Downloaded from orbit.dtu.dk on: Apr 28, 2024

DTU Library

Validation of a QTL for Flavobacterium psychrophilum resistance in rainbow trout Oncorhynchus mykiss

Mathiessen, Heidi; Duan, Yajiao; Marana, Moonika H.; Zuo, Shaozhi; Karami, Asma M.; Jafaar, Rzgar; von Gersdorff Jørgensen, Louise; Kania, Per W.; Dalsgaard, Inger; Madsen, Lone

Total number of authors:
15

Published in:
Aquaculture Reports

Link to article, DOI:
10.1016/j.aqrep.2023.101573

Publication date:
2023

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Mathiessen, H., Duan, Y., Marana, M. H., Zuo, S., Karami, A. M., Jafaar, R., von Gersdorff Jørgensen, L., Kania, P. W., Dalsgaard, I., Madsen, L., Nielsen, T., Grammes, F., Ødegård, J., Macchia, V., & Buchmann, K. (2023). Validation of a QTL for *Flavobacterium psychrophilum* resistance in rainbow trout *Oncorhynchus mykiss*. *Aquaculture Reports*, *30*, Article 101573. https://doi.org/10.1016/j.aqrep.2023.101573

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Aquaculture Reports 30 (2023) 101573

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Validation of a QTL for *Flavobacterium psychrophilum* resistance in rainbow trout *Oncorhynchus mykiss*

Heidi Mathiessen [a,1], Yajiao Duan [a,1], Moonika H. Marana [a], Shaozhi Zuo [a], Asma M. Karami [a], Rzgar Jafaar [a], Louise von Gersdorff Jørgensen [a], Per W. Kania [a], Inger Dalsgaard [b], Lone Madsen [b], Torben Nielsen [c], Fabian Grammes [d], Jørgen Ødegård [d], Valeria Macchia [e], Kurt Buchmann [a,*]

[a] *Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, DK-1870 Frederiksberg C., Denmark*
[b] *Institute of Aquatic Resources, Technical University of Denmark, Kgs. DK-2800, Lyngby, Denmark*
[c] *AquaSearch ova ApS, DK-7300 Jelling, Denmark*
[d] *AquaGen AS, P.O. Box 1240, NO-7462 Trondheim, Norway*
[e] *Institute of Aquaculture, University of Stirling, FK9 4LA Scotland, UK*

A R T I C L E I N F O

Keywords:
QTL
Rainbow trout
RTFS
BCWD
Breeding

A B S T R A C T

Flavobacterium psychrophilum is the causative agent of Bacterial Cold Water Disease (BCWD)/ Rainbow Trout Fry Syndrome (RTFS) in rainbow trout *Oncorhynchus mykiss*. The disease is associated with significant mortality in trout farms and thereby responsible for severe economical losses in this part of the aquaculture industry. Vaccination of the very young life cycle stages is not successful due to the immature development of the adaptive immunity in yolksac larvae and early fry. This explains the extensive usage of antibiotics for control of BCWD/ RTFS. Selective breeding of RTFS resistant fish may be a solution, and identification of markers associated with natural susceptibility/resistance to the disease may elevate breeding efforts towards more robust strains. We suggest a QTL (SNP Affx-88941461) for partial disease resistance on chromosome 25 (Omy25) based on our experimental *F. psychrophilum* challenges of outbred fish and subsequent GWAS analyses. The favourable SNP allele was designated Q, whereas q indicated the unfavourable allele. We validated this QTL in two subsequent challenge experiments by *F. psychrophilum* exposure of QQ, Qq and qq trout. In the first trial we produced trout carrying at least one favourable allele (QQ and Qq) associated with partial resistance by fertilizing trout eggs from non-selected (outbred) females with sperm from homozygous QQ males. In the second trial we also produced homozygous offspring by fertilizing trout eggs from QQ females with sperm from QQ males. The resistance profiles of these groups were then determined in the laboratory by triplicate or duplicate challenge experiments (common garden water bath exposure to *F. psychrophilum*), which showed a significantly higher survival in trout carrying the favourable allele. Field observations supplemented the laboratory studies. Under farm conditions QQ and Qq trout showed a higher survival rate compared to qq trout. In the present study chromosome 25 (Omy25) QTL was found associated with a partial resistance to *F. psychrophilum.* However, we recommend to include additional SNPs in future breeding studies because several QTL are associated with resistance.

1. Introduction

The Gram-negative bacterium *Flavobacterium psychrophilum* is the causative agent of bacterial cold-water disease (BCWD), also known as rainbow trout fry syndrome (RTFS) (Avendaño-Herrera et al., 2020), a disease causing high mortality and substantial economic losses in salmonid aquaculture (Dalsgaard and Madsen, 2000; Nematollahi et al., 2003). The bacterium is psychrophilic explaining that outbreaks mainly occur at temperatures below 15 °C (Hesami et al., 2011). Mortality rates up to 90% have been observed in fry (Muñoz-Atienza et al., 2019), while larger fish mortality rates are low, although morbidity rates may be high (Madsen and Dalsgaard, 1999; Nilsen et al., 2011). *F. psychrophilum*

* Corresponding author.
E-mail address: kub@sund.ku.dk (K. Buchmann).
[1] These authors contributed equally to the study

https://doi.org/10.1016/j.aqrep.2023.101573
Received 20 December 2022; Received in revised form 7 April 2023; Accepted 16 April 2023
Available online 18 April 2023
2352-5134/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

septicemia is associated with anemia, lethargy, darkening and erosion of the skin along with muscle degeneration and necrosis (Avendaño-Herrera et al., 2020; Muñoz-Atienza et al., 2019). The exposed and infected fish clearly respond by activating genes encoding both innate and adaptive immune factors (Villarroel et al., 2008; Evenhuis and Cleveland, 2012; Langevin et al., 2012; Henriksen et al., 2015b; Marancik et al., 2015; Semple et al., 2018; Muñoz-Atienza et al., 2019). This suggests that vaccination may improve protection of immunocompetent fish. In accordance with this notion it was shown that an experimental immersion vaccine protect trout fingerlings (Hoare et al., 2017), and oil-adjuvanted injectable vaccines may confer immunity in larger salmonids (Hoare et al., 2019; Macchia et al., 2022; Marana et al., 2022). However, the efficacy of a vaccine for larvae and young fry is expected to be low, due to the immature adaptive immune system in these very young stages of rainbow trout fry (Buchmann, 2022). Therefore alternative approaches are needed to secure the health status of these early life cycle stages of fish. Previous studies clearly pointed to a genetic background for innate RTFS resistance (Wiens et al., 2013; Langevin et al., 2012; Marancik et al., 2015). Subsequently a range of targeted investigations (Vallejo et al., 2014; Liu et al., 2015, 2018; Palti et al., 2015b; Fraslin et al., 2018; Vallejo et al., 2022) have presented evidence for markers and genes associated with natural resistance (quantitative trait loci, QTL) for a possible application in breeding programmes. Similar approaches have been applied to select trout with a natural resistance against vibriosis (Karami et al., 2020), white spot disease (Jaafar et al., 2020), enteric redmouth disease (Zuo et al., 2020) and furunculosis (Marana et al., 2021). We have therefore performed *F. psychrophilum* challenge experiments with outbred trout and analysed survival data in order to search for a candidate QTL. We found a possible QTL candidate on Omy25 (SNP Affx-88941461), which is clearly in line with earlier studies (Liu et al., 2015, 2018, 2022; Palti et al., 2015b; Fraslin et al., 2018; Vallejo et al., 2017, 2022). We have validated this QTL by producing different SNP genotypes (QQ, Qq, qq) and exposed the fish to *F. psychrophilum* (bath exposure), whereafter we compared morbidity/mortality rates between groups. In order to elucidate possible immune genes involved in the natural resistance, and evaluate a possible association with the SNP, we performed a transcriptomic analysis of immune genes expressed in exposed rainbow trout (fish with clinical signs, fish without clinical signs and surviving fish).

2. Materials and methods

2.1. Challenge study 1: QTL discovery

2.1.1. Fish

Rainbow trout *Oncorhynchus mykiss* (average weight 1 g) was used. The challenge trial 1 for QTL search was conducted at VESO Vikan (Namsos, Norway) based on outbred fry originating from the AquaGen nucleus (n = 1500; 15 individuals from each of 100 families derived from 30 sires and 30 dams) and AquaSearch nucleus (n = 1176; 28 individuals from each of 42 families derived from 30 sires and 30 dams). Fish were fed a commercial Atlantic salmon feed (Skretting AS, Norway) continuously supplied by automatic feeders throughout the study (2% body weight per day). The fish were acclimatized in 120 L glass fibre tanks with well-aerated flow-through (0.8 L/Kg/min) fresh water (oxygenation >70% in effluent water). The fish were challenged together in common-garden set-up.

