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Breeding strategy and rearing environment effects on the disease resistance of cultured Chinook salmon (*Oncorhynchus tshawytscha*)

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ABSTRACT

Most Pacific salmon farms propagate fish by artificial random mating, which along with artificial hatchery rearing conditions may result in unintentional selection of undesirable traits. Alternatively, salmon can be propagated using outdoor semi-natural raceways that would provide the opportunity for both sexual and natural selection to act on offspring production. We performed a disease challenge on one-year-old smolts of Chinook salmon (*Oncorhynchus tshawytscha*) to test the effects of breeding strategy and rearing environment on immune function. Farmed sexually mature salmon were bred following traditional aquaculture methods whereas others were allowed to spawn semi-naturally in outdoor spawning channels. The offspring were reared in their natal environment for six months when they were subjected to a reciprocal environment transplant and held for 5 additional months. Subsequently, fish were exposed to a *Vibrio anguillarum* waterborne challenge. A strong environmental effect on mortality was found for the hatchery-bred fish whereas the channel-bred fish showed no such response, perhaps indicative of a more canalized immune response. A two-way ANOVA resulted in a significant interaction between the breeding/early-rearing strategy and the reciprocal transplanted environment factors ($F_{0.05,\,1,\,12} = 17.95$, $P < 0.0012$). In addition, humoral immune response measurements revealed a similar interaction between these two factors at 28 ($P = 0.0014$) and 42 days ($P = 0.0022$). These results are indicative of genotype-by-environment interaction effects on the immune system of Chinook salmon. Remarkably, these effects were observed after only one round of sexual/natural selection using fish that have been under artificial selection for at least nine generations. The benefits of environmental canalization of the immune response may be an important advantage of channel-bred over hatchery-bred fish for the aquaculture industry. We suggest that introducing sexual/natural selection through mate choice via semi-natural spawning channels may lead to fish with more robust immune systems across changing environments.

1. Introduction

Genetic quality of hatchery broodstock is of concern to the rapidly developing aquaculture industry (Gjedrem, 2012). Though there are some selective breeding programs based on quantitative genetic principles, this approach is rather expensive since it takes 10–20 years to have an economic impact on production traits, making it impractical for many new cultured species (Hulata, 2001). The majority of current salmon farms propagate fish stocks by artificial random mating, which may be accompanied by some selective breeding for desirable traits such as growth rate (Gjedrem, 2010). Though these procedures have resulted in improvements in some selected traits, long-term artificial breeding and hatchery rearing environments tend to diminish genome-wide genetic variation (Kraaijeveld-Smit et al., 2006). Such approaches may also unintentionally select for undesired traits in hatchery populations. For example, egg size has been shown to decrease in artificially bred and captive reared Chinook salmon (Heath et al., 2003). Fleming and Gross (1993, 1994) found a genetic basis for changes in salmonid reproductive behavior resulting from artificial mating. The hatchery rearing environment per se is known to contribute to the fixation of adaptive characters for artificial environments but those traits may be maladaptive under natural conditions (Lynch and O'Hely, 2001). Hatchery selection was also found to develop boldness in newly hatched brown trout (*Salmo trutta*), which may be linked to a risk-prone aggressive phenotype (Sundström, 2004). In addition, rearing environment prior to smolting showed profound effects on the development of major organs such as the brain (Kihslinger and Nevitt, 2006). For example, Lema et al. (2005) found smaller telencephalon in salmonids reared under hatchery conditions, compared to wild fish.

Those findings highlight an important aspect of captive breeding and rearing that should be taken into consideration when part of the life cycle is in an uncontrolled, natural environment, as is the case with salmonids reared in ocean net pens (Jonsson and Jonsson, 2009). These

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unpredictable and, in the long term, undesired consequences for salmonid aquaculture production make it critical that other approaches to freshwater breeding and rearing be considered for commercial culture. An alternative form of salmon propagation and early-rearing based on semi-natural spawning channels is being used by salmon enhancement programs for Pacific salmon on the west coast of North America. The semi-natural spawning channels are outdoor graveled-bottom raceways with regulated water flow. They have several potential advantages compared with common intensive hatchery practices. First, maintenance costs are significantly lower as they are basically limited to an annual cleaning of sediment before each breeding season. Second, despite being artificial constructs, semi-natural spawning channels offer a much more natural environment than the traditional vertical-stacked incubation trays and the fiberglass or aluminum tanks used for egg incubation and fry rearing in most hatcheries. The spawning channels are outdoors, therefore they are under natural photoperiod and natural organic matter enriches the bottom accumulations supporting protozoan and insect growth on which fish can feed. Third, the use of spawning channels allows the broodstock to spawn with little human intervention (other than selecting fish density), allowing the natural process of sexual selection to occur (Neff and Pitcher, 2008).

Mate choice is a well known process described in many vertebrate taxa, including salmonids (Quinn, 2005) which have been shown to have a non-random mating system involving major histocompatibility (MH) gene-linked female mate choice (Bernatchez and Landry, 2003; Evans et al., 2012; Garner et al., 2010). MH genes are the main component of the adaptive immune system (Murphy et al., 2007) constituting a highly polymorphic family of protein receptors that present exogenous peptides to T cells (Germain, 1994). The high polymorphism of their peptide binding regions (PBRs) in natural populations is thought to be maintained by natural and sexual selection, as has been shown for different teleost species (Fraser et al., 2010; Turner et al., 2009). It has been proposed that sexual and natural selection act through heterozygote advantage where each MH gene variant has an affinity for particular pathogen motifs, hence heterozygote individuals are able to recognize a broader spectrum of foreign peptides than their homozygote counterparts (Doherty and Zinkernagel, 1975). Another way natural selection could maintain variability at MH loci is frequency-dependent selection, under which common alleles and genotypes drive resistance against pathogens, thus favoring rare alleles that are novel for pathogen resistance. Individuals carrying particular alleles may be favored if they provide protection against widespread pathogens (Arkush et al., 2002; Grimholt et al., 2003; Lohm et al., 2002). Once the rare alleles become more common by selection on the host they will, in turn, drive selection for pathogen resistance, thus constituting an evolutionary "arms race" (Penn and Potts, 1999). In population genetic models, "good genes" in mate choice contribute to the additive genetic variation whereas "compatible genes" adds to non-additive genetic variation (Neff and Pitcher, 2004). Non-additive genetic variation includes interactions between homologs at a gene locus (dominance effects) and the interactions between genes at different loci (Neff and Pitcher, 2008). Because of their complex nature, MH genes can have both additive and non-additive genetic effects on fitness (Pitcher and Neff, 2006), and it has been hypothesized that MH gene polymorphism driven by sexual selection may have the opportunity to increase fitness (Evans et al., 2012). Nevertheless, despite the potential benefits of sexual/natural selection, the effectiveness of semi-natural spawning channel breeding has not yet been comprehensively assessed, and the salmon aquaculture industry still relies primarily on artificial mating and controlled tank-rearing environments.

