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Genotypic and phenotypic correlates with proliferative kidney disease-induced mortality in Atlantic salmon

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ABSTRACT: Heritable variation in resistance to pathogens has been reported in many fish species, but little is known about its genetic architecture. To extend understanding, an investigation was made of the association of resistance to proliferative kidney disease (PKD) in 4 second filial generation (F_2) families of Atlantic salmon with molecular markers from different genetic linkage groups in the species' genome, following a natural disease outbreak. PKD causes serious mortality in cultured salmonids. In addition to mortality, associations with growth-related traits were also examined, as immune responses are energetically costly and have been observed to reduce growth. Associations were investigated for 34 microsatellite markers and 5 restriction fragment length polymorphism (RFLP) loci from 3 regions of the growth hormone 1 gene (*GH1*). The phenotypic and genotypic character of survivors was compared with unexposed fish derived from the same families. Mortality was not size-selective, but growth in the survivors was reduced, and fish had a lower condition factor than unexposed fish, suggesting an energetic cost to resistance. Five markers showed significant allele frequency differences between survivors and unexposed fish, albeit in single families. Prior to correction for multiple tests, 2 of these markers were also linked to variation in growth-related traits among survivors, along with a further 7 markers. Though sample sizes constrained the power of the analysis, the study points to regions of the salmon genome that may contain quantitative trait loci related to PKD resistance, on which further work on the genetic architecture of PKD resistance in this species could focus.

KEY WORDS: Proliferative kidney disease · PKD · Atlantic salmon · Resistance · Growth · Marker-trait associations · Quantitative trait locus · QTL

INTRODUCTION

Heritable variation between and within populations in their resistance to pathogens (including parasites) has been reported in many fish species (Price 1985, Gjedrem 2000, Coltman et al. 2001), including Atlantic salmon *Salmo salar* (Gjedrem et al. 1991, Mustafa & MacKinnon 1999). However, our understanding of the genetic architecture of pathogen resistance, such as which genes and gene interactions underpin variation in these quantitative traits, remains limited. In Atlantic salmon, this is in partly due to the long generation times required to create informative second-generation families for gene mapping studies. Yet knowledge of the genetic architecture of resistance is essential to understand the genetic basis of adaptive differentiation among wild populations (e.g. for *Gyrodactylus salaries*) (Bakke et al. 2004), as well as to gain knowledge of the relevant quantitative trait loci (QTL) (Lande & Thompson 1990) to design more effective selective breeding programmes.

Insight into the basic genetic architecture of pathogen resistance/susceptibility can be gained through family-based experiments by examining associations between genotypic and phenotypic variation in resistance-related traits such as survival. Establishment of associations of molecular markers from different genome regions with a resistance-related phenotypic

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trait would help to identify regions of the genome containing potential QTL influencing this trait. This approach has been used successfully in a number of studies of salmonid fishes, in which differential mortality to disease was the trait examined (Moen et al. 2004a, Barroso et al. 2008, Houston et al. 2008). However, disease may or may not lead to increased direct mortality in a host population. Where pathogen virulence is low (Alexander 1981, Ewald 1983), overall fitness may be reduced indirectly through influences on other heritable traits, such as growth and body condition (Sheldon & Verhulst 1996, Lochmiller & Deerenberg 2000). Mounting an immune response to a disease is costly, as energy and resources are directed towards regulation of the immune system and become unavailable for other important somatic processes. Consistent with this view, reduced growth following infection has been observed in a number of species (Lochmiller & Deerenberg 2000), including Atlantic salmon (Ellis et al. 1985). Thus, understanding of the genetic architecture of resistance also needs to consider the importance of heritable variation at genes that underpin variation in growth and growth-related fitness (Robison et al. 2001, Gilbey et al. 2005), which may be affected by the resistance response, e.g. the growth hormone 1 gene (*GH1*) (Björnsson 1997, Kuhnlein et al. 1997).

Infection with the myxozoan endoparasite *Tetracapsuloides bryosalmonae*, which causes proliferative kidney disease (PKD), occurs seasonally in many salmonid species and generally results in high levels of mortality (Hedrick et al. 1993). However, mortality rates are highly variable and often mediated through secondary infections. Furthermore, surviving fish appear to develop immunity against a second *T. bryosalmonae* infection (Hedrick et al. 1993), suggesting the involvement of an acquired immune response. Interspecific comparisons show some salmonid host species to be more resistant than others (Seagrave et al. 1981, Brown et al. 1991, Feist & Bucke 1993), and the narrow sense heritability of resistance, following a natural outbreak, was estimated to be $h^2 = 0.19 \pm 0.08$, based on differences between 2 strains of rainbow trout *Oncorhynchus mykiss* (Butterfield 2008). Beyond this, genetical aspects of the disease remain poorly understood, including the extent of intraspecific genetic variation in resistance and the genetic architecture of the heritable response to the disease (Holland et al. 2003).

