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

3
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12
13 **Keywords:** *Coregonus*, *Pseudomonas fluorescens*, plasticity, reaction norms, genetic
14 variation, life history

15

21
22 **Abstract**

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

43 Introduction

44 The quantity of mRNA transcribed for a particular gene is one of the earliest observable
45 phenotypes (Aubin-Horth & Renn 2009; Hodgins-Davis & Townsend 2009), and, like many
46 other phenotypes, can be heavily influenced by environmental conditions (Picard & Schulte
47 2004; Nath *et al.* 2006; Fisher & Oleksiak 2007; Larsen *et al.* 2007; Croisetiere *et al.* 2010;
48 Debes *et al.* 2012), by genotype (Cavalieri *et al.* 2000; Townsend *et al.* 2003; Debes *et al.*
49 2012; Runcie *et al.* 2012), and by their interactions (e.g. Landry *et al.* 2006; Li *et al.* 2006;
50 Côté *et al.* 2007; McCairns & Bernatchez 2010; Hodgins-Davis *et al.* 2012). In cases where
51 variation in gene expression has a genetic component, the trait has the potential to quickly
52 evolve in response to changing selection pressures (Schlichting & Pigliucci 1998) if genetic
53 covariance between traits and selection acting on correlated traits do not inhibit the expected
54 evolutionary changes (Merilä *et al.* 2001).

55 The heritability of a trait is typically not constant but varies across environments
56 (Wilson *et al.* 2006). There appears to be a lack in consistency in the immediate effects that
57 different types of environmental conditions have on the amount of genetic variation that can
58 be observed at a given moment (Hoffmann & Merilä 1999). Unfavorable conditions, i.e.
59 situations leading to an immediate reduction in fitness, can either (i) decrease heritable
60 variation, for example, by changing the environmental variance component (Charmantier &
61 Garant 2005) or by preventing an organism from reaching its genetic potential (Merilä &
62 Sheldon 1999); (ii) increase heritable variation (Agrawal *et al.* 2002; Relyea 2005), for
63 example, by amplifying phenotypic differences between genotypes or by lowering the
64 threshold for trait expression and releasing cryptic genetic variation (Gibson & Dworkin
65 2004; McGuigan & Sgro 2009); or (iii) may have no detectable effect on heritable variation
66 (Pakkasmaa *et al.* 2003; Merilä *et al.* 2004; Clark *et al.* 2013). If the impact of environmental
67 change on trait heritability is likely dependent on both the stressor and trait at hand, and
68 perhaps even varies among types of genes, evaluation on a case-by-case basis seems
69 necessary. For genes of the immune system, whose expression can be strongly dependent on
70 the biotic environment (Frost 1999), understanding the impact of ecological stressors on
71 heritable variation for trait means and the norms of reaction (i.e. the function that relates the
72 phenotypes that can be produced by one genotype across environments; Pigliucci 2001) is
73 important, as it would indicate a population's ability to evolve in response to parasites and
74 infectious diseases. Parasites and pathogens are ubiquitous (Windsor 1998), but pressures
75 from microbial organisms are often expected to increase in wild populations, due to pollution,
76 habitat degradation, and climate change (Harvell *et al.* 1999; Daszak 2000; Dobson &
77 Foufopoulos 2001).

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

93 Wedekind *et al.* 2008; Clark & Wedekind 2011). Salmonid embryos usually show high
94 survivorship under benign laboratory conditions (e.g. von Siebenthal *et al.* 2009; Clark *et al.*
95 2013), but mortality rates in the wild can be high (Stelkens *et al.* 2012). While a number of
96 factors including pollution (Heintz *et al.* 1999), predation (Phillips & Claire 2011),
97 temperature (Tang *et al.* 1987), and oxygen deprivation (Silver *et al.* 2011) can contribute to
98 this mortality, pathogens likely exert strong selection pressures (Schreck *et al.* 2001; Arkush
99 *et al.* 2002), and seemingly benign microbial symbiotic communities can quickly turn
100 virulent under altered environmental conditions (Jacob *et al.* 2010; Wedekind *et al.* 2010).
101 The ecological relevance of microbial pathogens is corroborated by the observations that
102 salmonid embryos have evolved early immunological defense mechanisms (see below). They
103 are also able to perceive water-borne cues from microbial infections and to switch life-history
104 strategies accordingly (Wedekind 2002; Pompini *et al.* 2013).

