Biochemical reference intervals and pathophysiological changes in *Flavobacterium psychrophilum*-resistant and -susceptible rainbow trout lines

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ABSTRACT: Host genetic resistance against disease-causing pathogens can be enhanced through family-based selective breeding. At present, there is an incomplete understanding of how artificial selection of fish alters host physiology and response following pathogen exposure. We previously reported the generation of selectively-bred rainbow trout *Oncorhynchus mykiss* lines with either increased resistance (ARS-Fp-R) or susceptibility (ARS-Fp-S) to bacterial cold water disease (BCWD). This study (1) determined baseline reference-range intervals for packed cell volume (PCV) and 18 plasma biochemistry analytes, and (2) examined pathophysiological changes following infection between the genetic lines. PCV and biochemistry reference-range intervals did not significantly differ between genetic lines; thus data were pooled into a single reference-range population (n = 85). ARS-Fp-R and ARS-Fp-S line fish were intraperitoneally challenged with *Flavobacterium psychrophilum*, and plasma was collected on Days 1, 3, 6, and 9 post-challenge. Splenic bacterial load was measured using an *F. psychrophilum*-specific qPCR assay. In both genetic lines, changes were observed in mean PCV, total protein, albumin, glucose, cholesterol, chloride, and calcium, falling outside the established reference intervals and significantly differing from phosphate-buffered saline challenged fish, on at least 1 d post-challenge. Mean PCV, total protein, and calcium significantly differed between ARS-Fp-R and ARS-Fp-S line fish on Day 9 post-infection, with values in the ARS-Fp-S line deviating most from the reference interval. PCV, total protein, cholesterol, and calcium negatively correlated with bacterial load. These findings identify divergent pathophysiological responses between ARS-Fp-R and ARS-Fp-S line fish following laboratory challenge that are likely associated with differential survival.

KEY WORDS: Bacterial cold water disease · BCWD · Flavobacteriosis · *Oncorhynchus mykiss* · Disease resistance · Breeding program · Pathophysiology · Biochemistry

INTRODUCTION

Genetic enhancement of disease resistance in salmonid fish is possible through family-based selective breeding (reviewed by Gjedrem 1983, Fjalestad et al. 1993). Interfamily variation in rainbow trout disease resistance has been demonstrated through laboratory challenge for a number of disease etiologies, including viral (Dorson et al. 1995, Henryon et al. 2005), bacterial (Henryon et al. 2005, Silverstein et al.

2009, Overturf et al. 2010), and parasitic infection (Hedrick et al. 2003). Further support of a genetic basis for rainbow trout disease resistance is evidenced by an increasing number of quantitative trait loci (QTL) that have been identified as influencing survival following exposure to pathogens including infectious pancreatic necrosis virus (Ozaki et al. 2001), infectious hematopoietic necrosis virus (Khoo et al. 2004), viral hemorrhagic septicemia virus (Verrier et al. 2013), *Myxobolus cerebralis* (Baerwald et

al. 2011), and *Flavobacterium psychrophilum* (Wiens et al. 2013a, Vallejo et al. 2014). As the aquaculture industry begins to utilize genetic lines developed from breeding programs that select for disease resistance, limited mechanistic information exists regarding host physiology and pathogen response.

Pedigreed rainbow trout lines have been selectively bred for differential survival to bacterial cold water disease (BCWD) at the National Center for Cool and Cold Water Aquaculture (NCCCWA, Kearneysville, WV). A disease-resistant line, designated ARS-Fp-R, demonstrates significantly higher survival following *Flavobacterium psychrophilum* challenge under laboratory conditions (Hadidi et al. 2008, Silverstein et al. 2009, Leeds et al. 2010) and natural exposure conditions (Wiens et al. 2013b) compared to the survival of commercial fish stocks or a disease-susceptible line, designated ARS-Fp-S. Characterizing and comparing disease response in ARS-Fp-R and ARS-Fp-S line fish provides the opportunity to explore intrinsic factors that differentially affect survival in selectively bred fish.

Biochemical profiles have been used to explore the physiological response of rainbow trout against a number of bacterial (Byrne et al. 1995, Řehulka 2002, Del-Pozo et al. 2010), viral (Amend & Smith 1975, Řehulka 2003), and parasitic (Powell et al. 2006) diseases; however, pathophysiological changes have not been characterized in response to *F. psychrophilum* challenge or in selectively bred lines. In this study, packed cell volume (PCV) and plasma biochemistry reference intervals of 18 analytes were established for the ARS-Fp-R and ARS-Fp-S lines to determine normal health parameters and explore whether artificial selection has resulted in physiological differences between genetic lines. Changes in analyte levels were monitored after experimental infection with *F. psychrophilum* to examine differential disease response in ARS-Fp-R and ARS-Fp-S line fish. Changes in PCV and plasma biochemistry analytes in *F. psychrophilum*-infected fish reveal insights into disease pathogenesis and differential host morbidity between genetic lines.

