Rainbow trout spleen size is highly heritable, genetically correlated with specific disease resistance, and affected by multiple QTL

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ABSTRACT

Selective breeding of animals for increased disease resistance is increasingly employed as a strategy to reduce losses in aquaculture. However, implementation of selective breeding programs is limited by an incomplete understanding of host resistance immunogenetics and correlated traits. We previously reported results of a selection program in which family survival was increased, relative to control, following challenge with Flavobacterium psychrophilum, the causative agent of bacterial cold water disease (BCWD). In addition, a positive phenotypic correlation was identified between family average spleen somatic-index (SI) and family survival following F. psychrophilum challenge suggesting a link with disease resistance. In the current study, we report the inheritance of SI and genetic correlation with BCWD resistance in odd and even-year lines evaluated over five year-classes. A total of 322 pedigreed families (n=25,369 fish) were measured for disease resistance, and 251 families (n=5,645 fish) were evaluated for SI. Spleen index was highly heritable ($h^2=0.72 \pm 0.08$ odd-year line, and $h^2=0.84 \pm 0.10$ even-year line) and there was a positive genetic correlation with BCWD resistance ($r_g=0.14 \pm 0.08$ odd-year line, and $r_g=0.32 \pm 0.11$ even-year line). Complex segregation analyses provided evidence for genes with major affect on SI, and a genome scan of a single family using 314 microsatellite markers detected significant genome-wide QTL on chromosomes 5 and 19 in addition to 17 suggestive QTL. This is the first estimation of spleen size heritability, identification of chromosomal regions, and evidence of a genetic link between SI and specific disease resistance in a teleost fish.
Introduction

Rainbow trout (*Oncorhynchus mykiss*) is a widely cultured fish species produced in freshwater and marine environments with 2008 global production estimated at 576,289 metric tons and valued at $2.39 billion USD (FAO, 2010). An important disease impacting salmonid aquaculture is bacterial cold-water disease (BCWD). The etiological agent of BCWD is a gram-negative bacterium, *Flavobacterium psychrophilum*, which also causes rainbow trout fry syndrome (RTFS) in small fish (Nematollahi et al., 2003). Economic losses from *F. psychrophilum* are due to direct mortality and also to deformities in fish that survive infection (Groff and LaPatra, 2000; Nematollahi et al., 2003). Scientists at the NCCCWA established a selective breeding program in 2005 to improve rainbow trout BCWD survival for several reasons. First, the pathogen is widely distributed and disease prevention though biosecurity is not currently feasible. Second, there is no commercial vaccine for BCWD or RTFS, and salmonids can be infected at early life stages prior to typical vaccination (Brown et al., 1997; Cipriano, 2005; Nematollahi et al., 2003; Vatsos et al., 2006). Third, limited chemotherapeutics are currently available, and finally, additive genetic variation for survival following *F. psychrophilum* challenge has been demonstrated indicating a favorable potential for family-based selective-breeding for increased resistance (Henryon et al., 2005; Leeds et al., 2010; Silverstein et al., 2009).

The NCCCWA has implemented even- and odd-numbered year family-based selection programs with the separate goals of increasing disease resistance and increasing growth, respectively. Phenotyping to measure BCWD resistance was initiated in 2005 using 2.4-g sized fish. Disease resistance was demonstrated to be a moderately heritable...
trait that was not adversely correlated with growth performance (Silverstein et al, 2009). Furthermore, we found that most families maintained their relative resistant or susceptible phenotype as average body weight increased over 300-fold (Hadidi et al, 2008). These results demonstrated that the resistance mechanism(s) were not expressed in a transitory manner during development. In these studies, resistant families displayed larger-than-average spleen size normalized to body weight (spleen index, SI), while susceptible families had smaller-than-average SI. After challenge, there remained a significant difference in SI between the challenged-susceptible and the challenged-resistant families on days 3 and 5 post-infection. Furthermore, on day 5 post-challenge, resistant fish had a 14-fold lower bacterial load in the spleen indicating a greater capacity to limit bacterial growth or trafficking to the spleen following challenge. These results demonstrated that whether naïve or challenged, the SI of fish from resistant families was larger than that of the treatment-matched fish from susceptible families and that this trait was associated with a reduced load of the pathogen in the spleen. The association between resistance and SI was confirmed in families from our 2006 year-class where families were phenotyped first for SI, and this was found to predict *F. psychrophilum* post-challenge survival (Hadidi et al, 2008). Based on the results from the 2005 and 2006 year class fish, we postulated a genetic link between rainbow trout survival following *F. psychrophilum* challenge and SI.

In the current study, we tested the hypothesis that genetically-linked genes influence both spleen size and BCWD survival by quantifying the genetic correlation between both traits over five pedigreed year-classes. The stability of family-mean SI during fish growth was determined, and a model of inheritance is proposed based on parent to
offspring transmission and complex segregation analyses using phenotypic data. In addition, using molecular markers we report the identification of multiple QTL influencing SI, and we directly examine the number, genomic positions, effects and interactions of significant QTL and their contribution to genetic variance (i.e. genetic architecture).
Materials and methods

Animals

All fish were maintained at the NCCCWA and animal procedures were performed under the guidelines of NCCCWA Animal Care and Use Committee Protocols #029, 033, 047 and 053. Each year, rainbow trout were spawned in late December through February and the embryos were developmentally-synchronized using water temperature manipulation as previously described (Leeds et al., 2010) in order to synchronize hatch dates within an approximate 2-week period in late February or early March. Samples from random fish representing the five broodstock cohorts were screened for pathogens and found negative for the presence of salmonid bacterial pathogens *Renibacterium salmoninarum* and *Yersina ruckeri*, and viral pathogens viral hemorrhagic septicemia, infectious hematopoietic necrosis virus, and infectious pancreatic necrosis virus. Post-hatch, families were maintained in separate tanks receiving 12-14°C flow-through well and spring water and fed a standard commercial diet (Ziegler Bros, Inc., Gardners, PA). The pellet size and percent body weight were adjusted according to NCCCWA standard feeding protocol for rainbow trout and fish were maintained at biomass densities <50 kg/m³. After growth to 15g, feed was delivered to each tank using Arvotec (Huutokoski, Finland) automatic feeders. Tricaine methanesulfonate, MS 222 (Sigma) was used to anesthetize the fish at 100 mg L⁻¹ for routine handling and weighing; while for sampling, fish were sacrificed using a 200 mg L⁻¹ concentration for at least 5 minutes.