To quantify the potential advantages of spawning channels over traditional aquaculture practices we evaluated the effects of breeding strategy and rearing environment on the immune response of Chinook salmon (*Oncorhynchus tshawytscha*) under disease challenge (vibriosis exposure). We bred sexually mature Chinook salmon from a commercial salmon farm following traditional aquaculture methods, while others were allowed to spawn semi-naturally in spawning channels. Offspring from each of these two breeding groups were subjected to a reciprocal freshwater environment transplant for five months after a six-month rearing period in their natal environment. After a *V. anguillarum* challenge, we monitored mortality, assessed humoral immune response and analyzed the MH class II β1 genotypes of mortalities and survivors. This methodology allowed us to assess the effects of breeding and rearing environment (and their interaction) on the immune performance of the captive salmon. Although we found no evidence for clear performance differences between the breeding/rearing strategies, we did find differences in the genotype-by-environment (GxE) interaction, indicating that the semi-naturally bred fish had environmentally canalized immune systems, allowing them to better respond to changing environments. The incorporation of natural/sexual selection and semi-natural early rearing represents a valuable culture technique for salmon aquaculture.

2. Materials and methods

2.1. Fish

This study was conducted using Chinook salmon from a commercially farmed population maintained for nine generations at Yellow Island Aquaculture Ltd. (YIAL), Quadra Island, British Columbia, Canada. YIAL is an organic salmon farm that stopped using antibiotics in 1989. In the following years, between 1990 and 1994, the farm lost about 65% of their production stock in outbreaks due to two common Pacific salmon diseases: bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum* and vibriosis due to *V. anguillarum*. Thus, the YIAL stock has been likely selected for BKD and vibriosis resistance.

2.2. Breeding and early-rearing treatment

Two fish groups, "channel" (CH) and "hatchery" (H), were generated in November 2006 following two breeding strategies using YIAL broodstock. The CH fish group was the offspring of broodstock allowed to spawn semi-naturally in 3.5 × 15 m spawning channels of about one meter water depth with a partially recirculating flow of approximately 300 L min^{-1}. Spawning channels were populated with 20 females (in an equal ratio of 4- and 5-year-old) and 12 males each. "Jack Channels" received two 3- and two 4-year-old males along with eight "jacks" (2-year-old mature males) whereas "No Jack Channels" received an equal mix of 3- and 4-year-old males. Carcasses were removed daily from which fin clips and scales were collected. Fertilized eggs remained buried in the gravel until fry emerged in the spring and were reared in the channel until seining in May 2007 (~6 months post-spawning). Spawning channels were located outdoors subjected to natural photoperiod and surrounded with mesh nets to prevent the entrance of predators. Automatic feeders fed the fish to satiation with commercial pellets (Ewos Canada Ltd., Surrey, BC). On the other hand, the H fish group was obtained following standard hatchery techniques characterized by artificial mating. Eggs and milt from randomly chosen broodstock were mixed for fertilization in a 2 ♀ × 3 ♂ cross design using 4- and 5-year-old females along with jack, 3- and 4-year-old males. Fertilized eggs were incubated in vertical stack incubation (Heath) trays with a constant water flow of approximately 15 L min^{-1}. On 28 Feb. 2007, fry were transferred to two 1000 L fiberglass tanks. A "Jack Tank" received 1440 fry constituted by 60 fish from each of 24 families created with 8 females and 12 males, including jack males. A "No Jack Tank" received 1400 fry constituted by 70 fish from each of 20 families created with 10 females and 10 males, none of them jacks. Fish were maintained until May 2007 under artificial light with a 16:8 light:dark cycle and fed to satiation with commercial pellets (Ewos Canada Ltd., Surrey, BC). Both CH and H fish experienced their natal environment (i.e. early-rearing environment: spawning channel and hatchery, respectively) during their first six months from fertilization.

2.3. Environmental transplant treatment

The first week of May 2007, CH fish were collected by seine from the channels and subgroups of CH and H fish were subjected to a reciprocal transplant between the two freshwater environments. A total of four spawning channels and four hatchery tanks were set up for the environmental transplant. Two channels contained a total of 400 CH fish each: 100 from channels that did not receive male jacks plus 300 from channels that did include jack males. Two other spawning channels were stocked with 400H fish each, 100 fish from families that did not include male jacks in the crosses and 300 fish from families that included jack males. The hatchery counterpart consisted of four 600 L indoor tanks with a 16:8 light:dark cycle. Two tanks were set up with 400 CH fish each: 100 fish from channels that did not receive male jacks plus 300 fish from channels that did include jack males. Two other tanks received 400H fish each, 100 fish from families that did not include male jacks in the crosses and 300 fish from families that included jack males. All fish were fed to satiation with commercial pellets (Ewos Canada Ltd., Surrey, BC). In summary, four fish groups were created by a five-month reciprocal environment transplant: CH fish reared in the channels (CH/CH), CH fish moved to hatchery tanks (CH/H), H fish reared in hatchery tanks (H/H) and H fish moved to the channels (H/CH).

2.4. Disease challenge set-up

At the end of the five-month reciprocal transplant period, in early October 2007, fish from each of the four groups were uniquely finclipped and mixed in equal numbers in four replicate 600 L tanks. Fish were acclimatized in these tanks supplied with UV-treated pumped seawater for three weeks, during which they went through the smoltification process. Subsequently, the disease challenge was started with fish numbers in each replicate tank as shown in Table 1. Two replicate tanks, A1 and A2, contained 92 fish from each of the four groups totalling 368 fish per tank. The two other replicate tanks, B1 and B2, contained fewer fish from the CH/H group, 32 and 34 CH/H fish each, due to stock limitations of this treatment group. Extra fish from the other three groups were added to maintain similar density levels (Table 1). We used 2-way contingency table analyses to test for an effect of the differences in numbers from each treatment group on mortality.

