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VACCINATION OF HYBRID STRIPED BASS: GROWTH, IMMUNE REACTION AND GENE EXPRESSION

P.A. Cotter[1], E. McLean[2], S.R. Craig[3], J.C. Craig[4], M. Westerman[5]

Summary

Hybrid striped bass (42.6 ± 4.9 g wet wt; 139.3 ± 6.1 mm length), were randomly stocked into one of 6 tanks (n=6 tank^{-1}) of a custom designed, recirculating life support system (RLSS). Water quality was as follows: DO_2 (6.5 ± 0.6 mg l^{-1}), pH (7.7 ± 0.5), TAN (0.06-1.31 mg l^{-1}), nitrite (0.06-0.60 mg l^{-1}) and nitrate (2.0-32.1 mg l^{-1}), salinity 5 ppt, temperature 28 ± 1 ^{o}C. A 12:12 photophase:scotophase was used, with a 30 min. dusk-dawn dimming of lights. Fish were fed at 4% body wt d^{-1} as two separate feedings (08.00 and 16.00 h). Dietary crude protein and lipid levels were 40% and 10% respectively. Tanks were randomly paired and fish either left untreated, vaccinated, or sham injected. The vaccine employed was an experimental formalin killed *Streptococcus iniae* oil-in-water adjuvanted bacterin. Fish were weighed and measured bimonthly for 8 wk with group weights being employed to adjust feeding rates. At trial termination, all animals were weighed and measured, and their condition factor (CF) and feed conversion ratios (FCR) calculated. Visceral somatic (VSI) and hepatosomatic (HSI) indices and intraperitoneal fat (IPF) and muscle ratios (MR) were also assessed. Blood was taken from anaesthetized fish and hematocrit recorded. Blood was allowed to clot overnight at 5 ^{o}C after which serum protein levels were recorded and lysozyme activity measured. Livers were prepared for microarray evaluation using standard techniques using a *Danio rerio* genechip to assess global changes in gene expression.

No differences were observed with respect to final weights, lengths, CF, FCR, or HSI

1 Paul Cotter graduated with an M.S. specializing in hybrid striped bass aquaculture at Virginia Tech., Department of Fisheries & Wildlife Sciences, 100 Cheatham Hall, Blacksburg VA 24060-0321, USA;

2 Ewen McLean, Ph.D., e-mail: ewen.mclean@utt.edu.tt (corresponding author), tel: (868) 634 8888; fax: (868) 634 3008, Programme Professor, Marine and Environmental Sciences, at the University of Trinidad and Tobago, Marine and Environmental Sciences, Phase III, Maritime Campus, University of Trinidad and Tobago, Second Avenue, Northwestern Main Road, Chaguaramas, Trinidad W.I;

3 Steven R. Craig, Ph.D. is Director of Nutrition Research at Virginia Cobia Farms, Virginia Cobia Farms, LLC, 108 Battleground Avenue, Saltville, VA 24370, USA;

4 Johanna Craig, Ph.D. is Founder and Principal of GATACA LLC, GATACA, LLC, 180 Orchard Hill Lane, Newport, VA 24128, USA;

5 Mark Westerman, Ph.D. is President and CEO of Intrinsic LifeSciences LLC, Intrinsic Life Sciences, 505 Coast Boulevard South, La Jolla, CA 92037, USA

although differences ($P < 0.001$) were determined for VSI, which was higher in control animals. Packed cell volume and serum protein levels were similar across groups ($P > 0.05$). Time-course of changes in serum lysozyme activity described an initial reduction in lysozyme activity followed by a rebounding in activity which peaked 25 days post-treatments. Evaluation of lysozyme activity among time-points revealed differences ($P < 0.05$) between 11 and 25 days post-vaccination. Examination of the hepatic microarray datasets revealed only four immune-discrete genes that were impacted 53-days following vaccination when compared against control fish. These included the up-regulated TCIRG1, or T-cell immune regulator 1 ($P < 0.0467$) and IL20RA, or interleukin 20 receptor alpha ($P < 0.0433$), and down-regulated cytokine inducible kinase, plk3 ($P < 0.01$) and mouse immune responsive protein, IRG1 ($P < 0.01$).

Key words: lysozyme, transcriptomics, microarray, *Streptococcus iniae*

INTRODUCTION

Vaccination has become increasingly important to aquaculture as a means of alleviating losses from and preventing the spread of disease. Indeed, it has been suggested that almost every farmed salmon is vaccinated against several key pathogens (Engelstad, 2005). Such strategies improve not only the welfare (see Huntingford et al., 2006) of the cultured animal but also its plate safety (i.e., residue-free status). Other than providing protection against specific pathogens, vaccination appears to enhance nonspecific immunity in general, thereby increasing the fish's overall resistance to infections (Adams et al. 1988). Vaccination also potentially provides protection for fish that have not been injected, or which have not received the full dose of vaccine, due to "herd immunity": that is animals may be protected indirectly due to a reduced incidence of disease in fully vaccinated fish (see: Anderson and May, 1985a,b). A further benefit of vaccination has been a reduction in use of antibiotics and concomitant diminution of risks associated with the development of antibiotic resistant strains of pathogen (Alderman and Hastings 1998; Lorenzen and LaPatra, 2005). Vaccination can also result in savings for the producer by decreasing costs associated with the purchase of medications in the event of disease. These savings could be reinvested to improve husbandry and productivity (Horne and Robertson 1987). Along with the many benefits connected with vaccination however, there are some negatives. For example, intra-abdominal lesions caused by vaccination may persist throughout a production cycle and result in downgrading of fish at harvest by as much as 50% (Midtyling 1996, Midtyling et al. 1996). The stress experienced by fish during the vaccination process may also have an overall negative effect, albeit short-term, upon the immune system (Dunn et al. 1990; Press and Lillehaug 1995), growth performance (Lillehaug et al., 1992; Espersen et al., 1999) and appetite (Midtlyng et al., 1995). Because vaccination increases the value of fish, if mortality does occur post-treatment, greater economic loss is suffered (Rønsholdt and McLean 1999). Other problems relating to vaccines include accidental self injection, which may cause side effects in humans (see: Culora et al., 1996; Leira and Baalsrud 1997). This is of major concern especially where

a "per fish vaccinated" fee is employed since workers are generally more hurried and less careful during the vaccination procedure.