Phenotyping

In general, fish were challenged at the smallest feasible size as BCWD mortality is typically highest when fish are between 0.2 to 4 g body weights. Families were
separately maintained and evaluated for survival in a total of eight challenge experiments (Table S1). Fish age ranged from 82 to 190 days post-hatch at the time of challenge. *Flavobacterium psychrophilum*, strain CSF 259-93, was used for all challenges and was initially provided by Dr. S. LaPatra (Clear Springs Foods, Inc.). This strain is commonly utilized in challenge studies (Crump *et al.*, 2001; LaFrentz *et al.*, 2003; LaFrentz *et al.*, 2004) and the clone used in this study was selected as a single colony in 2003, expanded in broth culture, and aliquots frozen at -80°C. Thawed bacteria were grown on TYES plates at 15°C for 5 days as described previously (Hadidi *et al.*, 2008). Each fish was injected intraperitoneally at the base of the pelvic fin with a suspension of *F. psychrophilum* cells in PBS. Challenge dose and injection volume were determined based on the fish population average body weight. The number of viable bacteria injected per fish was determined by plate count either immediately before or after fish challenge and ranged from 0.2 x10^6 to 3.8 x10^6 CFU per g body weight (Table S1). All fish were examined for typical clinical signs of disease including injection site hemorrhage, tissue necrosis and associated lesion(s), and a limited number of moribund or dead fish were examined using either gram stain or plate culture to confirm the presence of the challenge pathogen.

Measurements of spleen somatic-index were performed using naïve fish that were not part of experimental challenges. Total body weight was determined using a PG5002-S DeltaRange balance (Mettler Toledo) to 0.01g, or for larger fish, weighed with a Champ CW11 digital stainless steel balance (Ohaus) to 5g. The spleen was then removed and care taken to completely cut away any attached fat and connective tissue. Spleens were
weighed with an AB104 balance (Mettler Toledo) to 0.0001 g. Spleen somatic-index was calculated by dividing organ weight (mg) by total body weight (g).

**Pedigree of odd-year class rainbow trout line**

BCWD survival evaluation was initiated in 2005 (G₀ population) using 71 full-sib (FS) families from the 2005 year class that have been described (Silverstein et al, 2009). From each of the 71 families, a random sample comprised of 40-80 fish were challenged with *F. psychrophilum* using one or two replicates per family, n=40 fish per tank (Table S1). Four families with high survival (denoted R) and four families with low survival (denoted S) were identified for further study (Hadidi et al, 2008). Spleen size was measured in 15 fish from each of the R and S families in 2005 (Table S2). The non-disease challenged siblings from six of the eight R and S families were used as breeders to generate the F₁ QTL (2007) population.

In 2007, two groups were phenotyped for BCWD resistance and spleen size. The first population G₁ (2007) consisted of 97 FS families, of which 63 were part of the 2007 select line (Leeds et al, 2010), and 34 were additional families created by outcrossing a 2005 select-line parent with one that was not. The second group F₁ BCWD QTL (2007) was created to detect QTL for BCWD resistance and consisted of 15 FS families from RxR, RxS and SxS parents (Vallejo et al, 2010). Five of the RxR families were also evaluated in the G₁ (2007) challenge. Spleen size was measured in both the G₁ and F₁ BCWD QTL (2007) families (n=107 FS families) (Table S2). The number of measured fish per family varied from 9 to 60.

In 2009, three populations were phenotyped in several challenge experiments. The first experiment (Table S1, challenge 5) included 10 families from a F₂ mapping
experiment designed to detect BCWD QTL (Vallejo et al., 2010). The second experiment (Table S1, challenge 6) included 114 families G2 (2009) of which 96 families were from the select line (Leeds et al., 2010), as well as 18 susceptible families derived from the same base population G0 (2005) as the select line. The BCWD select line has since been designated ARS-Fp-R line and the susceptible families as the ARS-Fp-S line and maintained as closed populations. The third experiment (Table S1, challenge 7) included a total of 8 families that were part of F2 BCWD QTL mapping experiments, QTL#2a and 2b (2009), as well testing 5 families of the ARS-Fp-R line and 5 families from the ARS-Fp-S line that were part of a field trial validation study (Wiens et al, unpublished data). The 10 families from the ARS-Fp-R and ARS-Fp-S lines were also represented in G2 2009 challenge experiment, and were phenotyped for spleen size (Table S2).

Pedigree of even-year class rainbow trout line

Families used in this study from the even-year line were a synthetic population developed from the crossing of six domesticated strains (listed in descending order of % contribution): 1) University of Washington, Donaldson; 2) Kamloops/Puget Sound Steelhead cross; 3) Ennis National Fish Hatchery, Arlee; 4) Ennis National Fish Hatchery, Shasta; 5) Idaho Department of Fish and Game, Hayspur; and 6) Kamloops. Phenotyping of 100 full-sib families from the even-year line was initiated in 2006 and has been described previously (Hadidi et al., 2008). In 2008, a total of 15 full-sib families were created to detect spleen size QTL, using parents that were from families evaluated in 2006 for SI, and categorized as high spleen index (H) and low spleen index (L). A total of five HxH, HxL and LxL crosses were created and offspring assayed for both post-challenge survival (n=120 fish per family, mean age 114 days) and spleen index in naïve
fish measured at five intervals over a 500-day growth period (mean posthatch age ± SD of the five intervals were 145 ± 7, 170 ± 6, 241 ± 7, 323 ± 6, and 633 ± 5 days with mean BW ranging from 6g to 3,716g; Table S2).