V. anguillarum was obtained from a stock culture maintained at 4 °C in the facilities of the Pacific Biological Station (Nanaimo, BC; case No. 2004-124). A small amount of the culture was streaked onto trypticase soy agar (TSA) and grown at room temperature (RT) for 48 h. A slide agglutination test with rabbit anti-*V. anguillarum* antibodies confirmed that the strain corresponded to the O1 serotype. In addition, Gram negative staining and motility on drop glasses were also performed. The bacteria cultures for the disease challenge were prepared by inoculating three colonies grown in TSA plates to 40 mL of TSB placed in 50 mL Falcon® tubes and grown for 30 h at RT. The number of bacteria the fish were actually exposed to was estimated with 10× serial dilutions to determine the number of colony forming units (CFU). Twenty-five microliter of each dilution factor was plated in replicate TSA plates and grown for 48 h. The number of colonies per plate was counted on plates containing the 10^{-5} dilution factor.

The disease challenge consisted of an initial bacteria exposure that resulted in a fewer than expected mortalities (between 80 and 92 individuals per tank), thus a second exposure was performed on the 22nd day of the experiment. The first exposure consisted of placing the fish from each replicate tank into 50 L water baths with an estimated bacteria concentration of 8.6×10^4 cells/mL supplied with an air stone that kept bacteria mixed and suspended. The exposure time was 15 min and fish were returned to their respective tanks. This methodology was applied to each of the four replicate tanks, preparing a new challenge bath each time. The second exposure was performed in a 600 L tank divided into four compartments with a mesh net so that each of these received the fish from one replicate tank. Fish were exposed for one hour in a 160 L water bath with an estimated bacteria concentration of 3.7×10^5 cells/mL and four air stones. Mortalities were collected on a daily basis every 8 to 12 h and presence of bacterial infection was confirmed in a total of 64 mortalities throughout the experiment by streaking head kidney tissue on TSA plates and grown for 48 h to allow colony formation. Fin clips, gill, spleen, head kidney and blood samples were collected before exposure and post-infection at 24 and 96 h, and then at 21, 28, 31, 35, 39, and 42 days by euthanization with an overdose of MS-222 (Syndel Intl. Inc., Vancouver, BC). The surviving fish were PIT-tagged for individual identification and moved to a sea cage, after which two additional blood samplings were performed at 210 and 350 weeks for antibody response measurement. Tanks containing fish from the same groups kept nearby in the same hatchery building showed no mortalities over the experimental period, so mortalities were presumed to all be induced by the disease exposure.

Table 1
Experimental set up of replicate tanks for the disease challenge.
Fish numbers from each group allocated in each of the four replicate tanks (named A1, A2, B1, B2) prior to the disease challenge. (*) CH/H fish group was limited therefore less fish were available for replicate tanks B1 and B2. Fish from the remaining groups were added to maintain similar density levels in all tanks. CH/CH: channel-bred fish reared in the channels. CH/H: channel-bred fish moved to hatchery tanks. H/CH: hatchery-bred fish moved to the channels. H/H: hatchery-bred fish reared in hatchery tanks.

	A1	A2	B1	B2
CH/CH	92	92	114	112
CH/H	92	92	32*	34*
H/CH	92	92	114	112
H/H	92	92	114	112
Total fish	368	368	374	370

2.5. MH class II β1 genotyping

A total of 31 mortalities and 31 survivors from the disease challenge (Mortalities: 7 CH/CH, 8 CH/H, 9H/CH, 7H/H. Survivors: 8 CH/CH, 6 CH/H, 8H/CH, 9H/H) were fin clipped followed by DNA extraction using a standard phenol-chloroform method (Sambrook and Russell, 2001). We modified the primers that amplify the hypervariable region (exon 2) of MH class II β1 described in Miller et al. (1997) by adding a 12-nucleotide sequence containing a recognition site for the HindIII restriction enzyme. A two-stage PCR (PCR + 1, Borriello and Krauter, 1990) was adapted to avoid undesired heteroduplexes that leads to the formation of mosaics of the true loci (L'Abbé et al., 1992). The protocol consisted of an asymmetric PCR (reverse primer concentration was 5× higher than the forward primer) followed by the addition of a second forward primer in excess and an extra PCR cycle run. Reactions with 100 ng of DNA were started in a total volume of 25 μL containing 1× reaction buffer, 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.02 U μL^{-1} Taq, 0.04 μM of the unmodified forward primer B1FA 5′-CTTGGTCTTGAC TTGMTCAGTCA and 0.2 μM of the modified reversed primer B1RAHindIII 5′-CCCGAGAAGCTTCCGATACTCCTCAAAGGACCTGCA (restriction site sequence underlined). The amplification conditions were 5 min. at 95 °C and 34 cycles of 45 s at 95 °C, 30 s at 57 °C, and 45 s at 72 °C, followed by an extension of 2 min at 72 °C. An extra cycle with the same amplification conditions but with a final extension of 10 min was run after supplying each tube with 1 μL of 10 μM of the modified forward primer B1FAHindIIIb 5′-ATAGAGAAGCTTGGTCTTGACTTG MTCAGTCA. This last PCR run incorporated the forward HindIII primer into the amplicons. PCR products were inserted into pGEM-T Easy vectors as per manufacturer instructions (Promega Corporation, Madison, WI, USA). Ligation products were then transformed into XL1-blue *Escherichia coli* competent cells following the Inoue procedure (Sambrook and Russell, 2001) and grown in Luria Bertani (LB) plates with ampicillin (100 μg/mL), X-gal (100 μg/mL) and 1 μM isopropyl β-D-1-thiogalactopyranoside. Individual white colonies were re-grown in LB tubes and plasmid DNA was extracted from bacteria using an

alkaline miniprep protocol (Sambrook and Russell, 2001). Plasmid DNA was digested for 30 min with HindIII (Fermentas, USA) and electrophoresed in a 1% agarose gel. Target fragments were extracted directly from gels using GenElute Agarose Spin Columns (Sigma-Aldrich, St. Louis, MO, USA). Eight to twelve fragment inserts were sequenced on a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using ABI BigDye Terminator v3.1 fluorescent dye-terminators with T7 and Sp6 primers. The sequences obtained were aligned manually using BioEdit v. 5.0.9 (Hall, 1999) and were deposited in GenBank (accession numbers KC859612–KC859619).