Irrespective of the problems associated with vaccination their use will clearly increase and in some instances, vaccines represent the only viable method for the control of specific diseases. Such is the case for *Streptococcus iniae*, which is recognized as one of the most problematic diseases for hybrid striped bass farmers (Shoemaker et al., 2001). In the US no FDA-approved drug is available to treat the disease and indeed, therapeutics are generally ineffective against this pathogen (Bercovier et al. 1997), possibly due to its intramacrophage existence (Zlotkin et al., 2003). *S. iniae*, which has been recorded in over 30 wild and cultured marine and freshwater species (Buchanan et al., 2005), gains access to the animal via the snout. It causes a systemic disease that spreads rapidly and can result in 50% mortality within 48 h (Evans et al. 2001). The lack of effective treatments has led to several groups examining the efficacy of various drugs and experimental vaccines (Li et al., 2004; Abutbul et al., 2005; Whittington et al., 2005; Buchanan et al., 2005; Martins et al., 2011). These include successful studies with a killed toxoid enriched *S. iniae* vaccine and a live-attenuated vaccine. However, while traditional evaluations of vaccine effectiveness (dose, efficacy, survival, etc.) have been undertaken, no studies have examined the impact of *S. iniae* vaccination upon other biological and production-related characteristics in fish.

Hybrid striped bass presently represent the fourth most valuable crop and fifth most farmed species in American aquaculture (Harvey, 2006). The species is generally farmed in ponds, with some harvested from cage operations. Since hybrids are yielding to intensive cultivation, an increasing tonnage has been derived from recirculating life support systems (RLSS). A major feature of RLSS is that they permit greater control over environmental and water quality parameters and thereby allow increased stocking density. Occasionally however, hybrid striped bass, like other species, and especially those reared at higher temperatures and densities, become more susceptible to disease outbreaks. For cultured hybrid striped bass, *S. iniae* has become an increasingly important pathogen with estimates of losses to the industry exceeding $100 million annually. Clearly, vaccination against streptococcus infections represents the preferred method of crop protection. However, prior to anticipating the use of approved commercial vaccine formulations against *S. iniae*, farmers must be informed of the possible negative consequences of vaccination upon the performance characteristics of reared fish. Accordingly, the present study examined the effect of an experimental *S. iniae* vaccine upon growth, immunity, and gene expression in hybrid striped bass. Availability of such information would provide the aquaculturist with a more complete portfolio of knowledge with which decision-making processes (i.e., to vaccinate or not) could be more easily made.

MATERIAL AND METHODS

System and animal husbandry

Juvenile hybrid striped bass (♂*Morone saxatilis* x ♀*M. chrysops*; 42.6 ± 4.9 g wet wt; 139.3 ± 6.1 mm length), purchased from a commercial hatchery (Keo Fish Farms, AR,

USA) were PIT tagged (Biomark Inc., ID, USA) and randomly stocked into one of 24 tanks (n=6 tank^{-1}) of a custom designed RLSS. The RLSS comprised 120 liter glass tanks that had black-painted walls to reduce fish stress, and incorporated a bubble-bead filter (BBF-2 Aquaculture Technologies Inc., Metaire, LA, USA) to remove suspended solids, UV light sterilizer (Emperor Aquatics, Pottstown, PA, USA), a KMT fluidized bed with media (Kaldnes Inc; Providence, RI, USA) for biological filtration and a protein skimmer (R&B Aquatic Distribution, Boerne, TX, USA) to remove smaller solids and decrease turbidity.

DO_2 (6.5 ± 0.6 mg l^{-1}) and pH (7.7 ± 0.5) were monitored daily using an Y85 Series dissolved oxygen meter (YSI Inc., Yellow Springs, OH), and Hanna Instrument 9024 (Aquatic Ecosystems, Apopka, FL.). Total ammonia nitrogen (TAN; range: 0.06-1.31 mg l^{-1}) was monitored daily by spectrophotometric analysis (Hach Inc., Loveland, CO, USA). Nitrite (range: 0.06-0.60 mg l^{-1}) and nitrate (range: 2.0-32.1 mg l^{-1}) levels were quantified once weekly. Salinity was maintained at 5 ppt and measured with a refractometer. A heater was placed in the biofiltration sump to maintain water temperature at 28 °C. Fish were exposed to a 12:12 photophase:scotophase through fluorescent lighting positioned 2.5 m above the life support system. A 30 min. dusk-dawn dimming of lights was employed.

Throughout the trial period, a fishmeal-based diet (Table 1) was fed at 4% body wt d^{-1} as two separate feedings (08.00 and 16.00 h). Crude protein and lipid levels were formulated at 40% and 10% respectively. All known nutritional requirements of hybrid striped bass were met by the experimental feeds (NRC, 1993).