105 We chose to use two isolates of the opportunistic fish pathogen *Pseudomonas*
106 *fluorescens* (Austin & Ausin 1999) as microbial stressors. This bacterium is found widely in
107 the aquatic environment (Austin & Ausin 1999; Spiers *et al.* 2000) and has been associated
108 with disease pathologies not only in adult fish (Zhang *et al.* 2009), but also in embryos of
109 whitefish (Wedekind *et al.* 2001; von Siebenthal *et al.*, 2009) and brown trout (Clark *et al.*
110 2013; Pompini *et al.* 2013). Moreover, previous studies suggest that this bacterium has strain-
111 dependent virulence effects on salmonid embryos, with certain isolates directly increasing
112 embryonic mortality (von Siebenthal *et al.* 2009; Pompini *et al.* 2013), and other isolates
113 causing sub-lethal effects, i.e. delayed hatching and reduced growth (Clark *et al.* 2013). We
114 assessed whether challenge with both “high” (i.e. induced mortality) and “low” (i.e. delayed
115 hatching) virulence strains resulted in similar MHC expression patterns.

116 The extent to which embryos can mount an immune response against pathogens at this
117 developmental stage is not clear yet. The immune system of teleost fish is generally thought
118 to only become completely functional after hatching (Fischer *et al.* 2005; Zapata *et al.* 2006).
119 However, the timing of maturation may vary between species (Magnadottir 2006; Mulero *et al.*
120 2007). Fischer *et al.* (2005) found that MHC class I transcription begins shortly after
121 fertilization in rainbow trout (*Oncorhynchus mykiss*). Mortality of salmonid embryos has
122 been demonstrated to be both MHC-allele specific (Pitcher & Neff 2006) and dependent on
123 nucleotide diversity at the MHC loci (Evans *et al.* 2010a), and mortality during an epidemic
124 of a non-specified strain of *P. fluorescens* has led to a significant shift of MHC allele
125 frequencies within one of seven families of another lake whitefish (Wedekind *et al.* 2004).
126 Hence, the MHC can already play a role in determining the susceptibility of salmonids to
127 pathogens at late embryogenesis, either through direct pathogen-binding action or via
128 pleiotropic interactions and/or linkages with other viability genes. Notably, both classes of
129 the MHC are not necessarily ideal candidates for an expression study during embryonic
130 development. In teleost fish, class I and class II genes are in separate linkage groups (Sato *et al.*
131 2000), and the beginning of transcription is not always synchronous (Rodrigues *et al.*
132 1998). We, therefore, first examined whether transcripts of both MHC classes were
133 detectable in the whitefish embryos in an effort to identify a suitable locus for our study. We
134 then assessed whether bacterial infection changed MHC expression patterns. As we
135 employed a full-factorial breeding design, we were able to provide first estimates of the
136 heritable variation for gene expression and examine whether this varied according to
137 environmental conditions. Finally, we determined whether there was heritable variation for
138 gene expression reaction norms and assessed whether there was a relationship between MHC
139 gene expression and embryo mortality or time to hatching.

