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ABSTRACT

An experimental contraceptive vaccine was evaluated in Atlantic salmon (*Salmo salar*). A peptide derived from the beta subunit of luteinizing hormone (LH) was conjugated to two different carrier proteins, bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), and formulated with one of four immunostimulants in a water-in-oil emulsion. Specific antibody responses to the peptide and each carrier protein were evaluated. While the antibody response to KLH was stronger than the response to BSA, both carrier proteins stimulated comparable antibody responses to the LH peptide. The immunostimulant proved to be more important for enhancing the LH peptide antibody response than the carrier protein selection; vaccines containing a combination of *Aeromonas salmonicida* and *Vibrio anguillarum* stimulated significantly greater LH peptide antibody production than any of the other three immunostimulants evaluated at 12 weeks post-vaccination. This study provides proof-of-concept for specific antibody production against a hapten-carrier protein antigen in Atlantic salmon and reinforces the importance of vaccine immunostimulant selection.

1. Introduction/background

Aquaculture is the fastest growing food-animal producing sector [1] and provides 47% of fish for human consumption [2]. The United Nations Food and Agriculture Association (FAO) predicts that the global demand for seafood will increase 25% through 2030. As wild fish stocks have diminished and food security continues to be a global issue, sustainable fish farming practices are of increasing

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importance and are being heavily scrutinized by governmental and environmental agencies.

More than 14% of fish produced globally are salmonids [2]. With aquaculture production of salmonids expanding at an average annual rate of nearly 10% [2], there is an increasing risk of farmed salmon inadvertently escaping into bodies of water inhabited by wild fish populations [3–6]. Farmed salmon all originate from a limited set of lineages and have substantially reduced genetic diversity due to founder effects, and other selection-related factors [7]. Asynchrony in release of sperm and eggs, a lack of dominance relationships among males, indiscriminate aggression, and various other behavioral dynamics lead to reduced reproductive success in farmed Atlantic salmon [8,9]. Despite the reduced breeding performance of escaped farmed salmon, they are still able to breed with wild salmon, thereby altering the genetics of those unique wild populations and negatively affecting population survival [7,9–12]. Even sterile, induced-triploid male Atlantic salmon have been observed to exhibit spawning behaviors and induce wild females to spawn in the absence of a wild male [13], potentially affecting seasonal reproductive success.

Immunocontraception could provide a means to prevent escaped salmon from cross-breeding with wild salmon. If administered at the proper stage in a fish's development, a contraceptive vaccine could also potentially prevent fish from sexually maturing [14].

Gonadotropin releasing hormone (GnRH) is an important regulator of sexual maturation and reproduction. Two forms of GnRH are synthesized in the hypothalamus and transported via neurons to the pituitary [15–18]. In the pituitary, GnRH stimulates secretion of two protein hormones, gonadotropin I (GTH-I) and gonadotropin II (GTH-II), also known as follicle stimulating hormone (FSH) and lutenizing hormone (LH) respectively, which are carried to gonads in the bloodstream [15,19]. 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 [20,21]. FSH and LH both consist of an alpha subunit, necessary for structural integrity, and a beta subunit, which is the biologically active portion of the molecule [14].

Vaccination against selected infectious diseases in large-scale aquaculture operations is standard practice, especially for high-value species like Atlantic salmon [22]. Both adaptive and innate immune responses are observed in teleosts [23], though the innate system is thought to be more highly developed [24]. Most aquaculture vaccine antigens are poorly immunogenic on their own and require modification or formulation with an adjuvant to sufficiently excite the immune system [22,25].

Thus, as both endogenous and very small molecules, most reproductive peptides in a vaccine would fail to elicit any sort of immune response. Large foreign molecules such as keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) are easily identified as non-self and are useful for increasing immunogenicity of small or minimally immunogenic antigens [26]. KLH and BSA are readily taken up by antigen presenting cells (APCs) in teleosts [26–28] and aid in antigen uptake by recruiting T-helper cells [29] which make them well suited as carrier proteins.

Oil-based adjuvants may prolong the duration of antigen presentation due to a depot effect [22] wherein clearance of the antigen from the injection site is slowed [29]. Antigen uptake is also facilitated by oil-adjuvanted vaccines through varying degrees of tissue damage at the vaccine injection site [30]. Water-in-oil emulsions are the most common form of oil-adjuvanted vaccines.

Other adjuvants, referred to as immunostimulants, stimulate non-specific immune responses and often amplify specific immune responses, thereby improving potency and efficacy [26,31]. This is usually accomplished via enhanced antigen presentation or stability [32], immunomodulation [29], or by eliciting easily recognized 'danger' signals that stimulate immune system activation [30].