2.6. Assessment of humoral immune response

Humoral immune response was assessed in a total of 279 serum samples collected throughout the experiment. The levels of anti-*Vibrio* antibodies in fish serum were measured using an indirect enzyme-linked immunosorbent assay (ELISA) with each sample analyzed by triplicate totaling eleven 96-well plates. Antigen was prepared by homogenizing *V. anguillarum* serotype O1 and O2 vaccine (Microtek Intl. Inc., Saanichton, BC, Canada) with 0.1 mm zirconia/silicone beads. Polycarbonate 96-well plates (Evergreen Scientific, CA, USA) were coated overnight at RT with 100 μL per well of antigen diluted in one volume of coating buffer (15 mM Na_2CO_3, 34 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6). Plates were rinsed with tris buffered saline containing 0.05% Tween 80 (T-TBS) and blocked for one hour at RT with 300 μL of 5% skim milk in T-TBS per well. Plates were rinsed and probed for two hours at RT using 100 μL of fish serum dilution in BSA blocking buffer. After rinsing, a secondary rabbit anti-salmonid antibody diluted 1:1000 was added and incubated for another two hours at RT. Following rinsing, a third antibody, goat anti-rabbit whole molecule alkaline phosphatase conjugate 1:2500 dilution was incubated at 37 °C for one hour and rinsed again. Detection was performed using 50 μL per well of p-nitrophenyl phosphate (Fast p-NPP; Sigma, MO, USA) in the dark at RT for 30 min. Reactions were stopped by adding 50 μL of 0.03 M NaOH and absorbance was read at 405 nm using a microplate reader (VERSAmax microplate reader, Molecular Devices).

We applied an unbalanced randomized complete block design (RCBD) that considered each 96-well plate as a block while at the same time allowed us to compensate for the unbalanced aspect of the analysis (i.e. the number of samples taken from each treatment group was not always the same at each particular sampling time). We used ANOVA to test for the effects of a) breeding and early rearing environment and b) transplant environment, as well as the interaction effects on the anti-*Vibrio* antibody levels. Specifically, we applied a factorial design Type III ANOVA with plate as a fixed factor and breeding and early rearing, and transplant environment, as random factors. The number of plates was fixed and their effects were not relevant for our study since plates carried the ELISA inter-assay variation. Statistical analyses to test random factor effects were performed using the Statistical analysis system (SAS) v. 9.2 (SAS Institute Inc., Cary, NC). A sequential Bonferroni correction for multiple tests (Holm, 1979) was performed to recalculate significance values. The results obtained were arranged in an ascending order and compared with a starting P value of $\alpha = 0.05/(n = 10) = 0.005$ (i.e. there were 10 hypotheses).

3. Results

3.1. Disease challenge mortality

All replicate tanks resulted in similar overall daily mortalities with cumulative percentages of 24, 19.9, 22 and 22.1% for tanks A1, A2, B1 and B2, respectively (Fig. 1). Mortality was independent of the differences in numbers from each treatment group as contingency table P values resulted not significant: 0.55, 0.34, 0.20 and 0.53 for H/H, H/CH, CH/H and CH/CH, respectively. When treatment groups were examined across replicate challenge tanks, a box and whisker plot revealed a strong environmental effect on mortality in the H fish (Fig. 2). Mean mortality (percent) of H/H was 15.4% (S.D. = 2.8) whereas H/CH fish reached 33.6% (S.D. = 5.0). On the other hand, mean mortality for CH fish was similar in both reciprocal transplanted rearing environments: 20.0% (S.D. = 3.4) and 23.4% (S.D. = 7.7) for CH/CH and CH/H respectively. A two-way ANOVA resulted in a significant interaction effect ($F_{0.05,\ 1,\ 12} = 17.95$, $P < 0.0012$).

3.2. MH class II β1 genotyping

Twelve MH class II β1 nucleotide alleles were identified in the 62 fish analyzed. These coded for eight amino acid alleles presenting two major sequence motifs (Fig. 3). This is a low number of alleles for this many individuals when compared with studies on wild populations, but the