**Estimation of heritability and genetic correlation**

Heritability of survival following BCWD challenge (binary trait; fish that died were assigned a value of 0 and fish that survived were assigned a value of 1) and spleen index of naïve fish, and the genetic correlation between these traits, were estimated with single and 2-trait linear animal models using MTDFREML (Boldman et al., 1995). The model for survival following BCWD challenge included fixed effects of family mean body weight at the time of challenge (linear covariate) and challenge date and a random effect of challenge tank nested within family. The model for spleen index included fixed effects of age at the time of measurement (linear covariate) and year. The environmental covariance between the two traits was fixed at 0 because only one trait was measured per fish and there is no known or postulated environmental correlation. Variance of the likelihood in the simplex less than $1 \times 10^{-9}$ was used as the convergence criterion. Global minimum was assumed when $-2 \times$ logarithm of likelihood was identical to the third decimal place after “cold” restarts of the simplex with prior estimates of variance components. The even and odd-year populations differed in genetic background, and thus the datasets were analyzed separately. A total of 4,472 and 26,828 animals were used in the calculation of the inverse of the numerator relationship matrix for the even- and odd-year datasets, respectively. Standard errors of the genetic correlation estimates were approximated as $[(1 – r_A^2)/\sqrt{2}] \times \sqrt{[(\sigma_{h^2_x} \cdot \sigma_{h^2_y})/(h^2_x \cdot h^2_y)]}$, where $r_A^2$ is genetic.
correlation (squared), $h^2$ is heritability, and $\sigma^2_{h2}$ is standard error of the heritability estimate (Falconer and Mackay, 1996).

**Complex segregation analysis of spleen index using iBay**

In order to examine the mode of inheritance and identify potential families for QTL mapping, complex segregation analyses were carried out using phenotypic data collected from even-year line fish. Records from 1,348 fish were used in these analyses and included 1048 fish from fifteen 2008 F$_1$ QTL families and 300 records from full sibs of the parents (2006 year class). Two models: A (total body weight, age and year) and B (total body weight and age) were subjected to multivariable regression analyses using STEPWISE model selection with SAS Proc REG (SAS, 2007) to identify fixed effects and covariates to include in the BSA models. Bayesian segregation analyses (BSA) was carried out using iBay and eight mixed-inheritance models were evaluated and compared as described previously (Vallejo et al, 2010). The major gene genotypes were predicted for parents of rainbow trout families with iBay version 1.46 (Janss, 2008).

**Genotyping and linkage analyses**

A single cross 2008132 was selected for linkage analyses based on phenotypes and predicted parental major gene genotypes from BSA. Over a 100-day growth period, a total of 327 fish from cross 2008132 were phenotyped for SI and fin-clipped. Selective genotyping was performed using 194 fish from the SI distribution: 89 fish had SI<1.22 (27.2% of fish sampled), 103 fish had SI>1.45 (31.5% of fish sampled), and 2 fish were sampled from the middle of the distribution. For linkage analysis, a total of 336 microsatellite markers were genotyped using standard methods, and from this data, a total of 314 markers were mapped onto 28 autosomes and one sex chromosome using
MULTIMAP version 2.0 (Matise et al, 1994). The markers selected for the genome scan were previously mapped to the NCCCWA genetic map (Palti et al, 2011; Rexroad et al, 2008) with high LOD score and with the spacing between pairs of adjacent markers smaller than 20 cM.

QTL analysis

Before performing the QTL analysis, we performed STEPWISE model selection with SAS Proc REG (SAS, 2007) to assess the affect of the covariate age on the response variables (body weight and spleen related traits).

The marker information content was calculated as described (Knott et al, 1998) and implemented in the web-based software QTL Express (Seaton et al, 2002). This provides an indication as to how informative the multiple markers are at any chromosome location. The marker information content combines the multilocus probability of individuals inheriting allele 1 or 2 from the common parent with marker segregation distortion (Knott et al, 1996).

We performed half-sib (HS) regression interval mapping with the web-based software QTL Express (Seaton et al, 2002) using the module for HS family analysis. This software implements a multi-marker approach of interval mapping in HS families (Knott et al, 1996). The QTL genome scan was performed using a sire-family and a dam-family, separately, without making any assumptions on the number and frequency of QTL alleles within the studied population.

Briefly, a QTL with a gene substitution effect is fitted at 1-cM intervals along the chromosome using the linear model,
where $y_{ij}$ is the trait score of individual $j$ from sire (or dam) $i$; $a_i$ is average effect for HS family $i$; $b_i$ is regression coefficient within HS family $i$ (substitution effect of QTL); $x_{ij}$ is the conditional probability for individual $j$ of inheriting allele 1 (or 2); $z_{ij}$ is the covariate age effect for individual $j$ from sire (or dam) $i$; and $e_{ij}$ is the random residual.

**Distribution of F statistics**

The test statistic was calculated as an $F$ ratio for every 1-cM map position. The $P$-value was calculated assuming an $F$-value distributed with numerator DF equal to the number of sires or dams, and denominator DF equal to the total number of offspring minus twice the number of sires or dams (Knott et al., 1996).