Toll-like receptors and pattern recognition receptors bind a wide range of molecules exhibiting conserved pathogen-associated molecular patterns (PAMPs) [30], such as muramyl dipeptide (MDP) [33,34] and beta-glucans derived from yeast and fungi. These compounds are proven immunostimulants and immunomodulators in fish vaccines [29,34]. Bacteria are often an antigen in aquaculture vaccines and are not conventionally classified as immunostimulants. However, bacteria can act as an adjuvant when mixed with protein antigens by inducing macrophages to stimulate differentiation and proliferation of T cells specific to the protein antigen [35]. In this manuscript, we will refer to bacteria included in vaccine as an 'immunostimulant' due to its intended purpose as adjuvant rather than antigen.

This manuscript reports the findings from a study that was conducted to evaluate antibody production in Atlantic salmon injected with experimental vaccine formulations containing a LH peptide conjugated to a carrier protein. The purposes of this study were to: 1) assess the feasibility of stimulating antibody production against an endogenous reproductive peptide, 2) compare immunogenicity of two different carrier proteins, 3) determine which of four immunostimulants is most efficient at exciting non-specific antibody production, and 4) evaluate the suitability of a particular oil adjuvant for use in Atlantic salmon.

2. Materials and methods

2.1. Fish

Atlantic salmon (n = 503; Bolaks breed), hatched at the Nofima Marin Sunndalsøra Research Station (Sunndalsøra, Norway), were used in this study. Upon study initiation, mean fish weight was approximately 174 g (±1.2 g), smoltification was underway but incomplete, and none of the fish had received any other vaccinations. After three weeks in quarantine, acclimation to the housing conditions and smoltification were complete. The salmon were anesthetized in a 50 mg/L solution of Finquel®/MS-222 (tricaine methanesulfonate) (Scan Aqua AS; Hvam, Norway), 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.

2.2. Rearing conditions

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

2.3. Vaccines

There is wide agreement in the literature that intraperitoneal vaccination with 100–200 μL of oil-adjuvanted vaccine is appropriate for immunocompetent salmonids [27,36,37].

2.3.1. Vaccine compositions

Eight different contraceptive vaccine formulations (treatments 1–8) and a control vaccine (treatment 0) were manufactured in a

cleanroom at the National Wildlife Research Center (Fort Collins, Colorado, USA). All vaccines (except the control) contained 0.222 mg/mL of an 18 amino acid peptide derived from the beta subunit of the salmon LH protein (referred to as LH-β peptide herein) (GL Biochem (Shanghai) Ltd.; Shanghai, China). The peptide was conjugated to a carrier protein, either BSA or KLH (Thermo Fisher Scientific; Waltham, Massachusetts), per the carrier protein manufacturer recommendations. Briefly, for each vaccine formulation, 10 mg of maleimide-activated BSA or KLH was incubated with 8 mg of LH-β peptide in 3 mL of 0.03 M phosphate buffered saline (PBS) at 24 °C for 2 h. The conjugate was incorporated in the vaccines at a final concentration of 0.50 mg/mL [27,38,39]. Each vaccine also contained one of four immunostimulants at a concentration of 0.50 mg/mL or 8.3×10^4 cells/mL, including muramyl dipeptide, beta glucans, killed *Aeromonas salmonicida* and *Vibrio anguillarum* mixture, and killed *Mycobacterium marinum*.

2.3.2. Immunostimulant preparation

Ac-muramyl-Ala-D-Glu-NH_2 muramyl dipeptide (MDP) was sourced from Bachem Americas, Inc. (Torrance, California) and used without modification.

MacroGard® purified beta-1,3/1,6-glucans (Biorigin Europe; Antwerp, Belgium) was treated for 20 min at 80 °C to inactivate bacterial and fungal constituents. Once cooled, it was resuspended at 9 mg/mL in sterile 0.01 M PBS on ice and sonicated for 30 s twice to minimize clumping.

Mycobacterium marinum ATCC® 927™ was cultured on Middlebrook and Cohn 7H10 agar slants (BD Diagnostic Systems; Sparks, Maryland) at 30 °C for 9 days. A single colony of *M. marinum* was then inoculated into 50 mL of pre-warmed Middlebrook 7H9 broth with glycerol and ADC enrichment in a sterile 250 mL culture flask and incubated with continuous shaking for 23 days in the dark. Once confluent and in the late log-phase of growth (3 passages), the cells were heated at 121 °C for 30 min. Approximately 45 mL of the killed cell suspension was centrifuged for 10 min at 2500×*g*. The supernatant was aspirated and the pelleted cells were resuspended in 30 mL of sterile 0.01 PBS. The cells were washed twice more and resuspended in 10 mL sterile 0.01 M PBS. The cell suspension was passed through a 26 gauge needle three times and then centrifuged at 100×*g* for 2 min to recover a single-cell population. The concentration of the single-cell suspension was determined using a Sceptre handheld automated cell counter (EMD Millipore; Billerica, Massachusetts).

Advantigen® vaccine (Microtek International Inc.; Saanichton, British Columbia) was used as the source of *A. salmonicida* and *V. anguillarum* serotypes 01 and 02. The Advantigen was aliquoted in 15 mL conical tubes and centrifuged at 4 °C for 20 min at approximately 3700×*g*. The pelleted cells were transferred to clean tubes, resuspended in sterile 0.01 M PBS, and centrifuged for another 10 min. Cell concentration was again determined using a Sceptre handheld automated cell counter (EMD Millipore).