2.1.2. Challenge

The challenge was conducted according to Hoare et al. (2017). In brief: Before bacterial exposure the fish were pre-treated with hydrogen peroxide (200 mg/L) for 1 h under static conditions with aeration. After replacing the water containing hydrogen peroxide the subsequent bath-challenge was also performed under static conditions with aeration using a final *F. psychrophilum* concentration of 2×10^7 CFU/mL. The bacteria used for the challenge were from a Scottish isolate of *F. psychrophilum* (isolate 19_5). Moribund/dead fish were collected twice a day and assigned 0 for dead and 1 for survivor. After the challenge DNA samples (fin-clip) from mortalities or moribund fry (0) were collected continuously for 30 days. After 30 days all remaining fish were assigned survivor status (1), euthanized and fin clips taken for DNA typing.

2.1.3. Genetic analysis

All fish in this study were genotyped with the rainbow trout 57 K SNP genotyping array, Axiom®Trout (Affymetrix), (Palti et al., 2015a). For the QTL search the analysis comprised a total of 1674 fish, including 1474 disease challenged samples and 200 samples with known origin. Raw genotyping data (CEL files) of the challenge trial were processed as batch using the Linux-based Analysis Power Tools (APT) pipeline applying best practice thresholds (Contrasts quality control (DQC) threshold = 0.92 and STEP1 = 0.97). (https://www.affymetrix.com/support/developer/powertools: ThermoFisher Scientific; 2020).

Genotypes were imported into PLINK v1.9 (v30 Nov 2019) (Chang et al., 2015) for subsequent processing: Variants were filtered out if they did fulfil any of the following criteria: i) Not classified as 'PolyHighResolution' or `NoMinorHom` variant by the R package SNPolisher; ii) Minor allele frequency (MAF) < 0.01; iii) Missing rate per SNP < = 3%. The final datasets consisted of 45,490 SNPs and 1674 samples of both populations: AquaGen and AquaSearch. SNPs were positioned on the Rainbow trout genome assembly Omyk_1.0 (GCF_002163495.1).

Principal Component Analysis (PCA) was used to assign individuals to their respective population (AquaGen/AquaSearch) within the challenge trial. Genotypes of 200 individuals from the same year class from either population (AquaGen/AquaSearch) were spiked into the dataset. Since PCA assumes independence of the variables, SNPs were LD pruned (*plink –indep-pairwise 50 10 0.1*), resulting in a subset of 2501 SNPs / 1674 samples (1474 plus 200 spiked in samples), which was subsequently analysed in a principle component analysis (*plink –pca*).

2.1.3.1. Genome wide association study. The GWAS study and heritability estimate were obtained using GCTA (Genome-wide Complex Trait Analysis) version 1.91.7 beta1. Heritability was estimated with *GCTA –greml* estimating the proportion of variance in a phenotype explained by all SNPs (i.e. the SNP-based heritability) (Yang et al., 2011).

The GWAS analysis was performed separately for each of the two populations using the *mlma-loco* algorithm, which conducts a mixed linear model based association analysis with the chromosome, on which the candidate SNP is located, excluded from calculating the genetic relationship matrix (GRM). The model is:

$$y = \mu + Xb + g^{-} + e$$

Where y is the phenotype vector (the binary trat survival), μ is the mean term, X is the SNP genotype matrix, b is the additive genetic effect of the tested SNP, g^{-} is the accumulated effect of all SNPs except those on the chromosome where the candidate SNP is located and e is the residual. The GRM (G) was computed according to (Yang et al., 2011):

$$G_{jk} = \frac{1}{N}\sum_{i=1}^{N}\frac{(x_{ij} - 2p_i)(x_{ik} - 2p_i)}{2p_i(1 - p_i)}$$

Where x_{ij} is the number of copies of the reference allele for the ith SNP of the jth individual and p_i is the frequency of the reference allele. The meta GWAS was conducted using the METAL software according to Willer et al. (2010).

2.2. Challenge study 2: QTL validation

2.2.1. Fish

Whereas the challenge for QTL discovery was performed in Norway (see above), the validation study was performed in Copenhagen,

Denmark. Thus, when the QTL had been selected (see below) based on the first QTL search trial in Norway, we validated the effect of the QTL by two exposure studies at the University of Copenhagen in the subsequent two years (Fig. 1). We used fish hatched from disinfected eyed rainbow trout eggs of the Aquasearch ova ApS fish strain (Jutland, Denmark), which were brought to a certified pathogen free hatchery, Bornholms Lakseklækkeri, Aqua Baltic, Nexø, Bornholm (Xueqin et al., 2012). The eggs were hatched at 7 °C within the following 14 d. Larvae and fry were reared at 12 °C to a body weight of approximately 1 g. The fish were then transported (in oxygenated plastic tank bags over 3 h) to the experimental fish infection facility at the University of Copenhagen (Frederiksberg, Denmark). Upon arrival, the fish were acclimatized for 14 d prior to challenge. The disease free status was confirmed by bacteriological (blood agar and TYE plates) and parasitological examination of subsamples of ten fish (Buchmann, 2007; Dalsgaard and Madsen, 2000). The fish received commercial pelleted dry feed (1% biomass daily) (INICIO 917, BioMar A/S, Brande, Denmark). Fig. 1 shows the over-all outlines of the experimental set-up. For the first validation study we used heterozygous trout (Qq) offspring produced by use of three homozygous (QQ) male parents (with the Omy 25 Affx-88941461, see below). Sperm from these males was used for fertilization of eggs from 30 outbred females (qq or Qq). For the second validation study sperm from two homozygous (QQ) male parents was used for fertilization of four homozygous (QQ) female eggs. Fish without the favourable allele (qq without Omy 25 Affx-88941461) were produced from three male parents and sixteen females, all negative (qq) for the SNP. For the first QTL validation study (triplicate) we challenged a total of 900 fish (450 QTL fish and 450 non-QTL fish). They were placed in three replicated fish tanks (volume 120 L) each containing 300 fish (150 QTL and 150 non-QTL fish). For the second validation study (duplicate) we challenged a total of 300 trout (100 QQ, 100 Qq and 100 qq), which were placed in two fish tanks (each with 150 fish, 3 ×50 fish of each genotype). The different genetic groups in the common garden set-up could be differentiated by minor clips (upper, middle, lower) in the tail fin. The morbidity/mortality rates in all groups were thereby compared in duplicate or triplicate challenge trials.

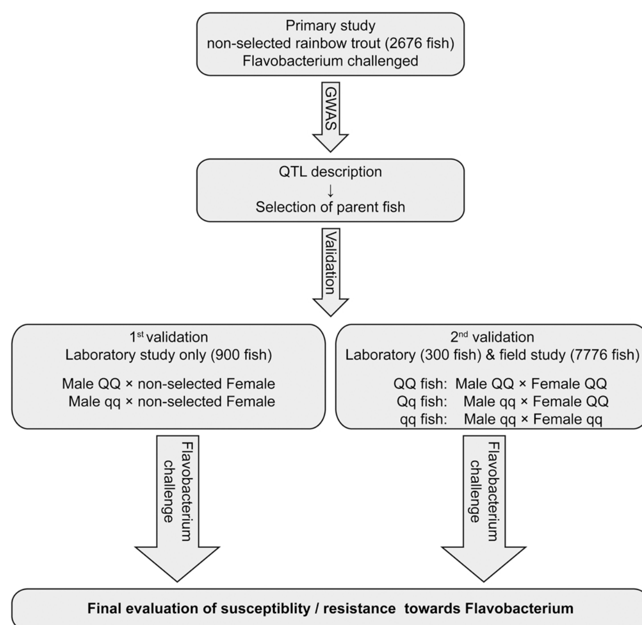

Fig. 1. Schematic diagram illustrating the flow sequence of elements and the number of fish used for each step in the investigation from QTL discovery via first and second validation to final evaluation of susceptibility/resistance.

2.2.2. Challenge

We challenged fish with known genetic status. In the first year we tested QQ and Qq fish against mainly qq and some Qq fish (Fig. 1) produced from DNA typed QQ male parents and non-typed female parents (unspecified qq, Qq). In the second year we included both heterozygous and homozygous fish in the test as both female and male parents were DNA typed and well characterized as QQ or qq (Fig. 1). The fish were primed by prior immersion in hydrogen peroxide for 60 min (100 mg/L first year and 200 mg/L the second year) in order to elevate the infection success (Hoare et al., 2017). The low concentration the first year was used due to welfare considerations, but as the treatment was tolerated very well, we increased the concentration the second year. The *F. psychrophilum* isolate 950106–1/1 serotype Fd used for bacterial exposure of trout in the validation trial was isolated from an outbreak in a Danish rainbow trout farm. ***Challenge procedure for the first validation study:*** Following hydrogen peroxide priming the fish were transferred to the challenge tank (volume 120 L containing 16 L solution). The fish were exposed to bacteria at a concentration of *F. psychrophilum* of 1.22×10^7 cfu/mL. Exposure time was 6 h, and subsequently water was added to each tank until a volume of 150 L. ***Challenge procedure for the second validation study:*** Fish were primed in hydrogen peroxide and then exposed for 7 h to a *F. psychrophilum* solution of 2.5×10^7 cfu/mL, whereafter water was added to each tank until a volume of 150 L. Morbidity/Mortality was continuously recorded every second hour throughout the Copenhagen laboratory experiments from exposure and until day 30. In brief, when a fish showed clinical signs it was removed and euthanized by immersion into an over-dosage of anaesthetic (300 mg/L) MS222 (Sigma-Aldrich, Denmark). In order to confirm the cause of disease we sampled the head kidney from dying fish and inoculated swabs on TYE (Tryptone Yeast Extract) plates to confirm presence of *F. psychrophilum* in the sick fish.