Here we report the results of a study of associations between the phenotypic and genotypic character of PKD resistance by analysing second filial generation (F_2) families of Atlantic salmon following a serendipitous natural outbreak of the disease. Survivors were analysed with regard to growth-related, phenotypic traits (length, weight, and condition factor) and variation at 34 microsatellite markers and 5 loci in *GH1*. Those data were compared with unexposed fish derived from the same families, as no analysis of mortalities was possible. A basic, single marker-single linkage group approach was used (Gilbey et al. 2006), whereby microsatellite markers were chosen to cover each linkage group of the linkage map of Gilbey et al. (2004), along with further markers from the linkage groups identified in SALMAP (www.asalbase.org/sal-bin/index) and Moen et al. (2008). Furthermore, a growth hormone gene marker, not mapped to any identified linkage group, was screened to assess association with this region of the genome. The main objective of the study was to identify genome regions likely to contain genes important to resistance on which to focus more detailed fine-scale QTL mapping studies.

MATERIALS AND METHODS

Atlantic salmon families. The 4 experimental families included in the study consisted of F_2 crosses between salmon originating from the Bristol Cove River (46° 63′ N, 53° 19′ W), Canada, and from the river Don (57° 11′ N, 3° 6′ W), Scotland. Mature adults from each source were crossed to produce first filial generation (F_1) families. At age 2+, 4 mature F_1 males were crossed to 4 mature F_1 females in a single mating crossing scheme. A total of 450 fish from each family (n = 1800) were reared in separate tanks at the MSS Freshwater Laboratory Rearing Unit at Almondbank, Scotland, until a proportion of fish were transported to the MSS Marine Laboratory Fish Cultivation Unit at Aultbea, Scotland, at 2 years of age. Prior to transport, a small piece of tissue was taken from different fins of fish from 3 of the families to allow external identification of the family of origin of each individual. Thereafter, the study fish were retained in 2 mixed family groups consisting of 300 and 150 fish from each family at Aultbea and Almondbank, respectively. Both groups were kept at equal densities and fed ad lib. The analysis of fish length and weight post-transport showed that the family groups, in both cases, consisted mainly of parr, but included some smolts.

Disease outbreak. Two weeks after the large mixed family group (n = 1200) had been transported to Aultbea, it incurred significant mortality (90%), and fish were treated with the antibiotic oxytetracycline for 10 d, based on an assumption that the mortality factor was furunculosis (caused by the bacteria *Aeromonas salmonicida*. However, oxytetracycline proved ineffective. Ten survivors were analysed by the Fish Health Inspectorate at the MSS Marine Laboratory, Scotland, and histological analysis revealed that *Tetracapsuloides bryosalmonae* was the causative agent. Further analyses were carried out, on a variety of tissues, to

assess potential infection with other viruses (infectious salmon anemia virus, viral haemorrhagic septicaemia virus, infectious haematopoietic necrosis virus, and infectuous pancreatic necrosis virus), bacteria (*A. salmonicida* and *Renibacterium salmoninarum*) and/or parasites (*Gyrodactylus salaris*), but those analyses did not reveal any signs of secondary infections by those pathogens. These are the most frequent disease vectors associated with mortality in Atlantic salmon culture in Scotland (Fish Health Inspectorate pers. comm.). The PKD pathogen is not treatable by antibiotics, and malachite green and fumagillin are the only 2 chemotherapeutants capable of controlling this disease (le Gouvello et al. 1999). Based on this analysis, it is reasonable to conclude that the surviving fish (10%, n = 120) were those that were resistant to PKD, as opposed to another pathogen.

A total of 27 d after the start of the infection, when there had been no mortality for 13 d, the standard length (mm), weight (g), and family of each surviving fish were recorded and a fin clip taken for DNA analysis. Condition factor (CF) was calculated following the method of Wootton (1990). The unforeseen nature of the infection meant that phenotypic data were not available for fish prior to the infection or from the fish that died of PKD, nor was there genotypic data for fish that had died. To address this problem, data of the PKD survivors were compared to phenotype (n = 201) and genotype (n = 305) information obtained from a subsample of fish from the small mixed family group that had been retained at Almondbank and had, consequently, not been exposed to the disease. The phenotypic data on those fish had been collected 5 d earlier, on Day 22 after the start of the infection of the Aultbea group.

Molecular markers. A total of 34 variable microsatellite loci were screened. These were chosen to mark the linkage groups identified in the map of Gilbey et al. (2004), along with markers from the SALMAP, Moen et al. (2008), and a number of unlinked markers (Table 1). To screen for variation at these loci, total genomic DNA was extracted from fin clips (n = 425) after Knox et al. (2002). PCR was performed in a 12.5 µl reaction volume containing 5 to 100 ng DNA, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, and 0.25 units of ABgene *Taq* DNA polymerase (ABgene). PCR cycling conditions consisted of a touchdown sequence detailed in Gilbey et al. (2004). Electrophoresis was carried out using a MegaBace 500 capillary sequencer (GE Healthcare).