2.3.3. Vaccine preparation

The vaccine aqueous phases, containing carrier-peptide conjugate and immunostimulant in sterile 0.01 M PBS, were combined with Seppic Montanide™ ISA 761 VG (Air Liquide; Puteaux, France) adjuvant (30:70 v/v) using a stand mixer with an impeller style blade to form a water-in-oil emulsion. The primary emulsion was passed one time through a Microfluidizer® Processor (Microfluidics Corporation; Westwood, Massachusetts). Sterile, rubber latex-free, single-use syringes were filled with individual doses of 0.2 mL. Each vaccine formulation contained the same peptide, one of two carrier proteins, and one of four immunostimulants, as designated in Table 1 as final concentrations.

2.4. Sampling and vaccination

Four weeks after PIT tagging, salmon were anesthetized as described above, and a 21 gauge needle was used to draw 0.5–1.0 mL of blood from the caudal vein. Following the blood draw, 0.2 mL of contraceptive vaccine was injected into the peritoneal cavity of each fish. Additional blood samples were taken at 8 and 12 weeks post-vaccination. All blood samples were collected in microcentrifuge tubes, allowed to clot at room temperature, and then centrifuged at 1000–2000×*g* for 10 min. Sera were transferred to clean microcentrifuge tubes and stored at −80 °C until analyzed. The weight and length of each fish were also measured at each sampling time point. Upon study termination, the fish were euthanized with an overdose of Finquel®.

2.5. Antibody responses

Three enzyme-linked immunosorbent assays (ELISAs) were developed to semi-quantitatively measure the presence of antibodies specific to the vaccine components; namely the LH-β peptide, BSA, and KLH.

Due to the small molecular size of the LH-β peptide, a conjugate was prepared for use as antigen. The LH-β peptide was conjugated to ovalbumin (OVA) (Thermo Fisher Scientific) using $SM(PEG)_2$ crosslinker (Thermo Fisher Scientific). Briefly, 4.5 mg of ovalbumin was reacted with 1 μmole of crosslinker for 30 min at 24 °C in 1 mL of molecular-grade water. Excess crosslinker was removed using a Sephadex G25 column (GE Healthcare; Little Chalfont, United Kingdom). The activated ovalbumin was then mixed with 8.3 mg of LH-β peptide in a total volume of 4.5 mL and incubated for 30 min at 24 °C. The resulting conjugate was loaded onto two PD10 Sephadex G25 columns, each eluted with 3.5 mL of 0.1 M PBS. The final protein concentration of the conjugate was determined by Bradford assay.

2.5.1. ELISA protocol

Salmon sera were diluted 1:10 in 'milk block' (5% w/v powdered skim milk in 0.01 M PBS with 5% Tween 20 (Amresco; Framingham, Massachusetts)) and stored at 4 °C overnight for pre-adsorbtion of serum antibodies [37]. 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 PBS (Sigma-Aldrich). Plates were stored overnight at 4 °C then washed three times with 200 μL of 0.01 M PBS with 0.05% Tween 20 (PBST) (Sigma-Aldrich). Each well was incubated with 200 μL of milk block for 1 h at 24 °C. Plates were washed three times with 200 μL PBST. Duplicate wells were loaded with 50 μL pre-adsorbed sera and plates were incubated for 1 h at 24 °C before being washed three times with 200 μL PBST. Monoclonal mouse anti-salmonid Ig antibody conjugated to 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 h at 24 °C, then washed three times with 200 μL wash buffer. Enzyme substrate was prepared by dissolving 1 mg of 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma-Aldrich) per 10 mL of 0.05 M phosphate citrate buffer with 0.014% hydrogen peroxide (Sigma-Aldrich). 50 μL of enzyme substrate was added to each well and plates were 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. Table 2 provides additional detail about the variations on this generic protocol that are specific to each antigen evaluated. All plates were run with positive control and negative control samples, identified during assay development.

Table 1
Comparison of Atlantic salmon contraceptive vaccine formulations.

Formulation #	Peptide	Peptide Conc. (mg/mL)	Carrier	Carrier Conc. (mg/mL)	Immunostimulant	Immunostimulant Conc.	Adjuvant
0 (sham)	none	0	none	0	none	0	ISA761 VG
1	LH-β 18mer	0.222	BSA	0.278	β-glucan	0.5 mg/mL	ISA761 VG
2	LH-β 18mer	0.222	KLH	0.278	β-glucan	0.5 mg/mL	ISA761 VG
3	LH-β 18mer	0.222	BSA	0.278	Muramyl dipeptide	0.5 mg/mL	ISA761 VG
4	LH-β 18mer	0.222	KLH	0.278	Muramyl dipeptide	0.5 mg/mL	ISA761 VG
5	LH-β 18mer	0.222	BSA	0.278	*A. salmonicida* + *V. anguillarum*	8.3×10^4 cells/mL	ISA761 VG
6	LH-β 18mer	0.222	KLH	0.278	*A. salmonicida* + *V. anguillarum*	8.3×10^4 cells/mL	ISA761 VG
7	LH-β 18mer	0.222	BSA	0.278	*M. marinum*	8.3×10^4 cells/mL	ISA761 VG
8	LH-β 18mer	0.222	KLH	0.278	*M. marinum*	8.3×10^4 cells/mL	ISA761 VG