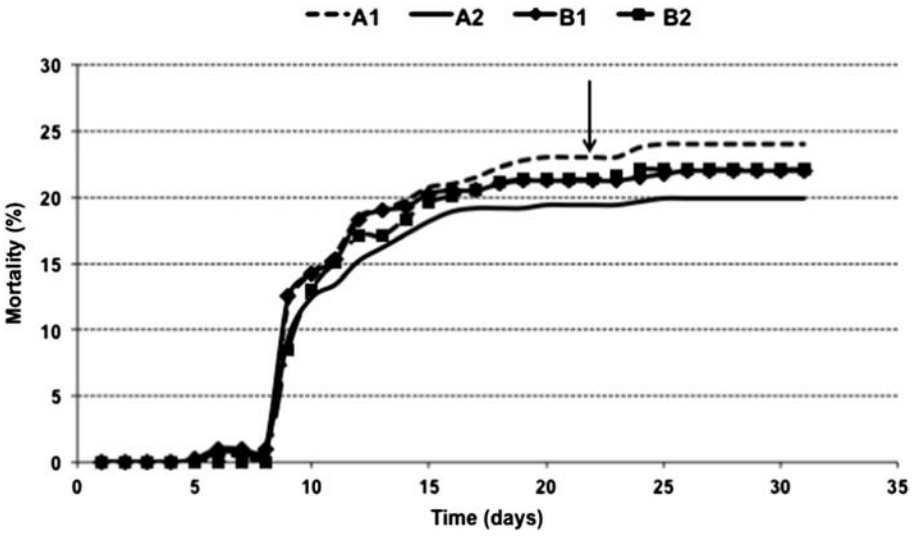

Fig. 1. Cumulative daily mortality (%) after a live *Vibrio* challenge. Cumulative mortality of Chinook salmon in four replicate tanks (A1, A2, B1 and B2) based on daily mortality counts resulting from a live *Vibrio* challenge. Replicate tanks presented similar trends in daily mortality patterns. Fish stopped dying by day 20 after initial exposure. A second exposure to live *Vibrio* produced a little mortality increase observed at day 23. Final mortality percentage in each tank ranged from 19.9 to 24%. Tanks A1 and A2 contained same number of fish from each fish group. Tanks B1 and B2 contained fewer H/CH fish, which was compensated by adding more fish of the remaining fish groups (H/H, CH/H and CH/CH). H/CH: hatchery-bred fish moved to the channels. H/H: hatchery-bred fish reared in hatchery tanks. CH/H: channel-bred fish moved to hatchery tanks. CH/CH: channel-bred fish reared in the channels. The arrow indicates the date of the second exposure to *Vibrio*.

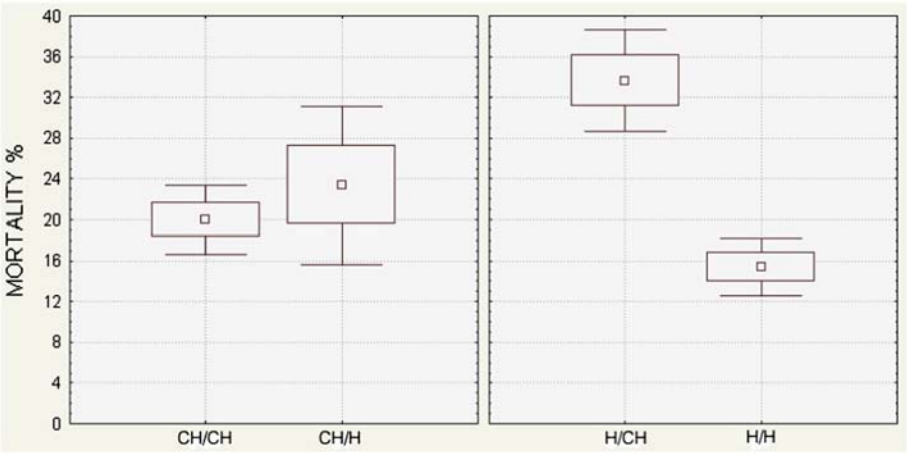

Fig. 2. Susceptibility (mortality) of Chinook salmon to *Vibrio anguillarum*. Box plot showing mean percent mortality of Chinook salmon to *V. anguillarum* after being subject to different breeding and rearing treatments. A two-way ANOVA resulted in a significant interaction ($P = 0.0012$) of the two factors: a) breeding and early-rearing strategy, and b) transplant environment. CH/CH = channel-bred fish reared in outdoor channels. CH/H = channel-bred fish moved to hatchery tanks. H/H = hatchery-bred fish reared in hatchery tanks. H/CH = hatchery-bred fish moved to outdoor channels.

YIAL stock has been through a bottleneck (see Section 2.1). Alleles of the main lineage *Onts*-DAB*01 were found in every fish analyzed, whereas alleles of the lineage *Onts*-DAB*02 were found in just 12 individuals. No bias in MH class II β1 alleles for disease resistance was detected since both survivors and mortalities showed similar *Onts*-DAB*01 allelic distributions (Fig. 3). As the primers amplified up to four MH class II β1 alleles per individual, we ran 2-way contingency table analyses to test for differences in the proportion of low allelic diversity (one and two alleles) and high allelic diversity (three and four alleles) between the CH and H fish groups, and also between mortalities and survivors. The CH fish presented relatively equivalent numbers of individuals with low ($n = 16$) and high ($n = 13$) allelic diversity, whereas the H fish group comprised many more fish with low ($n = 25$) than with high ($n = 8$) allelic diversity (Fig. 4). However, the contingency table analysis was not significant at the 0.05 level ($P = 0.09$). Moreover, looking at the relationship between susceptibility and allelic diversity, we found survivors with low ($n = 18$) and high ($n = 13$) MH allelic diversity, whereas there were 23 versus 8 mortalities with low and high MH allelic diversity respectively. However, the Chi-square test for this contingency table was also not significant ($P = 0.18$).

3.3. Humoral immune response

Following the sequential Bonferroni correction, the results of the statistics were arranged in ascending order and compared with a starting P value of $\alpha = 0.05/10$, as there were 10 hypotheses tested. Two sampling events (Table 2) showed significant interaction effects at 28 ($P = 0.0014$) and 42 days ($P = 0.0022$) after Bonferroni correction.

4. Discussion

The effect of the transplant rearing environment on the disease resistance of the H fish was notable, as mortality was significantly higher for fish reared in the spawning channels in comparison to those reared in hatchery tanks. Several studies have addressed how abiotic environmental parameters such as temperature, photoperiod, pH, oxygen level and salinity may affect the fish immune system (reviewed by Bowden, 2008). Many of those environmental parameters are, to some extent, altered under aquaculture rearing conditions such as those YIAL uses to rear Chinook salmon. Alleles associated with important fitness traits such as disease resistance may be selected against, making the fish unfit for the wild environment, while alleles better suited for the artificial environment may be favored. This phenomenon, which is associated with captive breeding and domestication, has been previously discussed in the literature related to salmon supplementation (Heath et al., 2003; Lynch and O'Hely, 2001; Petersson et al., 1996) but scarcely addressed in the salmon aquaculture industry. Therefore the disease susceptibility observed in the present study in the H fish after they were reared in a semi-natural environment, appears to be the consequence of long-term relaxed selection for an efficient immune system due to an artificial and relatively pathogen-free freshwater environment, although further experimentation would be required to definitively prove that. On the other hand, the CH fish showed moderate disease susceptibility in the two transplant rearing environments reflecting a canalized immune response that was capable of overriding environmental effects on the fish immune system. Environmental canalization refers to genotypes that exhibit small changes in phenotype when subject to environmental variation as opposed to phenotypic