MATERIALS AND METHODS

Experimental animals

The ARS-Fp-R and ARS-Fp-S genetic lines were derived from the same base population, and thus differed only as a result of artificial selection for BCWD post-challenge survival (Wiens et al. 2013b). Rainbow trout broodstock from each line were spawned during a 5 wk period in February and March 2012 at the NCCCWA. Single-sire × single-dam matings were made within genetic lines between 3 yr old females and 1 yr old neomales, and eggs were incubated separately by full-sib family in upwelling jars. The neomales had been previously intraperitoneally (IP) injected 3 times wk^{-1} with 1.5 mg salmon pituitary extract (Argent Laboratories) per kilogram of body weight beginning approximately 3 mo before spawning to induce sexual maturation at 1 yr of age. Water temperature in the incubation jars was manipulated so that all families would hatch within a 1 wk period (Leeds et al. 2010). Eggs were pooled within lines at the eyed stage and reared in 12.5°C flow-through spring water. The ARS-Fp-R pool consisted of 43 full-sib families (mean = 1489 eggs $family^{-1}$), and the ARS-Fp-S pool consisted of 11 full-sib families (mean = 393 eggs $family^{-1}$). A larger number of resistant line families were utilized as compared to susceptible line families, as more resistant families are generated within the breeding program (typically n = 100) in order to apply selection differential, and thus more accurately capture the genetic diversity within the resistant line. The resistant line fish used in this study were progeny of dams that had undergone 3 generations of BCWD selection while the sires had undergone 4 generations of selection. The susceptible line fish were progeny of parents that had undergone 1 generation of selection for increased susceptibility (2007 year class) and since that time had been randomly bred.

From swim-up through conclusion of the experiments, fish were fed a pelleted, commercial fish meal-based diet (Finfish G, Zeigler Bros.) consisting of 42% minimum crude protein, 16% minimum crude fat, 3% maximum crude fiber, 12% maximum moisture, and maximum 8% ash. All broodstock and fish used in this study were certified to be free of common salmonid bacterial and viral pathogens by 2 independent diagnostic laboratories as described previously (Leeds et al. 2010, Wiens et al. 2013b).

Fifty-five ARS-Fp-R line fish and 59 ARS-Fp-S line fish were PIT tagged (Avid Identification Systems and Biomark) for identification and divided between 12 tanks (55 l each) by genetic line. An additional 8 ARS-Fp-R and 8 ARS-Fp-S line fish were housed in separate 55 l tanks to be used as controls during the infectivity portion of the study. Tanks were supplied with 13°C flow-through water under indoor rearing conditions with 12 h cycles of artificial light. Water chemistries tested on in-flow water during the reference interval and infectivity studies are presented in Table 1.

Average starting fish weight was 296.5 ± 73.5 g. Fish were fed once daily and given a 3 wk acclimation period prior to sampling for the reference interval study.

Reference interval study

We sampled 43 fish from each genetic line to determine blood chemistry reference intervals for ARS-Fp-R and ARS-Fp-S line fish. Handling error occurred with 1 ARS-Fp-R line sample, reducing the sample size to 42 fish. Six or 7 randomly selected fish were rapidly netted from each tank into an anesthetic bath containing 100 mg l^{-1} tricaine methanesulfonate (Tricaine-S, Western Chemical). Fish were weighed and physically examined for gross lesions or signs of disease. Approximately 1 ml of blood was collected from the caudal vein using a 3 ml syringe and 25-gauge ½-inch needle (Kendall Monoject). Two micro-hematocrit capillary tubes (Fisher Scientific) were filled, and the remaining blood was placed into 3 ml lithium heparin tubes (Greiner Bio-One).

Micro-hematocrit capillary tubes were immediately centrifuged for 3 min at 3000 × g in an Autocrit Ultra-3 Direct Reading Centrifuge (BD Products), and the PCV was measured at the erythrocyte–plasma interface.

