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Authors: Clark E.S., Wilkins L.G., Wedekind C.,

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MHC class I expression dependent on bacterial infection and parental factors in whitefish embryos (Salmonidae)

Emily S. Clark[†], Laetitia G. E. Wilkins[†], and Claus Wedekind
Department of Ecology and Evolution, Biophore, University of Lausanne, 1015, Lausanne, Switzerland

[†]Shared first authorship

Correspondence to C. Wedekind, Tel: +41 21 692 42 50; Fax: +41 21 692 42 65; email: claus.wedekind@unil.ch

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Abstract

Ecological conditions can influence not only the expression of a phenotype, but also the heritability of a trait. As such, heritable variation for a trait needs to be studied across environments. We have investigated how pathogen challenge affects the expression of MHC genes in embryos of the lake whitefish *Coregonus palaea*. In order to experimentally separate paternal (i.e. genetic) from maternal and environmental effects, and determine whether and how stress affects the heritable variation for MHC expression, embryos were produced in full-factorial *in vitro* fertilizations, reared singly, and exposed at 208 degree days (late-eyed stage) to either one of two strains of *Pseudomonas fluorescens* that differ in their virulence characteristics (one increased mortality, while both delayed hatching time). Gene expression was assessed 48 hours post-inoculation, and virulence effects of the bacterial infection were monitored until hatching. We found no evidence of MHC class II expression at this stage of development. MHC class I expression was markedly down-regulated in reaction to both pseudomonads. While MHC expression could not be linked to embryo survival, the less the gene was expressed, the earlier the embryos hatched within each treatment groups, possibly due to trade-offs between immune function and developmental rate or further factors that affect both hatching timing and MHC expression. We found significant additive genetic variance for MHC class I expression in some treatments, i.e. changes in pathogen pressures could induce rapid evolution in MHC class I expression. However, we found no additive genetic variance in reaction norms in our study population.

Introduction

The quantity of mRNA transcribed for a particular gene is one of the earliest observable phenotypes (Aubin-Horth & Renn 2009; Hodgins-Davis & Townsend 2009), and, like many other phenotypes, can be heavily influenced by environmental conditions (Picard & Schulte 2004; Nath *et al.* 2006; Fisher & Oleksiak 2007; Larsen *et al.* 2007; Croisetiere *et al.* 2010; Debes *et al.* 2012), by genotype (Cavalieri *et al.* 2000; Townsend *et al.* 2003; Debes *et al.* 2012; Runcie *et al.* 2012), and by their interactions (e.g. Landry *et al.* 2006; Li *et al.* 2006; Côté *et al.* 2007; McCairns & Bernatchez 2010; Hodgins-Davis *et al.* 2012). In cases where variation in gene expression has a genetic component, the trait has the potential to quickly evolve in response to changing selection pressures (Schlichting & Pigliucci 1998) if genetic covariance between traits and selection acting on correlated traits do not inhibit the expected evolutionary changes (Merilä *et al.* 2001).

The heritability of a trait is typically not constant but varies across environments (Wilson *et al.* 2006). There appears to be a lack in consistency in the immediate effects that different types of environmental conditions have on the amount of genetic variation that can be observed at a given moment (Hoffmann & Merilä 1999). Unfavorable conditions, i.e. situations leading to an immediate reduction in fitness, can either (i) decrease heritable variation, for example, by changing the environmental variance component (Charmantier & Garant 2005) or by preventing an organism from reaching its genetic potential (Merilä & Sheldon 1999); (ii) increase heritable variation (Agrawal *et al.* 2002; Relyea 2005), for example, by amplifying phenotypic differences between genotypes or by lowering the threshold for trait expression and releasing cryptic genetic variation (Gibson & Dworkin 2004; McGuigan & Sgro 2009); or (iii) may have no detectable effect on heritable variation (Pakkasmaa *et al.* 2003; Merilä *et al.* 2004; Clark *et al.* 2013). If the impact of environmental change on trait heritability is likely dependent on both the stressor and trait at hand, and perhaps even varies among types of genes, evaluation on a case-by-case basis seems necessary. For genes of the immune system, whose expression can be strongly dependent on the biotic environment (Frost 1999), understanding the impact of ecological stressors on heritable variation for trait means and the norms of reaction (i.e. the function that relates the phenotypes that can be produced by one genotype across environments; Pigliucci 2001) is important, as it would indicate a population's ability to evolve in response to parasites and infectious diseases. Parasites and pathogens are ubiquitous (Windsor 1998), but pressures from microbial organisms are often expected to increase in wild populations, due to pollution, habitat degradation, and climate change (Harvell *et al.* 1999; Daszak 2000; Dobson & Foufopoulos 2001).

We have studied the effects of pathogen treatment on the expression of a major histocompatibility complex (MHC) gene, i.e. of a key component of the adaptive immune system of vertebrates. As a vertebrate model we chose a wild population of the lake whitefish *Coregonus palaea* (Salmonidae) (Kottelat & Freyhof 2007). *C. palaea* is an iteroparous, fast-growing, lake-dwelling Alpine whitefish that feeds mainly on zooplankton and insect larvae, and spawns once a year during a few days in early winter. Average body length of mature fish at the spawning place is 383 mm (SD = 27) as determined from a random sample of 30 fish. Like all Alpine whitefish, *C. palaea* has external fertilization and shows no parental care. Fertilized eggs simply cascade onto the lake floor where embryo development takes place over a period of approximately 300 degree days i.e. embryos are, over several weeks, exposed to microbes and further environmental stressors. Whitefish are excellent models for ecological and quantitative genetic studies because individuals produce large amounts of gametes that can be collected for experimental *in vitro* fertilization, including large-scale full-factorial breeding designs. Embryos can be reared singly or in groups under very controlled conditions and monitored until hatching (Wedekind *et al.* 2001; Wedekind & Müller 2005;

Wedekind *et al.* 2008; Clark & Wedekind 2011). Salmonid embryos usually show high survivorship under benign laboratory conditions (e.g. von Siebenthal *et al.* 2009; Clark *et al.* 2013), but mortality rates in the wild can be high (Stelkens *et al.* 2012). While a number of factors including pollution (Heintz *et al.* 1999), predation (Phillips & Claire 2011), temperature (Tang *et al.* 1987), and oxygen deprivation (Silver *et al.* 2011) can contribute to this mortality, pathogens likely exert strong selection pressures (Schreck *et al.* 2001; Arkush *et al.* 2002), and seemingly benign microbial symbiotic communities can quickly turn virulent under altered environmental conditions (Jacob *et al.* 2010; Wedekind *et al.* 2010). The ecological relevance of microbial pathogens is corroborated by the observations that salmonid embryos have evolved early immunological defense mechanisms (see below). They are also able to perceive water-borne cues from microbial infections and to switch life-history strategies accordingly (Wedekind 2002; Pompini *et al.* 2013).