140

141 **Materials and Methods**

142 *Whitefish sampling and rearing of embryos*

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

155

156 *Identification of pseudomonad sequence differences*

157 The two *P. fluorescens* isolates used were the “high virulence strain” DSM 50090 (“PF1”)
158 that had been linked to mortality in whitefish embryos (von Siebenthal *et al.* 2009) and a
159 “low virulence strain” (“PF2”) that had been isolated from whitefish gills and had been
160 observed to cause little embryonic mortality, but to delay hatching of smaller larvae in both
161 brown trout (Clark *et al.* 2013) and whitefish (E. Clark, unpublished data). PF2 had been
162 collected by swabbing gills with Amies agar gel transport swabs, followed by elution of
163 bacteria into phosphate buffered saline. A 10^{-2} dilution had been plated onto King’s B agar to
164 facilitate isolation of fluorescent pseudomonads. After incubation for 48 hours, a colony was
165 randomly selected and restreaked three times to obtain a pure culture. To compare sequences
166 of the two pseudomonads (and to confirm successful identification), DNA was first isolated
167 from both using the GenElute™ Bacterial Genomic DNA Kit, according to the
168 manufacturer’s instructions (Sigma-Aldrich). PCR was performed with a *P. fluorescens*-
169 specific primer set, 16SPSEfluF and 16SPSER (Scarpellini *et al.* 2004), which amplifies a
170 850 bp fragment of the 16S rRNA. The PCR was performed in a total volume of 25 µl and
171 contained 50 ng bacterial genomic DNA, 2.5 µl of 10X PCR buffer, 400 µM of each dNTP,
172 2.5 mM of MgCl₂, 0.6 µM of each primer, and 0.625 U of Taq polymerase (Invitrogen). The
173 thermal profile was modified from Scarpellini *et al.* (2004): 3 min at 94°C; 35 cycles of 94°C
174 for 30 s, 50°C for 30 s, 72°C for 1 min; and a final extension at 72°C for 10 min. Following
175 the PCR, the amplified products were purified with the Wizard® SV Gel and PCR Clean-Up
176 System (Invitrogen) and sequenced in the forward and reverse directions with
177 16SPSEfluF/16SPSER on a ABI Prism 3100 genetic analyzer (Applied Biosystems).
178 Sequences were edited and aligned with Geneious Pro™ version 5.3.4 (Biomatters).
179 Alignment of PF1 and PF2 showed a base pair difference (A-G) at position 733, relative to a
180 reference strain (strain CCM 2115; GenBank: DQ207731.2). To confirm the one base pair
181 difference in the 16S rRNA fragment between the two pseudomonads, two further PCR’s
182 were performed as described above, and the resulting fragments were sequenced.

183

184 *Preparation of bacterial inocula and treatment of embryos*

185 Once embryos had reached the late-eyed stage (208 degree days), two flasks, each containing
186 100 ml of nutrient broth (3 g meat extract, 5 g bactopectone, 1 L distilled H₂O), were
187 inoculated with either PF1 or PF2. As the two strains were observed to have different optimal
188 growth temperatures, they were incubated at 30 °C or 22 °C, respectively, for 36 hours on
189 shakers until reaching the exponential growth phase. The bacteria were transferred to 50 ml
190 conicals and spun at 4000 rpm for 15 minutes. The resulting pellet was washed three times
191 and resuspended in sterile water, standardized according to OECD guidelines (OECD 1992).
192 A Helber counting chamber was used to assess bacterial concentrations (see: Bast 2001, p.

193 280-285). The suspension was then diluted such that inoculation with 100 μ l would achieve a
194 concentration of 10^8 bacterial cells/ml in the wells. Prior to inoculation, nutrient broth was
195 added to the suspension to encourage bacterial growth, resulting in a 1:1000 concentration in
196 the wells. Thirteen replicates of every parental combination received PF1 and 13 received
197 PF2. The remaining plates served as controls and were either sham-treated with sterile
198 standardized water (N = 13) or were inoculated with nutrient broth (N = 13; 1:1000 dilution
199 per well).

