-116

COMPREHENSIVE LITERATURE REVIEW

Atlantic salmon background

Native to the North Atlantic and Arctic oceans and their influent rivers, Atlantic salmon (*Salmo salar*) have long been an important "social, economic, and cultural resource" (1, 2). Atlantic salmon are anadromous members of the salmonid family. Most populations spend the majority of their life at sea, although reproduction and the first few years of life require freshwater. Adaptation to sea water is facilitated by physiological changes during a process called smoltification (2). Unlike semelparous Pacific salmon (*Oncorhynchus spp.*) that die after spawning, Atlantic salmon are iteroparous and can survive to spawn over multiple years (3, 4).

Female Atlantic salmon lay approximately 1300-1600 eggs per kg body weight (3), in redds, nests of loose gravel that are formed by vigorous tail movements, in freshwater streambeds. As the female prepares to release the eggs, a male partner that has successfully courted the female will swim alongside the female and release the milt, the fish equivalent of semen, at the same time. This simultaneous release of eggs and milt helps to increase the odds that the eggs will be fertilized successfully and only by a single male's sperm. The eggs and milt are deposited into the gravelly hollow and loosely covered. This may take place multiple times as the female deposits hundreds to many thousands of eggs (3). The fertilized eggs develop into small hatchlings called alevin and remain in the redd for several weeks as they are sustained by their large yolk sacs. Eventually, the tiny fish swim up out of the redd and fill their swim bladders, at which point they are referred to as fry. The fry continue to grow, primarily feeding on insect larvae, and develop distinctive markings that aid in camouflaging and protecting the fish from predation. At this stage, they are called parr. After one to two years, parr undergo smoltification to become adapted to sea water. The resulting smolts migrate to

sea where they grow significantly and, after one to several winters, become sexually mature adults ready to migrate back to freshwater to spawn (Figure 1).

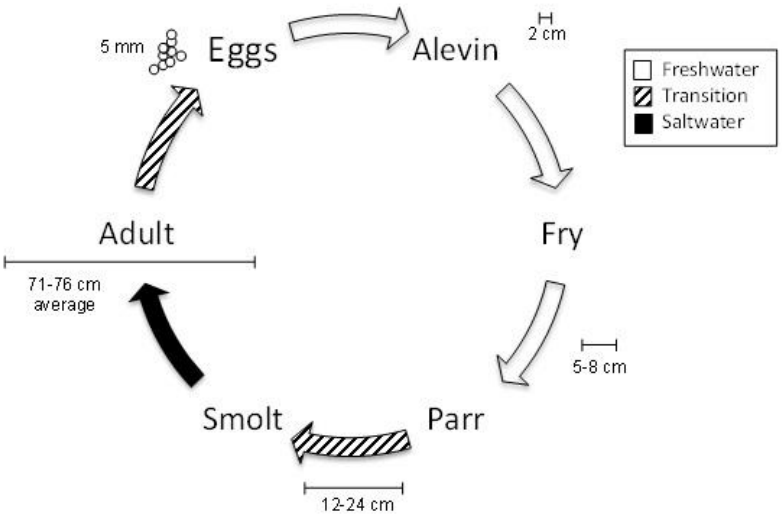

Figure 1: Atlantic salmon lifecycle

Reproduction of salmon for aquaculture is highly controlled. Eggs and milt are harvested from carefully selected broodstock and manually mixed together. The fertilized eggs incubate in freshwater tanks until they reach the parr stage. Parr are transferred to large freshwater tanks where they spend 1-2 years before undergoing smoltification and subsequent transfer to sea water. The next several years are spent in large sea pens, commonly consisting of a net that is suspended from floating walkways and anchored to the sea floor, until a commercial size of approximately 2-5 kilograms is reached (2).

Aquaculture

The demand for fish products has been rapidly increasing over the past few decades. As the fastest growing food-animal production industry (5), aquaculture holds an ever-increasing importance in the global food supply chain. Fish account for almost 17% of animal protein consumed by billions of people around the world (4). The Rio+20 UN Conference on

Sustainable Development included discussions on emphasizing sustainability and efficiency in agriculture, including aquaculture, towards the goal of global food security (4). In “The State of World Fisheries and Aquaculture 2012” publication released by The Food and Agriculture Organization (FAO) of the United Nations (UN), “Sustained demand, trade liberalization policies, globalization of food systems and technological innovations” are credited for the continued promotion and growth of worldwide fish trade. In 2010, the global aquaculture industry was valued at $119 billion USD (4).

More than 14% of fish produced globally are salmonids (4). The salmon farming business was revolutionized in the 1960’s when adult salmon raised in sea pens were first sold in Norway (2). The advent of injectable vaccines against common pathogens again transformed the salmon aquaculture industry in the 1990’s, simultaneously increasing productivity and fish health while drastically decreasing the need for heavy use of antibiotics (5). During that same time period, aquaculture production of fish began increasing at what has been an average rate of nearly 9% per year and capture fishing rates started to decline (4, 5). Now, aquaculture contributes approximately two thirds of salmon in the global market while only one third are wild caught (2).

The Atlantic salmon is the most popular salmon species for aquaculture and is the only type of salmon that aquaculture facilities are permitted to raise in the European Union. According to the European Commission on Fisheries and Aquaculture in Europe (2012), 93% of salmon produced in aquaculture are Atlantic salmon. Although Norway produces and exports more Atlantic salmon than any other country (1, 4), Canada, Chile, and the United Kingdon are also major contributors to the global supply while the United States, Australia, Japan, and Russia also contribute but to a much lesser extent (4, 5).

Sea pens, in which farmed Atlantic salmon spend most of their lives, are susceptible to damage since they are constructed from flexible netting. Small- to large-scale escapes of farmed salmon occur due to human error and natural causes, including routine handling, wear and tear of nets, and severe weather (6). Atlantic salmon escapes from aquaculture facilities are of particular significance in the North Atlantic (6), especially in Norway due to the unique geography of a lengthy coastline replete with hundreds of fjords and rivers flowing into the ocean.

It has been estimated that on average one third of the adult salmon entering rivers Norwegian are escaped farmed salmon (6). Liu, et al. (1) estimate that the spawning Atlantic salmon population in Norwegian rivers is composed of 14-36% farmed salmon on average and up to 80% in some cases. An estimated two million farmed Atlantic salmon escape annually in the North Atlantic (6). While these escapees only account for less than 1% of the Norwegian Atlantic salmon aquaculture population, they are estimated to amount to approximately 50% of the pre-fishery abundance of Atlantic salmon in the region (6). Most farmed Atlantic salmon stocks arise from common ancestry and are, thus, quite genetically homogenous. Domestication and selection of desirable traits has created a population of Atlantic salmon within the aquaculture industry that possesses very little genetic variation. When strictly confined to an aquaculture operation, this lack of genetic diversity is acceptable. However, it is inevitable that some farmed fish will escape.

Introduction of genetically-similar breeders to a wild population results in a trend towards homozygosity. Genetic variability is often reduced in endangered species as compared to non-endangered species, suggesting that this attribute is important for persistence of populations and species (7). Wild Atlantic salmon are now vastly outnumbered by farmed Atlantic salmon due to enormous growth of aquaculture. This balance continues to shift further towards aquaculture as the industry continues to grow and more wild Atlantic salmon populations in

Norway are under threat of extinction or endangerment (6). Despite reduced breeding performance relative to wild Atlantic salmon, the sheer number of farmed Atlantic salmon poses a huge threat to the genetic diversity of wild populations, even if only a small percentage of farmed fish successfully breed outside captivity (6).

Atlantic salmon are an important economic resource for anglers, tourism, and biodiversity of Norwegian rivers and fjords (6). The aspirations of growth and productivity within the salmon aquaculture sector are largely at odds with objectives to conserve wild Atlantic salmon (1).

Reproduction of Atlantic salmon

The major physiological changes involved in sexual maturation of Atlantic salmon require a large amount of energy (8) and take place over the course of one to two years (9). During the early stages of sexual maturation, appetite and feeding behaviors increase leading to increased growth rate (9). However, further into the process, food intake decreases significantly and the fish begin to produce energy from their fat, muscle, and viscera (9). In addition to sustaining normal physical activity, the fat, muscle, and viscera must also fulfill the substantial energy requirements of gonad development, among other physiological changes taking place. This diversion of energy towards sexual maturation depletes the valuable resources that make up the harvestable and consumable meat of the fish.

Consequences of sexual maturation are poorer quality meat and less of it, and, ultimately, fish of reduced commercial value. At peak weight, gonads alone account for over 25% of the average female Atlantic salmon's body weight (8, 9). After a certain point in the sexual maturation process, female Atlantic salmon begin to lose weight while the rate of weight gain for males tapers off (9). Fleming, et al. (8) estimate that females lose about 21% of their body weight and males lose approximately 12% of their body weight during the breeding

season. The skin color of both sexes changes from a characteristic silver color to a progressively darker brown during sexual maturation. More importantly, the meat undergoes significant changes that affect flavor, texture, and appearance. On average, sexual maturation results in a 4% reduction in protein content, 6% drop in fat content, and 4% increase in water content of the meat, collectively delivering decreased flavor and producing a watery, tough texture. A significant difference in pigmentation of the flesh between immature and sexually mature Atlantic salmon is also observed, fading the distinctive orange color of the flesh which is an important organoleptic property. As Aksnes, et al. (9) concluded, there is an "unmistakable deterioration in eating quality" in Atlantic salmon in late stages of sexual maturation versus their immature counterparts.