In addition to these linkage group markers, the association of resistance with variation in *GH1* region of the Atlantic salmon (Ryynänen & Primmer 2004a) was assessed. Sequences of a published 4712 bp fragment of the *GH1* (GenBank Accession #AY614002–AY614008) were aligned to investigate variation across European and North American populations. Four single nucleotide polymorphisms (SNPs) were identified and analysed using restriction fragment length polymorphism (RFLP) analysis, together with one insertion/deletion site (Indel) (Table 1). These were used to define composite haplotypes for this gene region, which served as markers of the gene and its associated genome region. PCR amplifications of the *GH1* loci were performed in a total volume of 20 µl containing 1 to 3 ng μl^{-1} DNA, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 250 µM of each dNTP, 0.5 µM of each primer (sequences obtained from Ryynänen & Primmer 2004b) and 0.25 units of ABgene *Taq* DNA polymerase. PCR cycling conditions were performed according to Ryynänen & Primmer (2004b). Restriction digests were carried out in a 20 µl reaction volume comprising 2 to 5 µl of PCR product and 2 U of restriction enzyme (Table 1). Digests were incubated overnight at the manufacturer's specified temperature and electrophoresis carried out on ethidium bromide stained 3% agarose gels.

Statistical analysis. Differences in survival rate between families were assessed for significance using a chi-square test. Due to the bimodal growth-related trait distributions observed here, a phenomenon common in most Atlantic salmon populations (Thorpe 1977, Heggenes & Metcalfe 1991), a principal component analysis (PCA), based on the correlation matrix between the phenotypic traits, was performed within both the PKD survivors and unexposed fish to split the fish into 2 groups according to their developmental status, a lower modal (parr) and an upper modal (smolts) group. Discrimination analysis was carried out to provide an estimate of the distance (Mahanalobis D^2) between the groups and to test for its significance. The PCA made it possible to compare differences in means (Mann-Whitney *U*-test) and distributions (2-sample Kolmogorov-Smirnov test) of fish size and condition between PKD survivors and unexposed fish within both developmental groups.

Potential size-selective mortality was investigated by: (1) testing for differences in the proportion of parr:smolts in the PKD survivors and unexposed fish, and (2) by comparing trait distributions (2-sample Kolmogorov-Smirnov tests) on standardised data. Distributions were standardised to obtain trait means of 0 for the PKD survivors and unexposed group, separately, by subtracting the trait means from the individual data. It was then possible to examine whether the character of the trait distributions had altered.

Single marker allele frequency differences between PKD survivors and unexposed fish were compared by chi-square analysis for dam and sire, separately. The

Table 1. *Salmo salar*. Details of Atlantic salmon (a) microsatellite and (b) growth hormone 1 gene (*GH1*) loci used. GenBank accession numbers are given for microsatellites, while locus name, restriction enzyme/Indel description, and fragment sizes are given for *GH1* loci. nd = no data

(a) Microsatellite markers

Marker	Linkage group	GenBank accession #
Ssa202	1[a]	U43695
Ssa171	2[a]	U43693
Ssosl311	3[a]	Z48597
Ssa42	3[a]	AF019174
Ssa48	4[a]	AF019176
Ssa11	5[a]	U58894
Ssa14	6[a]	–[d]
Ssa164	7[a]	AF019152
SSsp3016	8[a]	AY372820
SSsp2201	9[a]	AY081807
Ssosl444	10[a]	Z49997
CmrSS1.22	11[a]	AF020848
Ssa197	12[a]	U43694
Ssa213	13[a]	AF019163
Ssa9	14[a]	AF019197
SSsp2210	15[a]	AY081808
Ssa420	5[b]	AJ402737
Ssa64	6[b]	AF019183
Ssa417	11[b]	AJ402734
Ssa85	18[b]	U43692
Ssa405	28[b]	AJ402722
µ20.19	23[b]	nd
Ssa16	26[b]	AF019153
Ssa74	12[b]	AF019187
SSsp1605	18[c]	AY081812
SSsp2216	7[c]	AY081811
uF43	Unlinked	U37494
Ssleen82	Unlinked	U86706
Rsa172	Unlinked	AF271582
Hae050	Unlinked	AF271499
Ssa138	Unlinked	U58902
Ssa65	Unlinked	AF019184
Hae029	Unlinked	AF271486
Hae065	Unlinked	AF271510

