Parallelism and divergence in immune responses: a comparison of expression levels in two lakes

Shaun Robertson*, Janette E. Bradley and Andrew D.C. MacColl

School of Life Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, United Kingdom

*Corresponding author email: plxsr3@nottingham.ac.uk

ABSTRACT

Question: How do immune phenotypes differ between infected and uninfected wild individuals, and is the effect the same in different populations?

Organisms: Three-spined stickleback (*Gasterosteus aculeatus*) from two lake populations on the island of North Uist, Scotland, sampled in May 2015.

Methods: For each fish, we recorded length, sex, reproductive status, condition and parasitic infection. We measured the expression levels of eight genes which act as key markers of immune system function using qPCR, and then examined the relationship between measured factors and immune gene expression profiles within each population.

Conclusions: Populations differed significantly in their immune gene expression profiles. Within each population, multiple factors, including condition, reproductive status and *Schistocephalus solidus* infection levels, were found to correlate with expression levels of different arms of the immune system.

INTRODUCTION

Studies of the immune system to date have focussed primarily on humans and a few select model species (Maizels & Nussey, 2013; Pedersen & Babayan, 2011). Such studies are typically lab-based and performed in highly controlled settings where variation is minimised, so that cellular and molecular mechanisms can be identified. This approach, however, is disconnected from the natural world where 'real' immune systems have to operate (Pedersen & Babayan, 2011), where there is genetic diversity of both hosts and parasites, and variation in the abiotic environment. Such diversity may play an important role in shaping the function of the immune system in the wild (Lazzaro & Little, 2009; Martin *et al.*, 2011), and placing current knowledge of immunological mechanisms into this real-world context is a fundamentally important challenge in advancing our understanding of the immune system from an evolutionary perspective (Maizels & Nussey, 2013).

The expansion of the study of the immune system in the wild is constrained by a lack of appropriate assays and study species (Fassbinder-Orth, 2014), and studies applying modern immunological measurements to natural populations by simultaneously measuring multiple markers of the immune system are rare and primarily limited to rodents (Jackson *et al.*, 2014; Oko *et al.*, 2006; Schountz *et al.*, 2007). Expression assays represent an obvious starting point for the study of the immune response in organisms that lack reagents (particularly for protein-level analysis), although results can be difficult to interpret and data from wild populations inherently noisy. One way to address the possible limitations of such an approach is to compare expression profiles between different populations in relation to candidate causal factors, for example parasitic infection levels, sex or size. If signals are consistent across locations, we can have more confidence that the patterns identified are 'real'. Due to the restricted set of previous studies, there is a limited understanding of the role of an individual's environment in shaping immune function in a natural setting; the three-spined stickleback *Gasterosteus aculeatus* L. (hereafter 'stickleback') is ideally suited to address this issue.

Sticklebacks are a commonly studied model species in evolution and ecology (eg, see Hendry *et al.*, 2013). Populations of stickleback show stable differences in parasite community composition (De Roij & MacColl, 2012; Scharsack *et al.*, 2007a), and interactions with a range of parasites have been well documented (Barber, 2013; Kalbe & Kurtz, 2006; Konijnendijk *et al.*, 2013; MacColl, 2009). There is growing evidence for within and between population variation in parasite resistance (De Roij *et al.*, 2011), which probably has a genetic basis (El Nagar, 2014; Rauch *et al.*, 2006). This long history of study, combined with the ability to sample replicate populations in the wild and perform controlled infection experiments in the lab, has made the stickleback an excellent model in the study of evolutionary and ecological parasitology (Barber, 2013), and an ideal system in which to conduct immunological studies.

The sticklebacks found on the island of North Uist, Scotland, represent one well-studied system (De Roij & MacColl, 2012; Giles, 1983; MacColl *et al.*, 2013). A number of parasite species infect stickleback on North Uist, including *Gyrodactylus arcuatus*, an ectoparasitic trematode, and *Schistocephalus solidus*, a pseudophyllidean cestode. Infections with *S. solidus* can have a negative impact on host fitness (Barber & Scharsack, 2010; Barber *et al.*, 2008), and parasites have been shown to modulate the immune response of the host (Barber & Scharsack, 2010). Whilst the impact of infection on host fitness of a number of parasites has been well studied, the link between infection and a host's defence is less clear.

Past research has begun to examine the immune response of stickleback, particularly the role of immune-related cell populations (Scharsack *et al.*, 2004) and the major histocompatibility complex (MHC) (Kurtz *et al.*, 2004; Wegner *et al.*, 2006) . The genetic background of an individual appears to play a role in determining parasite load (Eizaguirre *et al.*, 2011; Rauch *et al.*, 2006), suggesting adaptation to local parasite populations, which could be modulated through the immune response.