Sexual maturation and reproduction are regulated by a signaling pathway that begins in the brain with gonadotropin releasing hormone (GnRH). As in mammals, fish GnRH is produced in the hypothalamus as a decamer peptide and is also detected in the gonads. As the first molecule in the signaling cascade that stimulates secretion of sex hormones such as testosterone, progesterone, and estrogen, GnRH is critical for reproduction (Figure 2). Atlantic salmon have two different circulating GnRHs, each produced by a different gene (10). Salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II), named for the species in which the sequence was first determined, are thought to perform similar functions but vary slightly in their amino acid sequences and distribution throughout the brain (10, 11).

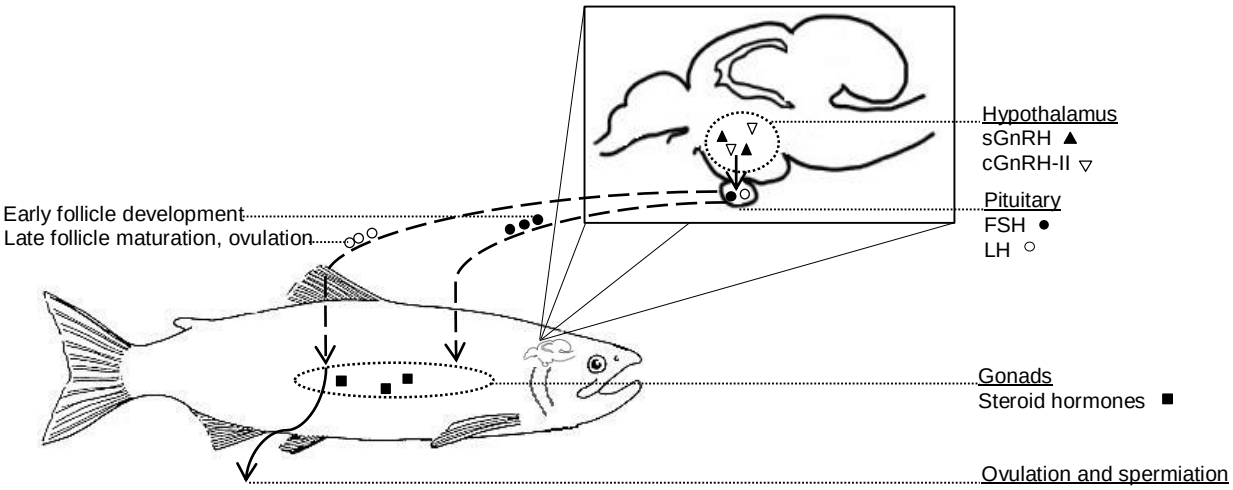

Figure 2: Atlantic salmon reproductive signaling; GnRH is produced in the hypothalamus then travels through neurons to the pituitary where it stimulates synthesis of FSH and LH; the FSH and LH act in the gonads to stimulate steroidogenesis; FSH is responsible for early stages of sexual maturation while LH acts predominantly in later stages of folliculogenesis and ovulation.

Teleosts lack the narrow hypothalamo-hypophyseal portal that mammals use to transport GnRH out of the hypothalamus (11, 12). Instead, neurons extending from the preoptic-hypothalamic region of the brain innervate the pituitary, delivering predominantly sGnRH (10, 11, 12). Binding of GnRH to its receptors in the pituitary stimulates release of gonadotropins I and II (GTH-I and GTH-II), also referred to as follicle stimulating hormone (FSH) and luteinizing hormone (LH) respectively, for their functions analogous to the molecules of the same name in mammals (10). Mature GnRH is produced at a very young age in Atlantic salmon, although it does not exert any physiological effects contributing to sexual maturation for the first few years (10) as a positive correlation exists between gonadal size and responsiveness of the pituitary to stimulus from GnRH (11).

FSH is recognized as providing an important stimulus for production of sex hormones in gonads (11) and contributes significantly to early stages of gonadal growth and gametogenesis (13, 14). LH plays an important role in fertility of a sexually mature fish and regulates late gametogenesis, release of sperm and eggs, and production of sex hormones (11, 15). While

FSH is involved in the early stages of follicle growth and development, LH doesn't appear until the follicles are in the final stages of maturation and ready for ovulation (12, 14, 15). LH stimulates production of the sex steroid 17,20β-Progesterone which is responsible for oocyte maturation (16). Follicular estrogen is produced in the ovary as a result of stimulation by FSH (16). The FSH receptor gene is highly expressed during early primary growth but expression decreases as vitellogenesis is approached (16). In coho salmon, ovary weight was found to have a positive correlation with both pituitary and plasma FSH concentrations (14).

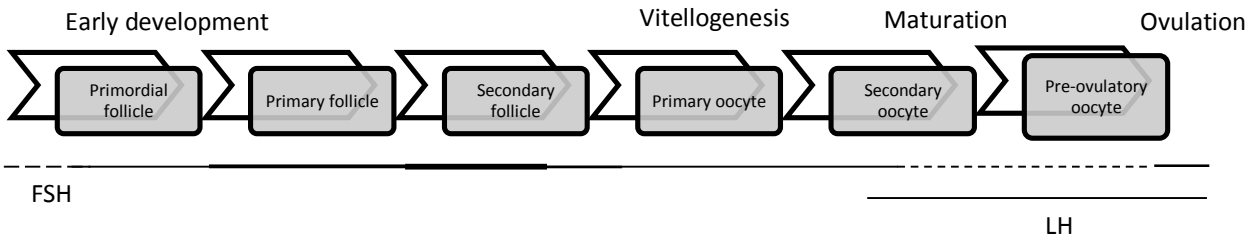

Figure 3: Development and maturation of female Atlantic salmon ovarian follicles, regulated by varying levels of FSH and LH

Fish gonadotropins are heterodimers consisting of an alpha subunit and a beta subunit which are covalently linked (12, 15). The alpha subunit is common between both gonadotropins. It is the beta subunit that importantly confers the specificity of the hormone and its activity (12). While the amino acid sequence of the LH beta subunit is well conserved among many fish, this is not the case for FSH.

Many years of research towards greater understanding of salmonid reproductive systems has led to development of methods for genetic modifications which modulate reproductive activity. AquAdvantage salmon, produced by AquaBounty Technologies, were recently approved for commercial production in landlocked tanks in the United States. Engineered to include the Chinook salmon growth hormone gene, the all-female, triploid AquAdvantage salmon grow nearly twice as fast as wild Atlantic salmon (17, 18). At present time, these fish are the only transgenic food animal approved for production and consumption in

the United States. However, The European Union has extremely stringent regulations on all genetically modified foods, and transgenic food animals are currently banned. Other common methods of contraception, although not used in fish, include mechanical, hormonal, and immune-mediated approaches (19).

Immune-mediated induction of infertility, also known as immunocontraception, makes use of a vaccine to produce antibodies against specific reproductive molecules (19). In this approach, the signaling cascade that leads to production or bioactivity of important hormones is inhibited. Without these hormones, reproduction certainly cannot occur and, depending on the time of vaccination, it is possible that sexual maturation could also be prevented. A vaccine against a molecule of importance in this signaling pathway could theoretically induce production of antibodies that would specifically bind to the endogenous molecule and prevent it from interacting with its receptors in the body. In the absence of receptor binding, the molecule goes unnoticed and fails to stimulate synthesis and secretion of other necessary molecules.

Atlantic salmon immune system

Vaccination against selected infectious diseases in large-scale aquaculture operations is now a standard practice, especially for high-value species like Atlantic salmon (20). Protection against regional and species-specific pathogens is regularly conferred by intraperitoneal injection, immersion, or oral administration via feed (20). Injectable vaccines typically produce the strongest protection and have the advantages of high potency, low dose requirement, flexibility to use adjuvants, and cost effectiveness (20). While immersion and oral vaccination may be less stressful for the fish, the vaccine efficacy, protection, and cost effectiveness provided by these strategies are still inferior to those achieved by injectable vaccines (20).

Both adaptive and innate immune responses are observed in teleosts (25) although the innate system is thought to be much more highly developed (24). The adaptive immune

response can be primed by vaccination or prior exposure to an antigen (21). The predominant immunoglobulin in teleost fish is tetrameric IgM, but a monomeric IgZ and an isotype related to IgD are also present (22, 23, 24). Though the IgM structure shares striking similarities with mammalian IgM, teleosts do not produce IgG (23, 24). Relative to mammals, fish exhibit a greatly slowed and reduced adaptive immune response in both magnitude and specificity (24). This lack of specificity combined with variability in IgM structure provides protection against a wide range of diseases and may allow for a more versatile immune response (24). Unlike mammals, teleosts do not have bone marrow or lymph nodes so the major immunological organ is the kidney (20, 26). The spleen, thymus, and mucosa-associated tissues also perform important immune functions (20, 24).