2.3. Challenge study 3: Gene expression

In order to describe expression of immune genes in rainbow trout fry exposed to *F. psychrophilum* we challenged outbred rainbow trout fry (1 g) (Aquasearch ova ApS eggs hatched at the Bornholm salmon hatchery, see 2.2.1) and sampled fish before, during and after the course of infection (see 2.3.1). A total of 1000 fish was exposed to the bacterial pathogen *F. psychrophilum* by simple bath challenge without any pretreatment with hydrogen peroxide as suggested by Hoare et al. (2017).

2.3.1. Tissue sampling

Samples for gene expression analyses (gills, liver and spleen) were taken at three different time-points, at day 0 (before exposure), at day 11–14 (when the mortality rate peaked) and at day 40 (termination of experiment). At day 0, samples were taken from 15 uninfected control fish. At day 11–14 we sampled from 15 uninfected control fish, 15 infected fish showing clinical signs (CS) and 15 fish infected fish showing no clinical signs (NCS). The samples at day 40 were from 15 uninfected control and from 15 surviving rainbow trout. All samples were fixed in RNAlater (cat. no. R90901. Sigma-Aldrich, Denmark) and stored at 4 °C for 24 h before being placed at − 20 °C until further analysis.

2.3.2. RNA extraction, cDNA synthesis and quantitative RT-qPCR (qPCR)

The transcriptomic study on fish organs was conducted as described previously by Zuo et al. (2020). In brief the gill, liver and spleen samples from the fish were homogenized (2 min, 20 Hz; Tissue-lyser II, Qiagen, Denmark) using a 2-mercaptoethanol (Sigma-Aldrich, Denmark) homogenization buffer after which RNA was extracted using the GenElu-teTM mammalian RNA kit (RTN350, Sigma-Aldrich, Denmark). A pre-treatment with Proteinase K (cat.no. P4850, Sigma-Aldrich, Denmark) was necessary for the liver samples. To remove genomic DNA DNase 1 (AMPD1, Sigma-Aldrich, Denmark) was applied. The concentration of RNA was determined using a Nanodrop 2000

spectrophotometer (Saveen & Werner, Sweden), whereas the quality was assessed by means of an agarose (cat.no. N8080234, Thermo Fischer Scientific, Denmark) gel electrophoresis. RNA was stored at − 80 °C until cDNA synthesis in a T100 Thermocycler (Biorad, Denmark) with a 20 µL reaction volume containing 1000 ng of RNA, oligo d(T)16 primer and TaqMan® Reverse Transcription Reagents (cat.no. N8080234, Thermo Fischer Scientific, Denmark). The cDNA was then stored at − 20 °C until further use. Quantitative PCR assays were performed using an AriaMx Real-Time PCR machine (cat.no. G88[30]A–[04]R-010, AH Diagnostics AS, Denmark) in the following cycles; one cycle of pre-denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 5 s with a combined annealing/elongation process at 60 °C for 15 s with endpoint measurement. Relevant primers and probes for rainbow trout can be found in S1 (supplementary Table S1). The reaction volumes used were 12.5 µL (2.5 µL cDNA, 6.25 µL Brilliant III Ultra-Fast QPCR Master Mix (cat. no. 600881, AH Diagnostics AS, Denmark), 1.0 µL primer-probe mixture (10 µM forward primer, 10 µM reverse primer and 5 µM TaqMan probe) and 2.75 µL RNAse-free water (cat. no. 10977049, Thermo Fischer Scientific, Denmark). Negative controls and reverse transcriptase minus were used for every plate setup. By applying Normfinder (Andersen et al., 2004) a combination of the three reference genes encoding elongation factor (ELF) 1-α, ARP and β-actin was chosen as endogenous control and stability values were 0.04, 0.03 and 0.03 for liver, gill and spleen respectively. The genes investigated in the study included those encoding interleukins (IL-1β, IL-2A, IL-4/13 A, IL-6A, IL-8 (isoforms A-E), IL-10A, IL-12 α chain, IL-17A/F2A, IL-17C1, IL-17C2, IL-22), cathelicidin 1, cathelicidin 2, immunoglobulins (IgDm, IgDs, IgM, IgT), complement factor 3 isoform 3 and 4 (C3–3 and C3–4), type II interferon (IFNɣ1 and IFNɣ2), lysozyme, serum amyloid protein A (SAA), T cell receptor β (TCRβ), transforming growth factor β (TGF-β1A) and tumor necrosis factor α (TNFα). The level of *F. psychrophilum* infection was estimated by the relative expression of the *F. psychrophilum* 16 S gene. Primers and probes were designed in this study. A PCR targeting a plasmid containing the 16 S gene was used to confirm the specificity of the assay. The determination was relative, as ΔCq values from samples taken from exposed fish were compared to values in the non-infected time point samples. No exact sensitivity was established from a calibration curve.

2.4. Field study in a Danish trout farm

Fish with the three SNP genotypes QQ, Qq and qq were also followed in a commercial trout farm (Jutland, Western part of Denmark), where RTFS outbreaks may occur. A total of 7776 rainbow trout were stocked into four fry fibre-glass raceways with a common natural water inlet (well water). These included QQ trout, comprising 1073 and 1133 fish (duplicate 1 and 2, respectively), Qq trout (2726 fish) and qq trout (2844 fish). A natural outbreak of flavobacteriosis arose when fish reached a body weight between 1 and 2 g, whereafter a florfenicol treatment (Florfenicol 15 mg/kg body mass/d for 10 days) was initiated on day 14 (following observation of the first disease signs) in order to reduce mortality. Morbidity and mortality was recorded daily throughout the entire course of infection.

2.5. Ethics and legislation

Exposure of fish was conducted both in Norway (VESO Vikan, Namsos) and in Denmark (University of Copenhagen, Frederiksberg Campus). The experimental procedures with live fish in Norway were carried out in accordance with the Norwegian Food Safety Authority guidelines and were approved by the Norwegian Food Safety Authority, National Assignments Department (approval no. 23188). The Danish infection procedure was performed under license number 2019–15–0201–01614 obtained from the Experimental Animal Inspectorate, Committee for Experimental Animals, Ministry of Environment and Food, Denmark. Ethical guidelines at the University of Copenhagen were followed and the study reviewed by the Institutional ethical review board. Fish showing clinical disease signs after challenge were taken out and euthanized by immersion into 300 mg/L MS222.

2.6. Statistical data analyses

2.6.1. Morbidity/Mortality analysis

The observed morbidity/mortality in the different challenge studies were compared by non-parametric statistical tests. Comparison of two groups were done by use of the Wilcoxon matched pairs signed rank test. When comparing multiple groups the Friedman's test with Dunn's multiple comparisons test was performed. The Log-Rank Mantel-Cox test was conducted as well. In all cases a probability level of 5% was applied ($p < 0.05$).

2.6.2. Gene expression data analysis

Data sets from the gene expression study were processed using Microsoft Office Excel and GraphPad Prism 9. The qPCR assays used had efficiencies at 100 ± 5% and data were analyzed using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). NCS, CS and non-exposed controls fish were compared to each other's using a One-way ANOVA with Tukey's multiple comparisons test. A Student's t-test were applied to compare gene expression of survivors compared to non-exposed controls. Results were only considered significant if differences were at least two-fold and $p < 0.05$. Less than three Cq values were obtained for transcripts of the IL17-C1 gene in the spleen samples at day 11–14. In that case, a non-parametric Kruskal-Wallis test with Dunn's multiple comparison was performed. Differences between CS and NCS were evaluated using a Student's t-test. In both tests differences were considered significant if they were two-fold and $p < 0.05$ (5% probability level).