**Significance thresholds**

The chromosome-wide significance thresholds were calculated empirically using a permutation method (Churchill and Doerge, 1994). The chromosome-wise $F$-value at $P=0.05$ and $P=0.01$ were calculated for each linkage group using 10,000 permutations with QTL Express (Seaton et al., 2002). The QTL was defined as suggestive (*) if the chromosome-wise $P$-value was $\leq 0.05$; as significant (**) if the genome-wide $P$-value was $< 0.05$, and highly significant (***) if the genome-wide $P$-value was $< 0.01$. Non-significant QTL with nominal $F$-values $\geq 4.0$ were considered as close to the suggestive level.

**Phenotypic and genetic variance explained by QTL**

The proportion of phenotypic variance explained by the QTL ($\sigma^2_{QTL}$) was calculated as $4[1 - (MSE_{full}/MSE_{reduced})]$ where $MSE_{full}$ is the mean squared error of the full model, accommodating one QTL effect for each informative mapping parent, while
\[ \text{MSE}_{\text{reduced}} \] is the mean squared error of the reduced model omitting the QTL effect (Knott et al, 1996). The estimated \[ \sigma^2_{\text{PQTL}} \] was corrected for selective genotyping (Darvasi and Soller, 1992). Briefly, the correction factor was estimated to be \( \gamma_p = 2.674 \) for the selected sample; then the uncorrected variance proportions were divided by this correction factor to obtain the corrected variance proportions.

**QTL allele substitution effect**

For QTL declared as significant, suggestive and close to the suggestive level, the parents were judged as segregating alternative QTL alleles (i.e., heterozygous) on the basis of the \( t \)-test of the estimated allelic effect. The allele substitution effect for each parent was tested using a one-sided \( t \)-test (absolute \( t \)-value) with one DF. For this test, where the overall QTL effect had already been declared, a parent was defined as QTL heterozygous using a nominal 10% significance threshold. We expressed the QTL allele substitution effect in phenotypic standard deviation units (i.e., estimated effect size / SD of the trait).

**QTL 95% confidence intervals**

QTL Express (Seaton et al, 2002) was used for estimating average QTL location and 95% confidence intervals for the significant and suggestive QTL using 10,000 bootstraps with re-sampling.
Results

Spleen Index heritability and genetic correlation with BCWD survival

If spleen size and disease resistance are influenced by common or tightly linked genes; we hypothesized there should be a positive genetic correlation between the two traits. To test this hypothesis, two separate breeding populations of pedigreed rainbow trout, from 5 year classes, were phenotyped. A total of 322 defined-pedigree families (n=25,369 fish) were measured for BCWD survival at sizes averaging from 2.4g to 21.7g and ages from 82 to 149 days post-hatch (Table S1). Heritability estimates for survival following BCWD challenge were 0.23 ± 0.09 in the even-year population and 0.25 ± 0.03 in the odd-year population. Spleen and body weight was determined for 5,614 fish from a total of 251 families. Average family body weights ranged between 6 and 3,716 g (Table S2). Heritability estimates for spleen index were 0.84 ± 0.09 in the even-year population and 0.72 ± 0.07 in the odd-year population. Estimates of genetic correlation between family SI and family BCWD survival were 0.35 ± 0.13 in the even-year population and 0.14 ± 0.07 in the odd-year population.

Mode of SI inheritance

The high heritability of SI and the presence of a positive genetic correlation with BCWD resistance led us to investigate the mode of inheritance and genetic architecture of spleen size. A total of 15 crosses were created in 2007 and 2008 by mating highly divergent animals for SI and BCWD survival (Table S3). In 2007, crosses were created using parents whose full-sibs were BCWD challenged in 2005 and identified as disease resistant or susceptible families. Five of each RxR, RxS and SxS crosses were made and offspring assayed for both post-challenge survival (n=200 fish per family) and spleen
index (n=20 per family) (Figure 1A). There was a positive phenotypic correlation $r=0.804$ between both traits across families. In RxS crosses, both SI and survival were similar to the RxR crosses suggesting that the pattern of inheritance of these traits may be co-dominant or dominant. In 2008, we utilized a similar experimental design in which 15 crosses were created from fish whose full-sibs were evaluated in 2006 for SI and categorized as high spleen index (H) and low spleen index (L). Five of each HxH, HxL and LxL crosses were created and offspring siblings assayed for both post-challenge survival (n=120 per family) and SI (n=20 per family). There was a positive phenotypic correlation between the two traits, $r=0.4603$, albeit lower than observed in 2007 with the disease selected families (Figure 1B) and with a greater phenotypic variation in average SI between families. Some HxL crosses survived at a higher percentage than HxH crosses while SI of the HxL crosses was similar or slightly lower than HxH crosses. These phenotypic data suggest that the pattern of spleen inheritance may be additive or dominant.

**Stability of SI during growth**

We previously observed that spleen size was a stable trait over a 50 day period during which fish weight increased 74% (Hadidi et al., 2008). Given the high heritability of spleen size, we examined whether relative family spleen size would be stable over the entire growth cycle. Fifteen families from the 2008 YC were measured at five intervals over a 500-day growth period and results averaged between cross types. Average family spleen index, apart from the initial measurements, was generally stable over time (Figure 1C), and growth of the 15 families was similar (Figure 1D). Correlations among family means for spleen index across the five intervals ranged from 0.48 ($P$-value = 0.08; 95%
CI = -0.07 to 0.81) to 0.86 (P-value < 0.001; 95% CI = 0.63 to 0.96) and generally decreased as time between measurements increased (Table 1). This pattern of correlation was less consistent at the oldest age, which may be partly attributable to smaller sampling sizes at that age (n = 81 animals, range = 0 to 12 animals per family).