Water temperature and stress are two of the most significant determinants of the timing and quality of teleost immune response (24, 27). Serum antibodies may be detected as early as 3 weeks post-vaccination but may not reach peak levels until up to 6 months thereafter (27). Compared to homeotherms, a low antibody response is seen in fish (28). This may in part be due to a heavy reliance on non-specific defense mechanisms (29).

Vaccines for aquaculture

Heat- or formalin-inactivated bacterial vaccines are commonly used to expose fish to a pathogen without causing clinical disease (30). Literature suggests that intraperitoneal vaccination with 100-200 µL of oil-adjuvanted vaccine is appropriate for immunocompetent salmonids (31, 32, 33). A typical immunostimulant dosage in fish vaccines is 50 µg (29, 31, 32). Fish bacterial vaccine concentrations seem to be less standardized but often range from 10^6 to 10^9 CFUs per dose (33, 34, 35).

Antigen presenting cells (APCs) in other teleosts, such as channel catfish (*Ictalrus punctatus*) (36) and rainbow trout (*Oncorhynchus mykiss*) (29, 31), readily take up large foreign

proteins such as keyhole limpet hemocyanin (KLH). KLH has been successfully used as a carrier protein presenting an average of three haptens per KLH molecule for an experimental vaccine administered to white sturgeon (*Acipenser transmontanus*) (31). Additionally, a KLH-dinitrophenol (DNP) hapten conjugate vaccine induced anti-DNP antibody production in rainbow trout (29). Carrier proteins such as KLH and bovine serum albumin (BSA) aid in antigen uptake by recruiting T-helper cells (37). When used in appropriate species, these large carrier molecules conjugated to a hapten antigen are readily taken up by APCs.

As both endogenous and miniscule molecules, reproductive peptides included in a vaccine would fail to elicit any sort of immune response. However, large foreign molecules such as KLH and BSA are easily identified as non-self, even when conjugated to numerous copies of a small peptide so they are useful for increasing immunogenicity of small or minimally immunogenic antigens (29). This technique has been researched and used more extensively in mammals, and an immunocontraceptive containing haptenated carrier protein is now registered through the United States Environmental Protection Agency (EPA) for multi-year sterilization of several mammalian species (38). Additionally, Sambroni, et al. (13) used phages displaying LH and FSH receptors to vaccinate rainbow trout. These peptide vaccines stimulated antibody production and caused reduced hormone levels and delayed spermiation and vitellogenesis (13).

Vaccines using carrier proteins conjugated to a peptide offer several advantages over those conjugated to a whole molecule. Peptides are often small enough that they can act as a single epitope which promotes production of antibodies that all function similarly by always binding the same region on the antigen. The relatively short amino acid sequence of a peptide helps to achieve a more consistent binding conformation between the peptide and the carrier protein which also supports production of antibodies against the target region of a molecule.

Additionally, custom synthesis of a small peptide is more economical than synthesis of a large peptide which is an important consideration for use in commercial aquaculture.

Most aquaculture vaccine antigens are poorly immunogenic on their own and require an adjuvant to sufficiently excite the immune system (20). Oil-adjuvanted vaccines may prolong the duration of antigen presentation due to a depot effect (20) wherein clearance of the antigen from the injection site is slowed (37). Antigen uptake is also facilitated by oil-adjuvanted vaccines through varying degrees of tissue damage at the vaccine injection site (39). Water-in-oil emulsions are the most a common form of oil-adjuvanted vaccines. An aqueous phase and an oil phase containing a surfactant are combined to create a stable, viscous suspension (37). Mineral oil is a commonly used adjuvant in many animal vaccines due to its immunostimulatory properties, ability to create a depot, and cost effectiveness. However, many mineral oil-based vaccines produce a visible lesion at the injection site that can remain for an extended time (20) so they are not used in humans or companion animals (37).

Other adjuvants, also referred to as immunostimulants, help to increase immune response but often do so without the potentially harmful side effects of mineral oil. Imunostimulants, administered either alone or incorporated in a vaccine, are substances that stimulate non-specific immune responses and often amplify specific responses (29). This is often accomplished via enhanced antigen presentation or stability, immunomodulation (37), or by eliciting easily recognized ‘danger’ signals that stimulate immune system activation (39).

Toll-like receptors (TLRs) and pattern recognition receptors (PRRs) bind a wide range of molecules exhibiting conserved pathogen-associated molecular patterns (PAMPs) that commonly signal danger from cell damage or invading pathogens (39). PAMPs often consist of repeated moieties such as glucose or fatty acids, a characteristic which is mimicked in other compounds that have proven to strongly stimulate the immune system when included in

vaccines or feed (39). Elevated white blood cell counts, indicative of immune stimulation, were observed in rainbow trout treated with vaccines that included muramyl-dipeptide (MDP) and beta-glucan (27, 39), both of which display repeated molecular patterns.

Beta-glucans, derived from yeast and fungi, are long polysaccharide chains that are common among many pathogens and environmental microflora so they readily stimulate non-specific immune mechanisms (29). Yeast beta-glucans are specifically recognized by a receptor on macrophages in Atlantic salmon (40). Activation of many components of the immune system is elevated in fish vaccinated with yeast beta-glucan and increased protection against several bacterial pathogens has been observed as a result (39). Intraperitoneally-administered fungal glucans have been used to increase resistance to bacterial infection in various fish species (40). Inclusion of yeast beta-glucan in fish feed also enhances growth and complement activation (39). Despite a great deal of variation among individuals, Aakre, et al. (32) showed that β-1,3-M-glucan increased mean antibody production in Atlantic salmon.

Peptidoglycan, a polymer of sugars and amino acids, provides structure as a conserved element of bacterial cell walls (40, 41). Muramyl dipeptide (MDP) is an important subunit of peptidoglycan (40) and has been shown to function as an immunostimulant and immunomodulator in fish vaccines (37, 41). MDP stimulates granulocyte activity, improves resistance to infection, increases antigen uptake (41), and generically stimulates non-specific defense (40). Increased resistance to *A. salmonicida* infection was conferred by inclusion of MDP in vaccines administered to rainbow trout and coho salmon (40).

Measurement of antibody response

Vallejo, et al. (36) assert that “Despite vigorous serum antibody production associated with the usage of these vaccines, ascertaining immune specificity has been difficult and the

demonstration of protective immunity has been unsuccessful in most cases…" Part of the difficulty in determining specificity of the immune response induced stems from challenges in development of assays that detect a specific antibody response with sufficient sensitivity.

Fish sera are notoriously difficult to work with due to high background levels and considerable non-specific binding of serum antibodies (29, 42). Kim, et al. (42) observed that non-specific binding of serum antibodies to blocking agents accounted for the majority of background noise on enzyme linked immunosorbent assay (ELISA) plates. Despite the lack of antigen in ELISA plate wells, a large amount of IgM was found to bind and remain, even after multiple wash steps (42). Pre-adsorption of the fish sera with 5% skim milk was shown to decrease the non-specific adsorption on the plate to reduce background noise without interfering with specific antibody detection (42).

REFERENCES

1. Liu Y, JO Olaussen, A Skonhoft. Wild and farmed salmon in Norway – A review. Marine Policy 2011; 35:413-418.
2. European Commission. Directorate-General for Maritime Affairs and Fisheries. *Fisheries and Aquaculture in Europe* [Salmon (*Salmo salar*)]. Brussels, 2012; 58.
3. Reid JE, G Chaput. Spawning history influence on fecundity, egg size, and egg survival of Atlantic salmon (*Salmo salar*) from the Miramichi River, New Brunswick, Canada. ICES Journal of Marine Science 2012; 69(9):1678-1685.
4. Food and Agriculture Organization of the United Nations, Fisheries and Aquaculture Department. *The State of World Fisheries and Aquaculture 2012*. Rome, 2012.
5. Sommerset I, B Krossøy, E Biering, and P Frost. Vaccines for fish in aquaculture. Expert Review of Vaccines 2005; 4(1):89-101.
6. McGinnity P, et al. Fitness reduction and potential extinction of wild populations of Atlantic salmon, *Salmo salar*, as a result of interactions with escaped farmed salmon. Proceedings of the Royal Society of London 2003; 270:2443-2450.
7. Reed DH and R Frankham. Correlation between fitness and genetic diversity. Conservation Biology 2003; 17(1):230-237.
8. Fleming IA. Reproductive strategies of Atlantic salmon: ecology and evolution. Reviews in Fish Biology and Fisheries 1996; 6:379-416.
9. Aksnes A, B Gjerde, and SO Roald. Biological, chemical and organoleptic changes during maturation of farmed Atlantic salmon, *Salmo salar*. Aquaculture 1986; 53:7-20.
10. Melamed P and N Sherwood. Molecular Aspects of Fish & Marine Biology, Volume 4: Hormones and Their Receptors in Fish Reproduction. Singapore: World Scientific Publishing Co. Pte. Ltd., 2005.