We chose to use two isolates of the opportunistic fish pathogen *Pseudomonas fluorescens* (Austin & Ausin 1999) as microbial stressors. This bacterium is found widely in the aquatic environment (Austin & Ausin 1999; Spiers *et al.* 2000) and has been associated with disease pathologies not only in adult fish (Zhang *et al.* 2009), but also in embryos of whitefish (Wedekind *et al*. 2001; von Siebenthal *et al*., 2009) and brown trout (Clark *et al.* 2013; Pompini *et al.* 2013). Moreover, previous studies suggest that this bacterium has strain-dependent virulence effects on salmonid embryos, with certain isolates directly increasing embryonic mortality (von Siebenthal *et al.* 2009; Pompini *et al.* 2013), and other isolates causing sub-lethal effects, i.e. delayed hatching and reduced growth (Clark *et al.* 2013). We assessed whether challenge with both "high" (i.e. induced mortality) and "low" (i.e. delayed hatching) virulence strains resulted in similar MHC expression patterns.

The extent to which embryos can mount an immune response against pathogens at this developmental stage is not clear yet. The immune system of teleost fish is generally thought to only become completely functional after hatching (Fischer *et al.* 2005; Zapata *et al.* 2006). However, the timing of maturation may vary between species (Magnadottir 2006; Mulero *et al.* 2007). Fischer *et al*. (2005) found that MHC class I transcription begins shortly after fertilization in rainbow trout (*Oncorhynchus mykiss*). Mortality of salmonid embryos has been demonstrated to be both MHC-allele specific (Pitcher & Neff 2006) and dependent on nucleotide diversity at the MHC loci (Evans *et al.* 2010a), and mortality during an epidemic of a non-specified strain of *P. fluorescens* has led to a significant shift of MHC allele frequencies within one of seven families of another lake whitefish (Wedekind *et al.* 2004). Hence, the MHC can already play a role in determining the susceptibility of salmonids to pathogens at late embryogenesis, either through direct pathogen-binding action or via pleiotropic interactions and/or linkages with other viability genes. Notably, both classes of the MHC are not necessarily ideal candidates for an expression study during embryonic development. In teleost fish, class I and class II genes are in separate linkage groups (Sato *et al.* 2000), and the beginning of transcription is not always synchronous (Rodrigues *et al.* 1998). We, therefore, first examined whether transcripts of both MHC classes were detectable in the whitefish embryos in an effort to identify a suitable locus for our study. We then assessed whether bacterial infection changed MHC expression patterns. As we employed a full-factorial breeding design, we were able to provide first estimates of the heritable variation for gene expression and examine whether this varied according to environmental conditions. Finally, we determined whether there was heritable variation for gene expression reaction norms and assessed whether there was a relationship between MHC gene expression and embryo mortality or time to hatching.

Materials and Methods

Whitefish sampling and rearing of embryos

Large-type adult whitefish were caught from their spawning grounds in Lake Geneva with gill nets and stripped of their gametes. These gametes were subsequently used for full-factorial *in vitro* fertilizations following the methods described in von Siebenthal *et al*. (2009). Embryos were distributed singly into 24-well plates (Falcon, Becton Dickinson) in a block-wise design and from then on stored in a 6.5°C climate chamber. Without additional challenge, this method for rearing embryos typically results in survival rates close to 100% (e.g. von Siebenthal *et al.* 2009; Clark *et al.* 2013; Pompini *et al.* 2013). Embryos were monitored weekly with a light table (Hama professional, LP 555) and a stereo zoom microscope (Olympus SZX9) until the start of hatching, at which point they were monitored daily. Thirteen offspring of 16 different sibships (resulting from a 4 x 4 cross) for each of the four treatments were randomly selected for subsequent work (N_{total} = 13 x 16 x 4 singly reared embryos).

Identification of pseudomonad sequence differences

The two *P. fluorescens* isolates used were the "high virulence strain" DSM 50090 ("PF1") that had been linked to mortality in whitefish embryos (von Siebenthal *et al.* 2009) and a "low virulence strain" ("PF2") that had been isolated from whitefish gills and had been observed to cause little embryonic mortality, but to delay hatching of smaller larvae in both brown trout (Clark *et al.* 2013) and whitefish (E. Clark, unpublished data). PF2 had been collected by swabbing gills with Amies agar gel transport swabs, followed by elution of bacteria into phosphate buffered saline. A 10^{-2} dilution had been plated onto King's B agar to facilitate isolation of fluorescent pseudomonads. After incubation for 48 hours, a colony was randomly selected and restreaked three times to obtain a pure culture. To compare sequences of the two pseudomonads (and to confirm successful identification), DNA was first isolated from both using the GenElute™ Bacterial Genomic DNA Kit, according to the manufacturer's instructions (Sigma-Aldrich). PCR was performed with a *P. fluorescens*-specific primer set, 16SPSEfluF and 16SPSER (Scarpellini *et al.* 2004), which amplifies a 850 bp fragment of the 16S rRNA. The PCR was performed in a total volume of 25 µl and contained 50 ng bacterial genomic DNA, 2.5 µl of 10X PCR buffer, 400 µM of each dNTP, 2.5 mM of $MgCl_2$, 0.6 µM of each primer, and 0.625 U of Taq polymerase (Invitrogen). The thermal profile was modified from Scarpellini *et al*. (2004): 3 min at 94°C; 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min; and a final extension at 72°C for 10 min. Following the PCR, the amplified products were purified with the Wizard® SV Gel and PCR Clean-Up System (Invitrogen) and sequenced in the forward and reverse directions with 16SPSEfluF/16SPSER on a ABI Prism 3100 genetic analyzer (Applied Biosystems). Sequences were edited and aligned with Geneious Pro™ version 5.3.4 (Biomatters). Alignment of PF1 and PF2 showed a base pair difference (A-G) at position 733, relative to a reference strain (strain CCM 2115; GenBank: DQ207731.2). To confirm the one base pair difference in the 16S rRNA fragment between the two pseudomonads, two further PCR's were performed as described above, and the resulting fragments were sequenced.

