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ORIGINAL ARTICLE

The susceptibility of Atlantic salmon fry to freshwater infectious pancreatic necrosis is largely explained by a major QTL

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Infectious pancreatic necrosis (IPN) is a viral disease with a significant negative impact on the global aquaculture of Atlantic salmon. IPN outbreaks can occur during specific windows of both the freshwater and seawater stages of the salmon life cycle. Previous research has shown that a proportion of the variation seen in resistance to IPN is because of host genetics, and we have shown that major quantitative trait loci (QTL) affect IPN resistance at the seawater stage of production. In the current study, we completed a large freshwater IPN challenge experiment to allow us to undertake a thorough investigation of the genetic basis of resistance to IPN in salmon fry, with a focus on previously identified QTL regions. The heritability of freshwater IPN resistance was estimated to be 0.26 on the observed scale and 0.55 on the underlying scale. Our results suggest that a single QTL on linkage group 21 explains almost all the genetic variation in IPN mortality under our experimental conditions. A striking contrast in mortality is seen between fry classified as homozygous susceptible versus homozygous resistant, with QTL-resistant fish showing virtually complete resistance to IPN mortality. The findings highlight the importance of the major QTL in the genetic regulation of IPN resistance across distinct physiological lifecycle stages, environmental conditions and viral isolates. These results have clear scientific and practical implications for the control of IPN.
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Introduction

Atlantic salmon is one of the most important farmed fish species, with a worldwide production of over 1.3 million tonnes at an estimated value of $6.6 billion (FAO, 2008). The viral disease infectious pancreatic necrosis (IPN) is widely considered to be the most serious viral disease currently impacting on salmon aquaculture (Ariel and Olesen, 2002). IPN outbreaks can cause significant mortality in salmon populations during two distinct lifecycle stages, which correspond to periods of immunological and physiological vulnerability. First, in the freshwater environment, fry can be susceptible to the disease shortly after hatching (mortality typically in the range of 30–80%), and second, post-smolt fish can also be susceptible shortly after transfer to the seawater environment (mortality typically in the range of 5–30%) (Roberts and Pearson, 2005). Vaccination can provide some protection against the disease for post-smolt fish (Ramstad and Midtlyng, 2008), but control in the freshwater environment is generally dependent on biosecurity measures and the innate resistance of the young fish.

The genetic basis of resistance to IPN in the seawater environment has been clearly shown, with estimates of heritability on the observed binary scale (that is, zero = survived, one = died) ranging from 0.07 to 0.56, with an average of 0.38 (Guy *et al*., 2009). In the freshwater environment, controlled challenge trials have shown that there is a similar level of heritability of resistance to IPN, with average estimates of 0.31 on the observed scale (Wetten *et al*., 2007) and 0.55 on the underlying scale (Kjoglum *et al*., 2008), with a high genetic correlation ($\sim$0.8) between resistance after a freshwater IPN virus (IPNV) challenge and resistance in a seawater IPN outbreak (Wetten *et al*., 2007). The results of these studies suggest that there is sufficient genetic variation to make selection for resistance a plausible control measure for both freshwater and seawater IPN. Experimental challenges on commercial populations have shown that genetic selection can indeed improve IPN resistance (Storset *et al*., 2007; Kjoglum *et al*., 2008). However, with a 4-year generation interval, traditional

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selective breeding strategies based on sib-testing routines, although effective, are costly and make relatively slow progress. Therefore, a reliable molecular genetic test for IPN resistance would be extremely valuable to the industry.

In an earlier study, we showed the effects of highly significant quantitative trait loci (QTL) for resistance to IPN in the seawater field environment, based on the analysis of 19 families of intermediate susceptibility. The largest QTL, on Atlantic salmon linkage group (LG) 21, was estimated to explain between 21 and 32% of the within-family phenotypic variation in IPN resistance during this field outbreak (Houston *et al*., 2008a, b), and was subsequently confirmed by Moen *et al*. (2009). A further genome-wide significant QTL was detected on LG 28 (LG denoted as given in the ASalBase map http://www.asalbase.org/sal-bin/map/index), with a suggestive QTL on LG 19 (Houston *et al*., 2008b). However, although these QTL clearly have an important role in determining IPN resistance in post-smolts and the largest QTL has been included in a commercial breeding programme (Landcatch Natural Selection Ltd, Ormsary, Scotland) by means of marker-assisted selection since 2007, their role in the genetic regulation of freshwater IPN resistance at the fry lifecycle stage in these populations is not yet known.

In the current study, we undertook a series of IPNV challenge experiments in salmon fry designed to provide us with the resources to thoroughly investigate the genetic regulation of IPN resistance in a controlled freshwater environment, and thereby move a step closer to effective and sustainable control of the disease through the exploitation of host genetic variation. This investigation includes estimation of the genetic contribution to variation in resistance to freshwater IPNV challenge in our population and its relationship to seawater IPN resistance. A particular focus was on the molecular genetic basis of fry IPN resistance and on the role of the previously identified major seawater IPN-resistance QTL in determining the levels of mortality after a freshwater IPNV challenge. In doing so, we are testing the effect of these QTL in distinct physiological lifecycle stages of Atlantic salmon, a completely different challenge environment (controlled freshwater challenge versus field seawater challenge) and a distinct (freshwater) viral isolate.