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 Conc. (ng/μL)	Coating Buffer	Substrate Dev. (minutes)
BSA	10	Carb-bicarb	15
KLH	5	Carb-bicarb	11
LH-β conjugate	3.74	PBS	11

2.6. Statistical analyses

There were 503 fish on trial initially but 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.

Antibody production was evaluated using optical densities (ODs) measured by the ELISAs. Samples were included in the dataset if the duplicate well ODs had a coefficient of variation (CV) less than 15%; if the CV exceeded 15% and one or both duplicate sample ODs were greater than the assay limit of detection, the sample was re-run.

Assay background was accounted for by subtracting the mean OD of wells incubated with PBS instead of serum, included on every plate. To account for inter-plate variation, results are presented as sample to positive ratios minus the mean pre-vaccination OD for that treatment [40–42].

A Kruskal-Wallis one-way analysis of variance (ANOVA) was used to compare the median pre- and post-vaccination ODs among all treatment groups [43]. In the event of a significant difference, pairwise comparisons of medians were made using Dunn's multiple comparisons test (among treatments at a given time, and within a single treatment over time). The data were not treated as repeated measures because treatment was applied between the first and second sampling times and sample sizes were unequal among treatments and within treatments over time.

Positive/negative thresholds for each ELISA were set to maximize sensitivity and specificity. Receiver operator characteristic (ROC) graphs were generated [43] for each ELISA using threshold values ranging from the minimum to maximum OD value for the assay in increments of 0.001. The OD corresponding to the optimum sensitivity and specificity was set as the positive/negative threshold for each assay.

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

Minitab (version 16, Minitab, Inc.; State College, Pennsylvania) and GraphPad Prism (version 6.07 GraphPad Software; La Jolla, California) statistical packages for Windows were used for the above statistical analyses.

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

Treatment Group	Pre-vaccination	8 wk Post-vacc.	12 wk 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

3. Results

3.1. Assay positive/negative thresholds

The ROC curves generated for each assay are shown in Fig. 1. Area under the curve (AUC) corresponds to the probability of a

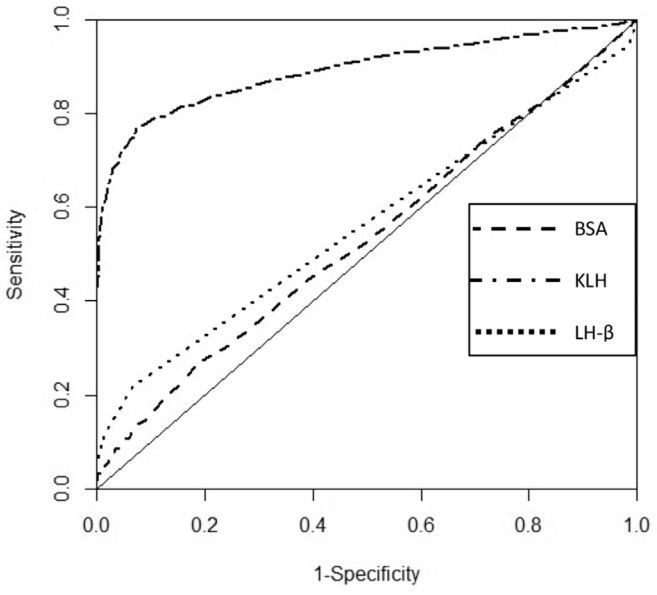

Fig. 1. ROC curves; the solid line from (0,0) to (1,1) represents a 50% chance of positive classification of a positive or negative sample.

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 [44]. 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 peptide assays were much lower at 0.5348 and 0.5648, respectively.

The optimum sensitivity and specificity for each assay and their corresponding thresholds, set as the positive/negative thresholds, are summarized in Table 4.

3.2. LH-β peptide antibody response

Overall, approximately 36% (303/849) of samples from fish vaccinated against the peptide were identified as positive (regardless of carrier; including 8 and 12 weeks post-vaccination). This is further broken down by treatment in Fig. 2. For all treatments, the proportion of samples positive for LH-β antibodies was highest at 12 weeks post-vaccination.

3.3. Carrier proteins antibody response

Approximately 96% (361/376) of samples from fish vaccinated against KLH and 76% (282/371) of samples from fish vaccinated against BSA were identified as positive for antibodies to the respective carrier protein. The proportion of positive samples was highest at 12 weeks post-vaccination for both carrier proteins.