Experimental treatments

Three experimental treatments were given: fish were either left untreated, vaccinated, or sham injected. The vaccine employed was an experimental formalin killed *Streptococcus iniae* oil-in-water adjuvanted bacterin (Kent Sea Tech Corporation, Mecca, CA). Injected fish received either vaccine (4 x 10^5 of cells/ml) or Courtland saline as a 100 µl volume. Fish were weighed and measured bimonthly for 8 wk with group weights being employed to adjust feeding rates.

Data collection

1. Morphological and feed data

At trial termination, all animals were weighed and measured, and their condition factor and feed conversion efficiencies were calculated according to the methods described in Weatherly and Gill (1986). Visceral somatic and hepatosomatic indices and intraperitoneal fat (IPF) and muscle ratios were calculated according to the methods described in Lunger et al. (2006).

2. Serum processing

Anaesthetized (MS-222; Sigma) fish were bled from the caudal artery-vein complex using 2 ml heparinized syringes. Collected blood was transferred into Eppendorf 2 ml microcentrifuge tubes. Blood packed cell volume (hematocrit) was immediately assessed following collection. Blood was drawn into microhematocrit tubes (Fisher Scientific, Pittsburgh, PA), sealed with Cristoseal (Fisher), centrifuged at 10,000 x *g* for 5 min (M

24 Micro-Hematocrit Centrifuge; LW Scientific, Lawrenceville, GA) and hematocrit read using the centrifuge's combo reader.

Table 1. Composition of the basal diet used in the present study. All diets provided 40% crude protein, 10% lipid and supplied 3.30 kcals/g

Tablica 1. Sastav osnovne hrane korištene u ovom istraživanju. Hrana se sastojala od 40% sirovog proteina, 10% lipida te je sadržavala 3,30 kcals/g

Ingredient (% dry matter basis) / Sastojak (% suhe tvari)	
Fishmeal[a]/ Riblja hrana[a]	58.8
Dextrin[c]/ Dextrin[c]	20.0
Lipid (Menhaden Oil)[b]/ Lipid (ulje vrste *Brevoortia tyrannus*)[b]	4.1
Mineral (Se free)[d]/ Mineral (bez Se)[d]	4
Vitamin[e]/ Vitamin[e]	3
Carboxymethyl Cellulose (CMC)[c]/ Karboksimetil Celuloza *(CMC)*[c]	1
Cellulose[c]/ Celuloza[c]	9.1
Selenium premix[g]/ Selenova smjesa[g]	0

[a] *Special Select® menhaden fish meal, Omega Protein, Inc., Hammond, LA, USA. 68% crude protein (dry); 0.915 dry matter / riblja hrana, Omega Protein, Inc., Hammond, LA, SAD. 68% sirovog proteina (suho); 0,915 suhe tvari;*

[b] *LCP ethoxyquin-free Omega Oils, Reedville, VA, USA / omega ulja bez etoksikvina, Reedville, VA, SAD;*

[c] *US Biochemical Corporation, Cleveland, Ohio, USA / Američka biokemijska korporacija, Cleveland, Ohio, SAD;*

[d] *Contained (g/5kg): Monocalcium Phosphate 680, Calcium Lactate 1742, Ferrous Sulfate 25, Magnesium Sulfate 7H20 660, Dipotassium Phosphate 1200, Monosodium Phosphate 440, Sodium Chloride 225, Aluminum Chloride 0.750, Potassium Chloride 0.750, Copper Sulfate 2.5, Manganous Sulfate 3.5, Cobalt Chloride 5, Zinc Sulfate 7H20 15.(MP Biomedicals, Aurora, OH, USA) / Sadrži (g/5kg): monokalcij fosfat 680, kalcij laktat 1742, željezo sulfat 25, magnezij sulfat 7H20 660, dikalij fosfat 1200, mononatrij fosfat 440, natrij klorid 225, aluminij klorid 0,750; kalij klorid 0,750; bakreni sulfat 2,5; manganski sulfat 3,5; kobalt klorid 5, cink sulfat 7H20 15. (MP Biomedicals, Aurora, OH, SAD);*

[e] *Contained (g/kg): Ascorbic acid 50.0, dl-calcium pantothenate 5.0, Choline chloride 36.2, Inositol 5.0, Menadione sodium bisulfite 2.0, Niacin 5.0, Pyridoxine HCL 1.0, Riboflavin 3.0, Thiamine mononitrate 0.5, dl-alpha-tocopherol acetate (250 IU/g) 8.0, Vit. A palmitate (500,000 IU/g) 0.2, Vitamin micro-mix 10.0, Cellulose 874.1. Micromix contained (g/mg) Biotin 0.50, Folic acit 1.8, Vitamin B12 .02, Cholecalciferol (40 IU/ug) 0.02, Cellulose 97.66 / Sadrži (g/kg): askorbinska kiselina 50,0; dl-kalcij pantotenat 5,0; kolin klorid 36,2; inozitol 5,0; menadion natrij bisulfit 2,0; niacin 5,0; piridoksin HCL 1,0; riboflavin 3,0; tiamin mononitrat 0,5; dl-alfa-tokoferol acetat (250 IU/g) 8,0; vit. A palmitat (500.000 IU/g) 0,2; vitaminska mikromješavina 10,0; celuloza 874,1. Mikromješavina sadrži (g/mg) biotin 0,50; folna kiselina 1,8; vitamin B12 .02; kolekalciferol (40 IU/ug) 0,02; celuloza 97,66;*