11. Trudeau VL. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*. Reviews of Reproduction 1997; 2:55-68.
12. Levavi-Sivan B, J Bogerd, EL Mañanós, A Gómez, JJ Lareyre. Perspectives on fish gonadotropins and their receptors. General and Comparative Endocrinology 2010; 165:412-437.
13. Sambroni E, L Abdennebi-Najar, J-J Remy, F Le Gac. Delayed sexual maturation through gonadotropin receptor vaccination in the rainbow trout *Onchorynchus mykiss*. General and Comparative Endocrinology 2009; 164:107-116.
14. Campbell B, et al. Previtellogenic oocyte growth in salmon: Relationships among body growth, plasma insulin-like growth factor-1, estradiol-17beta, follicle-stimulating hormone and expression of ovarian genes for insulin-like growth factors, steroidogenic-acute regulatory protein and receptors for gonadotropins, growth hormone, and somatolactin. Biology of Reproduction 2006; 75:34-44.
15. Swanson P, JT Dickey, and B Campbell. Biochemistry and physiology of fish gonadotropins. Fish Physiology and Biochemistry 2003; 28:53-59.
16. Zapater C, et al. Piscine follicle-stimulating hormone triggers progestin production in gilthead seabream primary ovarian follicles. Biology of Reproduction 2012; 87(5):1-13.
17. Van Eenennaam AL, WM Muir. Transgenic salmon: A final leap to the grocery shelf? Nature Biotechnology 2011; 29(8):706-710.
18. AquAdvantage® Fish, AquaBounty Technologies. 2012. Web. [4 Sept 2012].
19. Asa CS, IJ Porton. Wildlife Contraception: Issues, Methods, and Applications. Baltimore: Johns Hopkins University Press, 2005.
20. Evensen Ø. Developments in fish vaccinology with focus on delivery methodologies, adjuvants and formulations. Options Méditerranéennes, Series A 2009; 86:177-186.

21. Secombes CJ. Fish immunity: the potential impact on vaccine development and performance. Aquaculture Research 2011; 42:90-92.

22. Flajnik MF. The last flag unfurled? A new immunoglobulin isotype in fish expressed in early development. Nature Immunology 2005; 6(3):229-230

23. Morrison RN, B Nowak. The antibody response of teleost fish. Seminars in Avian and Exotic Pet Medicine 2002; 11(1):46-54.

24. Watts M, BL Munday, CM Burke. Immune responses of teleost fish. Australian Veterinary Journal 2001; 79(8): 570-574.

25. Cuesta A, MA Esteban, J Meseguer. Cloning, distribution and up-regulation of the teleost fish MHC class II alpha suggests a role for granulocytes as antigen-presenting cells. Molecular Immunology 2006; 43:1275-1285.

26. Press CMcL, Ø Evensen. The morphology of the immune system in teleost fishes. Fish and Shellfish Immunology 1999; 9:309-318.

27. Ackerman PA, GK Iwama, JC Thornton. Physiological and immunological effects of adjuvanted *Aeromonas salmonicida* vaccines on juvenile rainbow trout. Journal of Aquatic Animal Health 2000; 12:157-164.

28. Romstad AB, LJ Reitan, P Midtlyng, K Gravningen, Ø Evensen. Development of and antibody ELISA for potency testing of furunculosis (*Aeromonas salmonicida* subsp *salmonicida*) vaccines in Atlantic salmon (*Salmo salar* L). Biologicals 2012; 40:67-71.

29. Anderson DP. Immunostimulants, adjuvants, and vaccine carriers in fish: Applications to aquaculture. Annual Review of Fish Diseases 1992; 281-307.

30. Toranzo AE, JL Romalde, B Magariños, JL Barja. Present and future of aquaculture vaccines against fish bacterial diseases. Options Méditerranéennes, Series A 2009; 86:155-176.

31. Drennan JD, SE LaPatra, CM Swan, S Ireland, KD Cain. Characterization of serum and mucosal antibody responses in white sturgeon (*Acipenser transmonatanus* Richardson) following immunization with WSIV and a protein hapten antigen. Fish & Shellfish Immunology 2007; 23:657-669.

32. Aakre R, HI Wergeland, PM Aasjord, and C Endresen. Enhanced antibody response in Atlantic salmon (*Salmo salar* L.) to *Aeromonas salmonicida* cell wall antigens using a bacterin containing β-1,3-M-Glucan as adjuvant. Fish and Shellfish Immunology 1994; 4:47-61.

33. Nikl L, LJ Albright, TPT Evelyn. Influence of seven immunostimulants on the immune response of coho salmon to *Aeromonas salmonicida*. Diseases of Aquatic Organisms 1991; 12:7-12.

34. Marsden MJ, et al. A live (Δ*aroA*) *Aeromonas salmonicida* vaccine for furunculosis preferentially stimulates T-cell responses relative to B-cell responses in rainbow trout (*Oncorhynchus mykiss*). Infection and Immunity 1996; 64(9):3863-3869.

35. Kato G, H Kondo, T Aoki, and I Hirono. BCG vaccine confers adaptive immunity against *Mycobacterium* sp. Infection in fish. Developmental and Comparative Immunology 2010; 34:133-140.

36. Vallejo, et al. Antigen processing and presentation in teleost immune responses. Annual Review of Fish Diseases 1992; 73-89.

37. Spickler AR and JA Roth. Adjuvants in veterinary vaccines: modes of action and adverse effects. Journal of Veterinary Internal Medicine 2003; 17:273-281.

38. Gionfriddo JP, AJ DeNicola, LA Miller, KA Fagerstone. Health effects of GnRH immunocontraception of wild white-tailed deer in New Jersey. Wildlife Society Bulletin 2011; 35(3):149-160.

39. Dalmo RA, J Bøgwald. β-glucans as conductors of immune symphonies. Fish & Shellfish Immunology 2008; 25:384-396.

40. Robertsen B. Modulation of the non-specific defense of fish by structurally conserved microbial polymers. Fish & Shellfish Immunology 1999; 9:269-290.

41. Kodama H, et al. Activation of rainbow trout (*Oncorhynchus mykiss*) phagocytes by muramyl dipeptide. Developmental and Comparative Immunology 1993; 17:129-140.

42. Kim WS, T Nishizawa, M Yoshimizu. Non-specific adsorption of fish immunoglobulin M (IgM) to blocking reagents on ELISA plate wells. Diseases of Aquatic Organisms 2007; 78:55-59.

MATERIALS AND METHODS

Fish: Atlantic salmon (n = 503; Bolaks breed), hatched at the Nofima Marin Sunndalsøra Research Station (Sunndalsøra, Norway), were used in the study. At the time the study was started, the mean fish weight was approximately 50 g, smoltification was underway but incomplete, and none of the fish had received any vaccinations. A three week quarantine period allowed the fish to acclimate to the housing conditions and provided sufficient time for completion of smoltification. The salmon were anesthetized with Finquel®/MS-222® (tricaine methanesulfonate) according to the manufacturer's recommended dosages, and a passive integrated transponder (PIT) tag was inserted in the ventral area of the abdominal cavity (fatty tissue posterior to the pyloric ceca) for unique identification.

Tanks/Housing: Vaccine trials were conducted by Nofima Marin in Sunndalsøra, Norway in eight 250 L indoor tanks. The salmon were sustained on a diet of 3 mm Nutra Olympic feed (Skretting; Stavanger, Norway) provided by an automatic feeding system. Each salmon was randomly assigned, using a random number generator, to one of nine treatment groups (55-56 fish per treatment group) and randomly assigned to one of the eight tanks (62-63 fish per tank). Water temperature was maintained between 8 and 9°C for the duration of the study.

Vaccines: Eight different immunocontraceptive vaccine formulations and a control vaccine were manufactured in an ISO 6 cleanroom at the National Wildlife Research Center (Fort Collins, Colorado, USA). All vaccines (except the control) contained peptide conjugated to a carrier protein, immunostimulant, phosphate buffered saline (PBS), and Seppic ISA 761 VG (Air Liquide; Puteaux, France) adjuvant. The aqueous and oil phases were combined at a 3:7 ratio (v/v) to produce a water-in-oil emulsion. The final concentration of each component was the same in all vaccines. Syringes were filled with individual doses of 200 µL. Each vaccine

formulation contained the same peptide, one of two carrier proteins, and one of four immunostimulants, as designated in Table 1.

A peptide representing the amino acid sequence of a small portion of the LH molecule was synthesized for use in the vaccines. The amino acid sequence was selected based on several key characteristics important for conjugation with the cross-linker used in the present study.

Sampling and Vaccination: Four weeks after PIT tagging, salmon were anesthetized, as described above, and a 21 gauge needle was used to draw 0.5 to 1.0 mL of blood from the caudal vein. After the blood draw, a 0.2 mL dose of contraceptive vaccine was injected into the intraperitoneal cavity of each fish. Additional blood samples were taken 8 and 12 weeks post-vaccination. The weight and length of each fish were also measured pre-vaccination and 8 and 12 weeks post-vaccination. Upon study termination, the fish were euthanized with an overdose of Finquel®.