Genetic architecture determining spleen size

We next examined whether SI was influenced by the action of a segregating locus of large effect. We used complex segregation analyses to calculate and compare mixed inheritance models using the F1 QTL (2008) spleen size dataset. In this analysis, Mendelian transmissions of a single gene locus are simultaneously estimated with the patterns of covariance expected in polygenic inheritance (Vallejo et al, 2009; Vallejo et al, 2010). Analysis of spleen size models identified significant effects of BW, age and year (Table S4 and S5). For spleen index, BW was not significant in Model A, but was in Model B and vice versa for age. For the trait, \( \text{Ln}(\text{spleen index}) \) weight was a significant covariate in Models A and B, while Age was not significant. Estimated marginal posterior means for variance components (Table S6 and S7) and major gene parameters of \( \text{ln}(\text{spleen index}) \) using polygenic and mixed inheritance models indicated that Model 7 (Dominant \( A_1 \)) had the highest Bayes factor (1,495) (Table 2). These results suggest dominant inheritance of at least one major gene is segregating in the population.

The models assumed a single locus with autosomal Mendelian inheritance, and since the validity of this assumption was likely violated by the presence of multiple contributing loci and potential epistatic effects, we directly searched for the presence of QTL using a panel of microsatellite markers distributed across the rainbow trout genome. Based on predicted single-locus genotypes of parents of rainbow trout families (Table
A single putative backcross mapping family, cross ID 2008132, was chosen for QTL genome scan. This cross was highly divergent in SI (Figure 1B and Figure 2A and B) and was derived from two high SI parents. Cross 2008140 is included as a control that was created by mating two low SI parents (Figure 1B and Figure 2A and B). A total of 327 animals from cross 2008132 were phenotyped and 192 animals from the two tails of the spleen size distribution were chosen for genotyping (Figure 2B). Growth of these crosses were similar (Figure 2C) while BCWD resistance differed significantly (Figure 2D).

Before performing QTL analyses, we performed STEPWISE model selection and determined that the covariate age had significant effect on body weight, spleen weight and spleen index ($P < 0.05$); and the effect of the covariate age on log (SI) was close to the significance level ($P = 0.0507$) (Table S9 and S10). Subsequently, we decided to include the covariate age in the linear model used in the QTL analysis to minimize the variance in the sampled population. We also performed normality tests and estimated basic statistical measures for the response variables using SAS Proc UNIVARIATE (SAS, 2007). These results indicated that these response variables had significant departure from normal distribution (Table S11). However, the response variables SI and log (SI) had much smaller coefficient of variation, skewness and kurtosis than those of the variables body weight and spleen weight. The $de$ $novo$ built genetic maps using 314 informative markers (Table S12) were in good agreement with those previously reported (Rexroad et al, 2008). The QTL analysis using half-sib family regression, identified significant QTL influencing spleen size on chromosomes 5 and 19 ($P_{\text{genomewide}} < 0.01$) (Figure 3 and Table 3). The narrowest QTL confidence interval was 17 cm located on
A total of 17 suggestive QTL ($P_{\text{nominal}}<0.05$) influencing spleen size were identified on Chromosomes 1, 2, 3, 8, 9, 10, 11, 13, 16, 17, 18, 22, 26 and 27, (Tables 3 and 4). A single significant QTL for body weight was identified on chromosome 10 ($P_{\text{genomewide}}<0.01$) as well as suggestive QTL on chromosomes 2, 3, 9, 16, 17, 21, 22, 23 and the sex chromosome (Table S13).
Discussion

The genetic and mechanistic linkage between spleen somatic-index in naïve animals and BCWD resistance is of interest for several reasons. First, the contribution of the spleen to anti-bacterial immunity is poorly understood in lower vertebrates. The spleen first appears as a recognizable organ in shark and bony fish lineages while factors influencing its size and functions in lower vertebrates have received little attention (reviewed in (Brendolan et al, 2007; Fänge and Nilsson, 1985; Van Muiswinkel et al, 1991). In addition to evolutionary interest, spleen size has practical importance as an easy-to-measure surrogate trait that may have utility as a selection parameter for selective breeding programs. Herein, we report that spleen somatic-index is highly heritable and there are positive phenotypic and genetic correlations with specific disease resistance in rainbow trout cultured under disease-free conditions. These data provide evidence that spleen size of naïve rainbow trout and specific disease resistance are influenced by common or closely linked genes.

Heritability of disease resistance, spleen size and QTL segregation

Our previous modeling of BCWD resistance using Bayesian methods of segregation analysis suggested that 6-10 QTL explain 83-89% of phenotypic variance and the sum of these effects were best modeled as the mixed inheritance of co-dominant or dominant disease-resistant alleles plus polygenic effects (Vallejo et al, 2010). Here, we modeled the inheritance of spleen size using a similar approach and found evidence for dominant inheritance of small spleen size with at least one gene with major effect on the trait segregating within the even-year population. In order to search for responsible QTL, we utilized iBay to predict single-locus genotypes of fifteen 2008 families and identified a
single family that was likely informative for QTL mapping. Results from microsatellite mapping identified significant QTL on Chr 5 and 19 that each separately account for 18% of the phenotypic variance. The QTL interval on chromosome 5 has wider 95% CI than the chromosome 19 QTL interval because the male genetic linkage maps in salmonids is typically much smaller than female maps due to low recombination frequency during male meiosis (Phillips et al., 2009; Sakamoto et al., 2000). Studies investigating whether chromosome 5 or 19 SI QTL are pleiotropic or closely linked to BCWD resistance loci are ongoing.

In our study, a single genome-wide QTL for body weight was identified on chromosome 10 as well as nine suggestive QTL. Two other studies have also identified body weight QTL on chromosome 10 (linkage group 20) (Nichols et al., 2008; Wringe et al., 2010). Further study is required to determine if these are the same or separate QTL on chromosome 10.