Preparation of bacterial inocula and treatment of embryos

Once embryos had reached the late-eyed stage (208 degree days), two flasks, each containing 100 ml of nutrient broth (3 g meat extract, 5 g bactopeptone, 1 L distilled H_2O), were inoculated with either PF1 or PF2. As the two strains were observed to have different optimal growth temperatures, they were incubated at 30 °C or 22 °C, respectively, for 36 hours on shakers until reaching the exponential growth phase. The bacteria were transferred to 50 ml conicals and spun at 4000 rpm for 15 minutes. The resulting pellet was washed three times and resuspended in sterile water, standardized according to OECD guidelines (OECD 1992). A Helber counting chamber was used to assess bacterial concentrations (see: Bast 2001, p.

280-285). The suspension was then diluted such that inoculation with 100 µl would achieve a concentration of 10^8 bacterial cells/ml in the wells. Prior to inoculation, nutrient broth was added to the suspension to encourage bacterial growth, resulting in a 1:1000 concentration in the wells. Thirteen replicates of every parental combination received PF1 and 13 received PF2. The remaining plates served as controls and were either sham-treated with sterile standardized water (N = 13) or were inoculated with nutrient broth (N = 13; 1:1000 dilution per well).

RNA preservation and extraction from embryos

Three embryos per sibship were sampled from each treatment group at 48 hours post-inoculation, and the remaining ten embryos per sibship and treatment group were monitored for survival and time until hatching. All samples were placed in RNA*later*® (Ambion, Austin, TX), stored overnight at 6.5°C, and then at -20°C for long-term storage. Embryos were individually homogenized with a mixer mill (MM300; Retsch, Düsseldorf, Germany) using six tungsten beads (3 mm), five silica beads (1.5 mm) and 0.4 g silica powder (0.2 mm) (Qiagen, Valencia, CA). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was eluted with 40 µl of RNase-free water and an additional DNase treatment was performed in which the following was added to each sample: 40 u/µl RNasin® (Promega, Madison, WI, USA), Tris-HCl (pH 7.5, 1 M), $MgCl_2$ (100 mM), KCl (2.5 M), DTT (100 mM), and DNase I (10 u/µl) (Roche, Mannheim, Germany). Each sample was incubated for 15 minutes at 37°C, followed by a phenol-chloroform extraction and ethanol precipitation. The resulting pellet was resuspended in 10 µl of RNase-free water. RNA integrity was verified by measuring absorbance at 260-280 nm, and a random set of 12 extractions was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) to confirm that the ratio of 28s/18s rRNA was close to two.

Reverse transcription, PCR of cDNA, and real-time quantitative PCR

Total extracted RNA was reverse-transcribed using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and random hexamers in a 25 µl reaction according to the manufacturer's protocol. To assess whether MHC class I and class II transcripts were detectable in the whitefish embryos, a PCR was conducted to amplify the two different genes from cDNA of whole embryos (N = 16) and, as a positive control, from spleens of adult whitefish (N = 8; RNA preserved and extracted as described above). The PCR was performed in a total volume of 25 µl and contained 10-100 ng of cDNA, 2.5 µl of 10X PCR buffer, 400 µM of each dNTP, 2.5 mM of $MgCl_2$, 0.6 µM of each primer (Table 1), and 0.625 U of Taq polymerase (Invitrogen). As a negative control, water was added instead of cDNA. As an additional control, the two genes were verified to successfully amplify from genomic DNA of whole embryos (N=8) and also from fin clips of adult whitefish (N=8). DNA had been extracted using the DNeasy Blood and Tissue Kit (Qiagen) (according to the manufacturer's instructions). The thermal profile for both cDNA and DNA PCR consisted of: 3 min at 94°C; 32 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. Amplified products were subsequently run on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Expression of MHC class I, along with four housekeeping genes (G6PD, GAPDH, β-actin, NADH), was analyzed with real-time qPCR using primers developed for salmonids (Table 1). Primer efficiencies were verified for each pair with four-fold serial dilutions of cDNA. Each 10 µl reaction contained: 2 x SYBR® Green PCR Mastermix (Roche Diagnostics, Mannheim, Germany), 0.3 µM of each primer, and 1 µl of cDNA. Three technical triplicates of each reaction were run on a 7900HT Fast Real-Time PCR Sequence

Detection System (Applied Biosystems) with the following conditions: 94°C for 3 min, followed by 40 cycles of 94°C for 10 s, 60°C for 25 s, and 72°C for 30 s. No enzyme controls (NEC) and no template controls (NTC) from the cDNA reaction were run concurrently to test for genomic DNA contamination, and melting curve analysis was used to confirm primer specificity.

The stability of the four candidate reference genes was examined with the method by Vandesompele *et al.* (2002), using the "ReadqPCR" (Perkins 2011) and the "NormqPCR" packages (Perkins & Kohl 2011). All four candidate reference genes had been shown to be reliable housekeeping genes in previous expression studies in salmonids (Brzuzan *et al.* 2005; Olsvik *et al.* 2005; Brzuzan *et al.* 2007; Brzuzan *et al.* 2009; Benedetto *et al.* 2011). Expression of G6PD and GAPDH, but not β-actin or NADH, was confirmed to remain constant across our treatments, therefore meeting the stability criteria for reference genes. All samples were subsequently normalized to the geometric mean of G6PD and GAPDH, and relative expression of mRNA was determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001; Schmittgen & Livak 2008).