Lithium heparin tubes were centrifuged within 20 min of collection at 1000 × g (15 min at 15°C), and the plasma was collected into 1.5 ml microcentrifuge tubes. Plasma was frozen at −80°C until all fish were sampled. Plasma chemistries were analyzed on a Roche/Hitachi P 800 Modular Analytics System (Roche Diagnostics) at the Clinical Pathology Laboratory of the University of Georgia, College of Veterinary Medicine (Athens, GA). Total protein, albumin, glucose, cholesterol, blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), lactate dehydrogenase (LDH), total bilirubin, calcium, phosphorus, magnesium, bicarbonate, sodium, potassium,

Table 1. Incoming water quality ranges during reference interval sampling and infectivity trial

Parameter	Measurement
Temperature (°C)	13.1–13.2
pH	7.13–7.18
Nitrite (mg l^{-1})	0
Ammonia (mg l^{-1})	0–0.04
CO_2 (mg l^{-1})	38.72–39.6
O_2 (ppm)	12.1–16.4
Hardness (mg l^{-1})	254–295

and chloride were measured. Plasma osmolality was calculated using the equation 2 Na (mEq l^{-1}) + (Urea [mg dl^{-1}])/2.8 + (Glucose [mg dl^{-1}])/18 (Carlson 1997). Plasma cortisol was measured by radioimmunoassay following methods described by Feist et al. (1990), as modified from Redding et al. (1984), using cortisol antiserum (R4866; provided by Coralie Monroe, University of California Davis, School of Veterinary Medicine, Department of Reproduction), which was validated for use in rainbow trout (Barry et al. 1993). Reference intervals were determined following the American Society for Veterinary Clinical Pathology guidelines (Friedrichs et al. 2012).

Infectivity study

Flavobacterium psychrophilum CSF 259-93 was previously isolated from a BCWD field-case and maintained at −80°C in TYES media supplemented with 10% (v/v) glycerol and has been consistently used as the challenge strain within the selective breeding program (Silverstein et al. 2009, Leeds et al. 2010). Frozen stock was cultivated on TYES media for 5 d at 15°C prior to experimental infection.

Bacterial challenge was performed 25 d following reference interval sampling. Average fish weight was 297.3 ± 76.8 g. ARS-Fp-R and ARS-Fp-S line fish were anesthetized with MS-222 (100 mg l^{-1}), weighed, and challenged IP with 7.20 × 10^7 CFU of *F. psychrophilum* CSF 259-93 in 100 µl of chilled phosphate-buffered saline (PBS) using a repeater pipette (Eppendorf) and 26-gauge ½-inch needle. This dose was approximately 13-fold less, when adjusted for body weight, compared to the average dose used for 2005, 2007, and 2009 family challenge evaluations (Leeds et al. 2010). The lower challenge dose was used in order to reduce mortality in the susceptible line fish. Control fish were injected with 100 µl of chilled PBS.

Five fish from the ARS-Fp-R and ARS-Fp-S lines were sampled on Day 0 to serve as baseline controls. On Days 1, 3, 6, and 9 post-infection, 5 experimentally challenged fish and 2 control fish were sampled from each genetic line. These time points were chosen as Silverstein et al. (2009) observed 54% cumulative mortality in the baseline population by Day 9 post-challenge and (Hadidi et al. 2008) noted significant differences in *F. psychrophilum* splenic load between genetic lines on Day 5. Fish were anesthetized with MS-222 (100 mg l^{-1}) and weighed, and blood was collected for PCV and plasma biochemistry as described above. Fish were subsequently euthanized with MS-222 (>250 mg l^{-1}) following blood collection, and

splenic samples were collected for *F. psychrophilum*-specific qPCR, as previously described (Marancik & Wiens 2013). Changes in PCV and biochemical analytes post-infection were considered clinically significant if the observed mean fell outside the established reference interval and was significantly different than the mean from the PBS-challenged control fish.

Statistical analyses

Reference interval data were checked for normality using GraphPad Prism version 5.0 (GraphPad Software). The 90% confidence intervals were determined by a nonparametric bootstrap method following Box-Cox transformation and the central 95% robust method using Reference Interval Advisor (Geffré et al. 2011) with 1.5 interquartile range as the demarcation line for outliers. Correlation of analyte levels within the pooled reference population was performed using Spearman's rank order. Statistical comparison of analyte levels, fish weights, and bacterial loads in the infectivity study was performed by 2-way ANOVA and Pearson correlation using GraphPad Prism Version 5.0. Quantitative PCR data were $\log_{10}$-transformed prior to statistical analyses. A significance level of $p < 0.05$ was used for all mean separation tests and correlations.