200

201 *RNA preservation and extraction from embryos*

202 Three embryos per sibship were sampled from each treatment group at 48 hours post-
203 inoculation, and the remaining ten embryos per sibship and treatment group were monitored
204 for survival and time until hatching. All samples were placed in RNAlater[®] (Ambion, Austin,
205 TX), stored overnight at 6.5°C, and then at -20°C for long-term storage. Embryos were
206 individually homogenized with a mixer mill (MM300; Retsch, Düsseldorf, Germany) using
207 six tungsten beads (3 mm), five silica beads (1.5 mm) and 0.4 g silica powder (0.2 mm)
208 (Qiagen, Valencia, CA). Total RNA was extracted using the RNeasy Mini Kit (Qiagen,
209 Valencia, CA) according to the manufacturer's instructions. RNA was eluted with 40 μ l of
210 RNase-free water and an additional DNase treatment was performed in which the following
211 was added to each sample: 40 u/ μ l RNasin[®] (Promega, Madison, WI, USA), Tris-HCl (pH
212 7.5, 1 M), MgCl₂ (100 mM), KCl (2.5 M), DTT (100 mM), and DNase I (10 u/ μ l) (Roche,
213 Mannheim, Germany). Each sample was incubated for 15 minutes at 37°C, followed by a
214 phenol-chloroform extraction and ethanol precipitation. The resulting pellet was resuspended
215 in 10 μ l of RNase-free water. RNA integrity was verified by measuring absorbance at 260-
216 280 nm, and a random set of 12 extractions was analyzed on an Agilent 2100 Bioanalyzer
217 (Agilent Technologies, Waldbronn, Germany) to confirm that the ratio of 28s/18s rRNA was
218 close to two.

219

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

221 Total extracted RNA was reverse-transcribed using the SuperScript[™] III First-Strand
222 Synthesis System (Invitrogen, Carlsbad, CA) and random hexamers in a 25 μ l reaction
223 according to the manufacturer's protocol. To assess whether MHC class I and class II
224 transcripts were detectable in the whitefish embryos, a PCR was conducted to amplify the
225 two different genes from cDNA of whole embryos (N = 16) and, as a positive control, from
226 spleens of adult whitefish (N = 8; RNA preserved and extracted as described above). The
227 PCR was performed in a total volume of 25 μ l and contained 10-100 ng of cDNA, 2.5 μ l of
228 10X PCR buffer, 400 μ M of each dNTP, 2.5 mM of MgCl₂, 0.6 μ M of each primer (Table 1),
229 and 0.625 U of Taq polymerase (Invitrogen). As a negative control, water was added instead
230 of cDNA. As an additional control, the two genes were verified to successfully amplify from
231 genomic DNA of whole embryos (N=8) and also from fin clips of adult whitefish (N=8).
232 DNA had been extracted using the DNeasy Blood and Tissue Kit (Qiagen) (according to the
233 manufacturer's instructions). The thermal profile for both cDNA and DNA PCR consisted of:
234 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
235 at 72°C for 10 min. Amplified products were subsequently run on a 2% agarose gel, stained
236 with ethidium bromide, and visualized under UV light.

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

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

248 The stability of the four candidate reference genes was examined with the method by
249 Vandesompele *et al.* (2002), using the “ReadqPCR” (Perkins 2011) and the “NormqPCR”
250 packages (Perkins & Kohl 2011). All four candidate reference genes had been shown to be
251 reliable housekeeping genes in previous expression studies in salmonids (Brzuzan *et al.* 2005;
252 Olsvik *et al.* 2005; Brzuzan *et al.* 2007; Brzuzan *et al.* 2009; Benedetto *et al.* 2011).
253 Expression of G6PD and GAPDH, but not β -actin or NADH, was confirmed to remain
254 constant across our treatments, therefore meeting the stability criteria for reference genes. All
255 samples were subsequently normalized to the geometric mean of G6PD and GAPDH, and
256 relative expression of mRNA was determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen
257 2001; Schmittgen & Livak 2008).

258

259 *Cloning and sequencing of parental genotypes*

260 The gene of interest from each parent was cloned (Table 2) to confirm amplification of the
261 desired products in the qPCR reactions and the presence of two co-amplified loci in the MHC
262 class I sequences (Binz *et al.* 2001). Cloning was performed with the TOPO TA Cloning®
263 Kit (Invitrogen) using the pCR®2.1-TOPO vector and One Shot®TOP10 chemically
264 competent cells. Between seven and 14 positive clones per individual were selected and
265 amplified with M13 forward (5'-GTA AAA CGA CGA CCA G-3') and reverse (5'-CAG
266 GAA ACA GCT ATG AC-3') primers with the following amplification profile: 94°C for 2
267 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
268 extension at 72°C. Reactions were performed in a total volume of 15 μ l and contained: 10
269 μ M of each primer, 1.5 μ l of 10X PCR buffer, 0.5 μ M of each dNTP, 0.08 μ l Taq™ DNA
270 polymerase, 10 ng of cloned DNA, and water. Blank PCR reactions were included as controls.
271 Amplified products were purified with the Wizard® SV Gel and PCR Clean-Up System
272 (Invitrogen) and sequenced in the forward direction with M13 on an ABI Prism 3100 genetic
273 analyzer (Applied Biosystems). Each allele was confirmed to be present in at least three
274 clones to avoid cloning artifacts (Lenz & Becker 2008).