ELISAs: Three enzyme-linked immunosorbent assays (ELISAs) were developed to semi-quantitatively measure the presence of antibodies specific to the vaccines; each assay was optimized to detect antibodies against a single antigen. A plate washer was used for all wash steps in each ELISA protocol.

Due to the small molecular size of the LH peptide, a conjugate was prepared for use as antigen in the anti-LH assay. LH was conjugated to ovalbumin using a different cross-linker than the one used in the vaccines to prevent detection of any antibodies to the vaccine cross-linker. The resulting conjugate was purified via column chromatography and the concentration was determined using a Bradford assay.

Generic ELISA protocol: Salmon sera were diluted 1:10 in 'milk block,' 5% (w/v) powdered skim milk in 0.01 M phosphate buffered saline with 0.05% Tween 20 (PBST) (Sigma

Aldrich; St. Louis, Missouri), and stored at 4°C overnight for pre-adsorbtion of serum antibodies that non-specifically bind the milk proteins. 96-well high-bind polystyrene plates (Santa Cruz Biotech; Santa Cruz, California) were coated with 50 µL of antigen in either 0.05 M carbonate-bicarbonate buffer (Sigma Aldrich; St. Louis, Missouri) or 0.01 M phosphate-buffered saline (PBS) (Sigma Aldrich; St. Louis, Missouri). PBS was only used for coating plates with LH-OVA conjugate in order to maintain a neutral pH and prevent hydrolysis causing dissociation of the conjugate. Plates were stored overnight at 4°C then washed three times with 200 µL of 0.01 M PBS with 0.05% Tween 20 (Amresco; Framingham, Massachusetts) (PBST). Each well was blocked with 200 µL of milk block for 1 hour at 24°C. Plates were washed three times with 200 µL PBST. The pre-adsorbed sera were loaded in 50 µL volumes in duplicate. Plates containing 1:10 sera were incubated for 1 hour at 24°C then washed three times with 200 µL PBST. Monoclonal mouse anti-salmonid Ig antibody conjugated with horseradish peroxidase (HRP) (US Biological; Salem, MA) was diluted 1:1000 in 0.01 M PBS and 50 µL was added to each well. Plates were covered and incubated for 1 hour at 24°C, then washed three times with 200 µL wash buffer. Substrate was prepared by dissolving 1 mg of 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB 2HCl) (Sigma Aldrich; St. Louis, Missouri) per 10 mL of 0.05 M phosphate citrate buffer with 0.014% hydrogen peroxide (Sigma Aldrich; St. Louis, Missouri). Substrate was passed through a 0.45 µm filter to remove particulate and 50 µL was added to each well. Each plate was incubated at 24°C for a fixed time before the reaction was stopped with 50 µL of 2 M sulfuric acid and optical density was measured at a single wavelength of 450 nm. All plates for a given assay were run with the same 'positive' control sample identified during assay development. All plates were also run with six wells of PBS loaded instead of sera as the sample so that background levels of the assay could be determined. Table 2 provides additional detail about the variations on this generic protocol that are specific to each antigen that was evaluated in the present study.

Statistical Analyses: Although there were 503 fish in the trial initially, some of the fish were lost due to complications with the blood draws at each sampling time. As such, the number of samples from each treatment group and each time point decreased as the study progressed for a final count of 418 fish upon study termination, as shown in Table 3.

All analyses of antibody production were done using the optical densities (ODs) derived from the ELISAs. The ODs were accepted for use in statistical analyses if the duplicate ODs had a coefficient of variation (CV) less than 15%. If the CV exceeded 15% and one or both duplicate sample ODs were over the LOD, the sample was re-run at a later date and the original OD data was excluded from all analyses.

The mean OD of six PBS wells on each plate was subtracted from the individual OD values for all other samples on that plate to obtain corrected ODs. The mean corrected OD for duplicate wells of each sample was calculated and divided by the mean corrected OD of duplicate positive control wells on each plate to get an adjusted OD for each sample. The adjusted ODs accounted for both assay background levels and inter-plate variation so that data from different plates could be better compared. The mean adjusted pre-vaccination OD was also calculated for each treatment group and subtracted from the adjusted post-vaccination ODs in the respective treatment group (1, 2, 3) to generate subtracted ODs.

A Kruskal-Willis one-way analysis of variance was used to compare the median difference in pre- and post-vaccination adjusted ODs among all treatment groups (4). In the event of a significant difference, pairwise comparisons of means were made using Tukey's honestly significant difference (HSD) method (among treatments at a given time and within a treatment over time). The data were not treated as repeated measures because treatment was applied between the first and second sampling times, baseline antibody levels were otherwise accounted for, our interest was primarily in comparing mean antibody levels among treatments,

and sample sizes were unequal among treatments at a given time and within treatments over time due to complications with sampling.

Vaccine response rates were evaluated using the subtracted ODs. Positive/negative thresholds for each ELISA were set to maximize sensitivity and specificity.

$$Sensitivity = True\ Positive\ Rate = \frac{positives\ correctly\ identified}{total\ positives}$$

$$Specificity = 1 - \ False\ Positive\ Rate = 1 - \frac{negatives\ incorrectly\ classified}{total\ negatives}$$

Receiver operator characteristic (ROC) graphs were generated (5) for each ELISA using threshold values ranging from the minimum to maximum subtracted OD value for the assay in increments of 0.001. The OD corresponding to the maximum sum of sensitivity and specificity was set as the positive/negative threshold for each assay. Subtracted ODs greater than or equal to the threshold were classified as positive and the rest negative. The percentage of samples classified as positive within a treatment group was calculated using only post-vaccination samples for treatment groups vaccinated against the antigen of interest, and using pre- and post-vaccination samples for the other groups. The response rates were then calculated as the number of samples classified as positive (at any time point) divided by the total number of fish that were vaccinated against a particular antigen.

Mean fish weights and lengths were compared among treatment groups using one-way ANOVA.

REFERENCES

1. Townsend MB, et al. Humoral immunity to smallpox vaccines and monkeypox virus challenge: proteomic assessment and clinical correlations. Journal of Virology 2013; 87:900-911.
2. Wang F, et al. Phase I trial of a MART-1 peptide vaccine with Incomplete Freund's Adjuvant for resected high-risk melanoma. Clinical Cancer Research 1999; 5:2756-2765.
3. Wee JLK, et al. Pulmonary delivery of ISCOMATRIX influenza vaccine induces both systemic and mucosal immunity with antigen dose sparing, Mucosal Immunology 2008; 1:489-496.
4. Baldi B and DS Moore. *The Practice of Statistics in the Life Sciences*, 2nd edition. New York, W.H. Freeman and Company, 2012.
5. Fawcett T. An introduction to ROC analysis, Pattern Recognition Letters 2006; 27:861-874.

RESULTS

Prior to vaccination, there was no significant difference in median baseline antibody levels among treatment groups for BSA or KLH ($p = 0.707, 0.996$ respectively). There was a difference in median baseline antibody levels among the treatment groups for the LH assay ($p = 0.023$). However, upon subsequent analyses of means, there was no significant difference in mean baseline antibody levels among treatment groups for any of the three antigens/assays evaluated ($p \cong 1$ for all three assays).

No significant difference in median antibody responses (MARs) to BSA was detected among treatment groups 8 weeks post-vaccination ($p = 0.171$). However, a statistically significant difference in MARs was detected by 12 weeks post-vaccination ($p < 0.0001$). The mean response of group 5 was significantly different from the mean of other groups 12 weeks post-vaccination and was significantly greater than it was 8 weeks post-vaccination ($p = 0.0001$) (Figure 4, Table 4).

A statistically significant difference in MARs to KLH was detected among treatment groups at 8 and 12 weeks post-vaccination ($p < 0.0001$). At 8 and 12 weeks post-vaccination, treatment groups 2, 4, 6, and 8 (vaccinated against KLH) had mean responses that were significantly different than non-vaccinates receiving treatments 0, 1, 3, 5, and 7 ($p < 0.0001$). Additionally, the mean anti-KLH response of treatment group 6 was significantly different from groups 4 and 8 after 8 weeks ($p < 0.0001$) and was significantly different from treatments 2, 4, and 8 by 12 weeks post-vaccination ($p < 0.0001$) (Figure 5, Table 5).

No significant difference was detected in MARs to the LH peptide 8 weeks post-vaccination ($p = 0.210$). However, a statistically significant difference in MARs among treatments was detected by 12 weeks post-vaccination ($p < 0.0001$). The mean anti-peptide

response produced by treatment group 6 was significantly different than that of groups 0, 1, 2, and 4 (p = 0.001) (Figure 6, Table 6). The mean adjusted OD for treatment 6 was significantly higher at 12 weeks post-vaccination compared to 8 weeks (p = 0.002). However, the increase in OD from pre-vaccination to 8 weeks post-vaccination was not significant (p = 0.184).

There was not a significant difference in mean fish length between treatment groups at any time point (p = 0.214, 0.178, 0.260 for pre-vaccination, 8 and 12 weeks post-vaccination, respectively). Similarly, no significant difference in mean fish weight was detected between treatment groups at any time (p = 0.158, 0.154, 0.080 for pre-vaccination, 8 and 12 weeks post-vaccination, respectively).