The proportion of samples classified as positive are shown further broken down by treatment group to compare performance of the formulations (Figs. 3 and 4). High background levels in the anti-BSA and anti-peptide ELISAs are revealed by the large proportion of samples in treatment 0 that are classified as positive, despite the control group fish never having been vaccinated against these antigens. A dashed line extends horizontally from the top of the control group's bar to show the misclassification rate associated with the assay at its positive/negative threshold.

3.4. Comparison of immunostimulants

Prior to vaccination, there was no significant difference in median BSA or KLH antibody levels among treatment groups ($p = 0.707$, 0.996 respectively).

No significant difference was detected in median BSA antibody levels among treatment groups at 8 weeks post-vaccination ($p = 0.171$). However, a statistically significant difference in the median BSA antibody response was detected at 12 weeks post-vaccination ($p < 0.0001$); by this time, the median BSA antibody level of treatment group 5 was significantly different from that of all other groups except treatment 3 ($p < 0.05$) (Fig. 5, Table 5). Interestingly, treatment 5 was the only one that produced a significant difference between pre-vaccination and post-vaccination BSA antibody levels and this was only seen at 12 weeks post-vaccination ($p < 0.001$).

A statistically significant difference in the median KLH antibody response was detected among treatment groups at 8 and 12 weeks post-vaccination. All treatment groups vaccinated against KLH had median responses that were significantly different than those of the non-vaccinates (Fig. 6, Table 6). There was a significant difference between median pre-vaccination and post-vaccination (both 8 and 12 weeks) KLH antibody levels in all four treatment groups vaccinated with KLH ($p < 0.001$). The 8 week and 12 week post-vaccination median KLH antibody responses were significantly different from each other in only two treatment groups, 2 and 6 ($p < 0.05$).

Prior to vaccination, there was a significant difference in median LH-β antibody levels between treatment groups 1 and 5 ($p < 0.01$; treatment 1 sum of ranks and mean of ranks greater than treatment 5). This leveled out by 8 weeks post-vaccination and no significant difference was detected in the median LH-β antibody responses among treatment groups at this time ($p = 0.210$). However, a statistically significant difference in the median LH-β antibody response was detected at 12 weeks post-vaccination ($p < 0.0001$)

Table 4
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

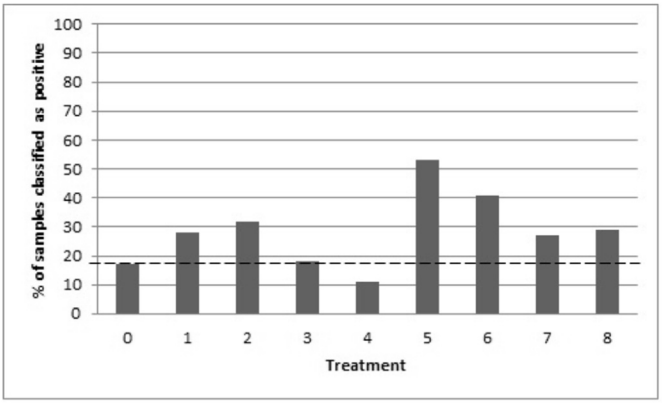

Fig. 2. Proportion of samples classified as positive for antibodies to LH-β peptide; Treatments 1 through 8 contained the LH-β peptide. Treatment 0 was a sham vaccine included as a control. 8 and 12 week post-vaccination samples are grouped together and represented by a single bar for each treatment. A dashed line extends horizontally from the top of the treatment 0 bar as an approximation of the misclassification rate associated with the assay at its positive/negative threshold. (Treatment carrier protein/immunostimulant content: 1: BSA/β-glucan, 2: KLH/β-glucan, 3: BSA/muramyl dipeptide, 4: KLH/muramyl dipeptide, 5: BSA/*A. salmonicida* + *V. anguillarum*, 6: KLH/*A. salmonicida* + *V. anguillarum*, 7: BSA/*M. marinum*, 8: KLH/*M. marinum*).

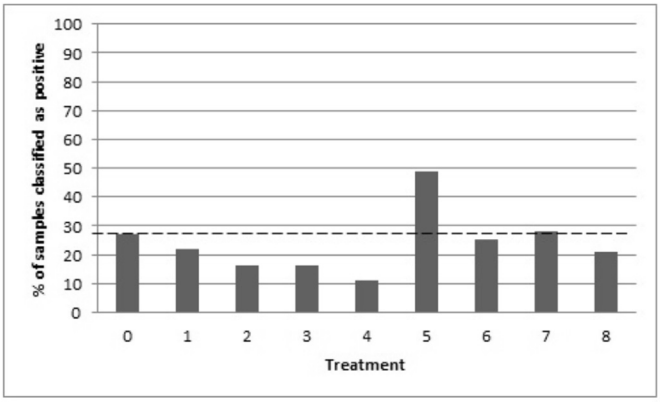

Fig. 3. Proportion of samples classified as positive for anti-BSA antibodies; Treatments 1, 3, 5, and 7 contained BSA. 8 and 12 week post-vaccination samples are grouped together and represented by a single bar for each treatment. The horizontal dashed line shows the misclassification rate associated with the assay at its positive/negative threshold. (Treatment carrier protein/immunostimulant content: 1: BSA/β-glucan, 2: KLH/β-glucan, 3: BSA/muramyl dipeptide, 4: KLH/muramyl dipeptide, 5: BSA/*A. salmonicida* + *V. anguillarum*, 6: KLH/*A. salmonicida* + *V. anguillarum*, 7: BSA/*M. marinum*, 8: KLH/*M. marinum*).