3. Results

3.1. QTL discovery

3.1.1. Challenge study 1

Challenge study 1 comprised fish from the AquaGen and AquaSearch populations, challenged in a common garden setup. Since fish were below tagging weight, PCA was used to assign the fish to their respective population. PCA revealed two distinct clusters (S4. Supplementary Fig. 2), which overlapped with the "spike-in" samples. Based on this, a total of 675 animals were assigned to the AquaGen and 799 animals to AquaSearch population. The first morbidity/mortality observations in the challenge study 1 were taken within 3 days post exposure and then increased exponentially until day 12, whereafter it decreased (S3. Supplementary Fig. 1). Overall mortality was 54.3%, mortality within the subset of genotyped animals was 49.6%. After population assignment we found that mortality within AquaGen and AquaSearch population was 57.3% and 43% respectively.

3.2. QTL validation

3.2.1. Laboratory validation

First validation: The mortality of rainbow trout fry in the triplicate tanks followed the same pattern and did not differ significantly. The onset of disease started after 14 days and continued for a week until plateauing (Fig. 2). Second validation: The morbidity/mortality of fry in the duplicate groups did not differ. Morbidity was evident in some fish after 10 d and the overall mortality reached 47% in qq fish, whereas QQ fish showed 34% and the Qq fish an intermediate morbidity (Fig. 3).

3.2.2. Field validation

Homozygous QQ, heterozygous Qq and qq trout fry were followed in separate fry raceways in a commercial trout farm concomitant with the second validation study in the laboratory. A natural infection in the farm

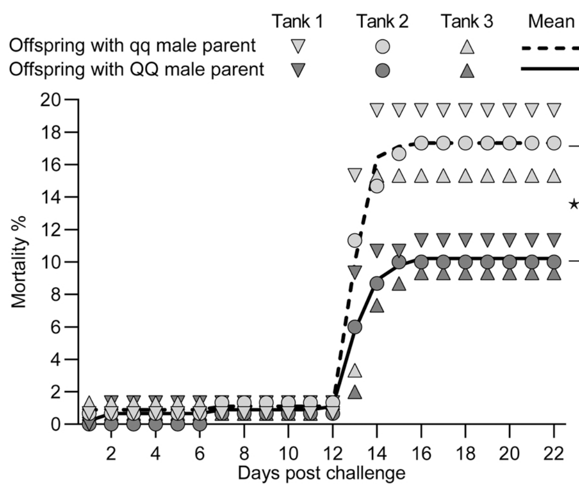

Fig. 2. QTL validation (first laboratory trial). Rainbow trout mortality curve following *F. psychrophilum* exposure. Laboratory exposure. Chemical priming by hydrogen-peroxide 100 mg/L for 60 min. Fish were either produced by fertilizing eggs from non-selected (outbred) trout by sperm from homozygous QQ male parents or from homozygous qq male parents. Triplicate common garden study. Wilcoxon matched-pairs signed rank test, * : $p < 0.05$. Log-Rank Mantel-Cox test, * : $p < 0.05$.

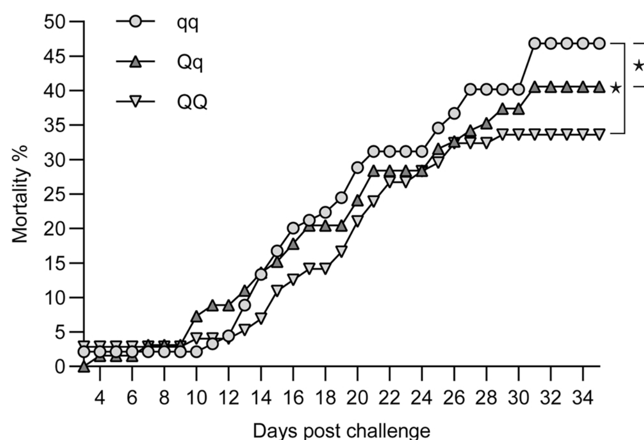

Fig. 3. QTL validation (second laboratory trial). Rainbow trout mortality curve following *F. psychrophilum* exposure. Laboratory exposure. Chemical priming by hydrogen-peroxide 200 mg/L for 60 min. Homozygous QQ, heterozygous Qq QTL-fish and non-QTL-fish qq are compared. Duplicate common garden study. Friedman's test and Dunn's multiple comparison test, * : $p < 0.05$. Log-Rank Mantel-Cox test, * : $p < 0.05$.

developed in the month of July, when fish reached a body weight between 1 and 2 g. Antibiotic treatment (florfenikol) had to be initiated 14 d after appearance of the first symptoms. However, the mortality data showed different susceptibility and survival between groups before and after treatment initiation. The QQ fish (duplicate 1 and 2) differed and showed 0.5% and 16% mortality but in both cases better survival than qq fish (Fig. 4).

3.2.3. Heritability and genetic correlation between the two populations

Heritability estimates (on the observed scale) for the trait survival of *F. psychrophilum* infection in the two populations were 0.32 and 0.38 (Table 1). The estimated "genetic" correlation (i.e., SNP marker effect correlation) for survival between both populations was not significantly different from zero based on likelihood ration test (data not shown). A likely explanation is that the associations between SNP markers to (unknown) functional mutations (QTL) differ in the two populations

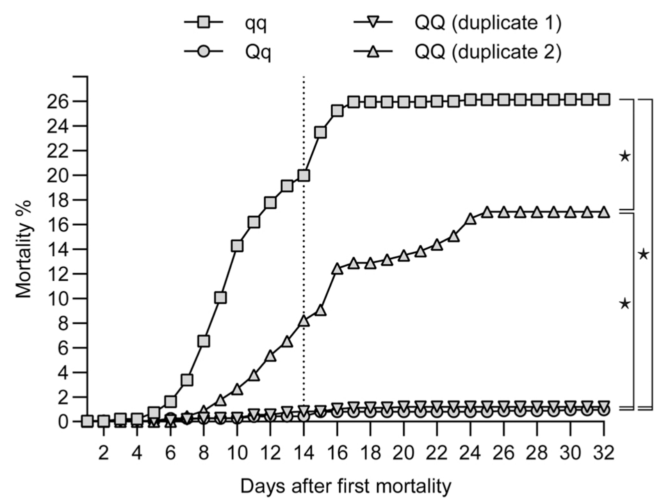

Fig. 4. QTL validation (field trial). Rainbow trout mortality curve following *F. psychrophilum* exposure. Field exposure at the farm. Homozygous QQ (duplicate 1 and 2), heterozygous Qq fish (one replicate) and qq fish (one replicate) are compared. Fish were florfenicol treated (for 10 d) in order allow survival (initiated at 14 d after appearance of first symptoms, shown by the dashed vertical line). Treatment was initiated. Friedman's test with Dunn's multiple comparison test, * : $p < 0.05$.

Table 1
Variance components and heritability estimates (S.E. standard error in brackets). The binary phenotype (dead = 0, survived = 1) was used.

Population	V_G	V_p	V_e	V_G/V_p
AquaGen	0.08 (0.02)	0.25 (0.01)	0.17 (0.01)	0,32 (0,06)
AquaSearch	0.09 (0.02)	0.24 (0.01)	0.15 (0.01)	0,38 (0,06)

VG genetic variance; Vp phenotypic variance; Ve residual variance; VG/Vp heritability (h2)

(but the functional mutations may still be the same).

3.2.4. GWAS and QTL identification

GWAS analysis of the two populations (Table 2) resulted in the identification multiple peaks of SNPs exceeding genome wide significance levels (after Bonferroni correction for multiple testing). The most notable peaks were located on chromosome 17 and 25. Manhattan plots are shown for both populations combined (S5. Supplementary Fig. 3) and for the two individual populations (S6. Supplementary Fig. 4). GWAS results for both populations were subsequently combined in a meta-analysis, which (again) showed multiple peaks of SNPs exceeding genome wide significance levels (after Bonferroni correction), most notably located on chromosome 25 where 45 SNPs exceeded genome wide significance. Two more SNPs that exceeded genome wide significance were located on chromosomes 9 and 24, however these were isolated SNPs and not associated to a peak. Although also a QTL on

Table 2
Summary statistics for the QTL marker Affx-88941461 across all datasets. The marker is located on chromosome 25 (NC_035101.1:20868713_G/T; G/T is the SNP with respect to the reference genome, the snpchip probe is reverse complement: A/C). *Proportion of the phenotypic variance (*Vp*) explained by the SNP ($V_p^{snp} = 2p(1-p)\quad b^2/V_p$).

Population	A1	A2	Freq (A1)	Beta (A1)	p-value	V_p^{snp}*
AquaGen	A	C	0.26	0.21419400	1.97460e-09	7.0797e-02
AquaSearch	A	C	0.33	0.20584300	1.47326e-10	7.7474e-02

Omy17 was detected on AquaGen fish, and not on Aquasearch fish, the overall most significant SNP, that was present in all datasets, was Affx-88941461 located on chromosome 25 (NC_035101.1:20868713_G/T). It was noted that chromosome 25 was the chromosome that was most frequently associated to QTL regions related to *F. psychrophilum* resistance by other studies. Based on the results of the meta GWAS the SNP Affx-88941461 located on chromosome 25 was selected for our subsequent validation study. It is located within the region of chromosome 25 reported in Valejo et al. (2017) and maps to these positions: NC_035101.1:20868713_G/T (Omyk_1.0) - NC_050571.1:24889755_G/T (USDA_OmykA_1.1).