Recent advances allow the expression levels of immune-related genes to be measured directly. Next-generation sequencing of the transcriptome of stickleback has shown adaptation of expression levels of immune genes to local, co-evoloved, parasite strains (Lenz *et al.*, 2013), and changes in gene expression which are dependent upon the genotype of the infecting parasite (Haase *et al.*, 2014) in controlled, laboratory-based, infection experiments. This whole-transcriptome sequencing approach has provided new insights into the response of individuals to controlled infections, but there is a relatively high cost associated with sequencing the large numbers of biological replicates required for such studies of wild populations. An alternative approach to whole transcriptome sequencing is to use quantitative real-time PCR (qPCR) to measure the expression of pre-selected genes (Fassbinder-Orth, 2014), chosen based on *a priori* knowledge of the function of the immune system. Such studies have proved fruitful in the study of rodents (Jackson *et al.*, 2011;

Jackson *et al.*, 2009), and qPCR has successfully been applied to stickleback (Dittmar *et al.*, 2014; Hibbeler *et al.*, 2008; Robertson *et al.*, 2015).

In order to give a cohesive overview of the function of the immune system, we developed a set of qPCR assays which measure the innate response, the Th1-type and Th2-type adaptive responses, and the regulatory response (Robertson *et al.*, 2015). In this study, we employ these assays to measure the immune response of wild stickleback from two lake populations on the island of North Uist, Scotland. We selected populations that typically have similar high rates of infection with *S. solidus* (MacColl, unpublished data), but are geographically isolated from each other. In this way, we could investigate whether we could detect changes in immune gene expression levels that were associated with *S. solidus* infection, above the natural variation expected in data from wild individuals. Furthermore, we examine which factors relate to immune gene expression levels within each population and look at whether there are common factors which may be involved in shaping the immune response in the wild. This research adds to the growing use of stickleback as an alternative species in the study of the immune system, and begins to examine which factors play a role in shaping immune gene expression in a natural setting.

MATERIALS AND METHODS

Sample Collection

All work involving animals was approved by the University of Nottingham ethics committee, under UK Home Office licence (PPL-40/3486), and sampling on North Uist was conducted with the permission of North Uist Estates. Fish were sampled from Loch a'Bharpa ('Bhar', $57^0$34'20"N; $7^0$18'11"W) and Loch Hosta ('Host', $57^0$37'30"N; $7^0$29'8"W) on the island of North Uist, Scotland, in May 2015. Forty six fish were sampled from each population, split between two sampling days per population. Fish were caught in Gee's Minnow Traps, set overnight for approximately 16 hours. All individuals caught in a trapping session were pooled, and a subset was selected for inclusion in this study. Fish were transported directly to the

laboratory in darkened conditions and processed in a haphazard order within four hours of collection. All fish were collected and processed before midday. No correlations were found between sampling order or sampling day and gene expression levels, indicating that our sampling procedure had no effect on expression levels.

Fish were euthanized using the appropriate schedule 1 technique according to UK Home Office regulations, then measured and weighed. Whole spleens, an immunologically important tissue in fish (Zapata *et al.*, 2006), were removed, weighed, and placed into RNAlater (Life Technologies). Sex was recorded along with reproductive status, determined by visual examination of the gonads. Male (M) and female (F) reproductive status was split into two categories: individuals with no apparent investment in reproduction (small gonads and kidneys in M, small ovules only in F), versus individuals investing in reproduction or ready to breed (gonads and/or kidneys showing enlargement in M, some or all ovules enlarged in F). The liver was removed and weighed, as was any adipose tissue in the body cavity.

Parasites were identified and counted under a dissection microscope. Where individuals were infected with *S. solidus*, the total weight of all plerocercoids (the life stage infecting stickleback) was recorded, and the parasite index calculated as the total proportion of a fish's body weight accounted for by the parasite (Arme & Owen, 1967). The relative weights of the spleen (spleen somatic index, SSI), liver (hepato-somatic index, HSI), and adipose tissue (adipose index, AI) were calculated. Individual condition scores were calculated using Fulton's condition factor, $K=(10^5 \times \text{weight(g)})/\text{Length(mm)}^3$ (cited in Nash *et al.*, 2006), adjusted for individuals infected with *S. solidus* by using individual weight minus the total weight of all plerocercoids.

Sample Preparation

All qPCR work was performed in accordance with the MIQE guidelines (Bustin *et al.*, 2009; Taylor *et al.*, 2010). Spleens were stored in RNAlater (Life Technologies) at -20^{o}C for no

longer than 3 months. RNA was extracted using the GeneJET RNA purification kit (Thermo Scientific) according to the manufacturers' standard protocol. Purity of RNA samples was assessed on a NanoDrop 1000 Spectrophotometer (Thermo Scientific), with a desired 260/280 absorbance ratio > 1.80. Integrity of RNA was assessed by incubating 5µl of sample at 65^{o}C for 10 minutes, followed by visualisation on a 2% Agarose gel stained with Ethidium Bromide.

All samples were DNase treated using Precision DNase (Primer Design), following the manufacturers' protocol. Reverse transcription reactions were performed on approximately 1.5µg of total RNA using the nanoScript2 RT kit (Primer Design), with a combination of random nanomer and oligo-dT priming, following the manufacturers' standard protocol. Periodic no-enzyme controls indicated that genomic DNA contamination was negligible. cDNA samples were diluted 1:10 with nuclease free water before further use.