(b) *GH1* loci

Polymorphism	Restriction enzyme/ Indel description	Fragment sizes
SNP2	*Mnl*I	A: 164, 131 B: 295
SNP3	*Xba*I	A: 102, 550 B: 652
SNP4	*Tsp509*I[e]	A: 392, 105, 69, 46, 40 B: 432, 105, 69, 46
SNP5	*Dde*I	A: 212, 440 B: 652
Indel1	TGTGTGTGTGTA	A: 217 B: 205

Linkage groups from the linkage map of [a]Gilbey et al. (2004), [b]SALMAP, or [c]Moen et al. (2008). [d]From McConnell et al. (1995). [e]Isozyme of *TAS*1 (see Ryynanen & Primmer 2004b)

linkage relationships of the *GH1* gene variants resolved were established by comparing observed offspring genotypes in the control group with those predicted for different phase relationships in the parents in each family. As no recombination was detected, the composite parental haplotypes could then be used as markers for the *GH1* gene and its associated gene region in the same way as the allelic variants of the microsatellite loci screened. The results of the tests for each marker for each dam and sire can be viewed as independent tests of the same hypothesis, i.e. that the gene region linked to that marker has been differentially selected for among PKD survivors. Thus, the results obtained for each family can be considered collectively for each marker using Fisher's method for combining probabilities (Sokal & Rohlf 1995), to test for the overall significance of changes in allele frequency observed. These significance levels were then corrected for carrying out multiple tests of marker association with phenotypic variation (i.e. 35 independent overall tests) using a sequential Bonferroni procedure.

The relatedness of the F_1 parents that were used to produce the informative families was determined by carrying out likelihood analysis using the Kinship program (Goodnight & Queller 1999). This program was run for 100 000 iterations under the null hypothesis that the parental individuals were unrelated, the alternative hypothesis being that the parents were full sibs.

Within the PKD survivors, associations between each growth-related trait and genetic marker were examined using a generalised linear model (GLM). In Atlantic salmon, there is a potential complication in such analyses because recombination in males is severely reduced compared to females (Gilbey et al. 2004, Moen et al. 2004b). This is dealt with in the model used by looking at the male and female effects separately, as well as their interaction. Due to the low numbers of survivors (n = 8), Family 3 was not included in the analysis. An initial GLM was created to test for the effects of family, development status, and their interaction on each phenotypic trait. The model was then extended to include genetic information. The 2 models were:

Partial model:

$$Y_{ijkl} = c + f_i + d_j + (f_i \times d_j) + \varepsilon_{ij} \quad (1)$$

Full model:

$$Y_{ijkl} = c + (f_i + d_j + (f_i \times d_j))/ (MF_k + MM_l + MF_k \times MM_l) + \varepsilon_{ijkl} \quad (2)$$

where Y_{ij} is the phenotypic parameter of the ith individual with the jth development status; c is a constant; f_i is the effect of family; d_j is the effect of development status, MF_k is the effect of the marker allele inherited from the female parent, MM_l is the effect of the marker

allele inherited from the male parent, and ε is the random residual associated with each individual.

The significance of the difference between the partial (Eq. 1) and the full (Eq. 2) models was analysed for individual markers and growth-related traits using an *F*-test, and the difference in R^2 between the 2 models was taken as the amount of variance explained by the trait-marker association. In cases where there was a significant difference between the models, sex-specific analysis was carried out by creating 2 additional GLMs of the same design, which were then used to investigate the dam and sire components, separately. To address the issue of significance levels where multiple tests are carried out, a sequential method, based on Fisher's approach for testing the overall significance of a set of probabilities, was used. Fisher's method is based on testing the distribution of probabilities observed compared to the distribution expected if the probabilities are purely the result of chance, in this case chance associations. Where a significant departure in the distribution of probabilities across markers was detected, the most extreme probability was removed sequentially and the set of probabilities retested; this was repeated until the overall test was no longer significant. This made it possible to demonstrate whether the extreme values, and how many of them, contributed to obtaining the significant departure of the probability distribution from that expected under the null hypothesis, namely that the distribution was due to random associations. This distributional test was applied to each growth-related trait individually, as the 3 growth-related traits used were not independent of each other.

RESULTS

The overall survival of the exposed fish was 10% (n = 120), but this varied significantly among families (χ^2_3 = 35.96, p < 0.0001), with highest survival in Family 4 (17.67%), followed by Family 1 (12%), Family 2 (7%), and the lowest in Family 3 (2.67%). PCA of the data revealed a single discriminatory factor that explained 97.36% and 98.72% of phenotype variation of the PKD survivors and unexposed fish, respectively. Discriminant analysis showed significant differences between the PCA-split groups in both PKD survivors (Mahanalobis D^2 = 35.14, F = 620.2, p < 0.0001) and unexposed fish (Mahanalobis D^2 = 18.99, F = 191.5, p < 0.0001).