The significance and allelic substitution effect of the 45 SNPs from the QTL on Omy25 are shown in S7 (supplementary Table S3).

3.3. Gene expression following F. psychrophilum exposure

The expression level of the 16 S sequence from *F. psychrophilum* was used to indicate the relative infection level in the three different organs sampled in the study. No Cq values for NCS or surviving fish were detectable. In the fish showing clinical signs the bacteria were detected. The expression was significantly higher in the spleen compared to the gill and the liver in CS fish (Fig. 5).

Immune genes. Gills – We saw a significantly higher expression in the CS fish (fish with clinical signs) compared to NCS fish (fish without clinical signs) for the genes encoding the interleukins, IL-1β, IL-4/13 A, IL-6, IL-8, IL-10, IL-12, 17 A/F2A, IL-22 as well as IgM, cathelicidin 1 and 2, IFNɣ, lysozyme, SAA and TNFα. Only for one gene (IL-17C2) a significantly lower transcript was recorded for CS compared to NCS. The genes encoding IL-10, IL-12, IL-17A/F2A, IL-17C1, IL-17C2, IL-22, IgM and C3 were significantly upregulated in fish surviving the infection (Figs. 6 and 7 with detailed data in S2 (supplementary Table S2).

Spleen – An infection with *F. psychrophilum* resulted in a significant upregulation of IL-1β, IL-8, IL-10, IL-12, IL-22, C3, cathelicidin 1 and 2 and SAA and a significant downregulation of IL-17A/F2A, IgDm, IgDs, IgT, and TCRβ in CS fish compared to NCS fish. A significant upregulation of IL-6, IL-17C1 and lysozyme was found in the CS fish. However, genes encoding IL-6 and IL-4/13 A showed a significant downregulation for both NCS and CS. Fish that survived the infection showed a significant downregulation of IL-10, 17 A/F2A, C3 and SAA (Figs. 6 and 7).

Liver – The number of transcripts of the genes encoding IL-1β, IL-6, IL-8, IL-10, IFNɣ, TNFα, IgM, C3, lysozyme, SAA were a significantly higher while IgDs were significantly lower when comparing CS to NCS fish. Cathelicidin 1 and 2 genes were significantly downregulated both in NCS and to some extent in the CS groups. A significant downregulation of the IL-4/13 A gene was found for the NCS fish while IL-17C2 and IL-22 genes were significantly upregulated in the CS group. Surviving fish exhibited a significant upregulation of genes encoding IL-22, IFNɣ, TNFα and IgDs, whereas IL-8 and SAA genes were significantly downregulated (Figs. 6 and 7).

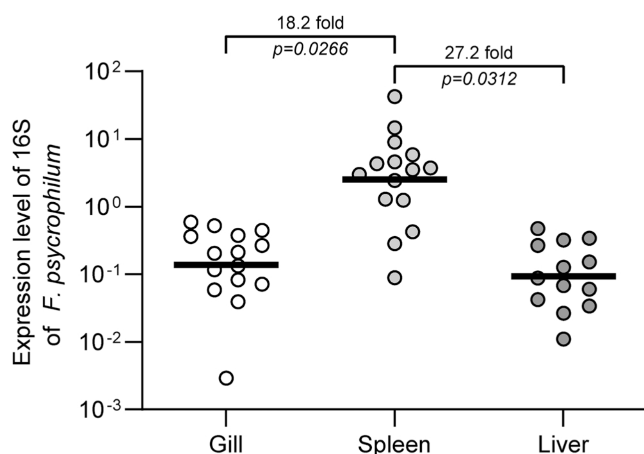

Fig. 5. Relative expression of the 16 S gene of *F. psychrophilum* rainbow trout exposed compared to non-exposed time point controls. CS: Fish showing clinical signs, NCS: Fish without clinical signs at the same time point. Survivors were sampled at the end of the exposure period.

4. Discussion

4.1. The QTL selection and validation

In the present study we addressed two questions related to *F. psychrophilum* infection in rainbow trout. First of all, based on the challenge study 1, we identified a possible QTL associated with resistance to *F. psychrophilum* on chromosome 25 with the peak SNP marker being Affx-88941461. It is noteworthy that markers on this chromosome previously have been associated with natural resistance of rainbow trout towards BCWD/RTFS (Palti et al., 2015b; Liu et al., 2015, 2018, 2022; Vallejo et al., 2017, 2022; Fraslin et al., 2018), which suggested the relevance of a validation test. It is known that also other QTL may add to the overall host susceptibility/resistance of rainbow trout to RTFS (Fraslin et al., 2018), but we decided to test this specific QTL on Omy25 for its effects. We then tested the hypothesis, regarding the association between the SNP Affx-88941461 and natural resistance to *F. psychrophilum* infection, by producing homozygous (QQ) and heterozygous (Qq) trout carrying this favourable allele, and subsequently, following bacterial exposure, evaluating their survival compared to qq fish, without the favourable allele. The elevated survival observed in QQ and Qq trout (both in laboratory and field tests) suggests that this marker can be applied in future breeding experiments. This may supplement previous breeding studies focusing on selection of *F. psychrophilum* resistant trout (Johnson et al., 2008; Wiens et al., 2013; Vallejo et al., 2014, 2017, 2022; Campbell et al., 2014; Liu et al., 2015, 2022; Palti et al., 2015; Kutyrev et al., 2016; Fraslin et al., 2018, 2019). The mortalities of the duplicate QQ trout groups which were tested at farm level differed. This may both be ascribed to difficulties in producing completely identical conditions in field studies and by the use of only one QTL in marker assisted breeding studies. Thus, the QQ and Qq fish experienced some mortality when tested both in laboratory and field environments, which suggests an oligogenic/borderline polygenic nature of the trait. We therefore recommend the inclusion of additional markers in future breeding studies. Thus, apart from the QTL on Omy25 also QTL on rainbow trout chromosome 3, 8, 11 were suggested to be associated with BCWD by (Liu et al., 2022; Vallejo et al., 2022). In addition, a QTL on Omy19 was one of the first QTL to be reported (Wiens et al., 2013; Vallejo et al., 2014; Liu et al., 2015). In our initial QTL search we also indicated the presence of a QTL on Chromosome 17.

However, it should also be noted that the present study applied bacterial bath challenge to infect the fish. Other ways of administering the pathogen to the fish may include i.p. injection, and it is noteworthy, that the use of such a challenge method suggest a range of other QTL associated with BCWD/RTFS resistance (Fraslin et al., 2018).

4.2. QTL associated genes

Host factors explaining the differential susceptibility/resistance are at present unknown (Semple et al., 2018), but several genes have been suggested to the associated with elevated resistance of rainbow trout (Palti et al., 2015b; Vallejo et al., 2017; Fraslin et al., 2018; Liu et al., 2022; Vallejo et al., 2022). It is tempting to suggest that one or more genes related first of all to innate immune reactions and secondarily to adaptive responses may play a role in the natural resistance to RTFS in rainbow trout. Some of these were previously pin-pointed (Palti et al., 2015b; Vallejo et al., 2017, 2022; Fraslin et al., 2018; Liu et al., 2022), and we therefore investigated a range of well-studied genes encoding central innate and adaptive factors. These genes were not found associated with the selected SNP on Omy25 but may none-the-less reflect if

