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Reduction of rainbow trout spleen size by splenectomy does not alter resistance against bacterial cold water disease

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ABSTRACT

In lower vertebrates, the contribution of the spleen to anti-bacterial immunity is poorly understood. We have previously reported a phenotypic and genetic correlation between resistance to *Flavobacterium psychrophilum*, the causative agent of bacterial cold water disease (BCWD) and spleen somatic index (spleen weight normalized to body weight, SI). Fish families with larger pre-challenge SI values were found to have greater BCWD survival (resistance) following intraperitoneal injection of a lethal dose of *F. psychrophilum*. Since the mammalian spleen is known to be crucial for capture and destruction of encapsulated bacteria, we tested the hypothesis that reduction of spleen size, by surgical splenectomy, should reduce the survival advantage of the larger-spleen, disease-resistant fish. Experiments were performed using two separate lines of fish that had previously been selected either based on BCWD survival (resistant and susceptible), or selected based on spleen size (high and low SI). Following 65 to 81 days post-surgical recovery, fish were challenged with *F. psychrophilum* and mortality monitored for a minimum of 21 days. No significant difference in the relative survival was detected between splenectomized or sham-operated groups, while SI of splenectomized fish was reduced to an average of 8–12% of control animals. A positive correlation was observed between the SI, measured at the time of splenectomy, and time-to-death post-challenge. In summary, these experiments argue that larger spleen size alone is not sufficient for greater BCWD resistance, but rather it is an indirect indicator of immunological status.

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1. Introduction

The basis of genetic disease resistance remains poorly understood in fish. Breeding programs offer unique opportunity to create divergent phenotypes for mechanistic study of innate disease resistance mechanisms (Gjedrem, 2010; Moen, 2010; Stear et al., 2012; Wiegertjes et al., 1996). The National Center for Cool and Cold Water Aquaculture (NCCCWA) has bred pedigreed, genetic lines of rainbow trout for divergent survival following challenge with a common aquaculture pathogen, *Flavobacterium psychrophilum*, that causes bacterial cold water disease (BCWD) (Leeds et al., 2010; Silverstein et al., 2009). An intriguing observation from the initial breeding studies was that in naive animals, BCWD resistant fish families displayed 29% larger-than-average spleen size normalized to body weight (spleen index, SI), while susceptible families exhibited a 22% small-than-average spleen size (Hadidi et al., 2008). Even after challenge, on days 3 and 5 post-infection, the SI of challenged-resistant fish was significantly larger than the challenged-susceptible fish. Furthermore, resistant fish displayed 14-fold greater reduction in splenic bacterial load by day 5 post-challenge suggesting greater clearance of *F. psychrophilum*. These results indicate that whether naive or challenged, the average SI of fish from resistant families was larger than that of the treatment-matched fish from susceptible families and that this phenotype associated with greater survival and reduced bacterial loads. The phenotypic association between resistance and spleen size was confirmed in even-year spawning families (2006 year class) where families were first separated into high, medium or low average spleen index and then the respective groups challenged with *F. psychrophilum*. Spleen index of naive animals predicted *F. psychrophilum* post-challenge survival of the full-sib cohorts (Hadidi et al., 2008). Subsequently, analysis of five year-classes of even- and odd-year NCCCWA spawning fish identified that SI is a moderately heritable trait and there was a significant genetic correlation with BCWD resistance within the even-year line ($r_g = 0.45 \pm 0.20$) (Wiens et al., 2013b). In sum, these data demonstrate a genetic basis for the phenotypic correlation between SI and BCWD resistance. Our

Abbreviations: BCWD, bacterial cold water disease; SI, spleen index; R-line, ARS-Fp-R; S-line, ARS-Fp-S; Fp, Flavobacterium psychrophilum.

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prior efforts to directly determine the mechanistic linkage were confounded as we were unable to measure both traits in the same animal.

The role of the fish spleen in disease resistance is not well understood (Fange and Nilsson, 1985; Van Muiswinkel et al., 1991). In contrast, it is well documented that the mammalian spleen is the crucial site required for the removal of encapsulated bacteria. Splenectomy (spleen removal) in humans increases the incidence of infections from encapsulated pneumococcus, *Hemophilus influenzae*, and meningococcus (Rushton et al., 2002). Furthermore, splenectomized mice and rats exhibit slowed clearance of injected bacteria resulting in fatal sepsis (Leung et al., 1972; Loggie et al., 1985). Thus, given the filtering function of the mammalian spleen and the observation that *F. psychrophilum* possesses a prominent surface glycocalyx (LaFrentz et al., 2007), we sought to test the hypothesis that larger SI might confer increased functional capacity and thus increase post-challenge survival. A prediction of this hypothesis is that removal/reduction of splenic tissue should diminish the survival advantage of fish with larger SI relative to fish with smaller SI. Herein, we directly examined the contribution of the spleen to the disease resistance phenotype following surgical splenectomy. We describe experiments carried out with fish from two distinct genetic lines of odd-year and even-year spawning populations of pedigreed rainbow trout families.