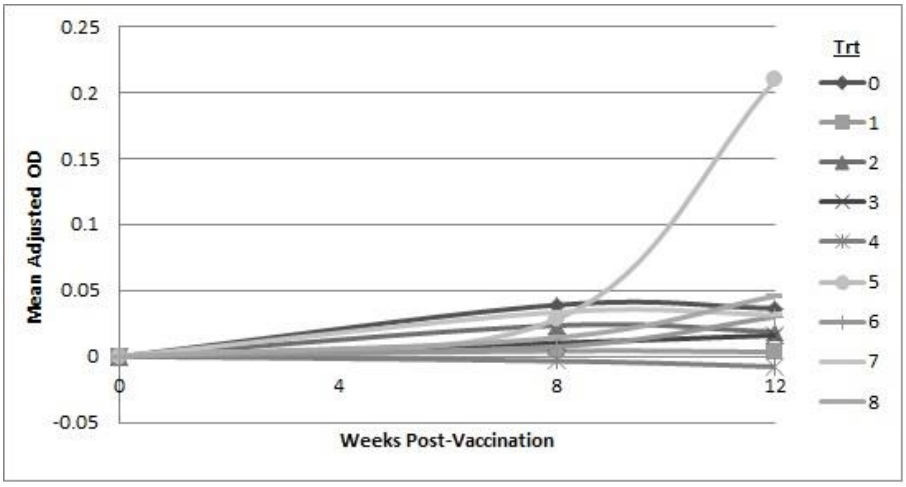

Figure 4: Mean anti-BSA antibody production over time; treatments 1, 3, 5, and 7 contain BSA.

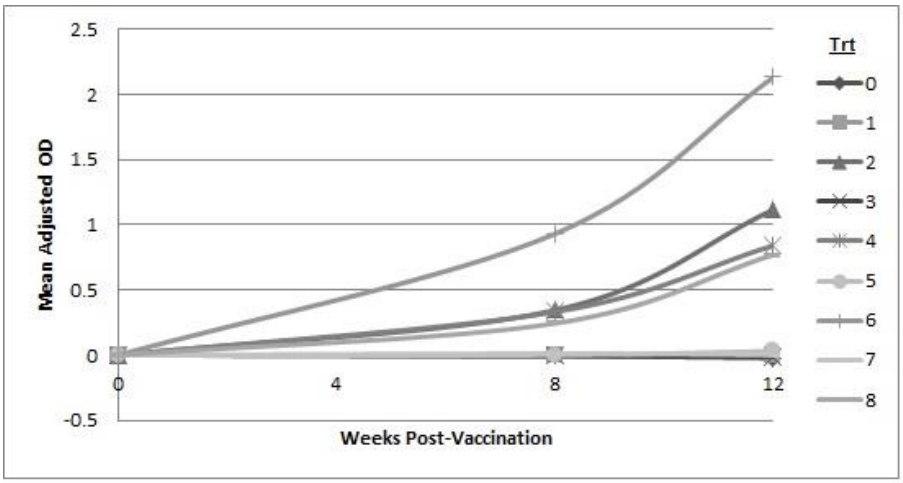

Figure 5: Mean anti-KLH antibody production over time; treatments 2, 4, 6, and 8 contain KLH.

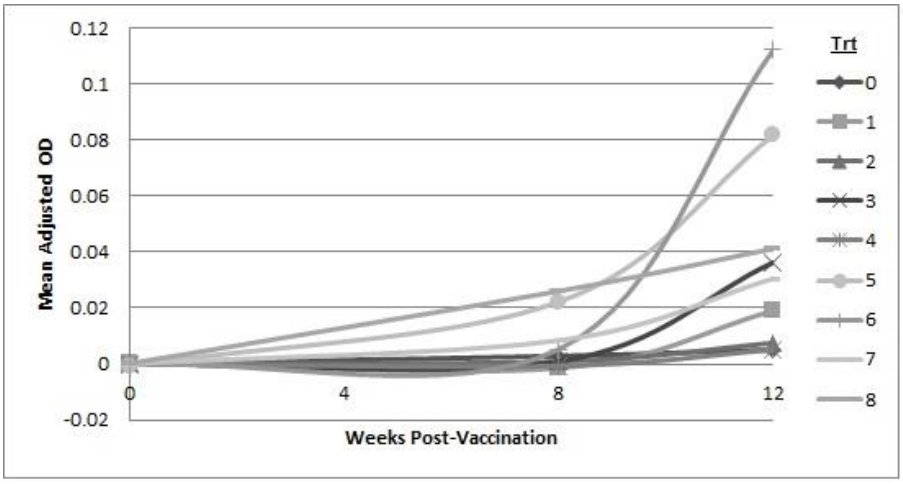

Figure 6: Mean anti-LH antibody production over time; treatments 1 through 8 contain LH peptide.

The ROC curves generated for each assay are shown in Figure 7. Area under the curve (AUC) corresponds to the probability of a randomly selected vaccinate being classified as positive compared to a randomly selected non-vaccinate receiving the same classification using the same assay with a fixed threshold. The AUC for KLH was calculated to be 0.8930, suggesting that the assay does an excellent job discriminating between positives and negatives. The AUCs for the BSA and LH assays were much lower at 0.5348 and 0.5648, respectively.

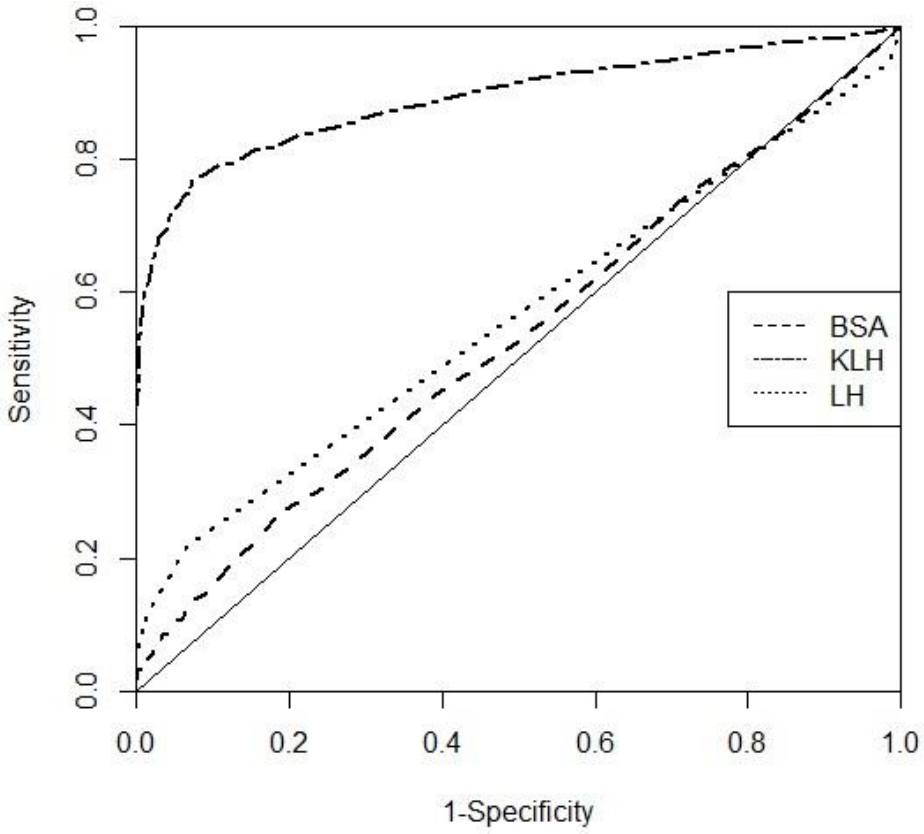

Figure 7: ROC curves; the solid line from (0,0) to (1,1) represents an equal 50% chance of positive classification of a positive or negative sample; the three assays evaluated offer improved criteria for positive/negative classification.

The maximum sums of sensitivity and specificity for each assay and their corresponding thresholds, set as the positive/negative thresholds, are summarized in Table 7.

Approximately 96% (361 of 376) samples from fish vaccinated against KLH, 76% (282 of 371) of samples from fish vaccinated against BSA, and 36% (303 of 849) of samples from fish vaccinated against LH were classified as positive (Figure 8).

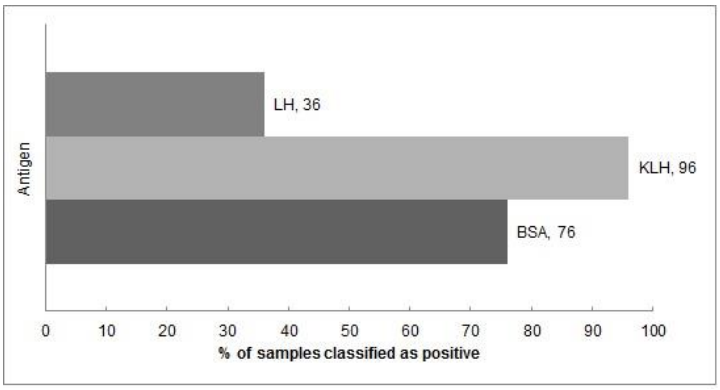

Figure 8: Percentage of salmon serum samples classified as positive for specific antibodies

The response rates were highest 12 weeks post-vaccination for all antigens (Figure 9).