Materials and methods

Animals

The families chosen for the challenge experiments were selected from the Scottish breeding nucleus of Landcatch Natural Selection Ltd. To ensure that a wide range of resistance genotypes were included in the study, 20 families were chosen based on the estimated breeding values (EBVs) for IPN mortality at the post-smolt, seawater lifecycle stage. A total of 4 of the most resistant elite (breeding program) families of the 2007 class from the breeding nucleus (top 25% EBV for IPN resistance), 12 elite families of intermediate resistance (selected from close to the median of IPN-resistance EBV) and 4 specially created IPN-susceptible families (from bottom 25% for IPN resistance of commercial females crossed to specially selected males with low EBV for IPN resistance) were challenged. The families were spawned in December 2007, which, given the 4-year generation interval, meant that the families used were from the same sub-line as for the QTL mapping study in smolts that were spawned in December 1999 (Houston *et al*., 2008b). In total, 20 dams and 17 sires were used to create the families (14 sires mated to a single dam and three sires mated to two dams each). The eggs were kept in full-sib family incubators under standard commercial rearing conditions at the family unit site of Landcatch Natural Selection Ltd. Approximately 2000 eyed ova per family were transferred in March 2008 from Ormsary to the disease challenge facilities at Cefas Weymouth Laboratory, England.

Virus preparation

The IPNV isolate V0512-1 (serotype A2 (Sp)) used for the challenge experiments was recovered from infected tissue of hatchery-reared Atlantic salmon fry experiencing high IPN mortalities in May 2005. Briefly, tissue from fry mortalities was homogenized using a sterile mortar, pestle and sand, and diluted (1:10) with Hanks balanced salt solution (Sigma–Aldrich, St Louis, MO, USA) supplemented with 2% fetal bovine serum (Sigma–Aldrich). The homogenate was centrifuged at 2500 *g* for 15 min at 4 °C and the supernatant filtered through 0.45 μm filter (Whatman, Maidstone, UK) before inoculation at 10^{-3} dilution onto a 25 cm^2 tissue culture flask containing a 24-h old confluent monolayer of RTG-2 cells (Wolf and Quimby, 1962) grown on L-15 media (Sigma–Aldrich) supplemented with 2 mM L-glutamine, 2% fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The virus-inoculated cells were incubated at 15 °C until a full cytopathic effect was evident. Previous experience with IPNV has shown that repeated passage in CHSE-214 cells is associated with loss of virulence of IPNV (Song *et al*., 2005); therefore, a low passage number (P2) in RTG-2 cells was used for the challenges described here. The first passage flask was used to inoculate further 75 cm^2 flasks of RTG-2 cells at 10^{-3} dilution and these were incubated at 15 °C. On reaching full cytopathic effect, the cell culture medium containing the virus from these flasks was harvested, centrifuged at 2500 *g* for 15 min at 4 °C to remove cell debris and the supernatant diluted (1:1) with glycerol and stored at −20 °C. Presence of IPNV was confirmed by enzyme-linked immunosorbent assay (ELISA) (test-line) as per manufacturer's instructions. This viral stock was then titrated on CHSE-214 cells in a 96-well cell culture plate and the titre was calculated after 7 days by the method of Kärber (1931). On the day of challenge, the harvest was titrated again to confirm the titre at the time of challenge.

Main challenge

After transport of the 20 selected full-sib families from Landcatch, Ormsary to Cefas on the 6 March 2008, the eggs were transferred to 20 separate 15 l family-specific holding tanks, all supplied with dechlorinated freshwater (pH of 7.3 and 11 dH hardness) at a flow rate of between 0.3 and 0.4 l^{-1} min^{-1} throughout the experiment. Water temperatures in all the tanks were raised from 10 °C to 12 °C within 2 days of arrival of the eggs. Egg hatching occurred in the tanks ~7 days after their arrival, and the resultant fry were maintained at 12 °C

until 64–65 days after their arrival, when the tank water temperatures were gradually reduced to 10 °C. For each of the 20 families, three replicates of ~100 first-feeding fry were then transferred to separate half-height 30 l (15 l) aerated challenge tanks (60 tanks in total) on day 68 after arrival (13 May 2008). They were acclimated there for 7 days before challenge on day 75 after egg arrival (20 May 2008). The challenge tanks were supplied with freshwater, from the same source as that supplying the family-holding tanks, at a flow rate of $0.4\,l^{-1} \pm 0.1\,l\,min^{-1}$ with oxygen levels of at least 80% saturation. The fish were kept under a 12-h light and 12-h dark photoperiod, with light levels between 150 and 200 lux. The tank water temperatures were maintained at 10 ± 0.5 °C for the duration of the challenge.

An immersion challenge protocol was chosen after a series of pre-challenge experiments (data not shown) determining the optimal challenge method and dose necessary to provide mortality data suitable for genetic analyses. The flow of water was stopped to two of the replicate tanks for each family and the water levels of the tanks adjusted to 5 l. The stock virus suspension (80 ml) was then added to the tank water, giving a predicted challenge dose of $\sim 5.0 \times 10^5$ $TCID_{50}\,ml^{-1}$. For the third replicate tank for each family, the fish were sham challenged by stopping the water flows and lowering the water levels to 5 l for 4 h, as for the challenged tanks. Instead of addition of the virus suspension, these tanks had 80 ml of cell culture and glycerol (1:1) added at the start of the sham challenge period. After 4-h immersion with close monitoring, the water flows were returned to all the treatment tanks. Temperatures were retained within ±2 °C during the period the flows were stopped by addition of ice packs to the tanks. Developmentally, the fry were at day 45 (520 degree days) post first feeding when they were challenged. IPNV infection in each tank was allowed to proceed without intervention, and the experiment was terminated when there were less than three mortalities per day (summed across all the challenged tanks) for three consecutive days.