On average, both parr and smolts of the PKD survivors were significantly smaller and had a lower condition factor compared to the unexposed group (Table 2). Mean parr length of the PKD survivors was 8% less, mean weight was 30% lower, and mean condition factor 3% lower; mean smolt length of the PKD survivors was 16% less, mean weight was 47% lower, and mean condition factor 7% lower. The trait distributions for both parr and smolts were 'shifted' towards smaller individuals in the PKD survivors compared to unexposed fish in all families, resulting in significant differences in the distributions (Table 2). Though parr and smolts of the PKD survivors were smaller and had lower condition factors than the unexposed group, the proportion of parr to smolts did not differ between the two groups (χ^2_3 = 0.013, p = 0.91), with 21.67% (26 of 120) and 22.89% (46 of 201) of fish classified as smolts in the PKD survivors and unexposed group, respectively. Furthermore, the shapes of the standardised trait distributions were not significantly different in either parr (weight: p = 0.36; length: p = 0.87; CF: p = 0.45) or smolts (weight: p = 0.053; length: p = 0.41; CF: p = 0.09).

Prior to correction for multiple tests, significant allele frequency differences between the PKD survivors and the unexposed group were found for 4 microsatellite markers (*Ssa14*, *Hae050*, *Ssa85,* and *Ssleen82*) and the *GH1* locus (Table 3). These were all observed in single parents and were significant only in the dams and sires of Family 1 and Family 4. After combining the probabilities across families, sequential Bonferroni correction showed that significant allele frequency differ-

Table 2. *Salmo salar*. Differences in means and distributions of weight, length, and condition factor (CF) of parr and smolts between proliferative kidney disease (PKD) survivors and unexposed fish. Significance of means examined using Mann-Whitney *U*-tests; trait distributions analysed with Kolmogorov-Smirnov 2-sample tests (K-S test). n = no. of individuals. Significance levels: *p < 0.05, **p < 0.01, ***p ≤ 0.001

Trait	Group	Unexposed fish			PKD survivors			*U*-test	K-S test
		n	Mean	CI (95%)	n	Mean	CI (95%)	p	p
Weight	Parr	155	15.36	14.65–16.08	94	11.09	10.38–11.80	***	***
	Smolts	46	50.81	47.59–54.03	26	26.95	25.08–28.82	***	***
Length	Parr	155	97.14	95.49–98.78	94	91.92	89.94–93.91	***	**
	Smolts	46	156.78	153.86–159.71	26	131.27	126.35–136.19	***	***
CF	Parr	155	1.57	1.53–1.61	94	1.39	1.36–1.43	***	***
	Smolts	46	1.30	1.27–1.34	26	1.21	1.11–1.32	***	***

ences remained for the markers *Ssa14*, *Ssa85*, *Ssleen82,* and *GH1* (Table 3).

The kinship analysis revealed that the parental fish that were used to create Family 1 were full sibs (p = 0.001), whilest the parental fish used to create the other 3 families were not. Those individuals were, due to the single mating design, less inbred than half sibs. Interestingly, Family 1 showed the most and strongest associations.

For all 3 growth-related traits, variation was significantly associated with a number of microsatellite markers prior to correction for multiple tests (Table 4). These explained between 1.9 and 7.1% of the variance in weight, between 2.8 and 7.4% of the variance in length, and between 10.6 and 21.9% of the variance in condition factor. The significant component of these associations was variously associated with variation at either the dam or sire, with a significant dam component in 4 out of 5 associations with weight, explaining between 4.0 and 7.1% of its variance, in 2 out of 5 cases in relation to length, where it explained between 0.4 and 6.4%, and in 3 out of the 4 associations with CF, with between 5.0 and 7.6% of the variance explained (Table 4). Allelic variation in the sire explained between 2.0 and 5.0% of the variance in weight, 7.4% of the variance in length, and 12.9% of the variation in CF.

The distributions of probabilities for the markers showed a significant departure from expected in relation to weight (p = 0.003) and length (p = 0.007), and was nearly significant with respect to condition factor (p = 0.071). Sequential application of the Fisher's test indicated that the 3 most significantly associated markers in relation to weight (*µ20.19, SP3016, Ssa14*) contributed to the significant departure of the distribution of probabilities for this trait. Excluding these, the p-value for the Fisher's test was 0.099. This was also the case with regards to length, after the first 2 individually significant marker values (*Sp3016, Ssa48*) were excluded (p = 0.127). In the case of condition factor, the exclusion of the one most significant value (*Ssa16*) made the Fisher's test highly non-significant (p = 0.284). Similar results were found in the sex-specific analysis, though these were all related to the dam-based analysis. The p-values associated with the markers *µ20.19* and *Ssa11* remained significant in relation to weight after correction for multiple tests, while *SP3016* and *Ssa48* remained significantly associated with length and CF, respectively. The omission of those p-values turned the Fisher's test non-significant (weight: p = 0.164; length: p = 0.184; CF: p = 0.062). In the sire, none of the p-values remained significant after correction. The only marker appearing, prior to correction for multiple tests, to give a significant association in relation to all 3 traits is *SP3016* and 3 markers (*µ20.19, Ssa48,* and *Ssa9*) do so in relation to 2 of the traits.