Cloning and sequencing of parental genotypes

The gene of interest from each parent was cloned (Table 2) to confirm amplification of the desired products in the qPCR reactions and the presence of two co-amplified loci in the MHC class I sequences (Binz *et al.* 2001). Cloning was performed with the TOPO TA Cloning® Kit (Invitrogen) using the pCR®2.1-TOPO vector and One Shot ®TOP10 chemically competent cells. Between seven and 14 positive clones per individual were selected and amplified with M13 forward (5'-GTA AAA CGA CGA CCA G-3') and reverse (5'-CAG GAA ACA GCT ATG AC-3') primers with the following amplification profile: 94°C for 2 min, followed by 33 cycles of 94°C for 15 s, 54°C for 15 s, and 72°C 15 s, and a final 10 min extension at 72°C. Reactions were performed in a total volume of 15 µl and contained: 10 µM of each primer, 1.5 µl of 10X PCR buffer, 0.5 µM of each dNTP, 0.08 µl Taq™ DNA polymerase, 10 ng of cloned DNA, and water. Blank PCR reactions were included as controls. Amplified products were purified with the Wizard® SV Gel and PCR Clean-Up System (Invitrogen) and sequenced in the forward direction with M13 on an ABI Prism 3100 genetic analyzer (Applied Biosystems). Each allele was confirmed to be present in at least three clones to avoid cloning artifacts (Lenz & Becker 2008).

Statistical analysis

Mortality was analyzed as a binomial response variable in general linear mixed effect models (GLMM), while hatching time and MHC class I expression were analyzed as continuous response variables in linear mixed effect models (LMM). Prior to analysis, gene expression values, normalized to the housekeeping genes (i.e. ΔCt), were converted to the linear form with the $2^{-\Delta Ct}$ calculation (Livak & Schmittgen 2001; Schmittgen & Livak 2008) and were log transformed to avoid deviations from normality. Treatment was entered as a fixed effect, while sire and dam were entered as random effects. While dam effects encompass both genetic and maternal environmental effects, sire effects represent one-quarter of the additive genetic variance, assuming that epistatic effects are negligible (Lynch & Walsh 1998). Due to the fact that we had low replicate numbers (i.e. three) per fullsib family for the expression analysis (see Table S1 for mean expression levels per fullsib family and their associated variances), we did not include the sire x dam interaction as a random effect in the models. In the case of mortality and hatching time, the interaction term was also not included in the reference models, as it was not found to improve model fit to the data in any treatment. For the analysis, the nutrient broth treatment was used as the baseline control, as

both bacterial treatments received the same concentration of supplemental nutrients. While the addition of nutrients did not significantly elevate mortality in comparison to untreated embryos (1.8% increase; GLMM: $Z = 0.96$, $p = 0.34$), it did result in embryos hatching, on average, one day later (LMM: $T = 2.04$, $p = 0.04$).

To assess the importance of each effect, a reference model incorporating all relevant terms was compared to a model lacking the effect of interest. To examine the importance of interaction terms, a model incorporating the interaction was compared to the reference model. Akaike's information criteria (AIC), which provide a measure of model fit and model complexity (lower values indicate a better fit to the data) and likelihood ratio tests (LRT) were used to compare model fits. For models examining the importance of interaction terms (i.e. treatment x dam and treatment x sire), two models were run, one comparing the control to PF1, and one comparing it to PF2. As the two isolates differed in their virulence characteristics, we assessed for each pseudomonad separately whether a random slope-intercept term improved model fit.

To test for an effect of gene expression and treatment x gene expression on time until hatching, an analysis of covariance (ANCOVA) was performed. Average hatching time per fullsib family (by treatment) was entered as the dependent variable, with treatment and mean expression per fullsib family entered as independent variables. To examine the effect of gene expression on embryonic mortality, a general linear model (GLM) with a binomial distribution was used, with the proportion of dead embryos (out of the 10 initial replicates) per fullsib family entered as the response variable, and mean expression per fullsib family entered again as the independent variable. For this GLM on embryo mortality, we only looked within PF1, as survival in the control and in PF2 was close to 100%.

All analyses were done in the R environment (R Development Core Team 2011), using mainly the lme4 package (Bates *et al.* 2011). The MCMCglmm package (Hadfield 2010) was used as a means of verifying the significance of the treatments and the random effects in explaining variation in offspring phenotypes, as well as obtaining highest posterior density confidence intervals for the treatments and the random terms (see Supplementary Materials for details).

Results

Transcripts of MHC class I were detected in all whitefish embryos ($N = 16$) and all adults ($N = 8$), while MHC class II transcripts were only found in the tissue samples of the 8 adults (see Fig. S1 for amplification on agarose gels). Treatment with both *P. fluorescens* isolates resulted in decreased MHC class I gene expression (Fig. 1; Table 3; Table S2). While mortality was only significantly higher in PF1-treated embryos, hatching time was significantly delayed in both PF1 and PF2 (Fig. 2; Table 3; Table S2). Hatching time in PF1 was still significantly delayed after controlling for the likely confounding effects of potential non-random mortality in this treatment (Fig. S2).

We found significant additive genetic variance for MHC class I expression in the control and the PF1-treated groups, but not in the PF2-treated embryos (Table 4A; see Table S3 for the alternative MCMCglmm model). Analogous patterns could be observed with respect to the dam effects (Table 4A). Significant sire and dam effects on embryonic mortality were found after treatment with the virulent pseudomonad (PF1), but not in the PF2-treated embryos or the control group (Table 4B, Table S3). In contrast, we observed significant sire effects on hatching time in the control and PF2, but not in PF1-treated embryos (Table 4C, Table S3). Dam effects on hatching time was significant in PF2, but in neither of the other two treatments.