RESULTS

Reference interval study

Fish weight, PCV, and 19 plasma biochemistry analytes were measured in each fish. Twenty-eight of 85 (33%) creatinine measurements and 35/85 (41%) potassium measurements fell below the lower limits of detection (0.2 mg dl^{-1} and 1.5 mmol l^{-1}) using the Jaffe reaction and ion specific electrode methodology, respectively, and thus were excluded from further analyses. No significant differences in PCV or biochemical analyte concentrations were found between naive ARS-Fp-R line and ARS-Fp-S line fish, thus all data were compiled into 1 reference interval population (n = 85; Table 2). Data for ALT, AST, CK, LDH, and cortisol were not normally distributed, with 4 outliers detected for both ALT and AST, 3 outliers detected for both CK and LDH, and 1 outlier detected for cortisol. Between the 19 analyzed measurements obtained from each fish, a total of 35 significant positive correlations and 8 signifi-

Table 2. Reference intervals for packed cell volume and select plasma biochemical analytes from data pooled from *Flavobacterium psychrophilum*-resistant (ARS-Fp-R) and -susceptible (ARS-Fp-S) rainbow trout *Oncorhynchus mykiss* lines raised in high-density systems. BUN: blood urea nitrogen; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; ND: not determined

| Analyte	n	Min.	Max.	Med.	Lower limit (90% CI)	Upper limit (90% CI)
Packed cell volume (%)	85	33	51	42	33 (33, 35)	51 (48, 51)
Total protein (g dl^{-1})	85	3.3	5.8	4.70	3.5 (3.3, 3.8)	5.8 (5.6, 5.8)
Albumin (g dl^{-1})	85	1.5	2.6	2.0	1.6 (1.5, 1.7)	2.5 (2.4, 2.6)
Glucose (mg dl^{-1})	85	54	135	89.0	56 (54, 68)	121 (112, 135)
Cholesterol (mg dl^{-1})	85	255	561	385	260 (255, 280)	522 (509, 561)
BUN (mg dl^{-1})	85	0.8	3.3	2.10	0.8 (0.8, 1.2)	3.3 (2.9, 3.3)
Creatinine (mg dl^{-1})	85	<0.2	0.3	–	ND	ND
ALT (U l^{-1})[a]	85	6	107	17.0	6.6 (6.0, 11.0)	77.6 (35.0, 107)
AST (U l^{-1})[a]	85	163	1224	432	216 (163, 253)	1192 (957, 1224)
Creatine kinase (U l^{-1})[a]	85	1158	58002	5140	1222 (1158, 1584)	30529 (20230, 58002)
LDH (U l^{-1})[a]	85	335	4188	841	335 (335, 388)	4038 (2662, 4188)
Total bilirubin (mg dl^{-1})	85	0	0.1	0	0.0 (0.0, 0.0)	0.0 (0.0, 0.1)
Calcium (mg dl^{-1})	85	11	14.8	12.8	11.6 (11.0, 11.8)	14.7 (14.2, 14.8)
Phosphorus (mg dl^{-1})	85	11.3	19.6	15.1	12.1 (11.3, 12.6)	18.9 (17.9, 19.6)
Magnesium (mg dl^{-1})	85	2.3	3.5	2.80	2.3 (2.3, 2.4)	3.5 (3.2, 3.5)
Bicarbonate (mmol l^{-1})	85	26.5	42.6	33.60	26.2 (26.0, 26.7)	43.0 (41.3, 43.0)
Sodium (mmol l^{-1})	85	148	159	154.0	148 (148, 151)	158 (157, 159)
Potassium (mmol l^{-1})	85	<1.5	4.4	–	ND	ND
Chloride (mmol l^{-1})	85	95	112	102.0	96 (95, 97)	110 (106, 112)
Osmolality	85	301	323	313	302 (301, 307)	322 (319, 323)
Cortisol (ng ml^{-1})[a]	85	0.3	14.7	1.7	0.3 (0.3, 0.4)	6.7 (5.6, 14.7)

[a]Outliers present
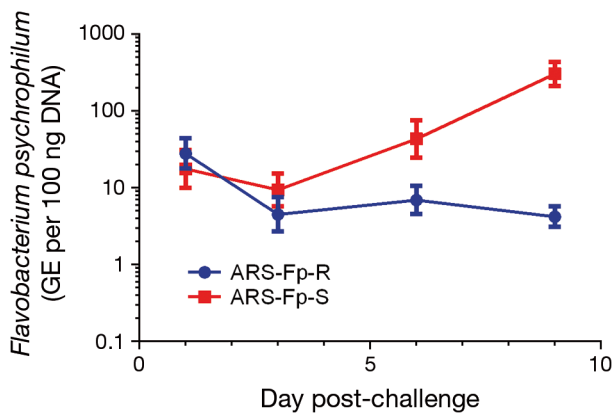
Fig. 1. Splenic bacterial loads in *Flavobacterium psychrophilum*-resistant (ARS-Fp-R) and -susceptible (ARS-Fp-S) rainbow trout *Oncorhynchus mykiss* lines sampled on Days 1, 3, 6, and 9 after experimental infection with *F. psychrophilum* and measured by qPCR (mean ± SE, n = 5). Numbers of *F. psychrophilum* genomic equivalents (GE) were significantly lower in the ARS-Fp-R line compared to the ARS-Fp-S line on Day 9 post-challenge (2-way ANOVA, p < 0.05). Control fish challenged with phosphate-buffered saline contained no detectable *F. psychrophilum*

cant negative correlations were identified (see Table S1 in the Supplement at www.int-res.com/articles/suppl/d111p239_supp.pdf).