DISCUSSION

The present study shows significant phenotypic and genotypic differences in the PKD survivors compared to unexposed fish from the same families. PKD survivors were, on average, smaller and condition factor was lower in both parr and smolts. Mortality did not appear to be size-selective, as the parr:smolt ratio and the character/shape of the trait distributions were similar in both groups, suggesting rather that there had been a difference in growth performance between the 2 groups. Additionally, differences were observed in the allele frequencies between the groups with respect to 5 markers, 4 microsatellite and 1 *GH1* markers. Furthermore, there was evidence supporting associations of at least 6 markers with growth-related traits.

Table 3. *Salmo salar*. Significant χ^2 tests for allele frequency differences between proliferative kidney disease (PKD) survivors and unexposed fish, assessed for dam and sire separately within each family. p-values in *italics* are significant. Fisher's test for combining probabilities was used to test for significance across all families. p-values in **bold** are significant (p < 0.0015) after correction for multiple tests (sequential Bonferonni) of the combined p-values. nd = no data; na = dam/sire was homozygous for that particular marker

Marker	Parent	Family 1	Family 2	Family 3	Family 4	Across all families
Ssa14	Dam	0.645	0.114	0.198	*0.033*	***<0.0001***
	Sire	*<0.0001*	na	0.832	na	
Hae050	Dam	0.313	0.131	0.713	1.000	0.1712
	Sire	*0.004*	0.885	0.732	0.330	
Ssa85	Dam	*0.0001*	nd	0.652	0.110	*0.0019*
	Sire	na	nd	0.652	na	
Ssleen82	Dam	0.630	0.106	na	0.133	*0.0088*
	Sire	0.924	0.073	0.317	*0.002*	
GH1	Dam	*0.004*	0.139	0.572	0.344	*0.0351*
	Sire	0.309	0.308	0.459	0.216	

Table 4. *Salmo salar*. Generalized linear model (GLM) of associations between markers and weight, length, and condition factor of the proliferative kidney disease (PKD) survivors. Linkage group (LG) is divided into markers derived from the linkage maps of Gilbey et al. (2004) and SALMAP. p-values are associated with differences between the proportion of variation (R^2) explained by the partial (Eq. 1) and full (Eq. 2) models, which encompassed analysis of both dam (D) and sire (S) components combined and analysed separately. See text for GLM details. p-values in **bold** are significant after correction for multiple tests. nd = no data

Marker	LG	R^2 partial	R^2 (D + S)	Difference	p	R^2 (D)	Difference	p	R^2 (S)	Difference	p
Weight											
µ20.19	23[b]	67.9	71.9	4.0	**0.002**	71.9	4.0	**0.002**	nd	nd	nd
SP3016	8[a]	67.9	75.0	7.1	**0.016**	75.0	7.1	0.005	71.1	3.2	0.406
Ssa14	6[a]	67.9	72.6	4.7	**0.033**	73.6	5.7	0.004	68.5	0.6	0.878
Ssa9	14[a]	67.9	72.9	5.0	0.04	71.4	3.5	0.062	72.9	5.0	0.040
Ssa42	3[a]	78.4	80.3	1.9	0.05	78.0	−0.4	0.511	80.4	2.0	0.022
Ssa11	5a	67.9	73.1	5.2	0.05	73.8	5.9	**0.002**	68.8	0.9	0.526
Length											
SP3016	8[a]	77.1	83.3	6.2	**0.002**	83.5	6.4	**<0.001**	79.1	2.0	0.434
Ssa48	4[a]	77.1	80.5	3.4	**0.006**	77.5	0.4	0.026	75.1	−2.0	0.777
µ20.19	23[b]	77.1	79.9	2.8	0.012	79.9	2.8	0.12	nd	nd	nd
Ssa9	14[a]	77.1	84.5	7.4	0.035	83.3	6.2	0.109	84.5	7.4	0.035
Condition factor											
Ssa16	26[b]	25.4	47.3	21.9	<0.001	31.7	6.3	0.008	38.3	12.9	0.003
Ssa48	4[a]	25.4	41.1	15.7	0.002	33.0	7.6	**0.003**	21.9	−3.5	0.689
SP3016	8[a]	25.4	36.0	10.6	0.009	30.4	5.0	0.034	27.6	2.2	0.083
Ssa74	12[b]	25.4	37.3	11.9	0.036	29.0	3.6	0.459	32	6.6	0.072