While we found evidence of additive genetic variance for MHC class I expression, embryonic mortality, and hatching time in certain environments, we found no indications of

heritable variance for their reaction norms, as indicated by the lack of sire x treatment interaction effects (Fig. 3; Table 3, see Table S4 for the alternative MCMCglmm model). In contrast, we did find evidence that the virulence of a given pseudomonad was modulated by dam effects, as suggested by the significant dam x treatment effects on embryo mortality in response to PF1 (Table 3A; Table S4) and hatching time in response to PF2 (Table 3B; Table S4).

We found no relationship between mean MHC gene expression and survival per full sibship in PF1, i.e. in the one bacterial strain that increased embryo mortality (GLM: $T = 0.72$, $p = 0.47$). However, there was an overall effect of gene expression on hatching time (ANCOVA: $F_{1, 41} = 5.0$, $p = 0.03$), with faster hatching embryos expressing less MHC (Fig. 4). No interaction between treatment and gene expression was found (ANCOVA: $F_{2, 41} = 0.90$, $p = 0.41$), i.e. the link between MHC expression and hatching time was similar in all treatment groups.

Discussion

We have described the experimental infection of whitefish embryos with two isolates of the opportunistic pathogen, *P. fluorescens*, and resultant effects on MHC expression, embryo mortality, and hatching time. A full-factorial experimental design was used to disentangle treatment from sire and dam effects on embryo traits, to assess whether and how environmental stress affects heritable variation for these traits, and to test for parental effects on embryo reaction norms. In addition, comparisons of full-sib families allowed testing whether MHC expression was linked to embryo survival or timing of hatching.

MHC expression in whitefish embryos and the effects of pathogen challenge

Transcripts of MHC class I, but not II, were detectable in the whitefish embryos under our experimental conditions. While our observations do not entirely exclude the possibility that MHC class II is expressed in the embryos at this point in development, as biases can be introduced during reverse transcription (Bustin & Nolan 2004), they nevertheless suggest that class I expression perhaps begins before class II. A study by Evans *et al*. (2010a) similarly suggested that the MHC class I pathway plays a significant role in survival before the MHC class II pathway, as their results provide strong support for a nucleotide diversity advantage at the MHC class I during the embryonic stage in another salmonid, i.e. the Chinook salmon (*O. tshawytscha*). Notably, Wedekind *et al*. (2004) found the allelic specificity on an MHC class II locus to influence survival until hatching in another whitefish during an epidemic with *P. fluorescens*. Pitcher and Neff (2006) also found evidence of MHC class IIB allele- and genotype-dependent survivorship during early developmental stages (i.e. through endogenous feeding) in the Chinook salmon. These findings, combined with the present ones, suggest that genes of MHC class I and class II can both be expressed prior to hatching, but that during the course of embryogenesis, class I genes are actively transcribed before class II.

Traditionally, MHC class I has been associated with the presentation of endogenous antigens (i.e. viruses and obligate intracellular bacteria), with class II binding proteins of exogenous origins. However, it is now well established that a significant amount of cross-over occurs between the two pathways (Kovacsovics-Bankowski & Rock 1995; Norbury *et al.* 1995; Yewdell *et al.* 1999; Ackerman & Cresswell 2004). Moreover, recent studies in both Atlantic cod (*Gadus morhua*) (Star *et al.* 2011) and pipefish (*Syngnathus typhle*) (Haase *et al.* 2013) have demonstrated a complete absence of MHC class II, suggesting that MHC class I can play an integral role in teleost adaptive immunity.

We found expression of MHC class I in whitefish embryos to vary according to environmental conditions. Specifically, treatment with the two different pseudomonads resulted in an average decrease in expression 48 hours post-inoculation. A very similar

expression phenotype was elicited, despite the fact that the two isolates showed different virulence characteristics (i.e. one induced mortality, as in von Siebenthal *et al*. 2009, while the other only delayed hatching, as in Clark *et al*. 2013). It is possible that a different pattern may have emerged had we sampled embryos at other time points post-inoculation, because gene expression can be very dynamic. However, the observed down-regulation of MHC expression confirms a number of previous observations in other fish: Koppang *et al*. (1999) found down-regulation of MHC class I following immuno-stimulation with lipopolysaccharide in Atlantic salmon (*Salmo salar*), and Reyes-Becceril *et al*. (2011) reported a reduction in MHC class II expression in gilthead seabream (*Sparus autata*) after infection with *Aeromonas hydrophila*. While these studies did not specifically investigate the reasons for this down-regulation, one explanation could involve pathogen-mediated suppression of transcription. Specifically, a number of bacteria and viruses seem capable of inhibiting expression of MHC as a means of immune evasion (e.g. Finlay & McFadden 2006; Antoniou & Powis 2008; Lapaque *et al.* 2009).

Another possible explanation for the observed down-regulation of MHC in our experiment is that a trade-off exists with other metabolic functions or immune pathways (Lochmiller & Deerenberg 2000). Life-history theory predicts that the immune response is a trait whose expression exacts an important cost on the organism (Moret & Schmid-Hempel 2000). Consequently, resource investment in this response will come at the expense of other traits (e.g. growth or reproduction) (Norris & Evans 2000; Schmid-Hempel 2003). In the context of our experiments, we found an overall positive relationship between MHC gene expression and hatching date. While fish, among other vertebrates, can alter hatching age to mitigate the fitness consequences of environmental stressors (e.g. pathogens and predators) (Warkentin 2011), late hatching is typically selected against in salmonids (Koho *et al.* 1991; Einum & Fleming 2000; Skoglund *et al.* 2012), and has been shown to be associated with reduced larval survival in another coregonid (*C. albula*) (Koho *et al.* 1991). Decreased transcription of MHC could, therefore, potentially reflect a strategic decision to invest resources into growth and an earlier life-history transition. Somewhat paradoxically, embryos raised in benign experimental conditions still managed to hatch earlier and express the most MHC. However, as they were not subjected to the burden of a pathogen challenge, they perhaps were in a better position to attain optimal phenotypes with respect to both traits. A comprehensive examination of the transcriptional changes of a wider array of genes, involved in both immunity and development, could help clarify the mechanisms behind this possible trade-off. Such an examination could also help shed light on whether MHC class I expression and developmental time are both affected by further factors that explain the somewhat counterintuitive observation that MHC class I expression declines with infection, hatching is delayed by infection, but there is a positive correlation between MHC class I expression and hatching date within each treatment group.