[f] *Selplex® (Alltech Inc., Nicholasville, KY, USA);*

[g] *Selenium premix: 0.1 g sodium selenite (Sigma Chemical Co., St. Louis, Missouri, USA) and 499.9 g cellulose / Selenova smjesa: 0,1 g natrij selenit (Sigma Chemical Co., St. Louis, Missouri, SAD) i 499,9 g celuloza*

Remaining blood samples were transferred to a refrigerator maintained at 5 °C and allowed to clot overnight. Resultant serum was stored in 2 ml Eppendorf tubes at -20 °C until further analyses. Serum samples were employed to evaluate protein levels using a hand-held VET360 temperature-compensated refractometer (Leica Optical Products Division, Buffalo, NY) and protein assay kit (Bio-Rad, Hercules, CA). Serum lysozyme activity was measured using the turbidimetric assay described by Parry et al. (1965). The assay employed 0.2 mg/ml *Micrococcus lysodeiktcus* suspended in sodium phosphate buffer (pH 7.2). 100 µl test sera were added to 100 µl phosphate buffer and 500 µl of suspended *M. lysodeiktcus* for a final volume of 700 µl. The reaction (24 °C; absorbance 540 nm) was measured at 30, 60 120 and 180 s, and the unit of lysozyme activity defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per min. Ceruloplasmin ferroxidase activity was quantified using a method based upon the catalytic oxidation of ferrous ions or ferrous complexes to the ferric state by ceruloplasmin (Cerón and Martínez-Subiela, 2004).

3. RNA isolation and microarray preparation

Total RNA was isolated from collected livers with the RNeasy RNA isolation kit (Qiagen, Valencia, CA). Each sample was precipitated with ethanol to concentrate the total RNA, and the resultant pellet was brought up to volume in RNase-free distilled water. The quality and the amount of starting mRNA was confirmed with a bioanalyser/agarose gel system (Agilent Technologies, Palo Alto, CA). The quality-checked total RNA was used for synthesizing biotin-labeled cRNA. Briefly, we used 10 µg of total RNA to genereate first-strand cDNA with a T7-linked oligo(dT) primer. After second-strand cDNA synthesis, *in vitro* transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY), resulting in approximately 100-fold amplification of cRNA. The cRNA was fragmented (15 µg per sample), spiked with internal controls (Affymetrix Inc., Santa Clara, CA) and hybridized overnight to the zebrafish gene chips (n = 9 chips total; i.e. three chips per treatment). The chips were washed and stained with streptavidin-phycoerythrin, before being scanned on the GeneChip scanner (Affymetrix, Inc.).

4. Data preprocessing

After import into the Bioconductor package (version 1.5) in R (version 1.9), the CEL files (containing the probe-level data, 22 gene spots per gene) were preprocessed using the RMA method to adjust the background, and to perform within- and between-chip (quantile) normalizations. Preprocessing of the data with RMA was more sensitive and specific and thus provided a more robust dataset than if the standard Affymetrix MAS 5.0 scaling or dCHIP techniques were used. The normalization process reduced unwanted technical variation. For an explanation and comparison of these methodologies, as well as their importance in microarray data analysis, the reader is referred to Saviozzi and Calogero (2003).

5. Filtering

Uninformative genes were eliminated from the dataset after implementing the preprocessing steps. Thus, genes with signals very near background, those that were considered absent by the Affymetrix scanner and genes that did not change expression values appre-

ciably across conditions were excluded. Since we were primarily interested in discovering genes with robust expression, eliminating the genes at the low end of the expression scale should not curtail this discovery. Filtering was performed in GeneSpring (version 7.2, Silicongenetics, Redwood City, CA).

6. Data analyses

After arriving at our quality-checked dataset (number of genes = 1,210), the individual gene chips were examined for reproducibility within the given conditions using two-dimensional scatter plots and hierarchical clustering in GeneSpring (Silicongenetics, Redwood City, CA). Principal components analysis (PCA) is a technique that can be used to simplify a dataset; more formally it is a linear combination of the variables (genes) that explains the maximum variation among these genes. PCA chooses a new coordinate system for the data set such that the greatest variance (determined by axis projections of the data set) comes to lie on the first axis (then called the first principal component, or PC1), the second greatest variance lies on the second axis (PC2), and so on. All PCAs were performed on GeneSpring (version 7.2, Silicongenetics, CA) using the full data set (roughly 15,000 genes). Thus, 15,000 variables were transformed linearly by the analysis into 15,000 new variables, or principal components. Herein, we used the first two PCs, describing 89% of the variability among the genes, to evaluate our treatment conditions.

7. Data analyses

All data were subjected to analysis of variance and means were compared by Duncan's Multiple Range Test with differences considered significant at the $P \leq 0.05$ level (SAS Inc., Cary, North Carolina).

RESULTS

No mortalities were recorded during the present trial. Irrespective of control, placebo or vaccination treatments hybrid striped bass grew at the same rate over the 8 wk of observation. Treatment therefore, had no effect upon weight, length, or condition factor. Final weights of fish from the control, placebo and vaccine groups were 150.4±30.4g, 149.4±21.1g and 157.1±35.8g respectively (Table 2), representing a tripling in animal weight over initial values.