[a]Gilbey et al. (2004)
[b]SALMAP

The observed phenotypic change indicated a 'shift' in the means and distributions of the size of fish towards smaller individuals in the PKD survivors compared to the unexposed group of fish. These 'shifts' could be explained either by environmental influences associated with the different conditions under which the fish were retained, or by a cost associated with mounting an immune response, with resources reallocated to this response rather than to growth. Although fish were kept at equal densities and were fed ad lib, both groups of fish were kept in different environments and were treated differently with respect to the antibiotic, which could have affected growth performance through loss of appetite (Villareal et al. 1988, Björnsson et al. 1989, Solbakken et al. 1994). However, despite the environmental differences between the hatcheries, it may be assumed that the influences on growth were similar for both sites, i.e. the environmental conditions and hatchery practices were not such as to favour growth at a particular site. As such, growth differences between the 2 groups due to environmental and treatment differences would have been expected to be less marked than was observed. In other words, it is unlikely these factors could have led to the extreme growth differences and pattern observed.

In both environments, growth may have been expected to be similar or perhaps even faster in smolts than in parr, as the former would have been more competitive and would therefore have acquired more food (Thorpe 1977, Metcalfe et al. 1989). However, this is contrary to what was observed, with smolts growing more slowly than parr in the PKD survivors, which suggests that the reduced growth was due to the PKD infection. Studies on pigs (van Heugten et al. 1996), Japanese quail *Coturnix coturnix japonica* (Fair et al. 1999), and salmonids (Ellis et al. 1985) have also reported weight loss and reduced growth associated with mounting an immune response. Ellis et al. (1985) found that the first signs of infection with PKD coincided with a significant reduction in growth rate in brown trout *Salmo trutta* and Atlantic salmon parr. As such, it is likely that the PKD infection resulted in the observed reduction in growth of both parr and smolts. Therefore, the reduced growth observed is consistent with the view that a cost is associated with mounting an immune response. Indeed, other studies support the view that a trade-off exists between allocating resources and energy towards pathogen resistance or to other fitness-related traits such as growth and maturation/reproduction (Sheldon & Verhulst 1996, Bakke & Harris 1998, Lochmiller & Deerenberg 2000, Colditz 2002). The larger mean differences in fish size and condition observed in smolts compared to parr could be explained by the added energetic/metabolic cost associated with the parr-smolt transformation (Mesa et al. 1999, Glover et al. 2006), which these fish are likely to have been undergoing at the time of the

PKD outbreak. Consequently, resources might have been allocated towards completion of the various changes undertaken during smoltification, resulting in there being less available for growth in smolts than in parr.

Where testable, allele frequency differences between PKD survivors and unexposed fish were always found to be significant in relation to allelic variation associated with one parent and were not always significant within and across families. Furthermore, the direction of the change in allele frequencies between the groups was variable among parents, with no consistent association of specific allele frequency changes with mortality across families. As such, this makes it impossible to say much about the nature and biological significance of the frequency differences observed, other than that the genetic architecture of the resistance response is probably complex. Also, due to the outbred nature of the families, it would be expected that most parents would not be informative for most markers and that the associations, therefore, were family-specific. Furthermore, it points to polygenic control of disease resistance, as found in other studies (Gilbey et al. 2006, Barroso et al. 2008), with the likelihood that loci with useful variation in mounting an immune response vary among families.

Nine markers showed significant trait associations prior to correcting for multiple tests, despite the low numbers of PKD survivors and the 1 marker per linkage group approach used. Of these, 4 (*µ20.19, SP3016, Ssa14,* and *Ssa48*) continued to show statistically significant associations when the adjustment for multiple tests was made, representing 4 different linkage groups. Interestingly, Gilbey (2003) reported the markers *Ssa14*, *Ssa48,* and *µ20.19* to be significantly associated with QTL affecting fish size during development from parr to smolt in backcrosses between Scottish and Canadian Atlantic salmon. Additionally, Boulding et al. (2008) found SNP markers on linkage groups AS9, AS18, and AS22 (derived from ASalBASE; http://www.asalbase.org) to be associated with weight and condition factor and markers on AS10 and AS12 to be linked to morphometric variables in trans-Atlantic crosses of Atlantic salmon. Those linkage groups contain the microsatellites markers *SP3016*, *Ssa85*, *Ssa16*, *Ssa48,* and *Ssa74*, respectively, which were found to be associated with growth-related traits in the present study. Similarly, *Ssa42* was found to be linked to QTL involved in resistance to *Gyrodactylus salaris* (Gilbey et al. 2006), and population differentiation between Scottish and Baltic populations was markedly higher at that marker in comparison with 15 other microsatellite markers (C. Thompson et al. unpubl. data), of which 5 were identified as being significantly associated with resistance to *G. salaris* (Gilbey et al. 2006).