Components of phenotypic variation and the consequences of environmental stress

We found significant heritable variation for MHC class I expression and hatching time under benign experimental conditions. As both of these traits are closely tied to fitness, one would traditionally expect them to be characterized by reduced additive genetic variance due to directional selection (Mousseau & Roff 1987). However, a number of studies have demonstrated that significant heritable variation is often maintained in fitness-related traits (e.g. Laurila *et al.* 2002; Jacob *et al.* 2007; Jacob *et al.* 2010), with high residual variance sometimes giving the impression of depleted heritable variation (Houle 1992). On the contrary, little additive genetic variance was observed for embryonic mortality under these conditions, although our power to detect such variation was limited by the overall lack of mortality.

We found that the significance of the sire effect changed for some of the monitored traits and not for others in the *Pseudomonas* treatments, suggesting environmental dependencies. Heritable variation for gene expression and for hatching time seemed to decrease after inoculation with one of the *Pseudomonas* isolates; however, both isolates did not seem to have the same effect on heritable variation for a given trait, suggesting that different strains of the same bacterium can differentially affect a trait's evolutionary potential. Somewhat analogous declines in the heritability of expression have been observed in another set of immune genes, i.e. cytokines, post-immunostimulation with a *Vibro* vaccine in Chinook salmon (Aykanat *et al.* 2012). Heritable variation for embryonic mortality seemed to increase under stressful conditions (i.e. in PF1), due potentially to a release of cryptic genetic variation that, under different circumstances, would be phenotypically neutral (Gibson & Dworkin 2004; McGuigan & Sgro 2009). These observations support the view that the effect of environmental stressors on additive genetic variance is not only trait-dependent, but also dependent on the stressor at hand (for other examples see Hoffmann & Merilä 1999; Laugen *et al.* 2005). However, none of the possible environmental dependencies on heritability could be confirmed in pair-wise comparisons between the controls and the pathogen strain (i.e. the sire x treatment interaction terms were never statistically significant).

As with sire effects, the importance of dam effects on offspring phenotype can also vary according to the ecological conditions (Einum & Fleming 1999; Laugen *et al.* 2005 and references therein). In the case of MHC class I expression, the significance of dam effects appeared to remain stable after treatment with one isolate of *P. fluorescens* (PF1), but to decrease following inoculation with the second (PF2). No dam x treatment interaction on gene expression was found, suggesting that the decrease in transcription across environments was uniform across females. The significance of dam effects on embryonic mortality and hatching time increased under certain pathogen conditions, as confirmed also in significant dam x treatment interaction terms in the pairwise comparisons between the controls and the pathogen strains. Maternal sibgroups must have varied in their response to treatment either due to characteristics of the maternal environmental contributions (e.g. immune compounds (Magnadottir 2006)) or due to genetic effects.

Heritable variation for embryo reaction norms

While we found evidence of additive genetic variance for all monitored traits and under some environmental conditions, we found no indications of heritable variation for reaction norms. As only four males were used in the current study, our power to detect such interactions was limited. Nevertheless, the observed additive genetic variance generally appeared to be context dependent. While certain studies have provided evidence of gene x environment interactions on reaction norms, including survival/length (Evans *et al.* 2010b), body mass (Crespel *et al.* 2013), developmental time (Clark *et al.* 2013; Pompini *et al.* 2013), and gene expression (Côté *et al.* 2007) in salmonids, others have not for some of the same traits (larval length: Clark *et al.* 2013; embryonic survival: Pompini *et al.* 2013). These discrepancies may be reflective of differential selective pressures imposed by environmental stressors, or of the costs of plasticity varying between species and traits (DeWitt *et al.* 1998).

With respect to gene expression reaction norms, differences may also arise as a consequence of the genes' functions. For example, a study by Landry *et al*. (2006) demonstrated that in yeast, heritable variation for expression reaction norms was biased towards genes with low ties to fitness. This bias was presumably due to the fact that such genes are expected to have fewer constraints on their possible responses to environmental change, and therefore have less canalized reaction norms. A wider survey of the transcriptional responses to pathogen stressors would be needed to confirm if the same were true in our whitefish population.

Conclusions

Treatment of whitefish embryos with two distinct isolates of the opportunistic pathogen *P. fluorescens* resulted in decreased expression of MHC class I. The more the MHC gene was transcribed, the later embryos hatched, suggesting a tradeoff between expression and an earlier life-history transition or the existence of further factors that influence both MHC expression and timing of hatching. Significant heritable variation was found for gene expression, embryo mortality, and hatching time under certain experimental conditions, but not for others. However, no evidence of gene by environment interactions on the reaction norms was found for any trait of interest. As heritable variation in reaction norms can play a key role in determining a population's ability to cope with unpredictable environments (Hutchings 2011), its absence could have important implications concerning the whitefish's capacity to adapt to changing ecological conditions.

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Data Accessibility

Data package with one file including hatching success and time until hatching for each monitored embryo and one file including $2^{-\Delta CT}$ values for each gene and embryo: DRYAD entry doi:10.5061/dryad.5sp75

Author Contributions

EC, LW, and CW designed the experiment. EC and LW executed the experiment, and EC, LW, and CW analyzed the data and wrote the manuscript.