**Can spleen size be used as a surrogate marker to improve disease resistance?**

Efforts to selectively breed Atlantic salmon and rainbow trout for increased disease resistance have progressed rapidly since the late 1980’s (Gjedrem, 2010). Genetic variation in survival following challenge has been demonstrated for a number of pathogens with heritability estimates generally ranging from 0.2 to 0.4 (Moen, 2010). Interestingly, the genetic correlation in resistance between any two pathogens is low, thus dictating the need for separate phenotyping efforts for each disease. In general, disease challenge experiments are time consuming and require biosecure isolation from brood stock. Since broodstock are generally maintained in a certified disease free status, the potential for utilizing within-family genetic variation for selection of disease resistance is
limited. For these reasons, there is interest in identifying quantitative traits that predict disease resistance, although this strategy has generally met with limited success in the past (Gjedrem, 2010; Wiegertjes et al, 1996). For an indirect selection parameter to be a useful measure of disease resistance, the trait should show genetic variation and be genetically correlated with resistance (Lund et al, 1995). In order to quantify the efficacy of using SI as a surrogate marker, we calculated the correlated response in survival using the formula: \[ CR_{BCWD} = i \times h_{BCWD} \times h_{SI} \times r \times \sigma_{SI} \], where \( i \) is selection intensity, \( h \) is the square root of the heritability estimate for the respective trait, \( r \) is the genetic correlation between both traits, and \( \sigma \) is the phenotypic SD of SI (Falconer and Mackay, 1996). The measured phenotypic SD of spleen index was 0.36 index units, and assuming selection of the top 10% of families, we predict a per generation increased survival of 9.2% in our even year population and 3.7% in our odd-year population if spleen index was used as the sole selection parameter to increase BCWD survival phenotype. While these values are encouraging, we have also quantified the response to selection using an injection challenge test and found an average 22% response per generation (Leeds et al, 2010). If the high response to direct BCWD phenotyping continues to be predictive of future genetic gain, then the utility of measuring spleen size as a sole selection parameter is limited. However, if the ability to perform challenge tests is restricted, or if spleen size can be used to facilitate within-family selection (e.g., using non-destructive estimates based on ultrasound, or by destructively measuring SI in parents at the time of spawning), this trait may have future utility in selection programs. Our finding of the stability of spleen size during growth of the 2008 year class cohort indicates a positive potential and warrants further investigation.
The recent identification of major QTL influencing viral and parasitic disease in rainbow trout and Atlantic salmon offers encouraging prospects for the use of marker assisted selection in aquaculture (Baerwald et al., 2010; Houston et al., 2008; Houston et al., 2010; Moen et al., 2009; Moen et al., 2007). It is possible that spleen size QTL may have utility in the future for marker assisted selection. However, fine mapping needs to be carried out with higher marker density surrounding the QTLs on Chr 5 and 19, and additional families and progeny are needed to validate these QTL. Studies are underway testing the progeny of cross 2008132 as well as other families to more precisely map and determine the contribution of these QTL to disease resistance.

In summary, this is the first report of a positive genetic correlation between spleen somatic-index and resistance to challenge with *F. psychrophilum* strain CSF259-93. Spleen index is a highly heritable trait in our population of rainbow trout as determined using two independent methods: 1) Bayesian segregation analysis using a polygeneic model estimated heritability at 0.88 in our even year population, and 2) maximum likelihood calculations using MTFREML estimated heritability at 0.72 and 0.84 in our odd-year and even-year respective populations. In this manuscript, we report the first identification of two QTL that each influence 18% of the phenotypic variation in spleen size. We suggest that rainbow trout may serve as a model system for mechanistic study of factors controlling spleen size and further dissection of the genetic basis of disease resistance.
ACKNOWLEDGEMENTS

This research was supported by Agricultural Research Service CRIS project 1930-32000-005 “Integrated Approaches for Improving Aquatic Animal Health in Cool and Cold Water Aquaculture”. We thank Drs J. Silverstein, M. Purcell and W.B. Van Muiswinkel for manuscript review and discussion. Biological science technicians K. Hovatter, J. Thompson, J. Harper, R. Lipscomb, and T. Moreland and summer student D. Williams participated in animal phenotyping. Fish were bred and maintained by J. Everson, J. Kretzer, J. Leasor, and J. Rollins. Genotyping was performed by K. Shewbridge and R. Long, and G. Gao streamlined the genotyping analyses pipeline. RLV acknowledges Dr. S. J. Hasstedt for suggestions developing mixed inheritance models. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S Department of Agriculture. USDA is an equal opportunity provider and employer.
Footnotes

2 G.D.W., R.L.V, T.D.L., and Y.P. contributed equally to this work.

3 Current Address: The Biomedical Research Centre, 2222 Health Sciences Mall, Vancouver, BC V6T 1Z3

4 List of abbreviations  BCWD, bacterial cold-water disease; RTFS, rainbow trout fry syndrome; SI, Spleen somatic-index; NCCCWA, National Center for Cool and Cold Water Aquaculture.

References


FAO. (2010). Cultured Aquatic Species Information Programme. Oncorhynchus mykiss. 


Table 1. Correlations among family mean SI.

<table>
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<tr>
<th>mean age¹, d</th>
<th>170</th>
<th>241</th>
<th>323</th>
<th>633</th>
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<tbody>
<tr>
<td>145</td>
<td>0.86</td>
<td>0.82</td>
<td>0.70</td>
<td>0.71</td>
</tr>
<tr>
<td>170</td>
<td>0.83</td>
<td>0.76</td>
<td>0.76</td>
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</tr>
<tr>
<td>323</td>
<td></td>
<td></td>
<td>0.48</td>
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</table>

¹Days post-hatching.
Table 2. Estimated marginal posterior mean for variance components and major gene parameters of ln(spleen index) using polygenic and mixed inheritance models in Bayesian segregation analysis.