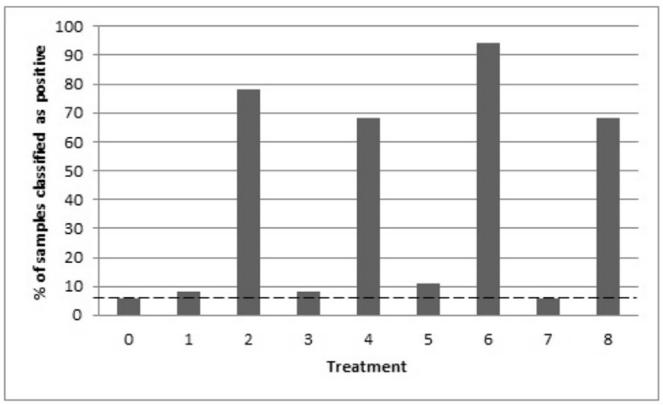

Fig. 4. Proportion of samples classified as positive for anti-KLH antibodies; Treatments 2, 4, 6, and 8 contained KLH. 8 and 12 week post-vaccination samples are grouped together and represented by a single bar for each treatment. The horizontal dashed line shows the misclassification rate associated with the assay at its positive/negative threshold. (Treatment carrier protein/immunostimulant content: 1: BSA/β-glucan, 2: KLH/β-glucan, 3: BSA/muramyl dipeptide, 4: KLH/muramyl dipeptide, 5: BSA/*A. salmonicida* + *V. anguillarum*, 6: KLH/*A. salmonicida* + *V. anguillarum*, 7: BSA/*M. marinum*, 8: KLH/*M. marinum*).

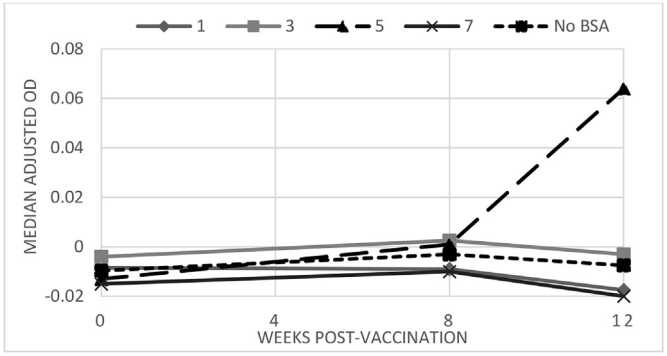

Fig. 5. Median BSA antibody production over time; treatments 1, 3, 5, and 7 contain BSA; treatment group 5 shows a significant increase from 8 weeks to 12 weeks post-vaccination. (Treatment immunostimulant content: 1: β-glucan, 3: muramyl dipeptide, 5: *A. salmonicida* + *V. anguillarum*, 7: *M. marinum*).

when treatment groups 5 and 6 were different from the control group ($p < 0.01$) (Fig. 7, Table 7). Additionally, the median LH-β antibody response of treatment group 5 was significantly different from that of groups 2, 3, and 4 at 12 weeks post-vaccination. Treatments 1 and 7 produced a significant jump in median LH-β antibody production from 8 weeks to 12 weeks post-vaccination ($p < 0.001$, $p < 0.05$ respectively) while treatment 8 had a significant difference between pre-vaccination and 12 week post-vaccination levels ($p < 0.01$). Treatments 5 and 6 produced a significant difference in median LH-β antibody response between pre-vaccination and 12 weeks post-vaccination ($p < 0.001$) and

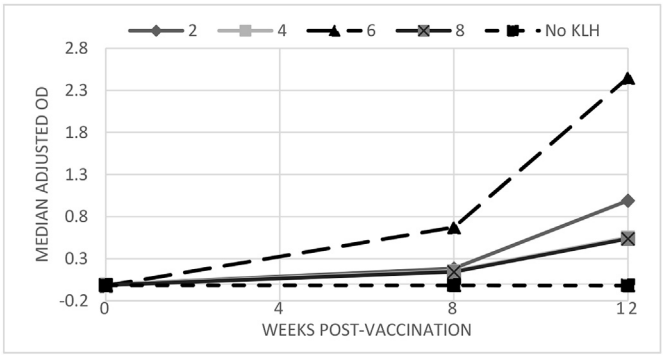

Fig. 6. Median KLH antibody production over time; treatments 2, 4, 6, and 8 contain KLH; group 6 anti-KLH levels are the highest at 8 and 12 weeks post-vaccination, but treatments 2, 4, and 8 also have elevated antibody levels compared to the groups which were not vaccinated against KLH. (Treatment immunostimulant content: 2: β-glucan, 4: muramyl dipeptide, 6: *A. salmonicida* + *V. anguillarum*, 8: *M. marinum*).

between 8 and 12 weeks post-vaccination ($p < 0.001$).