275

276 *Statistical analysis*

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

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

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

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

315 All analyses were done in the R environment (R Development Core Team 2011),
316 using mainly the lme4 package (Bates *et al.* 2011). The MCMCglmm package (Hadfield
317 2010) was used as a means of verifying the significance of the treatments and the random
318 effects in explaining variation in offspring phenotypes, as well as obtaining highest posterior
319 density confidence intervals for the treatments and the random terms (see Supplementary
320 Materials for details).

321 322 **Results**

323 Transcripts of MHC class I were detected in all whitefish embryos ($N = 16$) and all adults (N
324 $= 8$), while MHC class II transcripts were only found in the tissue samples of the 8 adults (see
325 Fig. S1 for amplification on agarose gels). Treatment with both *P. fluorescens* isolates
326 resulted in decreased MHC class I gene expression (Fig. 1; Table 3; Table S2). While
327 mortality was only significantly higher in PF1-treated embryos, hatching time was
328 significantly delayed in both PF1 and PF2 (Fig. 2; Table 3; Table S2). Hatching time in PF1
329 was still significantly delayed after controlling for the likely confounding effects of potential
330 non-random mortality in this treatment (Fig. S2).

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

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

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

348 We found no relationship between mean MHC gene expression and survival per full
349 sibship in PF1, i.e. in the one bacterial strain that increased embryo mortality (GLM: $T = 0.72$,
350 $p = 0.47$). However, there was an overall effect of gene expression on hatching time
351 (ANCOVA: $F_{1, 41} = 5.0$, $p = 0.03$), with faster hatching embryos expressing less MHC (Fig.
352 4). No interaction between treatment and gene expression was found (ANCOVA: $F_{2, 41} = 0.90$,
353 $p = 0.41$), i.e. the link between MHC expression and hatching time was similar in all
354 treatment groups.

355 Discussion

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

363 *MHC expression in whitefish embryos and the effects of pathogen challenge*

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

381 Traditionally, MHC class I has been associated with the presentation of endogenous
382 antigens (i.e. viruses and obligate intracellular bacteria), with class II binding proteins of
383 exogenous origins. However, it is now well established that a significant amount of cross-
384 over occurs between the two pathways (Kovacovics-Bankowski & Rock 1995; Norbury *et al.*
385 1995; Yewdell *et al.* 1999; Ackerman & Cresswell 2004). Moreover, recent studies in both
386 Atlantic cod (*Gadus morhua*) (Star *et al.* 2011) and pipefish (*Syngnathus typhle*) (Haase *et al.*
387 2013) have demonstrated a complete absence of MHC class II, suggesting that MHC class I
388 can play an integral role in teleost adaptive immunity.

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

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

406 Another possible explanation for the observed down-regulation of MHC in our
407 experiment is that a trade-off exists with other metabolic functions or immune pathways
408 (Lochmiller & Deerenberg 2000). Life-history theory predicts that the immune response is a
409 trait whose expression exacts an important cost on the organism (Moret & Schmid-Hempel
410 2000). Consequently, resource investment in this response will come at the expense of other
411 traits (e.g. growth or reproduction) (Norris & Evans 2000; Schmid-Hempel 2003). In the
412 context of our experiments, we found an overall positive relationship between MHC gene
413 expression and hatching date. While fish, among other vertebrates, can alter hatching age to
414 mitigate the fitness consequences of environmental stressors (e.g. pathogens and predators)
415 (Warkentin 2011), late hatching is typically selected against in salmonids (Koho *et al.* 1991;
416 Einum & Fleming 2000; Skoglund *et al.* 2012), and has been shown to be associated with
417 reduced larval survival in another coregonid (*C. albula*) (Koho *et al.* 1991). Decreased
418 transcription of MHC could, therefore, potentially reflect a strategic decision to invest
419 resources into growth and an earlier life-history transition. Somewhat paradoxically, embryos
420 raised in benign experimental conditions still managed to hatch earlier and express the most
421 MHC. However, as they were not subjected to the burden of a pathogen challenge, they
422 perhaps were in a better position to attain optimal phenotypes with respect to both traits. A
423 comprehensive examination of the transcriptional changes of a wider array of genes, involved
424 in both immunity and development, could help clarify the mechanisms behind this possible
425 trade-off. Such an examination could also help shed light on whether MHC class I expression
426 and developmental time are both affected by further factors that explain the somewhat
427 counterintuitive observation that MHC class I expression declines with infection, hatching is
428 delayed by infection, but there is a positive correlation between MHC class I expression and
429 hatching date within each treatment group.