	10 20 30 40 50 60 70	Surv.	Mort.
Onts-DAB*0101	GIEFIDSYVFNKAEYIRFNSTVGRYVGYTELGVKNAEAWNKGPQLGQEQAELERFCKPNAALHYRAILDKT	27	28
Onts-DAB*0102	.....E.....	10	10
Onts-DAB*0103	.....S.....	18	10
Onts-DAB*0104	.....E.....	8	11
Onts-DAB*0201	.....V.N.....	1	2
Onts-DAB*0202	.....V.N....G.....	2	0
Onts-DAB*0203	.....V.H.....H.....	1	0
Onts-DAB*0204	.....H.....V.H.....L.....V.....E.....	6	0

Fig. 3. Amino acid sequences of MH class II β1 alleles from Chinook salmon. Amino acid sequence alignment of the eight MH class II β1 alleles from 62 Chinook salmon (31 survivors and 31 mortalities) exposed to live *Vibrio anguillarum*. Sequences encompass 71 amino acids from the exon 2 of the MH class II β1 locus, containing a hypervariable region important in antigen presentation to T cells. Two main lineages (*Onts*-DAB*01 and *Onts*-DAB*02) can be distinguished. Columns on the right indicate number of survivors and mortalities having each allele.

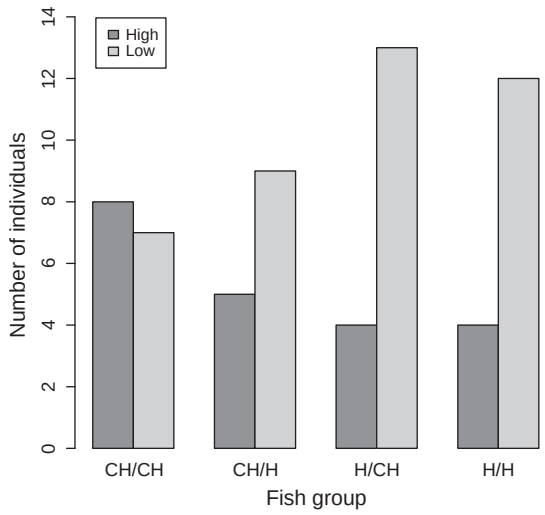

Fig. 4. Frequency of Chinook salmon with low and high MH class II β1 allelic diversity in each environmental transplant treatment fish group. Frequency histogram (counts) of individuals with one or two alleles (low allelic diversity), and those with three or four alleles (high allelic diversity), for each environmental transplant treatment group. The CH fish presented relatively similar number of individuals with low and high allelic diversity, whereas the H fish showed a higher proportion of fish with low allelic diversity, though it was not significant at the 0.05 level ($P = 0.09$). CH/CH = channel-bred fish reared in outdoor channels. CH/H = channel-bred fish moved to hatchery tanks. H/H = hatchery-bred fish reared in hatchery tanks. H/CH = hatchery-bred fish moved to outdoor channels.

plasticity, which refers to a significant change in phenotype (van Buskirk and Steiner, 2009). Hence, the contrasting mortality patterns of H and CH fish when reared in different environments are indicative of complex genotype-by-environment (GxE) interaction effects. Evidence for GxE interactions in survivorship at the larval and fry stages of Chinook salmon has been previously reported in translocation experiments between natural populations (Evans et al., 2010). A noteworthy result of our study is that this interaction effect was detected not only at the whole organism level (i.e. mortality due to vibriosis), but also at the molecular level (i.e. antibodies generated by the elicited humoral immune response).

The genotyping results expressed the complex nature of the immune response of Chinook salmon. The fact that 75% of the mortalities analyzed had low MH class II β1 allelic diversity agrees with the common understanding that individuals with a greater number of different MH receptors are better at mounting an effective immune response (Doherty and Zinkernagel, 1975). However, individuals with low MH allelic diversity were also well represented among survivors (58%) indicating that a low number of MH alleles was not a precondition to succumb to disease. This could be a result of the genetic bottleneck experienced by the YIAL production stock when antibiotic treatment was stopped in the early 1990s, which produced massive mortalities due to BKD and vibriosis. Selection then may have favored alleles providing resistance to vibriosis, which would also explain the low number of mortalities (20 to 24% per tank) in our disease challenge. Contrary to the findings in some other studies on salmonids (Grimholt et al., 2003; e.g. Lohm et al., 2002; Wynne et al., 2007), we found no association between particular MH class II β1 alleles and susceptibility to the disease challenge. This observation is in accordance with Pitcher and Neff (2006) who found that, in addition to additive genetic variation, non-additive genetic variation at MH class II β1 loci also affects survivorship in Chinook salmon. Moreover, complex traits such as disease resistance are generally accepted as polygenic, having non-MH genes contributing to variation in resistance as well (Pitcher and Neff, 2007). The extent to which the epistatic effects of these genes contribute to variation in disease susceptibility is still unknown, but should not be neglected (Carlborg and Haley, 2004).

The contrasting, though not significant ($P = 0.09$), MH class II β1 allelic diversity found between CH and H fish groups could only have come from sexual and natural selection in the channels since the same broodstock was used to generate both groups. A previous study on Chinook salmon using spawning channels reported that spawners mated non-randomly, with an increase in genetic diversity at the MH class II β1 gene in the offspring, therefore contributing to additive genetic variation (Neff et al., 2008). Furthermore, recent publications have described MH-linked mate choice that contributed to non-additive genetic variation in different vertebrate taxa. For instance, in the freshwater fish Chinese rose bitterling, *Rhodeus ocellatus*, female mating preferences for non-additive benefits were found to correlate with MH dissimilarity (Agbali et al., 2010). The mechanisms of sexual selection are of particular importance for fish species without post-hatch parental care, as is the case for salmonids (Gross and Sargent, 1985). The potential role of sexual selection is even more critical for semelparous Pacific salmon species that die after spawning, as they have only one opportunity to mate and to leave descendants. For example, YIAL's founder broodstock was taken from Robertson Creek, a DFO-operated hatchery that has been artificially propagating Chinook salmon since 1972 (DFO, nd). Thus, the Chinook salmon population used in this study has been under artificial selection for at least nine generations. Remarkably, our results showed significant differences in the disease resistance of the YIAL stock after only one round of sexual selection and early rearing in a semi-natural environment. Our finding is highly relevant for commercial rearing of salmon in the aquaculture industry, as there may be other fitness-related traits favored by the introduction of more natural breeding methods. We therefore recommend that sexual selection should be incorporated into breeding strategies by the aquaculture industry when propagating and managing salmon stocks. Furthermore, we cannot rule out the possibility that the allelic diversity of the CH fish may have been under the influence of natural selection in the channel environment as well. Evidence of pathogen-driven selection in Atlantic salmon (*Salmo salar*) has been reported in natural environments (Dionne et al., 2009). As pathogens were not controlled for in our spawning channels, selection could have acted upon the genetic variation of the CH fish by eliminating maladapted genotypes during early rearing in the spawning channels. Therefore both, sexual and natural selection combined appears to have modeled the MH diversity of the swim-up and both have likely contributed to the observed canalized disease resistance of the CH fish.