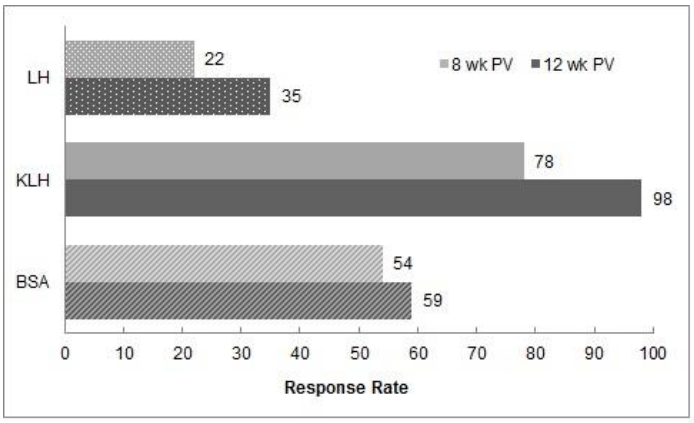

Figure 9: Response rates for BSA, KLH, and LH antigens at 8 and 12 weeks post-vaccination

The number of samples classified as positive are shown further broken down by treatment group to compare performance of the formulations (Figures 10, 11, 12). High ELISA background levels are shown by the percentage of samples in treatment 0 that are classified as positive for anti-BSA and anti-LH antibodies, despite the fish never receiving vaccines against these antigens. A dashed line extends horizontally from the top of the treatment 0 bar to show the misclassification rate associated with the assay at its positive/negative threshold.

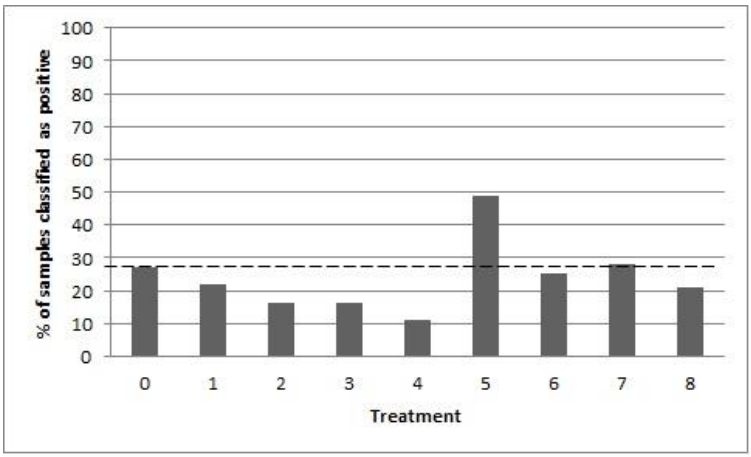

Figure 10: Percentage of samples classified as positive for anti-BSA antibodies; Treatments 1, 3, 5, and 7 contained BSA.

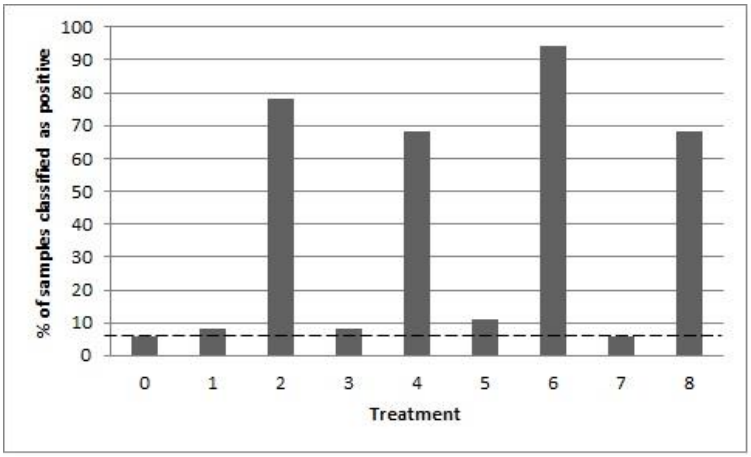

Figure 11: Percentage of samples classified as positive for anti-KLH antibodies; Treatments 2, 4, 6, and 8 contained KLH.

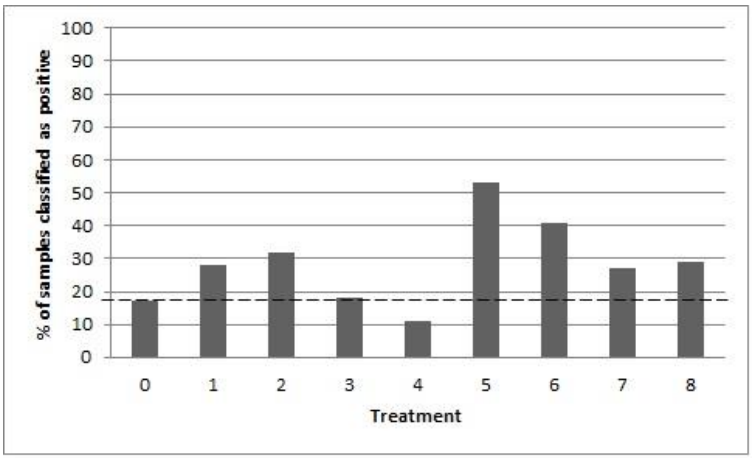

Figure 12: Percentage of samples classified as positive for anti-LH antibodies; Treatments 1 through 8 contained LH.

Tables

Table 1: Comparison of Atlantic salmon contraceptive vaccine formulations

Treatment	Peptide	Carrier Protein	Immunostimulant
0 (control)	None	None	None
1	LH	BSA	A
2	LH	KLH	A
3	LH	BSA	B
4	LH	KLH	B
5	LH	BSA	C
6	LH	KLH	C
7	LH	BSA	D
8	LH	KLH	D

Table 2: Antigen-specific modifications to the generic ELISA protocol provided for detection of antibodies to BSA, KLH, and LH in Atlantic salmon

Antigen	Antigen Concentration (ng/µL)	Coating Buffer	Substrate Development (minutes)
BSA	10	Carbonate-bicarbonate	15
KLH	5	Carbonate-bicarbonate	11
LH conjugate	3.74	PBS	11

Table 3: Number of salmon in each treatment group sampled at each time point in the study

Treatment Group	Pre-vaccination	8 weeks post-vacc.	12 weeks post-vacc.
0	56	51	48
1	56	49	46
2	55	51	49
3	56	51	46
4	56	51	43
5	56	43	42
6	56	47	43
7	56	51	49
8	56	50	48
TOTAL	503	452	418

Table 4: Mean anti-BSA antibody levels and standard deviation (StDev) about the mean by treatment, over time

Treatment	Pre-vacc.		8 wk post-vacc.		12 wk post-vacc.	
	Mean	StDev	Mean	StDev	Mean	StDev
0	0.0000	0.0386	0.0391	0.1211	0.0365	0.1127
1	0.0000	0.0539	0.0042	0.0555	0.0034	0.0810
2	0.0000	0.0618	0.0235	0.0732	0.0188	0.0849
3	0.0000	0.0242	0.0103	0.0358	0.0160	0.0660
4	0.0000	0.0412	-0.0034	0.0394	-0.0077	0.0436
5	0.0000	0.0462	0.0283	0.0716	0.2094	0.3280
6	0.0000	0.0533	0.0075	0.0581	0.0300	0.1104
7	0.0000	0.0816	0.0336	0.1529	0.0317	0.2000
8	0.0000	0.0473	0.0104	0.0539	0.0458	0.2244

Table 5: Mean anti-KLH antibody levels and standard deviations by treatment, over time

Treatment	Pre-vacc.		8 wk post-vacc.		12 wk post-vacc.	
	Mean	StDev	Mean	StDev	Mean	StDev
0	0.0000	0.0640	-0.0069	0.0517	-0.0212	0.0950
1	0.0000	0.0595	-0.0062	0.0707	-0.0008	0.0934
2	0.0000	0.0530	0.3447	0.4432	1.1161	0.8999
3	0.0000	0.0590	-0.0042	0.0665	-0.0096	0.0590
4	0.0000	0.0600	0.3325	0.4565	0.8411	0.7998
5	0.0001	0.0654	0.0021	0.0730	0.0302	0.0987
6	0.0000	0.0705	0.8311	0.7361	2.1330	0.9224
7	0.0000	0.0825	0.0120	0.1018	0.0027	0.1259
8	0.0000	0.0593	0.2444	0.3244	0.7706	0.7827

Table 6: Mean anti-LH antibody levels and standard deviations by treatment, over time

Treatment	Pre-vacc.		8 wk post-vacc.		12 wk post-vacc.	
	Mean	StDev	Mean	StDev	Mean	StDev
0	0.0000	0.0099	0.0029	0.0196	0.0051	0.0209
1	0.0001	0.0044	-0.0015	0.0112	0.0190	0.0746
2	0.0001	0.0104	0.0009	0.0105	0.0075	0.0346
3	-0.0001	0.0059	0.0009	0.0140	0.0363	0.1374
4	0.0000	0.0059	-0.0007	0.0083	0.0051	0.0195
5	-0.0006	0.0117	0.0222	0.0742	0.0818	0.2054
6	0.0000	0.0094	0.0052	0.0269	0.1124	0.2301

7	0.0000	0.0074	0.0084	0.0429	0.0305	0.0903
8	0.0001	0.0077	0.0261	0.1555	0.0421	0.2067

Table 7: Summary of values for maximum sum of sensitivity and specificity, corresponding positive/negative threshold, and individual sensitivity and specificity

Antigen	Max Sum	Threshold (OD)	Sensitivity	Specificity
BSA	1.0894	0.0633974	0.2776	0.8118
KLH	1.6951	0.168481013	0.7686	0.9265
LH	1.1654	0.012	0.2603	0.9051

DISCUSSION

The results of the present study lay the foundation for further research on Atlantic salmon immunocontraceptive schemes and indicate that a hapten-protein conjugate is functional as vaccine antigen in this species. This study also reaffirms the value of selecting an appropriate immunostimulant to enhance the non-specific immune response.