813 Table 1. Primers used in the present study.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
GAPDH [a]	ATG ACC ACT CCA TCT CCG TAT TC	ACG ACG TAA TCG GCA CCG
G6PD [b]	CCC TAT ATG AAG GTG GCA GAC TCT	GGC GTA CTT CCC ACT GAC ATA AG
β-actin [c]	GTG GCG CTG GAC TTT GAG CA	ACC GAG GAA GGA GGG CTG GA
NADH [d]	CAT CAC CAT CGC ACT ATC CA	CCT CCT TGG GTT CAC TCG TA
MHC class I [e]	TGT GGC TGT GGG GAT GGT GGA	TTT GGG CAC CGC TCT CTG GC
MHC class II [f]	ATG TTT TCC TTT TAG ATG GAT ATT TT	AGC CCT GCT CAC CTG TCT TA

814 [a] Atlantic salmon (Olsvik *et al.* 2005), [b] rainbow trout (Benedetto *et al.* 2011), [c] whitefish
815 (Brzuzan *et al.* 2007), [d] Atlantic salmon (Olsvik *et al.* 2005), [e] whitefish, modified from Binz
816 et al. (Binz *et al.* 2001), [f] salmonids (Pavey *et al.* 2011).

817 Table 2. Observed MHC I genotypes. Primers amplified a 68 bp fragment of the MHC gene.

Individual	Alleles	GenBank Accession Number
Male 1	Cosp-A1-H-6/8*	AF213305/AF2133
	Cosp-A1-H-1	AF213306
Male 2	Cosp-A1-H-15	AF213293
Male 3	Cosp-A1-H-6/8*	
	Cosp-A1-H-1	
	Cosp-A1-H-10	AF213296
Male 4	Cosp-A1-H-6/8*	
	Cosp-A1-H-1	
Female 1	Cosp-A1-H-6/8*	
	Cosp-A1-H-1	
	Cosp-A1-H-10	
	Cosp-A1-H-11	AF213303
Female 2	Cosp-A1-H-1	
	Cosp-A1-H-15	
	Cosp-A1-H-10	
Female 3	Cosp-A1-H-6/8*	
	Cosp-A1-H-15	
Female 4	Cosp-A1-H-1	
	Cosp-A1-H-11	

818 * matches to either Cosp-A1-H-6 or Cosp-A1-H-8 (Binz et al. 2001)

Table 3. Likelihood ratio tests on mixed model logistic regressions on MHC class I expression, embryonic mortality, and hatching time. Treatment was entered as a fixed effect (two levels including the control and PF1 (A) or the control and PF2 (B)), while sire and dam were entered as random effects. To test the effect of treatment, a reduced model was compared to the reference model (in bold). To test for interaction effects, a model incorporating the term was compared to the reference model. Akaike's information criteria (AIC), which provide a measure of model fit and model complexity (lower values indicate a better fit to the data) and likelihood ratio tests (LRT) were used to compare model fits (χ^2).

Model	Effect tested	AIC	χ^2	DF	p	AIC	χ^2	DF	p	AIC	χ^2	DF	p
		MHC I expression				Embryonic mortality				Hatching time			
A) Control vs. PF1													
t + s + d		66.3		5		135.1		4		1757.2		5	
s + d	t	70.5	6.2	4	0.01	160.0	26.8	3	<0.001	1785.5	30.3	4	<0.001
t + t x s + d	t x s	69.7	0.6	7	0.73	139.1	0.1	6	0.98	1761.2	0	7	1
t + s + t x d	t x d	69.5	0.8	7	0.68	123.8	15.3	6	<0.001	1760.1	1	7	0.59
B) Control vs. PF2													
t + s + d		40.4		5		82.2		4		1828.9		5	
s + d	t	42.5	4.1	4	0.04	80.7	0.5	3	0.48	1948.0	121.1	4	<0.001
t + t x s + d	t x s	42.4	2	7	0.36	86.2	0	6	1	1831.6	1.3	7	0.52
t + s + t x d	t x d	42.6	1.8	7	0.4	85.8	0.3	6	0.84	1820.8	12.1	7	0.002

t: treatment; s: sire; d: dam; t x s: treatment x sire; t x d: treatment x dam interactions; DF: degrees of freedom

Table 4. REML estimates of variance components (V_{sire}, V_{Dam}, and V_{Res}) for MHC class I expression, embryonic mortality and hatching time in each treatment (control, PF1, and PF2). Numbers in parentheses indicate percent of total variance explained by each separate component. The significance of each variance component was determined by comparing a mixed effect model incorporating all effects of interest to one lacking it (see Methods).

	V_{Sire}	V_{Dam}	V_{Res}
A) MHC I expression			
Control	0.06 (40.0)***	0.02 (13.3)*	0.07 (46.7)
PF1	0.05 (27.7)**	0.03 (16.7)*	0.10 (55.6)
PF2	0.02 (22.2)	0.01 (11.1)	0.06 (66.7)
B) Embryonic mortality			
Control	0 (0)	0 (0)	1$^{\sharp}$
PF1	2.1(21.6)**	6.6 (68.0)***	1$^{\sharp}$
PF2	0 (0)	0.5 (33.3)	1$^{\sharp}$
C) Hatching time			
Control	3.1 (18.2)***	0.4 (0.02)	13.5 (79.4)
PF1	2.6 (6.4)	0.8 (1.9)	37.1 (91.7)
PF2	5.8 (17.0)***	3.6 (10.5)**	24.8 (72.5)

$^{\sharp}$ Since mortality was a binomial response variable, residual variance was set to one.
*p < 0.05; **p<0.01, ***p < 0.001