In conclusion, our study showed that altering major aspects of the salmonid life cycle fundamentally affects fitness-related traits

Table 2
Humoral immune response of Chinook salmon infected with vibriosis.
Analysis of the humoral immune response of Chinook salmon subjected to different breeding/early-rearing strategies and an environmental transplant factor after infection with live *V. anguilarum*. Samples were collected at ten sampling points during the disease challenge and afterwards, when fish were transferred to sea cages. Plate number column lists the ELISA plates used for each sampling time analyzed. N indicates the total number of fish assessed by triplicate per sampling time. P values were obtained from a type III ANOVA model with unbalanced RCBD and were significant (*) for interaction effects at 28 and 42 days after Bonferroni correction.

Time (days)	Plate number	N	P values for interaction
0	1,2	15	0.1328
4	1,2	14	0.4375
21	1,2,3,4	32	0.1929
28	1,2,3,4,5	32	0.0014 (*)
31	5,6,7,8	32	0.1681
35	5,6,7,8	32	0.9095
39	5,6	12	0.3479
42	6,7,8	24	0.0022 (*)
210	7,8,9,10	36	0.1949
350	9,10,11	31	0.3464

important to commercial culture, ergo impacting production parameters. The high level of domestication and selection (intentional or not) that farmed salmon experience in hatcheries was shown to affect their immunocompetence. Artificial mating and early rearing in an artificial environment made offspring's immune system more sensitive to an environmental change after 6 months of age. Contrary to that, spawning channel technology led offspring to a moderate disease susceptibility regardless of the rearing environment experienced afterwards. Those effects were likely mediated by sexual and natural selection acting at immune-function related loci, such as MH, and other genes simultaneously. These results are indicative of complex GxE interactions affecting the disease resistance of Chinook salmon, leaving immune challenges impractical for broodstock selection efforts (Aykanat et al., 2012). Although our study primarily focused on the freshwater rearing environment, we should mention that the salmon aquaculture industry usually transfers artificially reared juveniles into sea cages, which exposes them to a natural environment that harbors a different suite of pathogens, and hence immune challenges (Kent, 2000). Stocking the spawning channels with semi-naturally bred broodstock instead of artificially bred broodstock, could help to elucidate long-term effects natural propagation processes have on fitness-related traits relevant to salmon farming. We suggest that more natural breeding protocols in addition to semi-natural rearing environments may provide commercial salmon industry with fish having more robust immune systems, capable to respond to changing environments.

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References

Agbali, M., Reichard, M., Bryjová, A., Bryja, J., Smith, C., 2010. Mate choice for nonadditive genetic benefits correlate with MHC dissimilarity in the rose bitterling (*Rhodeus ocellatus*). Evolution 64, 1683–1696.

Arkush, K.D., Giese, A.R., Mendonca, H.L., McBride, A.M., Marty, G.D., Hedrick, P.W., 2002. Resistance to three pathogens in the endangered winter-run chinook salmon (*Oncorhynchus tshawytscha*): effects of inbreeding and major histocompatibility complex genotypes. Can. J. Fish. Aquat. Sci. 59, 966–975.

Aykanat, T., Heath, J.W., Dixon, B., Heath, D.D., 2012. Additive, non-additive and maternal effects of cytokine transcription in response to immunostimulation with Vibrio vaccine in Chinook salmon (*Oncorhynchus tshawytscha*). Immunogenetics 64, 691–703.

Bernatchez, L., Landry, C., 2003. MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? J. Evol. Biol. 16, 363–377.

Borriello, F., Krauter, K.S., 1990. Reactive site polymorphism in the murine protease inhibitor gene family is delineated using a modification of the PCR reaction (PCR + 1). Nucleic Acids Res. 18, 5481–5487.

Bowden, T.J., 2008. Modulation of the immune system of fish by their environment. Fish Shellfish Immunol. 25, 373–383.

Carlborg, O., Haley, C.S., 2004. Epistasis: too often neglected in complex trait studies? Nat. Rev. Genet. 5, 618–625.

DFO (Ed.), n.d. Robertson Creek Hatchery. URL http://www.pac.dfo-mpo.gc.ca/sep-pmvs/projects-projets/robertson/robertson-eng.htm (accessed 2.13).

Dionne, M., Miller, K.M., Dodson, J.J., Bernatchez, L., 2009. MHC standing genetic variation and pathogen resistance in wild Atlantic salmon. Philos. Trans. R. Soc. B-Biol. Sci. 364, 1555–1565.

Doherty, P.C., Zinkernagel, R.M., 1975. A biological role for the major histocompatibility antigens. Lancet 1, 1406–1409.

Evans, M.L., Neff, B.D., Heath, D.D., 2010. Quantitative genetic and translocation experiments reveal genotype-by-environment effects on juvenile life-history traits in two populations of Chinook salmon (*Oncorhynchus tshawytscha*). J. Evol. Biol. 23, 687–698.

Evans, M.L., Dionne, M., Miller, K.M., Bernatchez, L., 2012. Mate choice for major histocompatibility complex genetic divergence as a bet-hedging strategy in the Atlantic salmon (*Salmo salar*). Proc. R. Soc. Lond. B 279, 379–386.

Fleming, I.A., Gross, M.R., 1993. Breeding success of hatchery and wild coho salmon (*Oncorhynchus kisutch*) in competition. Ecol. Appl. 3, 230–245.