Response to the LH peptide was low in both magnitude of the mean ODs and response rate. KLH was the strongest immunogen with the majority of fish responding positively to the treatments containing KLH.

Although KLH comes from mollusks which, like Atlantic salmon, belong to a marine phylum, it is a much larger molecule than BSA. The larger molecular size of KLH may have contributed to its superior immunogenicity as a carrier protein in Atlantic salmon.

Treatments 5 and 6, which contained the same immunostimulant, had the strongest immune response to their respective carrier proteins and to the LH peptide. This indicates that this particular immunostimulant may be able to enhance the specific immune response to the vaccine antigens to a much greater extent than any of the other three immunostimulants evaluated in the present study. The non-specific immune response to the vaccines was not evaluated but it is likely that the other immunostimulants excite different immune components and, thus, encourage the immune system to focus its efforts differently.

The anti-BSA and anti-LH ELISAs provided predictive capability greater than random chance and allowed for crude statistical comparison of the various treatments. However, a larger area under the curve, corresponding to better predictive ability of the assay, may prove helpful for comparing treatments in future research. Further optimization and refinement of the assays will hopefully help to improve both sensitivity and specificity.

Drennan, et al. (1) observed minimal and inconsistent antibody responses in white sturgeon (*Acipenser transmontanus* R.) vaccinated against a protein-hapten antigen. However, after administering a boost dose of the vaccine, a 16-fold titer increase was observed and the response rate jumped to 100%. In most species, a boost dose administered at the appropriate time has been observed to have this enhancing effect on antibody production and resulting protection (2). Despite the known benefits of boosting, it is desirable to achieve protection through a single vaccination due to the cost of vaccines and manpower required to administer those vaccines to fish. Additionally, the stress of being handled and vaccinated takes a toll on fish health so minimizing the number of different vaccines necessary is advantageous (3). However, a multiple vaccination scheme should not be ruled out until cost-benefit analyses are conducted.

In maximizing the sum of sensitivity and specificity, a large trade-off between the two was observed, particularly for BSA and LH which had low AUCs. This is not unexpected due to the nature of the relationship between sensitivity and specificity, but it does lead to low true positive rates for these antigens while the true negative rates are very high. Further optimization of these assays may be necessary in future studies in order provide improved differentiation between true and false positives.

Since response rates were greatest at 12 weeks and the study was terminated at that time, it is not possible to ascertain whether peak antibody production was reached during the span of the study. It will be necessary to conduct future trials in a manner that allows for determination of time post-vaccination when peak antibody production occurs for each antigen.

Within treatment groups, there appears to be an association between having a large mean OD and having a high response rate. Interestingly, the response rate for KLH was significantly higher than the response rate for BSA at both 8 and 12 weeks post-vaccination ($p <$

0.0001). The treatments with the best response rates and highest ODs for the carrier proteins (BSA and KLH) had the best response to the LH peptide.

Weight and length were tracked primarily to ensure that vaccination did not adversely affect growth of the fish. None of the fish developed gross lesions at the vaccine injection site and none of the fish deaths that occurred during the study can be definitively attributed to vaccination; most of the fish that died appeared to have suffered nerve damage resulting from blood sampling. These observations do not provide any reason to suspect that the adjuvant used in the study caused any severe adverse effects. The Seppic ISA 761 VG adjuvant appeared to be well accepted by the salmon but additional studies will be necessary to further evaluate any side effects not externally visible.

As indicated by large standard deviations, there was a great deal of variation in antibody response among fish. Standard deviation was higher among vaccinates than among non-vaccinates.

The results of this study indicate that it is possible to stimulate the immune system of Atlantic salmon to produce antibodies against a small endogenous peptide conjugated to a carrier protein. It was determined that KLH is a stronger immunogen and, thus, may be a better carrier protein than BSA for Atlantic salmon. Response rates and antibody levels were highest at 12 weeks post-vaccination suggesting that the water-in-oil vaccine provides a potentially extended release of antigen. Further research will be necessary to improve the formulation of the contraceptive vaccine candidates and determine the physiological significance of the resulting antibodies.

REFERENCES

1. Drennan JD, SE LaPatra, CM Swan, S Ireland, and KD Cain. Characterization of serum and mucosal antibody responses in white sturgeon (*Acipenser transmontanus* Richardson) following immunization with WSIV and a protein hapten antigen, Fish & Shellfish Immunology 2007; 23:657-669.
2. Zepp F. Principles of vaccine design – Lessons from nature. Vaccine 2010; 28S:C14-C24.
3. Anderson DP. Immunostimulants, adjuvants, and vaccine carriers in fish: Applications to aquaculture. Annual Review of Fish Diseases 1992; 281-307.

RECOMMENDATIONS FOR FUTURE RESEARCH

Proof of concept for hapten-protein vaccination of Atlantic salmon has been achieved. Now, additional research is necessary to address sub-optimal response rates, assess vaccine efficacy for immunocontraception, and evaluate the utility of a contraceptive vaccine for Atlantic salmon aquaculture. The suggestions below are provided as general guidelines for some of the next steps towards development of a contraceptive vaccine for farmed Atlantic salmon.

- Refine conjugation method to promote full saturation of the carrier protein with peptide
    - Try different cross-linkers
    - Compare different reaction conditions
        - Vary molar ratios of carrier protein, cross-linker, and peptide
        - Experiment with reaction times and temperatures
    - Evaluate saturation of carrier protein with peptide using column chromatography or a fluorescently-labeled peptide
    - Determine whether *Concholepas concholepas* hemocyanin (also known as 'blue carrier' or 'blue protein') is effective as a carrier protein for Atlantic salmon
        - Establish how many peptide molecules can be conjugated to this carrier proteins versus others (such as KLH) with the optimal crosslinking technique
- Assess immunogenic potential of peptide
    - Look at tertiary structure of different amino acid sequences
    - Look at immunogenicity of individual amino acids
- Increase conjugate concentration in vaccine
    - Increase antigen concentration to increase dosage with hopes of also increasing immune response

    - Evaluate dose-dependence of antibody response
- Evaluate physiological significance of antibodies against the peptide
    - Just because antibodies are there doesn't mean they're protective
    - What level of antibody is needed to achieve a contraceptive effect
- Conduct further experiments to determine if antibody levels continue to rise after 12 weeks post-vaccination or if antibody production peaks at that time
- Look into other immunostimulants that have properties similar to immunostimulant 3 used in the present study
    - Investigate potential for enhancing immune response with different concentrations of stimulatory compounds
    - Weigh importance of stimulating various immune components for creating a specific and lasting antibody response
- Explore alternate methods for presentation of an endogenous peptide in a fish immunocontraceptive
    - Bacterial plasmid, DNA vaccine, etc.
    - Oral or immersion vaccination?
- If sufficient vaccine efficacy achieved in trials, need to experiment with:
    - Determining optimal age at which fish should be vaccinated
        - Must be early enough to prevent initiation of early stages of sexual maturation but must be late enough that the necessary antibodies are still present when they are needed
    - Options for combining this vaccine with others already administered in aquaculture to simplify vaccination
    - Verifying that vaccination does not produce injection site reactions with unacceptable frequency, persistence, and size for meeting food animal quality standards

- Non-target risk assessments to determine potential hazard of vaccinated fish to environment and to natural predators
- Establish safety of human consumption of immunocontracepted fish
    - Guidelines for time of harvest relative to time of vaccination
- Altering production scale to accommodate bulk manufacture of vaccine
- 'Field' studies to evaluate vaccine efficacy in commercial aquaculture settings representative of those in which the vaccine would typically be used
- Consider market for a commercial contraceptive vaccine
    - Support for vaccine within aquaculture industry
        - Cost versus benefit for aquaculture application
    - Consumer acceptance of immunocontracepted fish
- Research applications of immunocontraception in other fish species that are intensively produced in aquaculture
    - Trout are similar to salmon so it might be possible to use the exact same vaccine for both
    - Cod and catfish contraceptive vaccine formulations would at least require different peptides but including these species could significantly expand the market