Final fish lengths and condition factors were 205±12.1 mm, 204.9±9.0 mm 207.1±14.1 mm and and 1.72±0.08, 1.73±0.06 and 1.74±0.05 for the control, placebo and vaccinated hybrid striped bass respectively (Table 2). Feed conversion ratios (FCR) throughout the trial averaged 2.33. Data relating to hepatosomatic and visceral indices, IPF and muscle ratios are presented in Table 3. Hepatosomatic indices ranged between 1.6 ±0.1 and 1.7±0.2 whereas those for IPF and muscle ratios ranged between 5.0±0.8 and 5.2±0.7 and 26.0±4.3 and 28.9±5.5 respectively. Differences ($P < 0.001$) were observed between treatments with regard to VSI, which were higher in control fish (Table 2).

Table 2. Morphological indices of hybrid striped bass 8 weeks following vaccination, placebo injection, or no treatment. Data with different superscripts in a column were different (P < 0.05).

Tablica 2. Morfološki indeksi hibridnog lubina 8 tjedana nakon cijepljenja, cijepljenja placebo ili bez cijepljenja. Podaci s različitim eksponentima u pojedinom stupcu su različiti (P < 0,05).

Treatment/Tretman	Weight / Masa (g)	Length / Dužina (mm)	CF[1]	IPF[2]	HSI[3]	VSI[4]
Control/Kontrolni	150.4±30.4	205.0±12.1	1.72±0.18	5.0±0.8	1.7±0.2	13.1±2.0[a]
Placebo	149.4±21.1	204.9±9.0	1.73±0.06	5.2±0.7	1.6±0.3	10.2±1.2[b]
Vaccinated/Cijepljeni	157.1±35.8	207.1±14.4	1.74±0.05	5.1±0.8	1.6±0.1	10.2±0.8[b]

[1]CF = condition factor / Faktor kondicije
[2]IPF = intraperitoneal fat ratio / odnos intraperitonealne masnoće
[3]HSI = hepatosomatic index / hepatosomatski indeks
[4]VSI = visceral somatic index / visceralno somatski indeks

Blood-related parameters are summarized in Table 3. Packed cell volume and serum protein levels were similar across groups.

Table 3. Hybrid striped bass packed cell volume (PCV), plasma protein (PP) and serum lysozyme activity (units ml-1) 8 weeks following vaccination, placebo injection, or no treatment.

Tablica 3. Volumen koncentriranih stanica (PCV) hibridnog lubina, plazma protein (PP) i aktivnost serumskog lizozima (jedinice ml-1) 8 tjedana nakon cijepljenja, cijepljenja placebo ili bez cijepljenja.

Treatment/Tretman	PCV	PP	Lysozyme/Lizozim
Control/Kontrolni	41.4±6.3	8.1±0.9	97.2±27.9
Placebo	40.4±7.1	7.8±0.9	71.5±30.7
Vaccinated/Cijepljeni	42.7±7.7	7.6±0.8	126.5±34.6

Time-course of changes in serum lysozyme activity of control animals and those receiving vaccination or placebo injection are presented in Fig. 1. Following vaccination, which incorporated a handling stressor, serum lysozyme activity decreased but subsequently rebounded. While no differences were detected between treatments at the specific time points examined, evaluation of lysozyme activity at specific time-points revealed differences (P < 0.05) between 11 and 25 days post-vaccination (Fig. 1). Examination of the hepatic microarray datasets revealed only four immune-discrete genes that were impacted 56-days following vaccination when compared against control fish. These included the up-regulated TCIRG1, or T-cell immune regulator 1 (P < 0.0467) and IL20RA, or interleukin 20 receptor alpha (P < 0.0433), and down-regulated cytokine inducible kinase, plk3 (P < 0.01) and mouse immune responsive protein, IRG1 (P < 0.01).

Figure 2 summarizes results derived from principal component analysis on 15,000

genes. Vaccinated fish were distinguished from non-treated controls along PC1, and from the non-treated and placebo-injected controls by genes encoding components of the oxidative phosphorylation pathway, ATP synthesis and ribosomal proteins. The predominant gene separating the groups was one that encoded for a low density lipoprotein receptor.

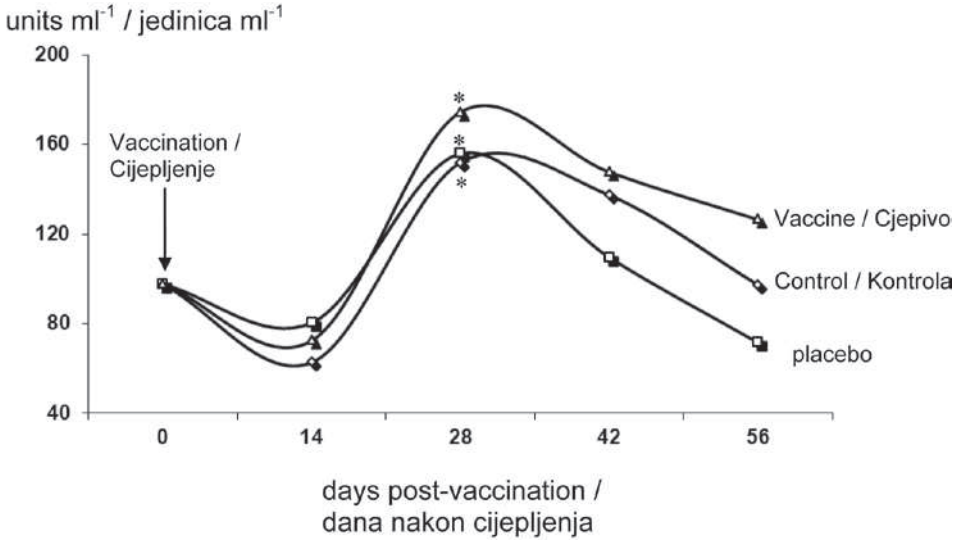

*Figure 1. Time-course of serum lysozyme response in hybrid striped bass following vaccination (Δ), placebo injection (□) or remaining untouched (◊) for a 56-day period. Vaccinated fish received a single dose (100µl) of an experimental oil-in-water Streptococcus iniae vaccine, whereas placebo injections comprised an identical volume of Courtland saline. No differences were discernable at specific time points for serum lysozyme activity, although significant elevations (P < 0.05; *) in activity were observed between days 11 and 25 post-vaccination (n = 5 per time and treatment)*