Behavioural observations and other routine tank management parameters were recorded at least daily, with water temperatures in all tanks automatically controlled by a central computer to be always within 0.5 °C of the set temperature, except for when the flows were stopped during the challenge. Mortalities during the experiments were removed and stored in ethanol for later DNA extraction and genotyping. For one pool of mortalities from each tank, caudal fins were removed and retained in ethanol for genotyping, whereas the remaining body was frozen (−70 °C) for subsequent testing for the presence of IPNV. At termination of the experiment, all surviving fish were killed by an overdose of anaesthetic (benzocaine/methanol) and stored in ethanol. Similarly, for one pool of survivors from each tank, the caudal fin was stored in ethanol for DNA extraction and genotyping, and the remaining body frozen for virological analysis.

IPN virology testing

Fry mortalities and survivors from the challenged tanks and control tanks were tested for the presence of IPNV. Fry were weighed, homogenized using sterile pestle, mortar and sand, and diluted (1:10) with cell culture media. The homogenate was centrifuged at 2500 *g* for 15 min at 4 °C, and the supernatant removed and filtered through 0.45 μm filter (Whatman) before inoculation onto 24-h-old confluent monolayers of CHSE-214 cells in 96-well cell culture trays for titration according to Kärber (1931). Culture trays were incubated at 15 °C and titres were read after 7 days. Wells showing positive cytopathic effect for each sample were further tested by ELISA (test-line) to confirm the presence of IPNV.

Genotyping

DNA extraction from all fin clips was performed using a Biosprint DNA kit (QIAGEN, Crawley, UK) following the manufacturer's protocol. Genotyping of the fish was performed for a set of seven microsatellite markers that were chosen from LGs 21, 28 and 19 (as denoted on the linkage map of ASalBase (http://www.asalbase.org/sal-bin/map/index)) based on their proximity to the three previously identified QTL affecting IPN resistance in post-smolt fish under seawater field conditions (Houston *et al*., 2008b). It should be noted that LG 28 was previously referred to as LG 26 in the seawater study (Houston *et al*., 2008b). A Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) was used for optimization and genotyping of the markers, and ABI-377-mediated fluorescent detection was used to create allelic profiles, which allowed the markers to be included in a single PCR multiplex. The 10 families with the highest average mortality levels across the two replicates of the challenge experiments were chosen for genotyping. In the remaining families, it was deemed that the very low mortality levels resulted in insufficient within-family variation to enable detection of QTL. All sampled IPN mortalities in the 10 chosen families were genotyped, with all survivors genotyped in some families, but only a subset of survivors genotyped in the lower mortality families (Table 2).

Quantitative genetic analysis

The trait data collected during the main challenge included binary data (mortality or survivor) and survival (time to death) data. Owing to the relatively low average mortality levels across the experiment (~10% average mortality across the 20 families), with approximately half the families showing virtually no mortality, the focus of the analysis was on the binary variable Y_i, where Y_i is the phenotype value of the ith fish (assigned as $Y_i = 1$ if the fish died and $Y_i = 0$ if the fish survived). As the challenge experiments were allowed to run until mortalities were negligible, this binary variable was assumed to be an indicator of relative susceptibility or resistance, which itself was assumed to be controlled by an underlying continuous liability variable (Falconer and Mackay, 1996). The data from the IPNV-challenged tanks were analysed fitting an animal mixed model using a pedigree that included all known direct ancestors of the challenged fish for four generations. The analysis was implemented using the ASReml package (Gilmour *et al*., 2006), with the additive genetic variance calculated across all 20 families (two tank replicates per family) using the model:

$$Y_{ijkl} = g_i + b_{nijk} + a_{ijkl} + e_{ijkl}$$

where Y_{ijkl} is the observation on the lth fish, g_i is fixed effect of the ith genetic group, n_{ijk} is the number of fish in

the *k*th tank of the *j*th family in the *i*th genetic group with *b* being a linear regression coefficient to correct for variations in the number of fish per tank, a_{ijkl} is the random effect of the *l*th fish in the *k*th tank and e_{ijkl} is the random residual term.

Genetic group was included in the model, as the deliberate choice of families from the extremes of the Landcatch Natural Selection breeding population (in terms of estimated IPN resistance/susceptibility) could have resulted in an overestimate of the additive genetic variance. As each full-sib family was split into three unique tanks (two IPNV challenged, one control), tank was confounded with animal genotype and, therefore, the individual tank effects could not be directly estimated in the model. However, in an alternative parameterization of this model fitting full-sib family as the genetic effect and nesting tank as a random effect within family, tank effects were negligible, explaining $<0.1\%$ of the total variation (the raw correlation between replicate tanks was 0.91).

The heritability (h^2) for IPN resistance was estimated as $h^2 = \sigma^2_A/(\sigma^2_A + \sigma^2_E)$, where σ^2_A is the estimated additive genetic variance and σ^2_E is the estimated residual variance.

The analysis was performed both on the observed (0,1) scale and on the underlying liability scale, fitting a logit link function to transform data from the binary scale to the continuous underlying liability scale. In addition, the observed-scale heritability estimate was also transformed to the liability scale using the approximate formula $h^2_{\text{liability}} = h^2_{\text{observed}}(1-p/i^2p)$, where p is the prevalence of mortality and i is the corresponding mean liability (Falconer and MacKay, 1996)

Linkage and QTL mapping analysis

The linkage between all microsatellite markers was evaluated using the 'two-point' option in Crimap version 2.4 (Green *et al*., 1990), with a logarithm of the odds ratio score of >3 considered as significant linkage. The marker order on LG 21 (three markers) was tested using the 'build' and 'flipsn' options. The genetic distance between the markers was then evaluated using the 'fixed' option, with separate maps created for male and female parents because of the large differences in recombination rate between the sexes in Atlantic salmon (Gilbey *et al*., 2004; Moen *et al*., 2004). The relative positions of the markers within the three LGs, based on the 10 genotyped families, are given in Table 1.