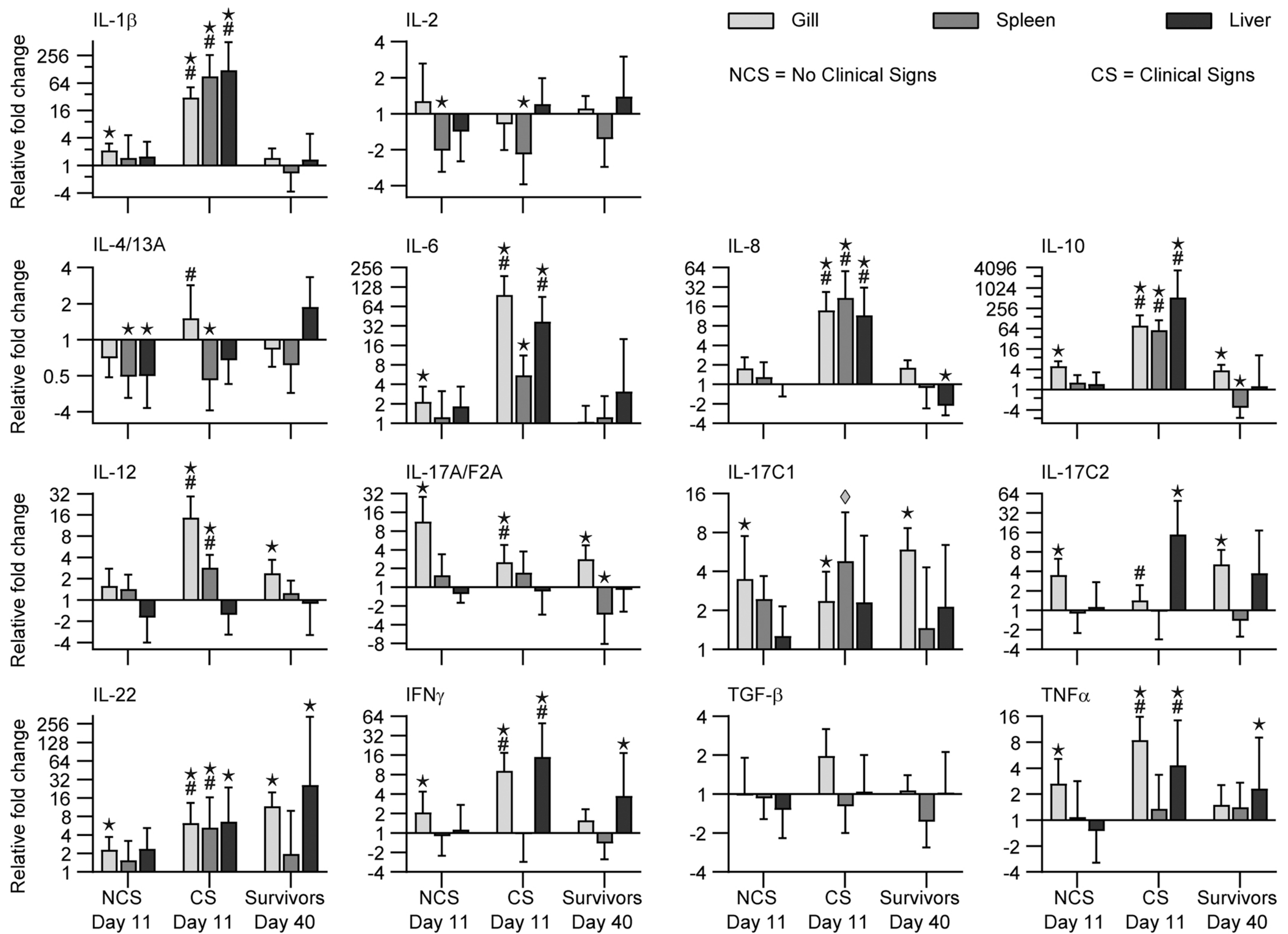

Fig. 6. Expression of cytokine genes of rainbow trout experimentally exposed to *F. psychrophilum.* CS: Fish showing clinical signs, NCS: Fish without clinical signs at the same time point. Survivors were sampled at the end of the exposure period.

responses occur. Thus, the putative decisive genes suggested (Palti et al., 2015, Vallejo et al., 2017, 2022; Fraslin et al., 2018; Liu et al., 2022) may interact with cascades and reactions responsible for the detected qPCR reactions detected in the present study.

However, it should not be excluded that physiological/anatomical elements may confer some natural resistance to a fish. Thus, fish without clinical signs and survivors were the fish without qPCR detectable pathogens. This may indicate that the resistant fish has some kind of ability to reject the bacterium at an early stage post-exposure. Various structural and physiological mechanisms could explain this, although immune genes probably are involved. Accordingly, it is at present unknown if genes encoding effector molecules and/or regulatory elements for immune reactions are involved in the natural resistance towards *F. psychrophilum*, but the investigation on the reactions may give a clue to the mechanisms involved. Our gene expression study showed that the susceptible fish, showing clinical signs, exhibited a strong inflammatory reaction with marked IL-1β gene expression associated with immediate production of antimicrobial peptides and complement. It is noteworthy that Vallejo et al. (2017) suggested that the gene encoding a receptor for this proinflammatory cytokine was associated with resistance. However, as the immune gene expression was very low in resistant fish, and high in susceptible fish, our results reflect that the immune gene expression is initiated when the pathogen invades the host successfully. Several studies have previously elucidated and confirmed similar immune response patterns in fish following exposure to *F. psychrophilum* (Avendaño-Herrera et al., 2020; Evenhuis and Cleveland, 2012; Henriksen et al., 2015a, 2015b; Kutyrev et al., 2016; Muñoz-Atienza et al., 2019; Semple et al., 2018; Villarroel et al., 2008), but the protective elements still remain elusive. It should be noted that the way of exposing fish to the pathogen may affect the results. Different challenge methods have been investigated including i.p. injection (Madsen and Dalsgaard, 1999; Semple et al., 2018), intra-rectal instillation (Chettri et al., 2018), cohabitation (Madsen and Dalsgaard, 1999) and pre-treatment with a stressor (e.g. formalin or hydrogen peroxide) before immersion (Garcia et al., 2000; Henriksen et al., 2015a, 2015b, 2013, Hoare et al., 2018). These different challenge models generally induce a higher morbidity/mortality compared to immersion alone. However, injection bypasses the first line of defence (Madsen and Dalsgaard, 1999; Nematollahi et al., 2003) and hydrogen peroxide may damage the gills and alter the immune response (Henriksen et al., 2015a). This may be one of the reasons that Fraslin (2018) presented evidence for a strong effect of the challenge method used with regard to BCWD/RTFS resistance QTL discovery. Along with this knowledge it should be stated that in all our QTL search challenges and the subsequent validation steps, we pre-treated the fish with hydrogen peroxide before immersion into the bacterial solution, which elicited a relatively high morbidity/mortality. Therefore, it cannot be excluded that this challenge procedure may influence also the QTL search and the validation challenge. This note of caution calls for future studies addressing this point. However, when we challenged rainbow in order to study the expression of immune relevant genes after *F. psychrophilum* exposure, we did not pre-treat fish with hydrogen peroxide. The response recorded is therefore with high

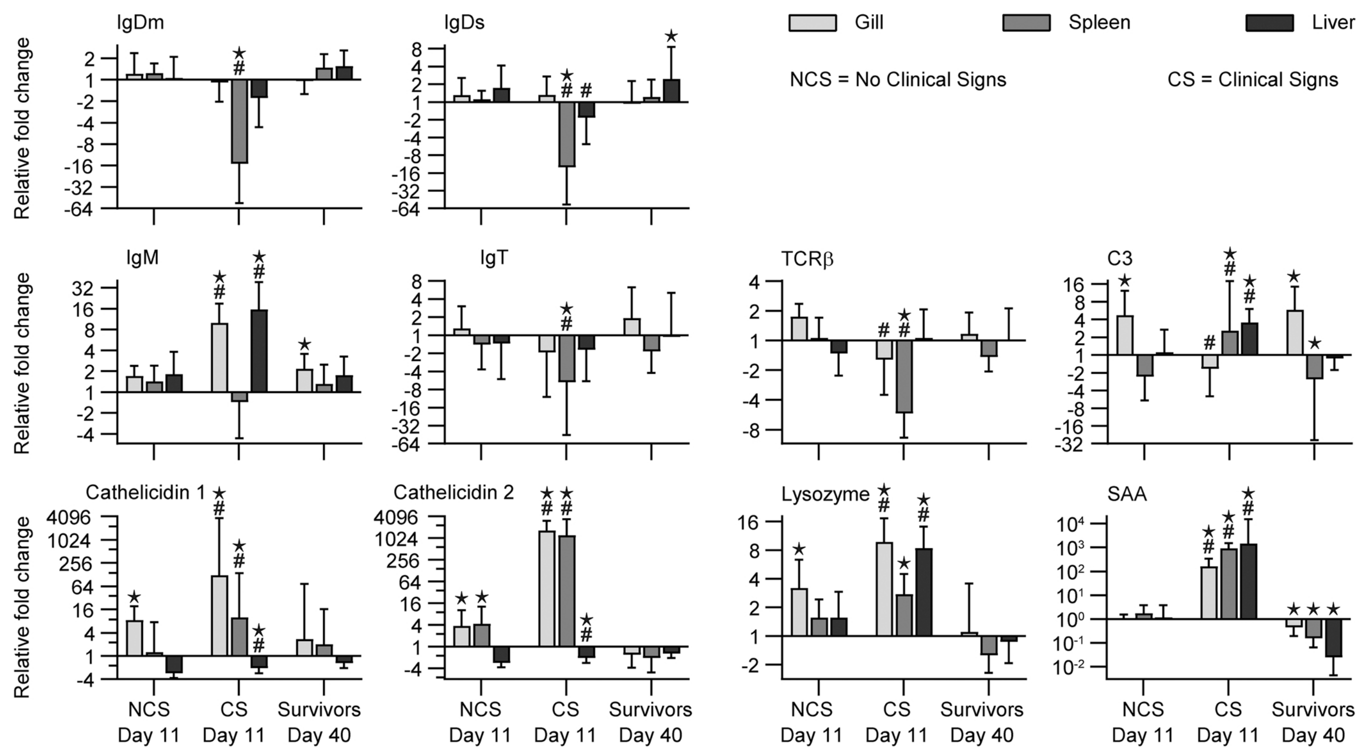

Fig. 7. Expression of immune effector genes of rainbow trout exposed to *F. psychrophilum.* CS: Fish showing clinical signs, NCS: Fish without clinical signs at the same time point. Survivors were sampled at the end of the exposure period.

probability the result of the bacterial interaction with the host. It was evident that the immune genes were induced by infection. Thus, the most susceptible fish, which displayed clinical signs, showed the strongest response, whereas the resistant fish and the survivors, which did not show clinical signs, showed no or very moderate change of immune gene expression.