<table>
<thead>
<tr>
<th>Model</th>
<th>$\sigma^2_e$</th>
<th>$\sigma^2_u$</th>
<th>$\sigma^2_g$</th>
<th>$a$</th>
<th>$d$</th>
<th>$p$</th>
<th>$\tau_{A_1/A_1A_1}$</th>
<th>$\tau_{A_1/A_1A_2}$</th>
<th>$\tau_{A_1/A_2A_2}$</th>
<th>$h^2_p$</th>
<th>$\log_e[p(y/H_i)]$</th>
<th>Model tested</th>
<th>BF $(H_2; H_1)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. General</td>
<td>0.011</td>
<td>0.110</td>
<td>3.408</td>
<td>1.36</td>
<td>-1.21</td>
<td>0.58</td>
<td>0.85</td>
<td>0.46</td>
<td>0.15</td>
<td>0.90</td>
<td>1,378.0</td>
<td>1 vs. 2</td>
<td>487.1</td>
</tr>
<tr>
<td>2. General fixed</td>
<td>0.087</td>
<td>[0]</td>
<td>35.834</td>
<td>5.72</td>
<td>-5.99</td>
<td>0.73</td>
<td>0.99</td>
<td>0.28</td>
<td>0.02</td>
<td>[0]</td>
<td>890.8</td>
<td>2 vs. 3</td>
<td>315.3</td>
</tr>
<tr>
<td>3. Sporadic</td>
<td>0.156</td>
<td>[0]</td>
<td>[0]</td>
<td>[0]</td>
<td>[0]</td>
<td>[0.0]</td>
<td>[0.0]</td>
<td>[0.0]</td>
<td>[0.0]</td>
<td>[0]</td>
<td>575.5</td>
<td>4 vs. 3</td>
<td>715.4</td>
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<td>4. Polygenic</td>
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<td>0.131</td>
<td>[0]</td>
<td>[0]</td>
<td>[0]</td>
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<td>[0.0]</td>
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<td>[0.0]</td>
<td>[0]</td>
<td>1,290.9</td>
<td>7 vs. 5</td>
<td>140.4</td>
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<tr>
<td>5. Dominant $A_2$</td>
<td>0.015</td>
<td>0.121</td>
<td>0.055</td>
<td>0.23</td>
<td>0.23</td>
<td>0.45</td>
<td>[1.0]</td>
<td>[0.5]</td>
<td>[0.0]</td>
<td>0.89</td>
<td>1,354.3</td>
<td>5 vs. 1</td>
<td>-23.7</td>
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<tr>
<td>6. Additive</td>
<td>0.015</td>
<td>0.107</td>
<td>0.035</td>
<td>0.29</td>
<td>[0]</td>
<td>0.55</td>
<td>[1.0]</td>
<td>[0.5]</td>
<td>[0.0]</td>
<td>0.87</td>
<td>1,337.6</td>
<td>6 vs. 1</td>
<td>-40.4</td>
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<tr>
<td>7. Dominant $A_1$</td>
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<td>0.110</td>
<td>0.031</td>
<td>0.20</td>
<td>-0.20</td>
<td>0.52</td>
<td>[1.0]</td>
<td>[0.5]</td>
<td>[0.0]</td>
<td>0.91</td>
<td>1,494.7</td>
<td>7 vs. 1</td>
<td>116.8</td>
</tr>
<tr>
<td>8. Codominant</td>
<td>0.012</td>
<td>0.115</td>
<td>0.405</td>
<td>1.18</td>
<td>-0.13</td>
<td>0.46</td>
<td>[1.0]</td>
<td>[0.5]</td>
<td>[0.0]</td>
<td>0.90</td>
<td>1,343.2</td>
<td>8 vs. 1</td>
<td>-34.8</td>
</tr>
</tbody>
</table>

1Bayesian segregation analysis of ln(spleen index) performed with software iBay version 1.46 (Janss, 2008). The Gibbs sampler had these characteristics: number of iterations per chain = 1,200,000; burn-in period = 600,000; thinning = 10,000; collected samples per chain = 60; total chains = 20; and total collected samples = 1,200.

2Model parameters: error variance $\sigma^2_e$; polygenic variance $\sigma^2_u$; major gene variance $\sigma^2_g$; major gene additive effect $a$; major gene dominance effect $d$; $p$ is frequency of spleen size-decreasing allele $A_i$; $h^2_p$ is the polygenic model heritability $\sigma^2_u / (\sigma^2_u + \sigma^2_e)$; and transmission probabilities defined as the probability that a parent with any of the three genotypes $(A_1A_1, A_1A_2, A_2A_2)$ transmits the allele $A_i$ to its offspring.

3$log_e$ of the marginal density under the fitted model $H_i$.

4Bayes factor $BF(H_2; H_1) = p(y/H_2)/p(y/H_1)$ is the ratio of the marginal likelihood under one model to the marginal likelihood under a second model, and $H_1$ and $H_2$ are the two competing models.

5Value between squared brackets indicates the parameter was fixed to the value shown.
Table 3. Significant and suggestive QTL for spleen index using half-sib family regression\(^1\) in rainbow trout.