To test whether QTL were segregating in the 10 families (20 mapping parents), a two-stage linear regression approach (Knott *et al*., 1998) was applied separately for male and female parents in the software package GridQTL (Seaton *et al*., 2006). The QTL mapping methodology is described in more detail in Houston *et al*. (2008b). The thresholds for significance were determined empirically by a permutation analysis (Churchill and Doerge, 1994), and the chromosome-wide 5% threshold (obtained using 10 000 permutations per LG) was considered an appropriate threshold level, as the analysis was focussed on specific LGs. For the overall significant QTL on LG 21, the test of QTL segregation in individual mapping parents was assessed using an absolute *t*-value (*t*-value $2.0 \approx P$-value of 0.05, for a two-tailed *t*-test), which is based on the comparison of mean phenotype values of offspring inheriting the alternative parental gametes.

QTL effect size

The size of effect of the significant QTL on challenge mortality was estimated by the GridQTL software. To estimate the proportion of phenotypic variation explained by the QTL, several methods were employed. First, within-family variation explained by the QTL was estimated from the regression analyses as: $h^2_{\text{QTL}} = 2\{[1-(\text{MSE}_{\text{full}}/\text{MSE}_{\text{reduced}})^{\text{Sire}}] + [1-(\text{MSE}_{\text{full}}/\text{MSE}_{\text{reduced}})^{\text{Dam}}]\}$, where MSE_{full} is the mean square error (MSE) of the model including the QTL and $\text{MSE}_{\text{reduced}}$ is the MSE of the model fitting only a family mean in the GridQTL software.

Second, at the best-estimated position of the QTL, the LG 21 marker information (for which a significant QTL was observed, see Results) was used to estimate the identity-by-descent (IBD) coefficients for all relationships in the pedigree following the two-step approach described by George *et al*. (2000). The gametic IBD matrix is a matrix that contains the IBD probabilities between the two gametes (the paternally and the maternally inherited alleles) of an individual, and between these gametes and all other gametes in the pedigree. The gametic IBD matrix was estimated using the recursive method described by Pong-Wong *et al*. (2001). The QTL effect and its associated IBD matrix at the best-estimated position of the QTL were then added to the additive genetic model (described above) resulting in the following mixed inheritance model:

$$Y_{ijklm} = g_i + bn_{ijk} + a_{ijkl} + q_{ijklm} + e_{ijklm}$$

Table 1 Details of the microsatellite markers genotyped on linkage groups 21, 26 and 19, and their positions on linkage groups (as calculated in the current study)

	Linkage group	*Position male (cM)*	*Position female (cM)*	*Number of het. (sires)*	*Number of het. (dams)*	*Forward primer*	*Reverse primer*	*Genbank accession no.*
BHMS217	21	0.0	0.0	6	6	GCTGTTCATTTCTGAGCAG	GACACACCGAATCAGTGC	AF256786
Rsa476	21	0.2	2.0	9	8	ATGGTGCGGACCTCATTC	CTTCATCGTTGTGTCGTC	AY544054
Alu333	21	0.3	8.9	5	6	TTCATAGTCCAAGAACAGTG	GCTGAGTTTACATTACACCTG	AY543859
SSA405UOS	26	0.0	0.0	10	10	CTGAGTGGGAATGGACCAGACA	ACTCGGGAGGCCCAGACTTGAT	AJ402722
BHMS437	26	0.0	81.9	9	7	AGAGAAGTATAAACCCTGC	AATATGGTAGGAAGACACAG	AF256816
BHMS235	19	0.0	0.0	1	6	AGCGAGCTTTCTTTCCAG	AGCTGTCTATTCACGACTC	AF256846
Rsa277A	19	6.8	5.0	10	6	GCTAATAGATACTGTGGCTC	TGAGTCATACACCATTTGTC	AY543949.1

Abbreviation: het., heterozygous.

where q_{ijklm} is the random effect of the mth QTL allele of the lth fish and all other terms are as above. The data were analysed either restricting the data set to the 10 genotyped families or including all 20 families. Fish without genotype information had their QTL genotype set to missing. The proportion of phenotypic variation explained by the QTL was then estimated as $h^2_{QTL} = \sigma^2_{QTL}/(\sigma^2_A + \sigma^2_{QTL} + \sigma^2_E)$, whereas the proportion of genetic variation explained by the QTL was estimated as $\sigma^2_{QTL}/(\sigma^2_A + \sigma^2_{QTL})$.

Lastly, the variance due to the QTL was also calculated directly from the estimates of additive (a) and dominance (d) effects as $2pq(a + d(q-p))^2$. The resistant allele frequency (p) was inferred from the family mortality levels (see Results).