430

431 *Components of phenotypic variation and the consequences of environmental stress*

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

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

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

471 472 *Heritable variation for embryo reaction norms*

473 While we found evidence of additive genetic variance for all monitored traits and under some
474 environmental conditions, we found no indications of heritable variation for reaction norms.
475 As only four males were used in the current study, our power to detect such interactions was
476 limited. Nevertheless, the observed additive genetic variance generally appeared to be context
477 dependent. While certain studies have provided evidence of gene x environment interactions
478 on reaction norms, including survival/length (Evans *et al.* 2010b), body mass (Crespel *et al.*
479 2013), developmental time (Clark *et al.* 2013; Pompini *et al.* 2013), and gene expression
480 (Côté *et al.* 2007) in salmonids, others have not for some of the same traits (larval length:
481 Clark *et al.* 2013; embryonic survival: Pompini *et al.* 2013). These discrepancies may be
482 reflective of differential selective pressures imposed by environmental stressors, or of the
483 costs of plasticity varying between species and traits (DeWitt *et al.* 1998).

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

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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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805 **Data Accessibility**

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

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810 **Author Contributions**

811 EC, LW, and CW designed the experiment. EC and LW executed the experiment, and EC,
812 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 <i>et al.</i> 2005), ^b rainbow trout (Benedetto <i>et al.</i> 2011), ^c whitefish	
815	(Brzuzan <i>et al.</i> 2007), ^d Atlantic salmon (Olsvik <i>et al.</i> 2005), ^e whitefish, modified from Binz	
816	et al. (Binz <i>et al.</i> 2001), ^f salmonids (Pavey <i>et al.</i> 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)

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

Model	Effect tested	MHC I expression				Embryonic mortality				Hatching time			
		AIC	χ^2	DF	p	AIC	χ^2	DF	p	AIC	χ^2	DF	p
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

827
 828 t: treatment; s: sire; d: dam; t x s: treatment x sire; t x d: treatment x dam interactions; DF:
 829 degrees of freedom

830 Table 4. REML estimates of variance components (V_{sire} , V_{Dam} , and V_{Res}) for MHC class I
 831 expression, embryonic mortality and hatching time in each treatment (control, PF1, and PF2).
 832 Numbers in parentheses indicate percent of total variance explained by each separate
 833 component. The significance of each variance component was determined by comparing a
 834 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 [#]
PF1	2.1(21.6)**	6.6 (68.0)***	1 [#]
PF2	0 (0)	0.5 (33.3)	1 [#]
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)

835 [#] Since mortality was a binomial response variable, residual variance was set to one.