2. Materials and methods

2.1. Genetic lines of rainbow trout used in splenectomy experiments 1 and 2

All fish were maintained at the National Center for Cool and Cold Water Aquaculture following Standard Operating Procedures for the Care and Use of Research Animals (Rainbow trout) and NCCCWA IACUC committee approval (Protocol #048). Experiments were performed utilizing fish derived from the odd- and even-year spawning populations. Experiment 1 (Exp. 1) utilized fish from the odd-year spawning population selectively bred based on full-sib survival following challenge with *F. psychrophilum* and which have been designated ARS-Fp-R (resistant, R-line) and ARS-Fp-S (susceptible, S-line) lines (Wiens et al., 2013a). The founder composition and response to selection have been previously described (Leeds et al., 2010; Silverstein et al., 2009; Wiens et al., 2013a). Fish were spawned in 2010 and progeny pooled at the eyed-egg stage from 1×1 matings of 2007 year-class females (1st generation selection) with 2009 year class (2rd generation selection), sex-reversed neomales. Five hundred eyed-eggs from cross ID 2010076, 2010159, 2010160, 2010161 and 2010164 were used to make the R-line pool, and 417 eggs from families 2010075, 2010156, 2010157, 2010158, 2010162 and 2010163 were used to make the S-line pool (complete pedigree information is available upon request from the NCCCWA database http://10.11.102.9:8080/apex/f?p=104:1). Eggs from each cross were temperature synchronized and hatched on March 3, 2010. Fish were subsequently cultured at ambient water temperature (12–13 °C) and fed standard trout diet rations. Evaluation of disease resistance was measured at 64 days post-hatch at mean body weight of 0.6 g (Appendix: Supplementary Table S1). Spleen index was measured in naive fish at an average of 170 days post-hatch at an average body weight of 28 ± 9 g. Growth was evaluated over a 168-day period between 64 and 238 days post-hatch.

Fish used in Experiment 2 (Exp. 2) were offspring of the even-year spawning population and the crosses made using a nested mating design. Parents were descendants of families originally identified in 2006 as either high or low spleen index families (Hadidi et al., 2008) and then subsequently propagated in 2008 by mating HxH or LxL families (Wiens et al., 2013b). Fish utilized in this experiment were generated in 2010 by first dividing the eggs of a female from family 2008132 (high SI), and then fertilizing with either a male from family 2008286 (producing an HxL cross, ID 2010235) or a male from family 2008098 (producing an HxH cross, ID 2010234). Similarly, egg lots from females from a family 2008140 (low SI) that were fertilized with either a male from a low SI family 2008286 (producing two LxL crosses, IDs 2010154 and 2010070) or a male from a high SI family 2008098 (producing an LxH cross, ID 2010153). The same male from family 2008098 was utilized for both crosses while the two males from family 2008286 were full sibs. Eggs from these crosses were not temperature synchronized and thus hatched on different dates: cross 2010070 on February 18, 2010, crosses 2010153 and 2010154 on February 25, 2010, and crosses 2010234 and 2010235 on March 6, 2010. Fish were subsequently cultured at ambient water temperature (12–13 °C) and fed standard trout diet (Zeigler Bros, Inc). Evaluation of disease resistance was measured at 101 days post-hatch at mean body weight of 3.1 ± 0.8 g. Spleen index was measured in naive fish at an average of 172 and 377 days post-hatch at an average body weight of 21 ± 11 g and 341 ± 93 g respectively.

2.2. Splenectomy

In Exp. 1, a total of 21 fish were randomly subjected to either surgical splenectomy (n = 11) or sham operation (n = 10). Fish average body weight was 259 ± 36 g and age 279 days post-hatch. Surgery time averaged 10 min per fish including anesthesia through initiation of recovery. One splenectomized and one sham-operated fish died from post-surgical complication (10% mortality). At the time of surgery or tank allocation, all fish were pit tagged. At 65 days post-surgery, fish were completely recovered from the operation as assessed by visual inspection of the incision site.

In Exp. 2, a total of 146 fish from HxH, HxL, LxH and LxL groups were randomly subjected to either surgical splenectomy (n = 74) or sham operation (n = 73). Fish average body weight was 380 ± 123 g and age of 354 d post-hatch. Surgery time averaged 11 min per fish. At the time of surgery or tank allocation, all fish were pit tagged. A total of 8 splenectomized and 10 sham-operated fish died post-surgery (11% mortality). Between 67 and 82 days post-surgery, fish were challenged with *F. psychrophilum* and survival recorded over 21 days. Post-challenge, two fish could not be assigned due to pit-tag loss, and two fish were excluded due to post-surgical complication.

2.3. Flavobacterium psychrophilum challenge

Flavobacterium psychrophilum strain CSF259-93 was cultured on TYES and prepared as previously described (Hadidi et al., 2008; Wiens et al., 2013a). Fish were challenged by i.p. injection of a dose that ranged between 3.9×10^5 and 5.1×10^5 CFU per g mean body weight and relevant parameters for each challenge are summarized in Appendix: Supplementary Table S1. Specific mortality post-challenge was recorded for either 21 or 28 days and randomly chosen mortalities were cultured to confirm the presence of *F. psychrophilum*. Mortality of PBS injected fish (at 0.6 g size) was less than 3%, and thus, the majority of post-challenge mortality was deemed to be specific to *F. psychrophilum* infection.

2.4. Statistical analyses

We compared *F. psychrophilum* post-challenge survival of splectomized resistant (or high SI) to susceptible (or low SI) animals and to genetic group or family-matched sham-operated controls. In Exp. 2, sample size was increased to provide a calculated power of (0.75) to distinguish a 30% difference in survival at an alpha value of 0.05 (G*Power v3.1.2). Differences in survival were determined using Log-rank (Mantel–Cox) Test calculated within GraphPad PRISM (v 5.01).