Results

Challenge experiments

A total of 20 full-sib families were IPNV challenged with two replicate tanks per family and a sham-challenged control tank. Titration of the challenge dose confirmed that the fish were exposed to 5.46×10^5 $TCID_{50}$ IPNV particles per ml at the start of the challenge. Mortality levels in the IPNV-challenged tanks started to increase at $\sim$ 10 days post-challenge, with a peak at $\sim$23 days post-challenge (Figure 1). There was a small rise in control mortalities at approximately the same time, the reason for which is unclear. At 44 days post-challenge, the experiment was terminated, as the mortalities in the challenge tanks had returned to close to the baseline level (less than three total mortalities per day for three consecutive days). The average cumulative mortality curves for the resistant, intermediate and susceptible families (with family category being based on EBVs of their parents, as determined through field IPN challenge at the post-smolt stage) are shown in Figure 2. Those families that were designated as susceptible before the challenge showed the highest average levels of mortality, followed by intermediate, with the lowest mortality levels in the families designated as resistant (Figure 2). The overall level of mortality in the experiment was $\sim$10%, averaged across all families. Nine of the families showed negligible mortality in the experiment, and seemed to be completely resistant to the challenge. Details of the mortality levels in each family, for both the challenged and control tanks are given in Table 2.

Virology

A total of 30 dead salmon fry recovered from 28 of the 30 tanks that experienced mortality were tested for IPNV by cell culture. Cytopathic effect was evident and significant viral titres were obtained from all the fish tested, and the samples were all confirmed positive for the presence of IPNV antigens when tested by ELISA. Viral titres in these fish varied between 8.9×10^4 and 5.0×10^8 $TCID_{50}\,ml^{-1}$.

A total of 84 surviving fish from 28 of the challenged tanks (three fish per tank) were tested for the presence of IPNV. The numbers of surviving fish testing positive for IPNV are given in Table 2. IPNV-positive surviving fish were detected in families with mortality and also in families without mortality (Table 2). Three fish from each of the 20 control tanks were also tested and were all negative for the presence of IPNV.

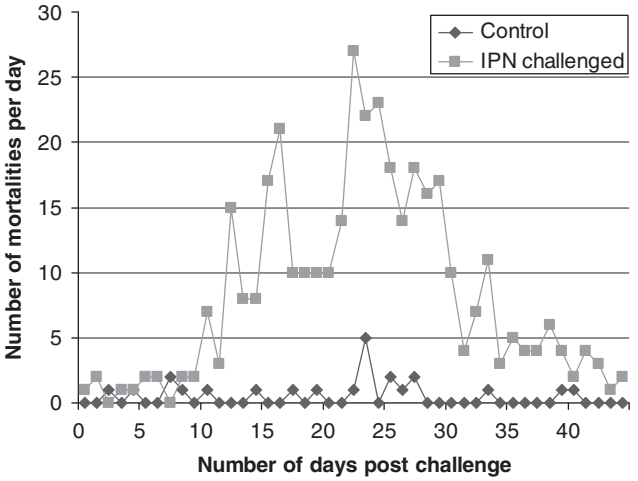

Figure 1 The daily mortality curve for the control and infectious pancreatic necrosis virus (IPNV)-challenged tanks.

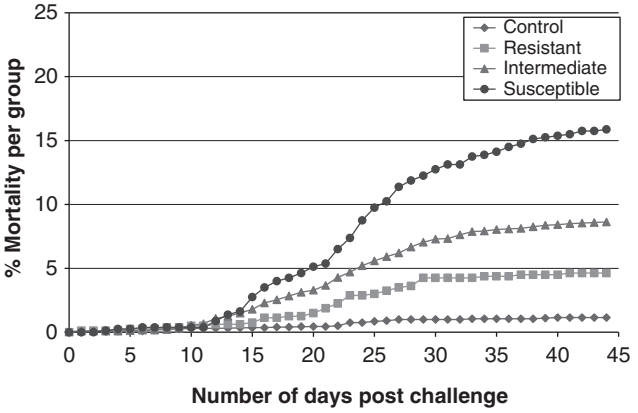

Figure 2 Cumulative mortality curves for the three groups of families according to smolt breeding value classifications (4 susceptible families, 12 intermediate families and 4 resistant families).

Quantitative genetic analysis

To estimate the contribution of polygenic additive variance to the observed variation in mortality levels in the 20 families, an animal mixed-model analysis was performed. A heritability of 0.26 (s.e. 0.09) was estimated on the observed scale (Table 3). This corresponds to 0.88 on the underlying liability scale, based on a mean prevalence of 10% mortality. When a logit link function was applied to the model, the estimate of heritability was 0.55 (s.e. 0.05) (Table 3). The full-sib model resulted in a heritability of 0.21 (s.e. 0.07) on the observed scale, and the estimated heritability on underlying liability scale exceeded 1.0 when fitting a logit link function.

Linkage and QTL mapping

The linkage maps were calculated for the three LGs previously shown to harbor QTL affecting seawater IPN resistance (Table 1). On LG 21, the best-estimated order of the markers BHMS217 and Rsa476 was reversed compared with the map described in Houston *et al*. (2008b). As these markers are tightly linked and lie towards the end of the LG, the difference in their

Table 2 Details of mortality levels, number of fish genotyped and virology results in the 20 families

Family code	*Smolt breeding value class*[a]	*Mortality in control tank (%)*	*Average mortality in challenged tanks (%)*	*Number of mortalities genotyped*	*Number of survivors genotyped*	*IPNV detected in survivors (prop. tested with $>1.0\times10^4$ $TCID_{50}$ viral particles per fish)*
J	I	1	34	71	128	− (0/3)
N	S	1	31	65	134	+ (1/3)
I	I	0	23	44	156	+ (3/6)
P	S	4	15	37	119	+ (3/6)
B	I	0	12	25	80	ND
O	S	0	12	24	80	− (0/6)
D	I	4	11	25	80	+ (4/6)
S	R	0	10	18	81	− (0/6)
C	I	0	8	16	60	− (0/6)
L	I	0	8	18	60	+ (3/6)
R	R	1	8	0	0	− (0/3)
M	S	2	4	0	0	+ (5/6)
H	I	6	3	0	0	+ (1/6)
A	I	2	2	0	0	− (0/3)
E	I	0	1	0	0	+ (1/3)
K	I	0	1	0	0	− (0/3)
F	I	1	0	0	0	+ (3/3)
G	I	0	0	0	0	− (0/3)
Q	R	1	0	0	0	− (0/6)
T	R	0	0	0	0	− (0/3)

Abbreviation: IPNV, infectious pancreatic necrosis virus.
[a]As designated from estimated breeding values of the parents based on natural sea water IPNV challenge data (see Materials and methods).