Infectivity study

Flavobacterium psychrophilum-challenged fish exhibited clinical signs of lethargy and anorexia by Day 3 post-infection, and necropsied fish demonstrated pathologic changes consistent with BCWD, including splenomegaly and ascites (Rangdale et al. 1999, Nilsen et al. 2011). One mortality in the ARS-Fp-S line occurred on Day 7 post-infection.

Quantitative PCR revealed significantly lower splenic levels of *F. psychrophilum* in the ARS-Fp-R line (mean ± SE, 4 ± 2 genome equivalents [GE] per 100 ng DNA, n = 5) compared to the ARS-Fp-S line (306 ± 133 GE per 100 ng DNA, n = 5) on Day 9 post-infection (p < 0.05; Fig. 1). The splenic bacterial load in the ARS-Fp-S mortality was 7079 GE per 100 ng DNA.

Rainbow trout used as PBS-injected controls (n = 16) showed no significant difference in PCV or biochemical analyte concentrations between genetic lines and did not significantly differ from Day 0 samples (Fig. 2a–g, see also Fig. S1 in the Supplement). Both ARS-Fp-R and ARS-Fp-S line *F. psychrophilum*-infected fish demonstrated clinically significant changes in PCV, total protein, albumin, glucose, calcium, cholesterol, and chloride that fell outside the established reference range on at least 1 post-infection sample day and significantly differed from mean values of PBS-challenged fish (p < 0.05; Fig. 2a–g). Packed cell volume, total protein, and calcium levels significantly differed between ARS-Fp-R and ARS-Fp-S line fish on Day 9 post-infection, with values in the ARS-Fp-S line deviating farther from the reference interval (p < 0.05; Fig. 2a,b,e). Sodium levels were also significantly different between genetic lines on Day 9, but mean values for ARS-Fp-R line fish remained within the reference range and the mean was considered low-normal for ARS-Fp-S line fish, although 3 of 5 fish were below the lower reference range limit (Fig. 2h). Values for osmolality and AST also fell outside the reference interval in 3/5 ARS-Fp-S line fish on Day 9, but the mean was considered low–normal (Fig. S1). Correlations between significantly changed analytes in the infectivity trial, including negative correlation between bacterial load and PCV, total protein, cholesterol, and calcium, are presented in Table 3 for pooled ARS-Fp-R and ARS-Fp-S samples (p < 0.05).

Table 3. Pearson's correlation coefficients (below diagonal) and p-values (above diagonal) for qPCR bacterial levels, weight, and analytes that were significantly changed (p < 0.05) after challenge with *Flavobacterium psychrophilum* in pooled resistant (ARS-Fp-R) and susceptible (ARS-Fp-S) rainbow trout *Oncorhynchus mykiss* lines. Dashes represent values that were not statistically significant (p > 0.05). *Fp*: *Flavobacterium psychrophilum*; PCV: packed cell volume