3.5. Adjuvant suitability

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

4. Discussion

The findings of this study lay the foundation for further research on development of contraceptive vaccines for Atlantic salmon and indicate that a hapten-protein conjugate could be functional as a vaccine antigen in this species. This study also reaffirms the value of selecting an appropriate immunostimulant to enhance the immune response to a vaccine antigen.

Antibody response to the LH-β peptide was low in both magnitude and the proportion of positive samples. A simple option for improving antibody response to the peptide may be to administer a boost dose of the vaccine. Drennan et al. [27] observed minimal and inconsistent antibody responses in white sturgeon (*Acipenser transmontanus* R.) vaccinated against a hapten-protein antigen. However, after administering a boost dose of the vaccine, a 16-fold titer increase was observed and the response rate jumped to 100%. Similarly, rainbow trout (*Onchorynkus mykiss*) vaccinated with a fluorescein isothiocyanate-KLH conjugate had low serum antibody levels that were not differentiable from the control group until after a booster vaccine was administered [38]. In most species, a boost dose administered at the appropriate time has been observed to have this enhancing effect on antibody production and

Table 5
BSA antibody production over time (presented as median adjusted ODs).

Trt	Pre-vaccination			8 wk Post-vaccination			12 wk Post-vaccination		
	Median OD	StDev	n	Median OD	StDev	n	Median OD	StDev	n
0, 2, 4, 6, 8	−0.010	0.049006	217	−0.002	0.074969	194	−0.009	0.131198	180
1	−0.009	0.053386	56	−0.009	0.055603	52	−0.018	0.080978	48
3	−0.004	0.024170	52	0.003	0.035719	48	−0.003	0.066019	51
5	−0.013	0.046256	55	0.001	0.071625	38	0.064	0.327983	42
7	−0.015	0.081633	52	−0.010	0.152953	44	−0.020	0.200036	48

Table 6
KLH antibody production over time (presented as median adjusted ODs).

Trt	Pre-vaccination			8 wk Post-vaccination			12 wk Post-vaccination		
	Median OD	StDev	n	Median OD	StDev	n	Median OD	StDev	n
0, 1, 3, 5, 7	−0.012	0.0661714	274	−0.011	0.073484	245	−0.018	0.097673	238
2	−0.007	0.0530410	55	0.185	0.443164	49	0.991	0.899854	50
4	−0.002	0.0599784	55	0.155	0.456520	51	0.553	0.799839	43
6	−0.019	0.0705586	56	0.675	0.736036	45	2.450	0.922341	43
8	−0.013	0.0593099	56	0.145	0.324416	47	0.539	0.782617	48

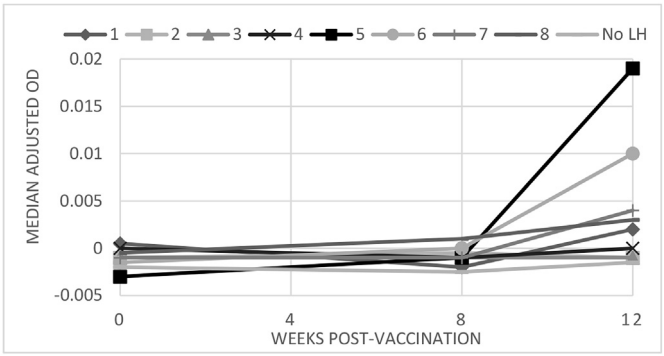

Fig. 7. Median LH-β peptide antibody production over time; treatments 1 through 8 contain peptide; groups 5 and 6 have the highest median antibody levels 12 weeks post-vaccination. (Treatment carrier protein/immunostimulant content: 1: BSA/β-glucan, 2: KLH/β-glucan, 3: BSA/muramyl dipeptide, 4: KLH/muramyl dipeptide, 5: BSA/*A. salmonicida* + *V. anguillarum*, 6: KLH/*A. salmonicida* + *V. anguillarum*, 7: BSA/*M. marinum*, 8: KLH/*M. marinum*).

resulting protection [45]. Despite the known benefits of boosting, it is desirable to achieve efficacy through a single vaccination due to the cost of vaccines and manpower required to administer those vaccines to fish [46]. Additionally, the stress of being handled and vaccinated takes a toll on fish health so minimizing the number of different vaccines necessary is advantageous [26]. Nevertheless, a multiple vaccination scheme should not be ruled out until cost-benefit analyses are conducted.