Table 3 Estimated heritabilities for IPN-resistance and proportion of the variance explained by inclusion of the LG 21 QTL in the model in the full data set of 20 families and in the subset of 10 families that were genotyped

	All 20 families		*10 Genotyped families*	
Parameter Model	*No QTL*	*QTL*	*No QTL*	*QTL*
$\sigma^2_{polygenic}$	0.023	0.013	0.002	0.000
σ^2QTL	—	0.010	—	0.023
$\sigma^2_{residual}$	0.068	0.057	0.013	0.010
h^2_a	0.26 ± 0.09	0.29 ± 0.09	0.02 ± 0.02	0.00 ± 0.00
h^2_{QTL}	—	0.13 ± 0.02	—	0.18 ± 0.03
%QTL	—	44.7[a]	—	99.8

Abbreviations: IPN, infectious pancreatic necrosis; LG, linkage group; QTL, quantitative trait loci.
[a]Proportion of variation due to QTL underestimated, as genotype information not available on the 10 families with the lowest mortality.

estimated order between the two studies is likely to be a statistical artifact. The expected discrepancy in distance between the male and female maps, due to the difference in recombination rate, was observed.

Quantitative trait loci analysis was performed on 10 families (20 mapping parents) with the highest levels of mortality in the main challenge (Table 2). Highly significant evidence for a QTL on LG 21 was shown in both the sire and dam analysis (Table 4). The QTL position was consistent with the previous seawater study, but as the genotyping was restricted to the markers predicted to be closest to the QTL and these markers were closely linked, a more precise estimate of QTL position was not possible. However, there was no significant evidence for QTL on either LG 19 or LG 28. The effect of the LG 21 QTL in the individual mapping parent is shown in Table 5, and all 10 families had at least one parent segregating for the QTL. However, three parents had estimated effects that were substantially smaller than those in the remaining significant parents, two of which had *P*-values greater than a Bonferroni-corrected threshold accounting for 20 independent tests and family sizes of ca. 100 fish (*t*-value $\approx$3.0). These two parents were deemed to have only suggestive evidence for a segregating QTL. The average size of the estimated QTL effect (not including the suggestive category) expressed in terms of mortality proportion was 0.39.

To examine within-family effects and to determine the possible mode-of-inheritance of the LG 21 QTL, the families in which both sire and dam were classified as segregating were further examined. The average mortality of offspring inheriting the QTL-susceptible allele from both parents was 63%, compared with 0% for those that inherited the QTL-resistant allele from both parents (Table 6). Offspring that were classified as heterozygous for the QTL showed mortality levels similar to the homozygous resistant class. When the mortalities in all 10 families are examined, it is clear that there is a low mortality (5%) in offspring ($n=689$) known to inherit at least one resistance allele from a QTL-segregating parent (compared with an overall mortality rate in all genotyped fish of 25%). This indicates that a single resistance allele is sufficient to protect against mortality due to IPNV challenge under the experimental conditions described above. From the results shown in Table 6, in which the fry genotypes are unambiguous, the additive effect (*a*) of the QTL is −0.32 and the dominance deviation (*d*) is −0.30.

The percentage of phenotypic variation explained by the QTL in the IPNV-challenged families is given in

Table 4 Results from the Sire and Dam QTL mapping analyses for linkage groups 21, 28 and 19

Linkage group	*Sire F ratio*	*Sire position (cM)*	*Dam F ratio*	*Dam position (cM)*	*5% significance threshold*	*PVE[a] (if significant)*
21	**20.7***	**0**	**20.0***	**1**	**1.8**	**50.9%**
28	0.6	0	0.6	81	1.8	NA
19	1.3	0	0.7	5	2.0	NA

Abbreviations: PVE, phenotypic variation explained; QTL, quantitative trait loci.
[a]PVE is the proportion of within-family variation explained.
Values given in bold are significant.
*$P<10^{-36}$.

Table 5 LG 21 QTL effects in individual mapping parents

Family code	*Sire QTL effect (s.e.)*	T-*value*	*Dam QTL effect (s.e.)*	T-*value*
J	0.17 (0.06)	2.9[a]	**0.58 (0.06)**	**9.9**
N	0.05 (0.06)	0.8	**0.19 (0.06)**	**3.2**
I	**0.44 (0.06)**	**7.3**	0.14 (0.06)	2.3[a]
P	**0.45 (0.07)**	**6.8**	0.10 (0.07)	1.4
B	**0.38 (0.08)**	**4.7**	**0.41 (0.08)**	**4.9**
O	**0.42 (0.08)**	**5.1**	0.02 (0.08)	0.2
D	0.03 (0.08)	0.4	**0.36 (0.08)**	**4.4**
S	**0.37 (0.08)**	**4.5**	**0.27 (0.09)**	**3.2**
C	**0.39 (0.09)**	**4.2**	**0.38 (0.09)**	**4.0**
L	**0.33 (0.10)**	**3.4**	**0.43 (0.11)**	**3.9**
Mean of segregating parents	**0.40**		**0.37**	

Abbreviation: QTL, quantitative trait loci.
Parent genotypes with significant evidence for QTL segregation are given in bold.
[a]Suggestive evidence for QTL segregation.