5. Conclusion

Based on the challenge trials conducted and the subsequent analysis of survival data we detected a SNP marker, Affx-88941461 SNP on chromosome 25, suggested to be associated with a partial resistance to *F. psychrophilum*. We subsequently validated this specific genetic marker in a series of *F. psychrophilum* challenge trials. QQ and Qq trout, carrying the favourable allele, exhibited lower mortality compared to qq trout, which only carried the unfavourable allele. We recommend that future breeding studies should include additional QTL because resistance towards *F. psychrophilum* may be encoded by several genes. The precise mechanisms responsible for protection in partially resistant trout are not known, but immune genes and/or their regulation may be involved. Susceptible fish showing clinical signs achieved the highest infection and exhibited a significantly higher expression of immune-related genes when compared to fish with no clinical signs and survivors.

CRediT authorship contribution statement

Yajiao Duan: Methodology. **Heidi Mathiessen:** Methodology, Investigation, Writing. **Moonika H. Marana:** Methodology. **Shaozhi Zuo:** Methodology. **Asma M. Karami:** Methodology. **Rzgar M. Jafaar:** Methodology. **Louise von Gersdorff Jørgensen:** Methodology. **Per W. Kania:** Visualization, Formal analysis. **Inger Dalsgaard:** Resources. Resources. **Lone Madsen:Torben Nielsen:** Resources, Conceptualization. **Fabian Grammes:** Formal analysis, Data curation, Writing. **Jørgen Ødegaard:** Formal analysis. **Valeria Macchia:** Methodology. **Kurt Buchmann:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kurt Buchmann reports financial support was provided by Danish Ministry of Environment and Food. Kurt Buchmann reports financial support was provided by Innovation Fund Denmark. The company AquaGen in Norway is involved in genetic research and breeding. The company Aquasearch ova ApS in Denmark is exporting trout eggs.

Data Availability

Data will be made available on request.

Acknowledgement

The present study was supported by Green Development and Demonstration Program GUDP (EGYR grant no. 34009–21-1917) under the Danish Ministry of Environment and Food and the Innovation Fund Denmark (grant no. 8090–00002B) as well as by the Sustainable Aquaculture Innovation Centre (SAIC; project RD_2017_03).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101573.

References

Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64, 5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496.

Avendaño-Herrera, R., Benavides, I., Espina, J.A., Soto-Comte, D., Poblete-Morales, M., Valdés, J.A., Reyes, A.E., 2020. Zebrafish (*Danio rerio*) as an animal model for bath

infection by *Flavobacterium psychrophilum*. J. Fish. Dis. 3, 561–570. https://doi.org/10.1111/jfd.13156.

Buchmann, K., 2007. An Introduction to Fish Parasitological Methods - Classical and Molecular Techniques. Biofolia, Frederiksberg, Denmark.

Buchmann, K., 2022. The ontogeny of the fish immune system. In: Buchmann, K., Secombes, C.J. (Eds.), Principles of Fish Immunology. SpringerNature, Switzerland, pp. 495–510.

Campbell, N.R., LaPatra, S.E., Overturf, K., Towner, R., Narum, S.R., 2014. Association mapping of disease resistance traits in rainbow trout using resitriction site associated sequencing. G3, 4, 2473–2481. https://doi.org/10.1534/g3.114.014621.

Chang, C.C., Chow, C.C., Tellier, L.C., Vattikuti, S., Purcell, S.M., Lee, J.J., 2015. Second-generation PLINK: rising to the challenge of larger and richer datasets. Gigascience 4 (1), 13742–015-0047-8.

Chettri, J.K., Al-Jubury, A., Dalsgaard, I., Heegaard, P.M.H., Buchmann, K., 2018. Experimental anal infection of rainbow trout with *Flavobacterium psychrophilum*: A novel challenge model. J. Fish. Dis. 41, 1917–1919. https://doi.org/10.1111/jfd.12888.

Dalsgaard, I., Madsen, L., 2000. Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. J. Fish. Dis. 23, 199–209. https://doi.org/10.1046/j.1365-2761.2000.00242.x.

Evenhuis, J.P., Cleveland, B.M., 2012. Modulation of rainbow trout (*Oncorhynchus mykiss*) intestinal immune gene expression following bacterial challenge. Vet. Immunol. Immunopathol. 146, 8–17. https://doi.org/10.1016/j.vetimm.2012.01.008.

Fraslin, C., Dechamp, N., Bernard, M., Krieg, F., Hervet, C., Guyomard, R., Quillet, E., 2018. Quantitative trait loci for resistance to *Flavobacterium psychrophilum* in rainbow trout: effect of the mode of infection and evidence of epistatic interactions. Genet. Sel. Evol. 50 (60), 1–16. https://doi.org/10.1186/s12711-018-0431-9.

Fraslin, C., Brard-Fudulea, S., D'Ambrosio, J., Bestin, A., Charles, M., Haffrey, P., Quillet, E., Phocas, F., 2019. Rainbow trout resistance to bacterial cold water disease: two new quantitative trait loci identified after a natural disease outbreak on a French farm. Anim. Genet. 50, 293–297. https://doi.org/10.1111/age.12777.

Garcia, C., Pozet, F., Michel, C., 2000. Standardization of experimental infection with *Flavobacterium psychrophilum*, the agent of rainbow trout *Oncorhynchus mykiss* fry syndrome. Dis. Aquat. Org. 42, 191–197.

Henriksen, M.M.M., Madsen, L., Dalsgaard, I., 2013. Effect of hydrogen peroxide on immersion challenge of rainbow trout fry with *Flavobacterium psychrophilum*. PLoS ONE 8, 1–7. https://doi.org/10.1371/journal.pone.0062590.

Henriksen, M.M.M., Kania, P.W., Buchmann, K., Dalsgaard, I., 2015a. Effect of hydrogen peroxide and/or *Flavobacterium psychrophilum* on the gills of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish. Dis. 38, 259–270. https://doi.org/10.1111/jfd.12232.

Henriksen, M.M.M., Kania, P.W., Buchmann, K., Dalsgaard, I., 2015b. Evaluation of the immune response in rainbow trout fry*, Oncorhynchus mykiss* (Walbaum), after waterborne exposure to *Flavobacterium psychrophilum* and/or hydrogen peroxide. J. Fish. Dis. 38, 55–66. https://doi.org/10.1111/jfd.12201.

Hesami, S., Metcalf, D.S., Lumsden, J.S., MacInnes, J.I., 2011. Identification of cold-temperature-regulated genes in *Flavobacterium psychrophilum*. Appl. Environ. Microbiol. 77, 1593–1600. https://doi.org/10.1128/AEM.01717-10.

Hoare, R., Ngo, T.P.H., Bartie, K.L., Adams, A., 2017. Efficacy of a polyvalent immersion vaccine against *Flavobacterium psychrophilum* and evaluation of immune response to vaccination in rainbow trout fry (*Oncorhynchus mykiss*). Vet. Res. 48, 1–13. https://doi.org/10.1186/s13567-017-0448-z.

Hoare, R., Jung, S.-J., Thao, P.H., Ngo, T.P.H., Bartie, K.L., Thompson, K.D., Adams, A., 2019. Efficacy of a polyvalent injectable vaccine against *Flavobacterium psychrophilum* administered to rainbow trout (*Oncorhynchus mykiss*). J. Fish. Dis. 42 (2), 229–236. https://doi.org/10.1111/jfd.12919.

Jaafar, R., Ødegård, J., Mathiessen, H., Karami, A.M., Marana, M.H., von Gersdorff Jørgensen, L., Buchmann, K., 2020. Quantitative trait loci (QTL) associated with resistance of rainbow trout *Oncorhynchus mykiss* against the parasitic ciliate *Ichthyophthirius multifiliis*. J. Fish. Dis. 43, 1591–1602. https://doi.org/10.1111/jfd.13264.