Fleming, I.A., Gross, M.R., 1994. Breeding competition in a Pacific salmon (Coho: *Oncorhynchus kisutch*): measures of natural and sexual selection. Evolution 48, 637–657.

Fraser, D.J., Houde, A.L.S., Debes, P.V., O'Reilly, P., Eddington, J.D., Hutchings, J.A., 2010. Consequences of farmed-wild hybridization across divergent wild populations and multiple traits in salmon. Ecol. Appl. 20, 935–953.

Garner, S.R., Bortoluzzi, R.N., Heath, D.D., Neff, B.D., 2010. Sexual conflict inhibits female mate choice for major histocompatibility complex dissimilarity in Chinook salmon. Proc. R. Soc. Lond. B 277, 885–894.

Germain, R.N., 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 76, 287–299.

Gjedrem, T., 2010. The first family-based breeding program in aquaculture. Rev. Aquac. 2, 2–15.

Gjedrem, T., 2012. Genetic improvement for the development of efficient global aquaculture: a personal opinion review. Aquaculture 344–349, 12–22.

Grimholt, U., Larsen, S., Nordmo, R., Midtlyng, P., Kjoeglum, S., Storset, A., Saebø, S., Stet, R.J.M., 2003. MHC polymorphism and disease resistance in Atlantic salmon (*Salmo salar*); facing pathogens with single expressed major histocompatibility class I and class II loci. Immunogenetics 55, 210–219.

Gross, M.R., Sargent, R.C., 1985. The evolution of male and female parental care in fishes. Integr. Comp. Biol. 25, 807–822.

Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Heath, D.D., Heath, J.W., Bryden, C.A., Johnson, R.M., Fox, C.W., 2003. Rapid evolution of egg size in captive salmon. Science 299, 1738–1740.

Holm, S., 1979. A simple sequentially rejective multiple test procedure. Scand. J. Stat. 65–70.

Hulata, G., 2001. Genetic manipulations in aquaculture: a review of stock improvement by classical and modern technologies. Genetica 111, 155–173.

Jonsson, B., Jonsson, N., 2009. A review of the likely effects of climate change on anadromous Atlantic salmon *Salmo salar* and brown trout *Salmo trutta*, with particular reference to water temperature and flow. J. Fish Biol. 75, 2381–2447.

Kent, M.L., 2000. Marine netpen farming leads to infections with some unusual parasites. Int. J. Parasitol. 30, 321–326.

Kihslinger, R., Nevitt, G.A., 2006. Early rearing environment impacts cerebellar growth in juvenile salmon. J. Exp. Biol. 209, 504–509.

Kraaijeveld-Smit, F.J.L., Griffiths, R.A., Moore, R.D., Beebee, T.J.C., 2006. Captive breeding and the fitness of reintroduced species: a test of the responses to predators in a threatened amphibian. J. Appl. Ecol. 43, 360–365.

L'Abbé, D., Belmaaza, A., Décary, F., Chartrand, P., 1992. Elimination of heteroduplex artifacts when sequencing HLA genes amplified by polymerase chain reaction (PCR). Immunogenetics 35, 395–397.

Lema, S.C., Hodges, M.J., Marchetti, M.P., Nevitt, G.A., 2005. Proliferation zones in the salmon telencephalon and evidence for environmental influence on proliferation rate. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 141, 327–335.

Lohm, J., Grahn, M., Langefors, A., Andersen, Ø., Storset, A., von Schantz, T., 2002. Experimental evidence for major histocompatibility complex-allele-specific resistance to a bacterial infection. Proc. R. Soc. Lond. B 269, 2029–2033.

Lynch, M., O'Hely, M., 2001. Captive breeding and the genetic fitness of natural populations. Conserv. Genet. 2, 363–378.

Miller, K.M., Withler, R.E., Beacham, T.D., 1997. Molecular evolution at Mhc genes in two populations of chinook salmon *Oncorhynchus tshawytscha*. Mol. Ecol. 6, 937–954.

Murphy, K.M., Travers, P., Walport, M., 2007. Janeway's Immunobiology. Garland Sciences.

Neff, B.D., Pitcher, T.E., 2004. Genetic quality and sexual selection: an integrated framework for good genes and compatible genes. Mol. Ecol. 14, 19–38.

Neff, B.D., Pitcher, T.E., 2008. Mate choice for non-additive genetic benefits: a resolution to the lek paradox. J. Theor. Biol. 254, 147–155.

Neff, B.D., Garner, S.R., Heath, J.W., Heath, D.D., 2008. The MHC and non-random mating in a captive population of Chinook salmon. Heredity 101, 175–185.

Penn, D.J., Potts, W.K., 1999. The evolution of mating preferences and major histocompatibility complex genes. Am. Nat. 153, 145–164.

Petersson, E., Järvi, T., Steffner, N., Ragnarsson, B., 1996. The effect of domestication on some life history traits of sea trout and Atlantic salmon. J. Fish Biol. 48, 776–791.

Pitcher, T.E., Neff, B.D., 2006. MHC class IIB alleles contribute to both additive and nonadditive genetic effects on survival in Chinook salmon. Mol. Ecol. 15, 2357–2365.

Pitcher, T.E., Neff, B.D., 2007. Genetic quality and offspring performance in Chinook salmon: implications for supportive breeding. Conserv. Genet. 8, 607–616.

Quinn, T., 2005. The Behavior and Ecology of Pacific Salmon and Trout. University of Washington Press, Seattle.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sundström, L.F., 2004. Hatchery selection promotes boldness in newly hatched brown trout (*Salmo trutta*): implications for dominance. Behav. Ecol. 15, 192–198.

Turner, S.M., Chaves-Campos, J., DeWoody, J.A., 2009. Parental relatedness and major histocompatibility effects on early embryo survivorship in Atlantic salmon. Genetica 137, 99–109.

van Buskirk, J., Steiner, U., 2009. The fitness costs of developmental canalization and plasticity. J. Evol. Biol. 22, 852–860.

Wynne, J.W., Cook, M.T., Nowak, B.F., Elliott, N.G., 2007. Major histocompatibility polymorphism associated with resistance towards amoebic gill disease in Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol. 22, 707–717.