<table>
<thead>
<tr>
<th>Chromosome(^2)</th>
<th>Location (cM)</th>
<th>LR</th>
<th>F-value</th>
<th>P-value(^3)</th>
<th>(F_{\text{chrwise}})(^4) (p &lt; 0.05)</th>
<th>(F_{\text{chrwise}})(^4) (p &lt; 0.01)</th>
<th>(p_{\text{genowide}})(^5)</th>
<th>(\sigma^2_{\text{QTL}})(^6)</th>
<th>(\text{QTL effect})</th>
<th>(\text{Mean})(^7)</th>
<th>SE</th>
<th>ABS (t)</th>
<th>(P)-value(^8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dam HS family:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>40.0</td>
<td>10.77</td>
<td>11.11</td>
<td>0.0010283</td>
<td>6.52</td>
<td>9.81</td>
<td>0.04683**</td>
<td>0.07</td>
<td>-0.51</td>
<td>0.076</td>
<td>3.33</td>
<td>0.093</td>
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<td>10.19</td>
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<tr>
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<td>0.36</td>
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<td>2.57</td>
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<td>5.27</td>
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<td>0.0059344</td>
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<td>0.22925*</td>
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<td>0.118</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>9.22</td>
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<td>8.45</td>
<td>0.09130*</td>
<td>0.06</td>
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<td>6.11</td>
<td>9.30</td>
<td>0.00001***</td>
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<td>-0.71</td>
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<td>6.92</td>
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<td>4.34</td>
<td>7.60</td>
<td>0.38396*</td>
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<td>12.70</td>
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<td>0.32</td>
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<td>0.32</td>
<td>0.070</td>
<td>2.26</td>
<td>0.133</td>
<td></td>
</tr>
</tbody>
</table>

\(1\)Half-sib regression interval mapping was performed with web-based software QTL Express (Seaton et al., 2002).
\(2\)Chromosome number is based on Rexroad et al. (2008).
\(3\)The \(P\)-value was calculated assuming an \(F\)-value distributed with numerator DF equal to the number of sires or dams, and denominator DF equal to the total number of offspring minus twice the number of sires or dams (Knott et al., 1996).
\(4\)Chromosome-wise \(F\)-value at \(P = 0.05\) and \(P = 0.01\) was estimated using 10,000 permutations with QTL Express (Seaton et al., 2002).
\(5\)Genome-wide \(P\)-value was estimated using expression from deKoning et al. (1999). QTL is defined as suggestive (*) if it was chromosome-wise significant at \(P = 0.05\); as significant (**) if it was genome-wide significant at \(P < 0.05\), and highly significant (***) if it was genome-wide significant at \(P < 0.01\). Non-significant QTL with \(F\)-value \(\geq \) 4.0 are considered as close to the suggestive level.
\(6\)The proportion of phenotypic variance explained by the QTL (\(\sigma^2_{\text{QTL}}\)) was calculated as \(4 \left[ 1 - \frac{\text{MSE}_{\text{full}}}{\text{MSE}_{\text{reduced}}} \right]\) where \(\text{MSE}_{\text{full}}\) and \(\text{MSE}_{\text{reduced}}\) are the mean squared error of the full and reduced model, respectively (Knott et al., 1996). The estimated \(\sigma^2_{\text{QTL}}\) was corrected for selective genotyping according to Darvasi and Soller (1992).
\(7\)The QTL effect is expressed in phenotypic standard deviation (PSD) units.
\(8\)The allele substitution effect for each parent was tested using a one-sided t-test (absolute t-value) with one DF.
Table 4. Bootstrap analysis\(^1\) and flanking markers for significant and suggestive QTL for spleen index using half-sib family regression\(^2\) in rainbow trout.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Average QTL location (cM)</th>
<th>95% CI (cM)</th>
<th>QTL position</th>
<th>Length</th>
<th>Flanking markers</th>
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</thead>
<tbody>
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<td></td>
<td></td>
</tr>
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<td>[4, 114]</td>
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\(^1\)The average QTL location and 95% confidence intervals were estimated using 10,000 bootstraps with resampling with QTL Express (Seaton et al., 2002).

\(^2\)Half-sib regression interval mapping was performed with web-based software QTL Express (Seaton et al., 2002).
Figure 1. Family phenotypic correlation between naïve-animal spleen index and survival following challenge with *F. psychrophilum* strain CSF259-93. (A) Trait correlation in fifteen 2007 year-class families selected based on BCWD resistance phenotype. Crosses were created using parents whose full-sibs were BCWD challenged in 2005 and identified as highly disease “resistant” (R) or “susceptible” (S) families. Five RxR (blue), RxS (green) and SxS (red) crosses were assayed for both survival (mean age 118 days) and spleen index (mean age 128 days). (B) Trait correlation in fifteen 2008 year-class families. Fifteen crosses were created from fish whose full-sibs were evaluated in 2006 for SI and categorized as high spleen index (H) and low spleen index (L). Five HxH (blue), HxL (green) and LxL (red) crosses assayed for both survival (mean age 114 days) and spleen index (mean age 145 days). (C) Stability of SI in HxH, HxL and LxL cross types. Values represent an average (SEM) of 5 families per cross type. (D) Equivalent growth rates of the 2008 F1 QTL families.

Figure 2. Trout crosses with divergent spleen indices: high family average SI cross 2008132, low average family SI cross 2008140. (A) Four randomly selected fish and respective spleens. (B) SI phenotype distribution of crosses 2008132 (n=327 fish) and 2008140 (n=70 fish). (C) Average body weights (not statistically different). (D) Survival following laboratory injection challenge with *F. psychrophilum* strain CSF259-93 (*P*<0.001, results are an average from triplicate tanks, n=40 fish per tank).

Figure 3. Genetic loci linked to spleen index using HS family regression interval mapping in trout cross 2008132. (A) Plots of quantitative trait loci for dam HS family. (B) Plots of quantitative trait loci for sire HS family.
Figure 1
Figure 2

A

B

Cross 2008132 (n=327)

Cross 2008140 (n=70)

C

D

Figure 2
Figure 3

(A) Dam HS Family

(B) Sire HS Family

Figure 3