Johnson, N.A., Vallejo, R.L., Silverstein, J.T., Welch, T.J., Wiens, G.D., Hallerman, E.M., Palti, Y., 2008. Suggestive association of major histocompatibility IB genetic markers with resistance to bacterial cold water disease in rainbow trout (*Oncorhynchus mykiss*). Mar. Biotechnol. 10, 429–437. https://doi.org/10.1007/s10126-007-9080-7.

Karami, A.M., Ødegård, J., Marana, M.H., Zuo, S., Jaafar, R., Mathiessen, H., Buchmann, K., 2020. A major QTL for resistance to *Vibrio anguillarum* in rainbow trout. Front. Genet 11. https://doi.org/10.3389/fgene.2020.607558.

Kutyrev, I., Cleveland, B., Leeds, T., Wiens, G.D., 2016. Proinflammatory cytokine and cytokine receptor gene expression kinetics following challenge with *Flavobacterium psychrophilum* in resistant and susceptible lines of rainbow trout (*Oncorhynchus mykiss*). Fish. Shell, Immunol. 58, 542–553. https://doi.org/10.1016/j.fsi.2016.09.053.

Langevin, C., Blanco, M., Martin, S.A., Jouneau, L., Bernardet, J.F., Houel, A., Boudinot, P., 2012. Transcriptional responses of resistant and susceptible fish clones to the bacterial pathogen *Flavobacterium psychrophilum*. PLoS One 7 (6), e39126.

Liu, S., Vallejo, R.L., Palti, Y., Gao, G., Marancik, D.P., Hernandez, A.G., Wiens, G.D., 2015. Identification of single nucleotide polymorphism markers associated with bacterial cold water disease resistance and spleen size in rainbow trout. Front. Genet. 6, 1–10. https://doi.org/10.3389/fgene.2015.00298.

Liu, S., Vallejo, R.L., Evenhuis, J.P., Martin, K.E., Hamilton, A., Gao, G., et al., 2018. Retrospective evaluation of marker-assisted selection for resistance to bacterial cold water disease in three generations of a commercial rainbow trout breeding population. Front. Genet. 9, 286. https://doi.org/10.3389/fgene.2018.00286.

Liu, S., Martin, K.E., Gao, G., Long, R., Evenhuis, J.P., Leeds, T.D., Wiens, G.D., Palti, Y., 2022. Identification of haplotypes associated with resistance to bacterial cold water disease in rainbow trout using whole-genome resequencing. Front. Genet. 13, 936806 https://doi.org/10.3389/fgene.2022.936806.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Madsen, L., Dalsgaard, I., 1999. Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*. Dis. Aquat. Org. 36, 169–176. https://doi.org/10.3354/dao036169.

Marana, M.H., Karami, A.M., Ødegård, J., Zuo, S., Jaafar, R.M., Mathiessen, H., Buchmann, K., 2021. Whole-genome association study searching for QTL for *Aeromonas salmonicida* resistance in rainbow trout. Sci. Rep. 11, 1–12. https://doi.org/10.1038/s41598-021-97437-7.

Marana, M.H., Dalsgaard, I., Kania, P.W., Mohamed, A., Hannibal, J., Buchmann, K., 2022. *Flavobacterium psychrophilum*: Response of vaccinated large rainbow trout to different strains (doi.). Biology 11, 1701. https://doi.org/10.3390/biology11121701.

Marancik, D., Gao, G., Paneru, B., Ma, H., Hernandez, A.G., Salem, M., Wiens, G.D., 2015. Whole-body transcriptome of selectively bred, resistant-, control-, and susceptible-line rainbow trout following experimental challenge with *Flavobacterium psychrophilum*. Front. Genet. 5, 453.

Muñoz-Atienza, E., Távara, C., Díaz-Rosales, P., Llanco, L., Serrano-Martínez, E., Tafalla, C., 2019. Local regulation of immune genes in rainbow trout (*Oncorhynchus mykiss*) naturally infected with *Flavobacterium psychrophilum*. Fish. Shell Immunol. 86, 25–34. https://doi.org/10.1016/j.fsi.2018.11.027.

Nematollahi, A., Decostere, A., Pasmans, F., Haesebrouck, F., 2003. *Flavobacterium psychrophilum* infections in salmonid fish. J. Fish. Dis. 26, 563–574. https://doi.org/10.1046/j.1365-2761.2003.00488.x.

Nilsen, H., Olsen, A.B., Vaagnes, O., Hellberg, H., Bottolfsen, K., Skjelstad, H., Colquhoun, D.J., 2011. Systemic *Flavobacterium psychrophilum* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), farmed in fresh and brackish water in Norway. J. Fish. Dis. 4, 403–408. https://doi.org/10.1111/j.1365-2761.2011.01249.x.

Palti, Y., Gao, G., Liu, S., Kent, M.P., Lien, S., Miller, M.R., Moen, T., 2015a. The development and characterization of a 57 K single nucleotide polymorphism array for rainbow trout. Mol. Ecol. Res. 15 (3), 662–672.

Palti, Y., Vallejo, R.L., Gao, G., Liu, S., Hernandez, A.G., Rexroad III, C.E., Wiens, G.D., 2015b. Detection and validation of QTL affecting bacterial cold water disease resistance in rainbow trout using restriction-site associated DNA sequencing. PLoS ONE 10, 1–14. https://doi.org/10.1371/journal.pone.0138435.

Semple, S.L., Kellendonk, C.J., Al-Hussinee, L., MacInnes, J.I., Lumsden, J.S., Dixon, B., 2018. Serum IgM, MH class IIβ genotype and respiratory burst activity do not differ between rainbow trout families displaying resistance or susceptibility to the coldwater pathogen, *Flavobacterium psychrophilum*. Aquaculture 483, 131–140. https://doi.org/10.1016/j.aquaculture.2017.10.020.

Vallejo, R.L., Palti, Y., Liu, S., Evenhuis, J.P., Gao, G., Rexroad 3rd, C.E., Wiens, G.D., 2014. Detection of QTL in rainbow trout affecting survival when challenged with *Flavobacterium psychrophilum*. Mar. Biotechnol. 16, 349–360. https://doi.org/10.1007/s10126-013-9553-9.

Vallejo, R.L., Leeds, T.D., Gao, G., Parsons, J.E., Martin, K.E., Evenhuis, J.P., Fragomeni, B.O., Palti, Y., 2017. Genomic selection models double the accuracy of predicted breeding values for bacterial cold water disease resistance compared to a traditional pedigree-based model in rainbow trout aquaculture. Genet. Sel. Evol. 49, 1–13. https://doi.org/10.1186/s12711-017-0293-6.

Vallejo, R.L., Evenhuis, J.P., Cheng, H., Fragomeni, B.O., Gao, G., Liu, S., Long, R.L., Shewbridge, K.L., Silva, R.M.O., Wiens, G.D., Leeds, T.D., Martin, K.E., Palti, Y., 2022. Genome-wide mapping of quantitative trait loci that can be used in marker-assisted selection for resistance to bacterial cold water disease in two commercial rainbow trout breeding populations. Aquaculture 560, 738574. https://doi.org/10.1016/j.aquaculture.2022.738574.

Villarroel, F., Casado, A., Vásquez, J., Matamala, E., Araneda, B., Amthauer, R., Concha, M.I., 2008. Serum amyloid A: a typical acute-phase reactant in rainbow trout? Dev. Comp. Immunol. 32, 1160–1169. https://doi.org/10.1016/j.dci.2008.03.004.

Wiens, G.D., LaPatra, S.E., Welch, T.J., Evenhuis, J.P., Rexroad III, C.E., Leeds, T.D., 2013. On-farm performance of rainbow trout (*Oncorhynchus mykiss*) selectively bred for resistance to bacterial cold water disease: Effect of rearing environment on survival phenotype. Aquaculture 388–391, 128–136. https://doi.org/10.1016/j.aquaculture.2013.01.018.

Willer, C.J., Li, Y., Abecasis, G.R., 2010. METAL: fast and efficient meta-analysis of genome wide association scans. Bioinformatics 26 (17), 2190–2191.

Xueqin, J., Kania, P.W., Buchmann, K., 2012. Comparative effects of four feed types on white spot disease susceptibility and skin immune parameters in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish. Dis. 35, 127–135. https://doi.org/10.1111/j.1365-2761.2011.01329.x.

Yang, J., Lee, S.H., Goddard, M.E., Visscher, P.M., 2011. GCTA: a tool for genome-wide complex trait analysis. Am. J. Hum. Genet. 88 (1), 76–82.

Zuo, S., Karami, A.M., Ødegård, J., Mathiessen, H., Marana, M.H., Jaafar, R.M., Buchmann, K., 2020. Immune gene expression and genome-wide association analysis in rainbow trout with different resistance to *Yersinia ruckeri* infection. Fish. Shell Immunol. 106, 441–450. https://doi.org/10.1016/j.fsi.2020.07.023.