|	*Fp* load	Weight	PCV	Total protein	Albumin	Glucose	Cholesterol	Calcium	Sodium
Fp load		–	<0.001	<0.01	–	–	<0.05	<0.01	–
Weight	–		–	<0.05	–	–	<0.05	<0.05	–
PCV	−0.56	–		<0.001	<0.05	<0.001	<0.01	<0.01	<0.01
Total protein	−0.42	0.34	0.48		<0.001	<0.01	<0.001	<0.05	<0.001
Albumin	–	–	0.38	0.87		<0.05	<0.001	<0.001	<0.001
Glucose	–	–	0.60	0.48	0.38		<0.01	<0.01	<0.05
Cholesterol	−0.39	0.40	0.47	0.88	0.79	0.46		<0.001	<0.001
Calcium	−0.51	0.40	0.52	0.94	0.82	0.51	0.88		<0.001
Sodium	–	–	0.50	0.66	0.54	0.35	0.55	0.65	
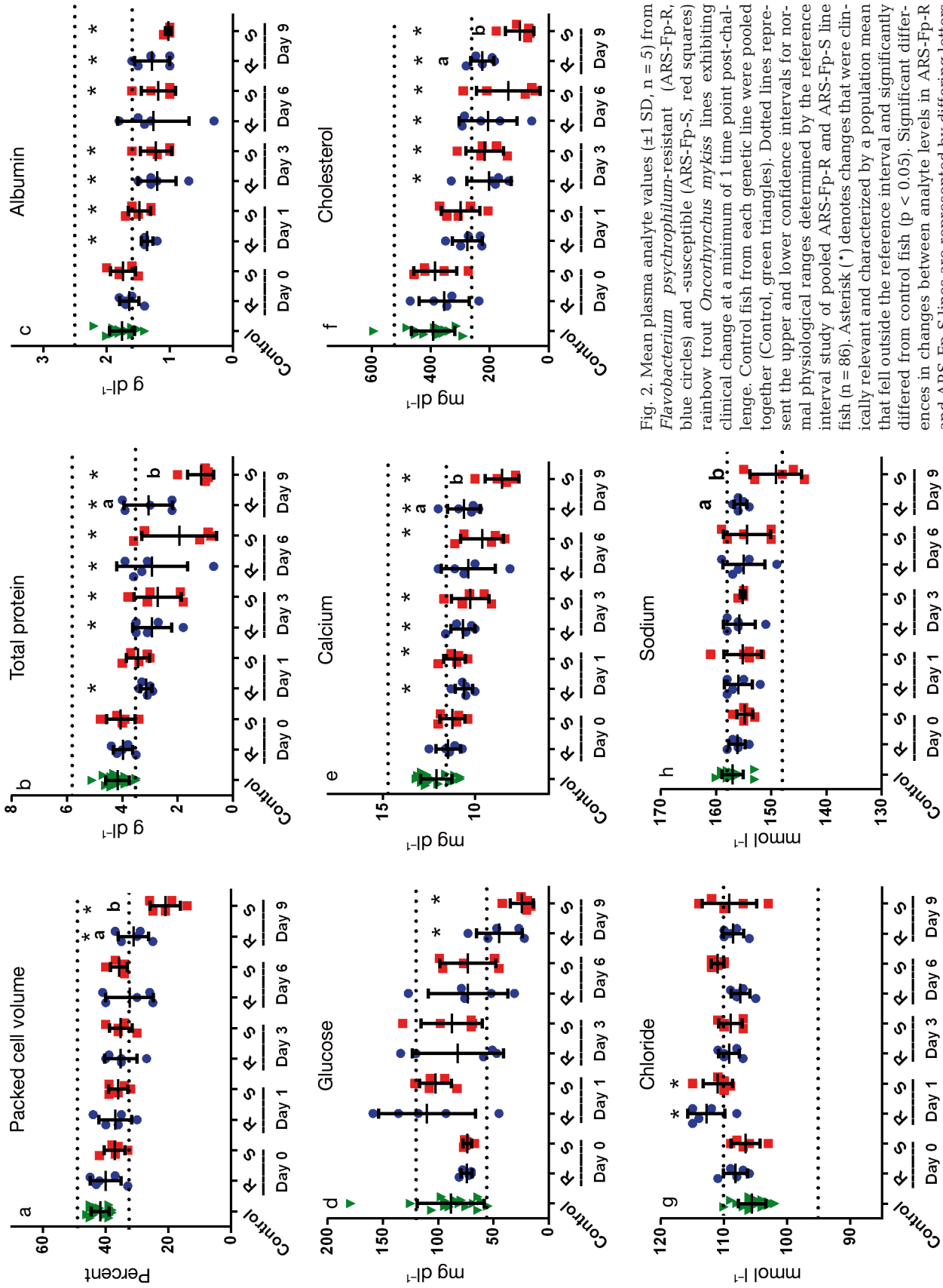
Fig. 2. Mean plasma analyte values (±1 SD, n = 5) from *Flavobacterium psychrophilum*-resistant (ARS-Fp-R, blue circles) and -susceptible (ARS-Fp-S, red squares) rainbow trout *Oncorhynchus mykiss* lines exhibiting clinical change at a minimum of 1 time point post-challenge. Control fish from each genetic line were pooled together (Control, green triangles). Dotted lines represent the upper and lower confidence intervals for normal physiological ranges determined by the reference interval study of pooled ARS-Fp-R and ARS-Fp-S line fish (n = 86). Asterisk (*) denotes changes that were clinically relevant and characterized by a population mean that fell outside the reference interval and significantly differed from control fish (p < 0.05). Significant differences in changes between analyte levels in ARS-Fp-R and ARS-Fp-S lines are represented by differing letters for (a) packed cell volume, (b) total protein, (e) calcium, (f) cholesterol, and (h) sodium on Day 9 post-infection