A nucleotide-nucleotide basic local alignment search tool (BLAST) search was carried out on the markers that showed either significant allele frequency differences between the 2 groups or marker-trait associations, using '*in silico* data mining' of the GenBank database, based on the procedure outlined by Cnaani et al. (2002). Though the chance of finding a QTL located next to a marker is very small, due to the approach taken, this was done to see if any of these microsatellites could be immediately linked to gene regions that could, by the nature of their function, be responsible for the observed associations. The flanking regions of only one marker, *Ssa42,* produced an interesting result. It showed a homology (94%; 48/51 bp; e value: 3×10^{-11}) with sequences of the immune-related genes *Synaptic Ras-GTPase-activating protein 1, PHD finger protein 1, and Ral guanine nucleotide dissociation stimulator-like 2* (GenBank accession #DQ246664) in rainbow trout. These genes belong to the extended major histocompatibility complex (MHC) class II region, described by Palti et al. (2007). Thus, this marker appears to be closely associated with a part of the MHC complex involved in pathogen resistance and may be a key element in the overall genomic architecture of resistance to a number of disease vectors, despite it no longer being significantly associated with weight.

The amount of variation explained by the associations of markers with growth-related traits varied between 1.9 and 21.9%, though this variation was lower, i.e. from 0.4 to 7.6%, when the dam and sire components were analysed separately. The variation explained by them did follow the expected exponential distribution (Orr 1998, Barton & Keightley 2002). However, due to the approach taken, where each microsatellite potentially marked a complete chromosome, it can be assumed that the observed trait variance potentially represented the cumulative effect of an unknown but potentially large number of QTL on that particular linkage group. Each growth-related trait measured was associated with variation at a number of markers on different linkage groups. A total of 8 linkage groups were identified as being associated with the traits, supporting a model of polygenic control of growth, as reported in other studies (Reid et al. 2005, Boulding et al. 2008).

Interestingly, in the sex-specific analysis, the significant associations, after correction for multiple tests, were observed in the dam, where recombination is more frequent that in males (Gilbey et al. 2004, Moen et al. 2004b). As such, despite the single marker-single linkage group approach taken here, whereby each marker represented a whole linkage group/chromosome, the distance between the marker and putative QTL may be expected to be smaller than if the associations were observed in the males.

One marker, *Ssa14*, showed both significant allele frequency differences between the unexposed and exposed groups and was significantly associated with weight within the PKD survivors. This could reflect real or apparent (due to linkage of the QTL involved in the resistance and growth responses) pleiotropy. The number of unexposed fish (n = 305) was large enough to accurately estimate the original distribution of alleles across the 4 families, even though data could not be collected from the PKD mortalities. The results for this marker may also indicate that, although different QTL might be involved, there may be an association between QTL influencing growth and resistance.

The analysis of *GH1* revealed allele frequency differences between the PKD survivors and the unexposed group. This suggests that the *GH1* gene itself could be linked to the observed pathogen resistance. Kuhnlein et al. (1997) reported consistent differences in allele frequencies of the *GH* gene in resistance-selected strains of white leghorn chickens, further evidence that allelic variation at this gene could affect resistance. However, it is more likely that the allele frequency differences observed in *GH1* were the result of linkage with other QTL that reside on the same linkage group. More detailed, fine-scale mapping of this linkage group could help to determine the extent to which the *GH1* locus itself is directly involved.

The observation that the allele frequency differences and marker-trait associations were not universal across families could reflect the fact that the genetic architecture of resistance is complex and variable among individuals, and is due to the outbred nature of the crosses. However, it could also reflect the relatedness of the F_2 crosses used. The majority of the allele frequency differences and highest significance levels were found in Family 1, the only family created by full sib parents. Families created by full sib parents, if containing relevant variation, have been shown to potentially be more informative with regards the trait of interest than less inbred families (Xie et al. 1998) and provide a more powerful mating design for association detection. This reinforces the probable biological significance of the observed associations.

Further large- and fine-scale mapping studies, focusing on the linkage groups identified, are needed to ascertain whether the markers identified here are closely linked and universal or just family-specific. More extensive studies of wild populations and aquaculture strains would also be helpful for establishing if the associations are widespread and consistent, focusing on the analysis of families from full-sib crosses. This would help with examining the potential usefulness of markers on these linkage groups in marker-assisted selective (MAS) breeding for pathogen resistance. The single marker-single linkage group approach used here points to some linkage groups that are potentially a part of the genomic architecture of resistance to PKD in Atlantic salmon. The reported findings should help guide future work aimed at finding the gene regions and QTL involved in the resistance response to PKD. Given the time taken to carry out informative family-based QTL-type studies, our findings will help to effectively focus future work.

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