836 *p < 0.05; **p < 0.01, ***p < 0.001

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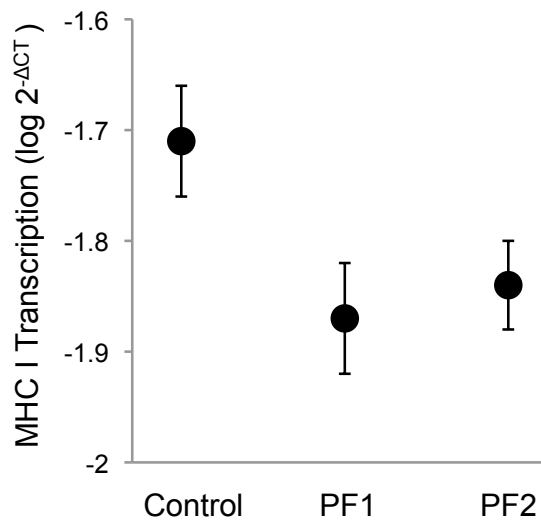
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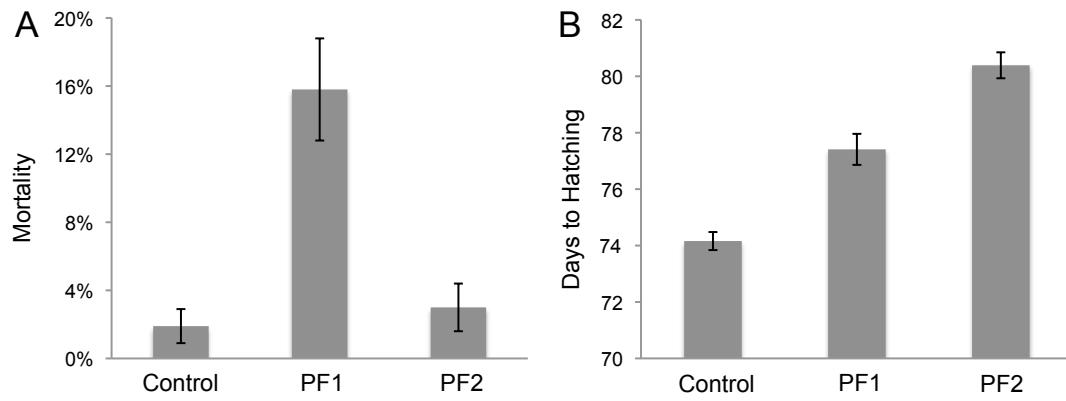
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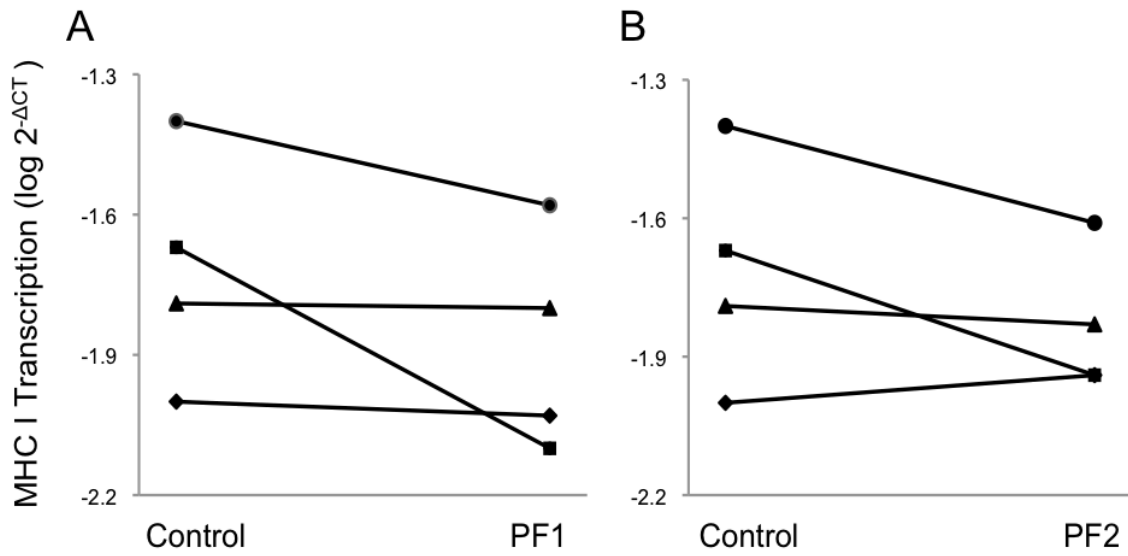


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Fig. 2. Treatment effects on (A) embryo mortality and (B) time until hatching (means \pm SE).



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).



853

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