DISCUSSION

This study established reference-range intervals for juvenile rainbow trout selectively bred for differential survival and monitored changes following laboratory injection challenge with *Flavobacterium psychrophilum* strain CSF259-93. Insignificant differences between ARS-Fp-R and ARS-Fp-S line reference range values of naive animals indicate that for the analytes evaluated, selective breeding did not measurably alter physiological parameters. Following challenge with *F. psychrophilum*, alterations in PCV, total protein, albumin, glucose, cholesterol, chloride, and calcium suggest changes in host physiology that likely contribute to morbidity and mortality in both ARS-Fp-R and ARS-Fp-S line fish. Less severe changes in PCV, total protein, calcium, and sodium in the ARS-Fp-R line compared to the ARS-Fp-S line suggest a lower severity of disease and thus may be associated with differential survival. Consistent with this observation, a trend toward divergent loads was evident by Day 6 post-challenge with significantly lower *F. psychrophilum* splenic loads identified in ARS-Fp-R line fish on Day 9 post-challenge.

There was significant negative correlation of PCV, total protein, cholesterol, and calcium levels with splenic bacterial load. The spleen has been identified as a major organ affected by BCWD (Nematollahi et al. 2005), with relatively high bacterial loads in fish that succumb to infection (Marancik & Wiens 2013). The correlations between bacterial load and PCV, total protein, cholesterol, and calcium suggest the utility of using select analytes as a non-lethal method to monitor infection in this challenge model. Currently, fish are euthanized and organs sampled for bacterial culture or qPCR to assess infection levels. Further validation of the relationship between infection level and biochemical analytes may allow non-lethal and multi-time point sampling in individual fish to assess infection levels during the critical periods of disease and recovery.

The decrease in PCV on Day 9 post-infection is consistent with previous case reports that describe pale gills and viscera in infected fish at necropsy (Nilsen et al. 2011). Similar declines in PCV have been observed in *Plecoglossus altivelis* experimentally infected with *F. psychrophilum*, although the cause of this change is unknown (Miwa & Nakayasu 2005). There were no signs of internal hemorrhage or skin ulcerations in necropsied fish to indicate anemia from loss of blood. Hemolysins capable of binding and lysing erythrocytes have been described for *F. psychrophilum* (Shah et al. 2008,

Högfors-Rönnholm & Wiklund 2010), possibly as a mechanism for iron acquisition by the bacteria. Other possible causes may include decreased red blood cell production due to necrosis of hematopoietic tissue in the spleen and kidney (Rangdale et al. 1999, Nilsen et al. 2011) or anemias of inflammation.

One of the earliest pathophysiological changes was a decline in total protein and albumin that was evident by Day 1 post-infection. Similar protein changes have been described in rainbow trout challenged with *Aeromonas* spp., mixed *Aeromonas* and *Streptococcus* spp., *Cryptobia* sp., and viral hemorrhagic septicemia virus (reviewed by Řehulka et al. 2005). The relatively early alteration in total protein and albumin in infected fish highlights these analytes as early biomarkers of disease in this challenge model. There was a significant negative correlation of total protein, but not albumin, with splenic bacterial load. Further studies identifying causative factors are needed, but an association of plasma total protein and albumin levels with nutritional status in fish (Barnhart 1969, Storebakken et al. 1991) suggests that anorexia may contribute to the low plasma levels.

We found a significant correlation of total protein and albumin with calcium and cholesterol levels, which is similar to previous reports of rainbow trout experimentally infected with *Arcobacter cryaerophilus* (Aydin et al. 2000, 2009). A substantial portion of calcium is protein-bound in rainbow trout (Björnsson & Haux 1985), and protein-bound cholesterol has been demonstrated in *Fundulus heteroclitus* (Jensen & Taylor 2001), although this relationship has not been established in rainbow trout. This suggests that the loss of protein and albumin as carrier-proteins may have contributed to hypocalcemia and hypocholesterolemia. The observed decrease in calcium in a subset of ARS-Fp-R and ARS-Fp-S line fish on Day 0 did not correlate with protein levels, although ionized calcium was not measured specifically due to sampling requirements. The cause of this change on Day 0 is unknown, and further studies to elucidate control of calcium transport across epithelial barriers during infection and stress are needed.