Table 6 Mortality levels in offsprings carrying the four alternative putative LG 21 genotypes, summed across the four families in which both sire and dam were classified as having significant evidence for QTL segregation

	Sire QTL haplotype	
	Resistant	*Susceptible*
Dam QTL haplotype		
Resistant	0/73 (0%)	1/74 (1%)
Susceptible	2/85 (2%)	69/109 (63%)

Abbreviations: LG, linkage group; QTL, quantitative trait loci.

Tables 3 and 4. When estimated using the comparison of the MSE of the model including a QTL with the MSE of the model only fitting a family mean, the phenotypic variation explained was 51%. This estimate is larger than the heritability estimated previously; however, the estimation depends on the relative proportions of survivors and the mortality of the fish genotyped. When the 10 genotyped families were analysed using the mixed-model approach described above, with the inclusion of the IBD coefficients at the best-estimated position of the QTL, the polygenic component was estimated to be negligible, whereas the QTL component was estimated to be 0.18 (s.e. 0.03). In other words, essentially, all the identifiable genetic variation in these 10 families was due to the QTL. When the IBD coefficients for the 10 families were included in the quantitative genetic analysis of all 20 families, the additive genetic component was estimated to be 0.31 (s.e. 0.10), of which 0.18 (s.e. 0.11) was because of the polygenic component and 0.13 (s.e. 0.02) was because of the QTL component (Table 4). However, because the non-genotyped families are almost certainly also affected by the QTL, this approach will underestimate genetic variation attributable to the QTL.

Discussion

The aim of the experiment described in this paper was to provide a detailed characterization of the genetic basis of resistance to freshwater IPN in Salmon fry. The IPNV challenge experiments were based on 20 full-sib families chosen for their diversity of genetic resistance to seawater IPN at the post-smolt, seawater lifecycle stage. In these families, a significant genetic component to IPN resistance was shown, with a significant estimated heritability of 0.26 on the observed scale (applicable to an average mortality level of 10%). In the 10 families chosen for targeted genotyping of putative IPN QTL LGs, a single major QTL was mapped to LG 21 with an overwhelming level of significance. The mixed-model analyses suggested that this QTL explained almost all the genetic variation in segregating families. The patterns of mortality within segregating families suggest that fish homozygous for the resistant allele show a completely resistant phenotype (virtually no mortality), whereas those carrying a single copy of the resistance allele show very low mortality under our experimental conditions, indicating possible dominance of the resistance allele.

Under the assumption that the QTL effects observed in the families in which both parents are segregating are consistent across the population as a whole, QTL genotypes can be inferred for the parents of ungenotyped families. Further, using the additive and dominance deviation effects estimated in the segregating

families, the expected mortality can be calculated for all families on the basis of these inferred genotypes. These calculations are shown in Table 7 under the assumption that zero mortality only occurs in RR families (under these specific experimental conditions), whereas the presence of IPN-related mortalities implies that at least one of the parents must carry at least one susceptibility allele. Further, in these calculations it is assumed that the two parents with 'suggestive' QTL effects are in fact false positives. A close relationship between observed and expected mortality is seen, although for three families, there is ambiguity over one of the parental genotypes that could affect expected mortality levels. From these calculations, it is expected that the 40 parents contributed 29 S alleles and 51 R alleles, that is, $p \sim 0.64$. Estimating genetic variation due to the QTL as $2pq(a + d(p-q))^2$ and correcting the additive genetic variance to a mean mortality of 11%, the QTL is predicted to explain 98% of the additive genetic variation. However, this value is very sensitive to changes in allele frequencies and population mean mortalities, as well as the precision of each estimated parameter.

The IPN mortality levels in our population are lower than expected based on previous challenge trials, which will result in slightly lower than expected heritabilities on the observed scale. However, the observed mortality levels do seem to be consistent with the apparent QTL effects and allele frequencies. In summary, our data suggest that genetic variation in IPN resistance in freshwater in the challenged population seems to be dominated by a single QTL, with the favourable allele having a frequency somewhat in excess of 0.5 (it is worth noting that this may have been biased by the choice of families based on smolt IPN-resistance EBVs classification). However, the heritability estimate of 0.26 compares with the average estimate of heritability (0.31) in the fry IPN challenge experiments described by Wetten *et al*. (2007). When a logit link function was applied to the data using an animal model, the estimate of heritability was 0.55, which is the same as the underlying scale heritability estimated for fry IPN resistance by Kjoglum *et al*. (2008).