*Slika 1. Promjene u reakciji serumskog lizozima tijekom vremena kod hibridnog lubina nakon cijepljenja (Δ), cijepljenja placebo (□) ili bez cijepljenja (◊) za 56-dnevni period. Cijepljena riba je primila jednu dozu (100µl) eksperimentalnog cjepiva „ulje u vodi" vrste Streptococcus iniae, dok su placebo injekcije sadržavale jednaku količinu otopine soli Courtland. Nisu primijećene nikakve promjene u aktivnosti serumskog lizozima u određenim vremenskim intervalima, iako je zabilježen značajni porast (P < 0,05; *) aktivnosti između 11. i 25. dana nakon cijepljenja (n = 5 po danima i cijepljenju)*

DISCUSSION

Previous challenge-based studies with hybrid striped bass and the vaccine used herein determined a high level of protection over extended periods (Buchanan et al., 2005). Fish in the present experiment were not challenged since the primary objective of this study was to determine whether the experimental *S. iniae* vaccine imposed negative effects

upon animal production characteristics. No differences were discerned between treatment groups for FCR, or weight and length growth indicating that treatment had no impacts upon animal performance. This finding contrasts to the observations of others who have employed oil-based vaccine preparations. In general, the use of oil, aluminum, and other types of adjuvant has been reported to negatively impact fish growth and appetite. However, until relatively recently, side-effect-based investigations were exclusively undertaken with salmonids (and thus at > 17 ºC). Nevertheless, studies with other species also indicate that vaccination has varying negative impacts upon farmed fish. While not affecting growth, vaccination caused injection site lesioning in cold water marine species such as Atlantic cod *Gadus morhua* (Mikkelsen et al., 2004) and turbot *Scophthalmus maximus* (Björnsdóttir et al., 2004) whereas in sea bass *Dicentrachus labrax* (Afonso et al., 2005), an apparent reduction in appetite was also noted although this feature was not explicitly monitored. In Arctic charr *Salvelinus alpinus* Pylkko et al. (2000) observed negative growth responses immediately following vaccination but over the entire study no negative growth impact was recorded. Studies with rainbow trout *Oncorhynchus mykiss* (Mulvey et al., 1995) and common whitefish *Coregonus lavaretus* (Lonnstrom et al., 2001) likewise record no effects of vaccination upon growth.

It has been hypothesized that the adjuvant component is not responsible for observed growth reductions in vaccinated fish. Rather, it is the antigen or antigen x adjuvant interaction that is liable (Rønsholdt and McLean 1999) - a suggestion that appears to be supported by the findings of Melingen and Wergeland (2002). Others have speculated that growth reduction and loss of appetite following vaccination results due to irritation of the gut or intrusion upon normal swim bladder function (Poppe and Brech, 1997). Mutoloki et al. (2004) discerned a correlation between the magnitude of the reaction of vaccinated fish and the release rate of antigen from the adjuvant. Further, it has been proposed that reduced growth might be anticipated due to the increased energetic costs of a stimulated immune system (Ackerman et al., 2000). Indeed, results with respect to visceral somatic index in hybrid striped bass may support this supposition, since comparison between vaccinated and unhandled control animals revealed greater lipid deposition in the latter. Conceivably, reduced intraperitoneal fat depots may have resulted due to the increased metabolic costs associated with the heightening of the immune state. But growth and FCRs were not influenced during the present study which would argue against this suggestion, as too would the apparent reduction in lysozyme activity immediately following vaccine injection.

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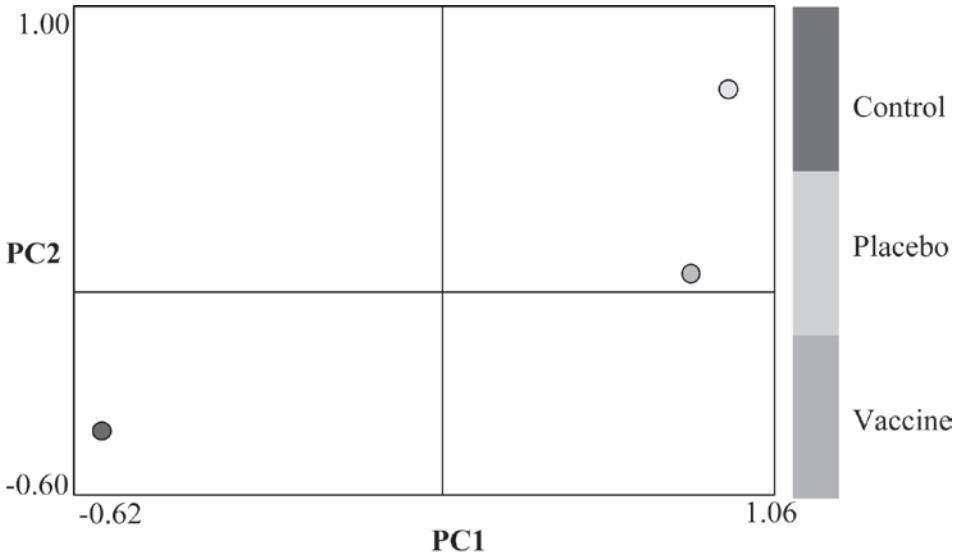

Fig 2. Principal components analysis on 15,000 genes. Plotted are the control (red), placebo (yellow) and vaccine (blue) conditions represented by all gene eigenvector values for principal components (PCs) 1 and 2. The vaccinated fish are distinguished from the non-treated controls along PC1, and from the non-treated and placebo-injected controls by genes encoding oxidative phosphorylation pathway components, ATP synthesis and ribosomal proteins. The predominant gene that separates these groups encodes a low density lipoprotein receptor.