Hypoglycemia observed on Day 9 post-infection is consistent with reports of decreased blood glucose in rainbow trout experimentally and naturally infected with bacteria, likely secondary to anorexia (Barham et al. 1980, Aydin et al. 2000, 2009). Plasma glucose did not significantly correlate with bacterial load, suggesting that levels may be affected by extrinsic factors such as diet (Bergot 1979) or a non-specific shift to glycolytic pathways during the acute phase of infection (Bolaños et al. 2010).

There was a transient increase in chloride, most notably on Day 1 post-infection, and a decrease in sodium and osmolality on Day 9 for a select number of ARS-Fp-S line fish. No change in potassium was observed, although levels could not be assessed in relation to reference intervals because of assay linearity limitations. Demonstrated electrolyte changes in rainbow trout challenged with bacterial (Barham et al. 1980), viral (Řehulka 2003), and parasitic (Rand & Cone 1990, Powell et al. 2006) pathogens have been shown to be variable with little consistency between sodium, chloride, and potassium fluctuations. No significant correlation was present between sodium and chloride levels post-challenge in this study. However, current osmoregulatory models in freshwater teleosts provide evidence for co-regulation of sodium, chloride, and potassium plasma levels in healthy fish (Le Bras et al. 2011), which is consistent with the observed positive correlation of sodium and chloride levels in reference interval samples.

The similarity between biochemical profiles in *F. psychrophilum*-challenged fish and rainbow trout infected with unrelated bacterial and viral pathogens (Barham et al. 1980, Aydin et al. 2000, 2009, Řehulka et al. 2005) indicates that the observed pathophysiological changes are not likely to be specific to BCWD. Changes may represent a general and non-specific host response to infectious disease. This suggests that plasma biochemistry may be useful as a general and early indicator of disease for experimentally and naturally infected fish, if defined reference ranges can be produced.

Biochemistry reference intervals deviated slightly from those described by Manera & Britti (2006), who used a similar statistical analysis to develop normal biochemistry ranges in 240 g rainbow trout. BUN, ALT, phosphorus, magnesium, and chloride levels from *F. psychrophilum*-infected fish fell outside the reference intervals produced by Manera & Britti (2006) on at least 1 day post-infection, whereas they demonstrated no significant deviation from normal using the reference range in this study. Variability in results between studies highlights the risk in applying reference-range intervals to fish populations raised under different environmental conditions (Wedemeyer & Nelson 1975) or when using different instrumentation.

We found no evidence to support stress as a component of this challenge model, based on insignificant alteration of cortisol levels in infected fish. Cortisol level reference intervals were consistent with previous studies exploring baseline cortisol levels in rainbow trout (Carballo et al. 1995, Jentoft et al. 2005, Lankford & Weber 2006). Insignificant change in cortisol after infection differs from previous studies that showed acute increases in cortisol levels in rainbow trout experimentally infected with *Vibrio anguillarum* (Ackerman & Iwama 2001) and in channel catfish bred for resistance and susceptibility to *Edwardsiella ictaluri*, with more pronounced changes in susceptible line fish after experimental challenge (Bilodeau et al. 2003).

We had difficulty assessing reference intervals for AST, ALT, LDH, and CK, as a substantial number of outliers resulted in positive skewed distributions. These results are in general agreement with previous observations in rainbow trout, although sample size and statistical analyses were not uniform between studies (Wedemeyer & Nelson 1975, Miller et al. 1983, Manera & Britti 2006). These analytes may be affected by extrinsic factors, and their utility as biomarkers of health and disease in intensively-reared rainbow trout is unclear.

In a few analytes, the population mean in *F. psychrophilum*-infected fish fell outside the established reference interval, but the value did not significantly differ from PBS-injected fish, precluding characterization as a clinically relevant change. This occurred for albumin and calcium in ARS-Fp-R line fish on Days 3 and 6, respectively, and for chloride in ARS-Fp-S line fish on Day 6. The lack of observed significance may represent a sample size that insufficiently accounted for variation in individual host response. Anecdotally, it was likely also affected by analyte values in PBS-injected fish that fell near the periphery of the reference range.

The degree of mortality was low in this study compared to previous challenge studies that used approximately 13-fold higher challenge dose (when normalized to fish body weight) of the CSF259-93 strain of *F. psychrophilum* (Leeds et al. 2010); thus, pathophysiological changes may deviate farther from the reference interval in perimortem and more severely infected fish.

Future studies will examine plasma analytes in healthy and diseased ARS-Fp-R and ARS-Fp-S line fish under natural field conditions. Exploration of resting plasma biochemistry values and changes in analytes after infection will help compare and contrast disease pathophysiology between this experimental challenge model and naturally infected fish. Characterization of biochemical changes in naturally infected fish may allow a more defined understanding of host–pathogen interactions and the pathophysiology of BCWD.

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