Table 7 Actual and inferred parental genotypes, and observed and expected mortalities

Family code	*Sire genotype*	*Dam genotype*	*Observed mortality*	*Expected mortality*
J	*SS*	**SR**	34	32.6
N	*SS*	**SR**	31	32.6
I	**SR**	*SS*	23	32.6
P	**SR**	*SS* or *RR*	15	16.8 (0.9,32.6)[a]
B	**SR**	**SR**	12	16.8
O	**SR**	*SS* or *RR*	12	16.8 (0.9,32.6)[a]
D	*SS* or *RR*	**SR**	11	16.8 (0.9,32.6)[a]
S	**SR**	**SR**	10	16.8
C	**SR**	**SR**	8	16.8
L	**SR**	**SR**	8	16.8
R	*RR* or *SR*	*SR* or *RR*	8	0.9
M	*RR* or *SR*	*SR* or *RR*	4	0.9
H	*RR* or *SR*	*SR* or *RR*	3	0.9
A	*RR* or *SR*	*SR* or *RR*	2	0.9
E	*RR* or *SR*	*SR* or *RR*	1	0.9
K	*RR* or *SR*	*SR* or *RR*	1	0.9
F	*RR*	*RR*	0	0.0
G	*RR*	*RR*	0	0.0
Q	*RR*	*RR*	0	0.0
T	*RR*	*RR*	0	0.0
Mean			9.15	11.0

Abbreviations: QTL, quantitative trait loci.
Actual parental genotypes are indicated by bold values, whereas inferred parental genotypes are indicated by italicized values.
[a]The numbers in parentheses refer to the expected probabilities of the alternative putative dam QTL genotypes.

Although the hypothesis that the overall low mortality levels in the experiments described in the current study are due to a single QTL with the resistance allele at high frequency is not definitive, it is consistent with the previous selection history of this population. The challenged families had been through two generations of selection for IPN resistance based on sib performance at the post-smolt, seawater stage, which could be expected to increase the frequency of the resistance allele. Therefore, one plausible reason for the intermediate allele frequencies in the population is that a relatively rare resistance allele in the population is becoming increasingly common through selection. This may suggest that the resistance allele is due to a relatively recent mutation or, as IPN outbreaks are much less frequent in wild fish, due to an old mutation that was not subjected to high selection pressure or possibly had pleiotropic effects on other fitness traits. Monitoring commercial populations for such possible pleiotropic effects is therefore important for the application of this QTL.

The LG 21 QTL located under field (seawater) conditions, showed a similar magnitude of effect on mortality, but differences in the pattern of inheritance. Heterozygous fish under the field conditions showed intermediate levels of mortality, with little suggestion of a dominance effect (Houston *et al*., 2008a). Therefore, the finding of very low mortality in heterozygous fish in the current study (Table 6) could suggest dominance of the resistance allele that is specific to the freshwater lifecycle stage or to the more controlled single-family tank challenge environment. Alternatively, as the overall mortality was much less in the freshwater study than in the seawater study, the low mortality in heterozygous fish could simply be a reflection of a dose–response relationship, in which heterozygous fish may be susceptible in an outbreak with higher overall mortality levels. Additional data from a mixed family freshwater tank environment with higher overall mortality levels suggests that this may be the case, as, apparently, heterozygous fish did indeed show intermediate mortality compared with the homozygous classes (AA Gheyas *et al*., unpublished data).

A notable difference between the findings under the current experimental conditions, compared with the previous seawater study, is that, we did not detect the QTL on LGs 19 and 28 in the current study. Further analyses restricted to fish homozygous for the susceptible allele on LG 21 also failed to find QTL on LGs 19 and 28 (results not shown). It is possible that these QTL were specific to IPN resistance at the post-smolt stage, which may be connected to an overall stress response associated with the transfer from freshwater to seawater. However, it is also possible that these QTL were false positives and confirmation of their effects in other IPN-challenged populations would be useful. The findings from a recently published study on Norwegian commercial salmon populations also suggest that a single QTL in the

same region of Atlantic salmon LG 21 explains much of the genetic variation in IPN resistance in fry (48%) and post-smolts (83%), and do not suggest a large role for any of the other LGs (Moen *et al*., 2009). The confirmation of the LG 21 major QTL by two independent research groups in two distinct populations is a strong evidence that the QTL effect is genuine and robust.

The finding of a single locus explaining the majority of genetic variation in susceptibility to such a critical disease has implications, from both a scientific and practical management standpoint. From a practical perspective, the overwhelmingly important role of the LG 21 QTL in the genetic regulation of IPN resistance has clear implications for marker-assisted selection. We have now shown that the QTL effects are robust across distinct lifecycle stages, environments (freshwater artificial challenge and seawater field challenge) and viral isolates. Rapid and substantial improvement in IPN resistance will be achieved if homozygous resistant parents could be identified for breeding. However, to allow population-wide screening of breeding candidate fish, it would be desirable to identify markers in strong linkage disequilibrium with the underlying causal mutation, and ideally, to identify the underlying causal mutation. Therefore, marker generation in the QTL region is pertinent, and the clear-cut classification of hundreds of fish from the QTL mapping families into distinct QTL classes, combined with the focused application of deep sequencing technology, may facilitate the identification of such markers.

From a scientific perspective, we can hypothesize that genetic resistance could be due to the effect of a mutant allele causing the presence or absence (or a fundamental modification) of a specific functional host protein. For example, the protein may form a component of a host barrier to infection or may be a component of the innate immune system affecting IPNV recognition or response. The fact that some of the fish from the lowest mortality tanks showed detectable viral titre implies that carrying a resistant allele does not necessarily prevent initial infection with the virus. To provide some insight into these hypotheses, and to highlight more generally which genes and pathways are differentially regulated between alternative QTL genotypes, we have run a concurrent series of challenge experiments in which we collected RNA and DNA samples from QTL-segregating families at various time points during the IPNV challenge. We plan to compare the transcriptomic response of the alternative QTL genotypes, which may also assist in identifying underlying pathways and candidate genes, which can be combined with a fine-mapping approach to progress towards identifying the underlying causal mutation. With such an overwhelming single QTL effect on such an important trait from an economic and welfare prospective, progression towards identifying the underlying mutation(s) is a priority area of research.

Conflict of interest

The authors declare no conflict of interest.

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