Slika 2. Analiza glavnih komponenata 15.000 gena. Predstavljeni su kontrolni (crveno), placebo (žuti) i cijepljeni (plavi) uvjeti koji su prikazani pomoću svih vrijednosti svojstvenog vektora gena za glavne komponente (PCs) 1 i 2. Cijepljena riba se razlikuje od kontrolne skupine koja nije cijepljena uzduž PC1, ribe koja nije tretirana te one koja je primila placebo po genima koji kodiraju oksidativne fosforilacijske komponente staze, ATP sintezi i proteinima ribosoma. Dominantni gen koji razdvaja ove grupe kodira lipoproteinski receptor niske gustoće.

vaccinated and unhandled control animals revealed greater lipid deposition in the latter. Conceivably, reduced intraperitoneal fat depots may have resulted due to the increased metabolic costs associated with the heightening of the immune state. But growth and FCRs were not influenced during the present study which would argue against this suggestion, as too would the apparent reduction in lysozyme activity immediately following vaccine injection.

That serum lysozyme activity was initially reduced in vaccinated hybrid striped bass contrasts to the reported actions of vaccines in a number of salmonids where plasma lysozyme increased. Lysozyme activity has also been reported to remain unchanged in goldfish *Carassius auratus* vaccinated against *Aeromonas salmonicida* (Robertson et al.,

2005) and Nile tilapia *Oreochromis niloticus* vaccinated against *S. iniae* (Whittington et al., 2005). In Nile tilapia immunized against *Mycobacterium* extracellular products, significantly higher plasma lysozyme levels were recorded 4 days post-vaccination (Chen et al. 1998) and similar responses have been reported in other species including whitefish (Koskela et al., 2004), bester *Huso huso* x *Acipenser ruthenus* (Kolman et al., 1999) and olive flounder *Paralichthys olivaceus* (Li et al., 2005). The magnitude of change and indeed presence of lysozyme recorded in the present study was in general much lower than that observed with other species. Indeed, the only other reports for lysozyme activity in hybrid striped bass are an order of magnitude higher (Li et al., 2004). This difference may reflect husbandry methods employed (water temperature, quality etc.), fish source, size and age, and immunological maturity and status (i.e., previous challenges), since each of these parameters are known to impact lysozyme activity (Schrock et al., 2001; Langston et al., 2002). Moreover, adjuvant and vaccine type (preparation and pathogen) have likewise been shown to affect lysozyme activity (Ackerman et al., 2000). This apparent broad variability in lysozyme response following (a)biotic challenges might thus be used to question the scientific value of assessing this apparently almost indifferent parameter as an indicator of immune response. Clearly, more sensitive markers of cause and effect can be gained by examining the impact of vaccination upon gene expression.

The use of heterologous microarrays requires rigorous validations although excellent correlations have been observed from human gene chips hybridized with distantly-related mammalian species (bovine, porcine and canine), the *Arabidopsis* chip for cross-species studies and a cichlid chip for research with various cichlid species, a salmonid, poeciliid and cyprinid fish (Becher et al., 2004; Ji et al., 2004; Renn et al., 2004). In the present study, a zebrafish *Danio rerio* microarray was employed to examine gene expression profiles 53 days post-vaccination. While a variety of genes expressed up-, and down-regulation only four, following rigorous data pre-processing and filtering, were deemed of direct significance to the immune response of fish. These included T-cell immune regulator 1, a V-ATPase. All cells must regulate their pH within very narrow limits and the V-ATPases, a family of ATP-driven proton pumps, act to regulate and maintain a reservoir of ions within various cellular organelles to enable this. V-ATPases are important constituents of macrophages because these cells often function in regions where pH may become restrictive (Brisseau, 1996), as exemplified by areas of inflammation which occur following vaccination of fish (Afonso et al., 1998; Mutoloki et al., 2006). Likewise, up-regulation of the interleukin-20 receptor alpha suggests a response to inflammation (Xu, 2004) but reports also indicate a role for IL20RA stimulated monocyte expression of IL-6, TNF-α and reactive oxygen species in T-cells (Wang et al., 2003). Polo-like kinase 3 (plk3) is intimately involved in cell cycle regulation and appears to be activated when cells are exposed to H_2O_2. Down-regulation of this enzyme may be considered beneficial since apoptosis would be arrested (Xie et al., 2001; Wang et al., 2002). IRG1 promotes the interferon response to viral infection and these genes may be down-regulated to enable focus upon non-viral defenses which may be anticipated in response to vaccination for bacterial diseases.

Clearly, injection of hybrid striped bass with the experimental *S. iniae* formulation had limited impact upon production performance. Such findings have economic significance to aquaculture since feed costs represent the largest operating variable during

the commercial fish farming and losses (feed and growth) would not be incurred with use of this vaccine. Indeed, appetite, as indicated by FCRs, remained unaffected, which contrasts to the situation for salmonids. The application of "aquanomic" (McLean and Craig, 2006) technology to pinpoint specific genes affected by vaccination represents an obvious target for future research and delineation of biomarkers that will enable more thorough investigations of the consequences that vaccination has upon directing whole animal response to (re)vaccination.