Treatments 5 and 6, which both contained *A. salmonicida* and *V. anguillarum* as the immunostimulant, produced the strongest antibody responses to both carrier proteins, and to the LH-β peptide. Using multiple strains of whole bacteria as a vaccine immunostimulant makes sense due to the number of different PAMPs presented. In fish, PAMPs have been shown to elevate white blood cell counts [30,47], stimulate non-specific immune mechanisms [26,33], increase antigen uptake [34], enhance activation of numerous immune components including granulocyte activity, and increase resistance to infection from several bacterial pathogens [30,33]. It is also possible that the enhanced antibody response observed may be partially attributed to an overall increase in antibodies, including those produced against the insoluble bacterial immunostimulants. It is worth noting that all four of the immunostimulants evaluated were insoluble bacteria or their derivatives, all of which would theoretically elicit an antibody response. Future studies should include groups of fish vaccinated with each individual vaccine component to evaluate respective contributions to the overall antibody response.

KLH was the strongest immunogen with the majority of fish responding positively to this carrier protein. The larger molecular size of KLH relative to BSA may have contributed to its superior immunogenicity in Atlantic salmon. However, there was not a statistically significant difference in the LH-β peptide response between the BSA and KLH carrier groups; the enhanced KLH antibody response was not accompanied by a corresponding increase in the LH-β response, indicating that both carrier proteins are suitable for use in this particular application. This also suggests that, relative to BSA, the stronger antibody response to KLH did not mask or overwhelm the LH-β peptide antibody response. Within the parameters evaluated in the present study, it appears that the immunostimulant choice has greater influence on the LH-β antibody response than does the choice of carrier protein.

Within treatment groups, there appears to be an association between having a strong antibody response and having a large proportion of responders. Interestingly, the proportion of responders to KLH was significantly higher than that for BSA at both 8 and 12 weeks post-vaccination. The treatments with the largest proportion of responders and strongest antibody responses for the carrier proteins also had the highest proportion of responders to the LH-β peptide. There was a great deal of variation in antibody response among fish. Standard deviation was higher among vaccinates than among non-vaccinates.

The anti-BSA and anti-LH-β ELISAs provided predictive capability greater than random chance and allowed for rudimentary statistical comparison of the various treatments. In maximizing the sum of sensitivity and specificity, a large trade-off between the two was observed, particularly for BSA and LH-β 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

Table 7
LH-β peptide antibody production over time (presented as median adjusted ODs).

Trt	Pre-vaccination			8 wk Post-vaccination			12 wk Post-vaccination		
	Median OD	StDev	n	Median OD	StDev	n	Median OD	StDev	n
0	−0.002	0.009880	53	−0.003	0.019656	48	−0.002	0.020910	48
1	0.001	0.004308	56	−0.002	0.011115	51	0.002	0.074569	49
2	−0.001	0.010397	55	−0.001	0.010483	50	−0.001	0.034649	47
3	−0.001	0.005855	53	−0.001	0.014000	51	−0.001	0.137381	46
4	0.000	0.005869	59	−0.001	0.008323	51	0.000	0.019441	41
5	−0.003	0.011769	59	−0.001	0.074170	42	0.019	0.205364	36
6	−0.002	0.009447	56	0.000	0.026871	53	0.010	0.230183	48
7	−0.001	0.007395	55	−0.001	0.042935	47	0.004	0.090234	49
8	−0.001	0.007682	54	0.001	0.155469	46	0.003	0.206755	46

positive rates for these assays while the true negative rates are very high. Further optimization of these assays may be necessary in future studies in order to provide improved differentiation between true and false positives.

Since response rates were greatest at 12 weeks post-vaccination 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.

Fish weight and length were tracked to ensure that the vaccine antigens did not adversely affect growth and to evaluate tolerability to the Montanide ISA 761 VG adjuvant. 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 complications during blood sampling. These observations do not provide any reason to suspect that the adjuvant used in the study caused any adverse effects. The Montanide ISA 761 VG adjuvant appeared to be well tolerated by the salmon but additional studies will be necessary to further evaluate any potential side effects not externally visible and to assess growth rate relative to an unvaccinated group.

The effects of vaccination on the reproductive physiology and hormone production of the fish were not evaluated. Sambroni et al. [14] vaccinated rainbow trout with phages displaying LH and FSH receptors, resulting in specific antibody production, reduced hormone levels, and delayed spermiation and vitellogenesis, suggesting that disruption of this signaling pathway could prevent sexual maturation of Atlantic salmon. Antibody responses are not always predictive or correlative to the physiological effects of vaccination, especially with immunocontraceptives, so a longer study duration and evaluation of additional reproductive metrics is necessary. This would be especially useful to determine whether any delay in sexual maturation had positive outcomes related to improved body condition and mass.

The findings 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 and BSA perform comparably as hapten carriers and that an immunostimulant combining *A. salmonicida* and *V. anguillarum* provides an improved antibody response. The proportion of positive samples and antibody levels were highest at 12 weeks post-vaccination suggesting that the water-in-oil vaccine provides a potentially extended release of antigen, possibly due to granuloma formation as observed by Evensen et al. [48] or persistent active inflammation [49]. Further research will be necessary to improve the formulation of the contraceptive vaccine candidates and determine the physiological significance of the resulting antibodies.

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