838 **Fig. 1.** Mean MHC class I gene expression per treatment (± SE).
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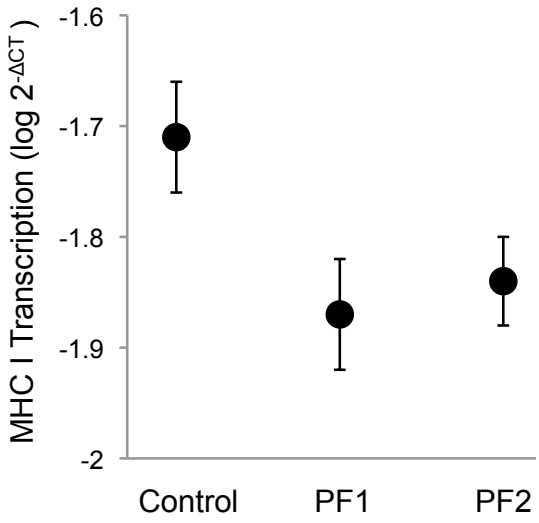
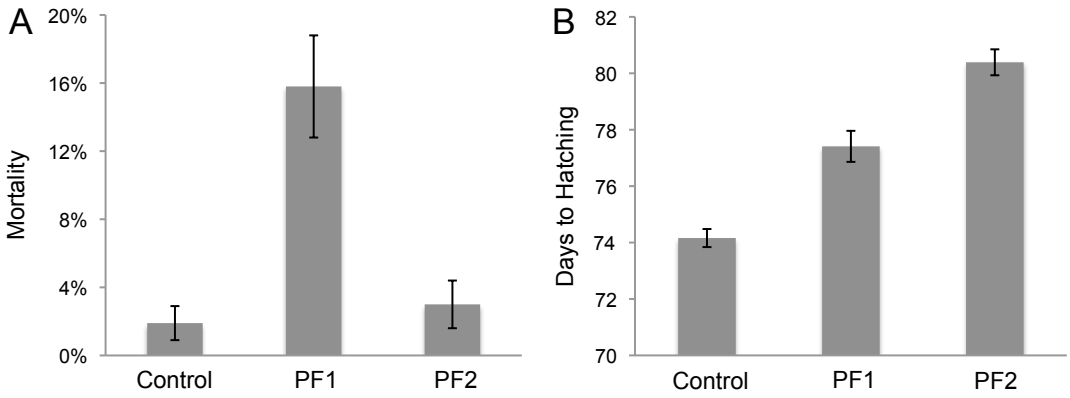


Fig. 2. Treatment effects on (A) embryo mortality and (B) time until hatching (means ± SE).



850 **Fig. 3.** Reaction norms of MHC class I gene expression across (A) control and exposure to
851 PF1 and (B) control and exposure to PF2. Lines correspond to means per paternal sibgroup
852 (N = 4).

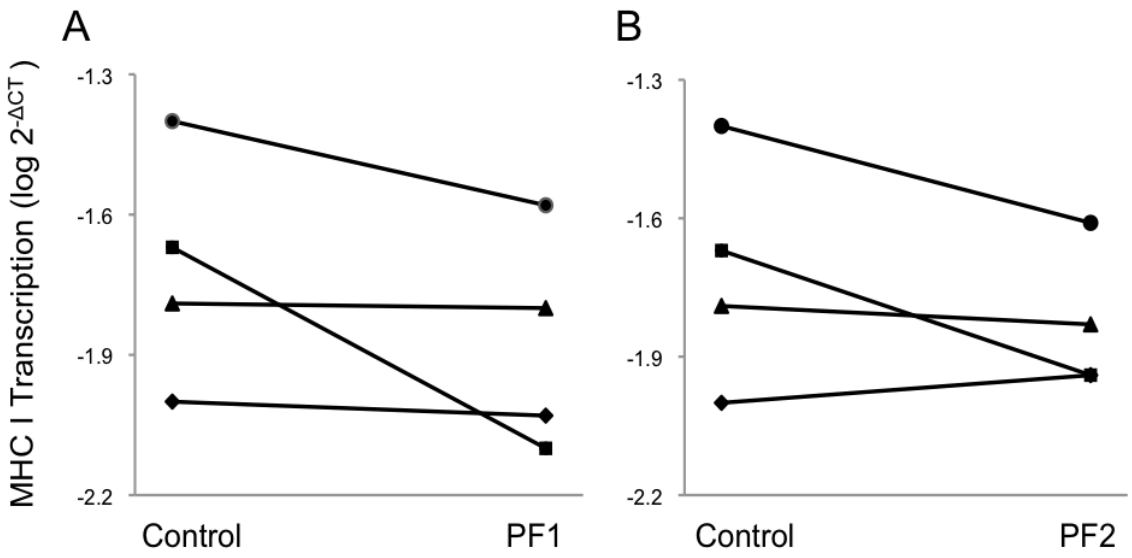


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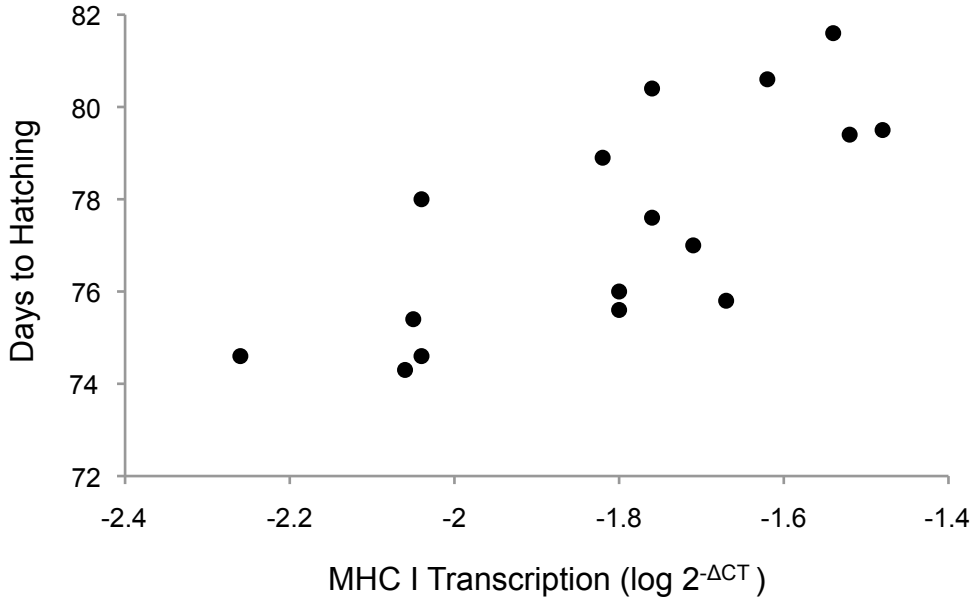
Fig. 4. Days to hatching versus MHC class I expression. Points represent means across treatments per fullsib family (N=16).



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