Sažetak

CIJEPLJENJE HIBRIDNOG LUBINA: RAST, IMUNOLOŠKA REAKCIJA I EKSPRESIJA GENA

P.A. Cotter[1], E. McLean[2], S.R. Craig[3], J.C. Craig[4], M. Westerman[5]

Hibridni lubini (42,6 ± 4,9 g mokre težine; 139,3 ± 6,1 mm dužine) su nasumce stavljeni u jedan od 6 spremnika (n=6 spremnik^{-1}) s recirkulirajućim sustavom za održavanje života (RLSS) izrađenim po mjeri. Kvaliteta vode je bila sljedeća: DO_2 (6,5 ± 0,6 mg l^{-1}), pH (7,7 ± 0,5), TAN (0,06-1,31 mg l^{-1}), nitrit (0,06-0,60 mg l^{-1}) i nitrat (2,0-32,1 mg l^{-1}), salinitet 5 ppt, temperatura 28 ± 1 °C. 12:12 fotofaza:skotofaza je korištena s 30-minutnim zatamnjivanjem svjetala u zoru i sumrak. Ribe su hranjene s udjelom od 4% tjelesne težine d^{-1} u dva odvojena hranjenja (u 08.00 i 16.00 sati). Razina hranidbenog sirovog proteina i lipida je bila 40%, odnosno 10%.

Spremnici su bili nasumce spajani te je riba bila netretirana, cijepljena ili cijepljena placebo injekcijom. Cjepivo koje je korišteno je eksperimentalni bakterin s adjuvansom „ulje u vodi" vrste *Streptococcus iniae* usmrćene formalinom. Riba je vagana i mjerena dvaput mjesečno tijekom 8 tjedana, a težinske grupe su se koristile za prilagođavanje količine hrane. Po završetku testiranja, sve ribe su vagane i mjerene te je izračunat njihov faktor kondicije (CF) i odnos promjene hranjenja (FCR). Visceralno somatski (VSI) i hepatosomatski (HSI) indeksi te intraperitonealna masnoća (IPF) i mišićni odnos (MR)

1 Paul Cotter graduated with an M.S. specializing in hybrid striped bass aquaculture at Virginia Tech., Department of Fisheries & Wildlife Sciences, 100 Cheatham Hall, Blacksburg VA 24060-0321, USA;

2 Ewen McLean, Ph.D., e-mail: ewen.mclean@utt.edu.tt (corresponding author), tel: (868) 634 8888; fax: (868) 634 3008, Programme Professor, Marine and Environmental Sciences, at the University of Trinidad and Tobago, Marine and Environmental Sciences, Phase III, Maritime Campus, University of Trinidad and Tobago, Second Avenue, Northwestern Main Road, Chaguaramas, Trinidad W.I;

3 Steven R. Craig, Ph.D. is Director of Nutrition Research at Virginia Cobia Farms, Virginia Cobia Farms, LLC, 108 Battleground Avenue, Saltville, VA 24370, USA;

4 Johanna Craig, Ph.D. is Founder and Principal of GATACA LLC, GATACA, LLC, 180 Orchard Hill Lane, Newport, VA 24128, USA;

5 Mark Westerman, Ph.D. is President and CEO of Intrinsic LifeSciences LLC, Intrinsic Life Sciences, 505 Coast Boulevard South, La Jolla, CA 92037, USA

su također procijenjeni. Anesteziranoj ribi se vadila krv i zabilježen je hematokrit. Dopušteno je da se tijekom noći krv zgruša na 5 °C nakon čega je zabilježena razina serumskih proteina i izmjerena aktivnost lizozima. Jetra su pripremljena za procjenu mikroniza koristeći se standardnim tehnikama pomoću genskog čipa *Danio rerio* kako bi procijenili globalne promjene u ekspresiji gena.

Nije primijećena nikakva razlika u konačnoj težini, dužini, CF-u, FCR-u ili HSI-u iako su razlike (P < 0,001) utvrđene za VSI koji je bio viši kod kontroliranih riba. Volumen koncentriranih stanica i razina serumskih proteina slični su u svim grupama (P > 0,05). Promjene u aktivnosti serumskog lizozima tijekom vremena ukazuju na početno smanjenje aktivnosti lizozima, nakon čega slijedi preokret u aktivnosti koji doseže svoj vrhunac 25 dana nakon tretmana. Procjena aktivnosti lizozima u vremenskim razmacima otkrila je razlike (P < 0,05) od 11 do 25 dana nakon cijepljenja. Ispitivanjem zbira podataka hepatičkog mikroniza otkrivena su samo četiri imuno-diskretna gena na koje je cjepivo djelovalo nakon 53 dana, u usporedbi s kontroliranom skupinom. Oni uključuju 'up-regulirani' TCIRG1, ili imunološki regulator T-cell 1 (P < 0,0467) i IL20RA, ili interleukin 20 receptor alfa (P < 0,0433), te 'down-reguliranu' kinazu koja inducira citokin, plk3 (P < 0,01) i protein s imunološkom reakcijom kod miševa, IRG1 (P < 0,01).

Ključne riječi: lizozim, transkriptomika, mikroniz, *Streptococcus iniae*

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Received: 4. 6. 2012.
Accepted: 7. 9. 2012.