Gene Expression Quantification

All qPCR reactions were performed in 10µl total volumes, containing 5µl of PrecisionFAST mastermix with SYBR green (Primer Design), 0.25µl of each primer, 2µl of template cDNA and 2.5µl of H_2O in 96-well optical PCR plates with optical seals (StarLab). Reactions were performed in an ABI 7500 FAST real-time thermocycler (Applied Biosystems) at 95^{o}C for 20 seconds, followed by 45 cycles of 95^{o}C for 3 seconds and 60^{o}C for 30 seconds. All runs included a post-PCR melt curve analysis.

Accurate normalization of gene expression is essential for the production of reliable data in qPCR experiments, with the optimal reference genes being specific to a particular set of experimental conditions (Dheda *et al.*, 2005). To select the most appropriate normalization strategy, a geNorm analysis was performed with six candidate reference genes (B2M, GAPDH, RPL13A, HPRT1, TBP and TOP1) on 12 cDNA samples, randomly selected from all experimental samples, using a custom stickleback geNorm kit for SYBR green (Primer Design), following the manufacturers' standard protocol. Analysis of the stability of

166 expression was performed in qbase+ (Biogazelle) with B2M and RPL13A identified as the
167 most stable combination of reference genes for this study.

168 Expression levels of eight genes of interest were measured, along with two reference genes.
169 Genes of interest were IL-1β, TNFα, Stat4, Tbet, Stat6, CMIP, FoxP3, and TGFβ (For full
170 details, see Robertson *et al.*, 2015). A reference sample was made by pooling cDNA from all
171 experimental samples, to control for between plate variation. A total of 92 cDNA samples
172 were split randomly between two plates, with reactions performed in duplicate for each
173 sample, and each plate also contained the reference sample and negative controls.

174 Relative expression values were calculated using the ΔΔCq method (Pfaffl, 2001), adjusted
175 for the amplification efficiencies of each primer pair and standardised against the geometric
176 mean Cq of the two reference genes for each sample (Vandesompele *et al.*, 2002).

177 **Data Analysis**

178 All expression values were $\log_{10}(x+1)$ transformed prior to analysis, due to the inherently
179 skewed distribution of relative expression data. Analysis was performed in R v.3.2.2 (R Core
180 Team, 2014). We first examined whether any factors relate to variation in individual
181 condition. Next we investigated whether any factors related to immune gene expression
182 variation in each population, by using principal components analysis (PCA) to summarise
183 immune gene expression levels, then fitted general linear models (glm's) containing
184 recorded factors which we expected to influence expression.

185 *Variation in Individual Condition*. Individual condition was summarised by using PCA on K,
186 HSI, SSI and AI, based on the correlation matrix. PCA was performed on both populations
187 combined to ensure that each condition principal component (PC) was summarising the
188 same variation in both populations, to allow direct comparisons of the relationships between
189 condition measures and immune gene expression levels in the models fitted to Host and
190 Bhar. PCs were retained for use in further analysis if they had a standard deviation ≥1 and
191 explained ≥10% of variance in the data. In order to examine what factors relate to the

condition measures, glm's were fitted separately to each population, with condition PC1 or PC2 as the response variable. Sex (2 levels), reproductive status (2 levels) *G. arcuatus* presence (2 levels) and *S. solidus* presence (2 levels) were included as factors, and length as a continuous variable, along with a sex by reproductive status interaction term. Non-significant terms were sequentially dropped from the full model to give a minimum adequate model, with significance of the remaining components determined by Wald F-tests.

Summarising Immune Expression. Profiles of the eight immune response genes were compared between the two lakes (Host and Bhar) using multivariate analysis of variance (MANOVA). Overall differences were assessed using the Pillai method, followed by examination of each immune type separately.

The expression of groups of functionally related components of the immune system was summarised using PCA, a widely used dimension reduction technique which converts a number of variables into principal components which summarises the variation present in the original measures. In this way, we can summarise the expression of the genes from each arm of the immune response in a single measure encompassing the majority of variation present in the original data. Genes were split into four functional groups based on their role: IL-1β and TNFα (Innate response); Stat4 and Tbet (Th1-type adaptive response); Stat6 and CMIP (Th2-type adaptive response); and FoxP3 and TGFβ (regulatory response). PCA was performed separately on each immune group in each population using the 'prcomp' function, based on the variance-covariance matrix. In most cases, except Th2-type expression in Bhar, the dominant first principal component (PC1) explained a high proportion of variation in the data (>69%), with both genes having positive loadings. Individual PC1 scores were used for subsequent analysis, except for the Th2-type response where Stat6 and CMIP relative expression values were fitted instead.

Factors Relating to Immune Expression. Glm's were fitted to both populations separately using each immune grouping PC1 score, or Stat6 and CMIP relative expression, as the

response variable, to examine which factors related to expression levels. Maximal models were fitted with sex (2 levels), reproductive status (2 levels), *G. arcuatus* presence (2 levels) and *S. solidus* parasite index (2 levels) as factors, and condition PC1 and PC2 scores and length as continuous variables. Biologically relevant interaction terms were also fitted. Non-significant terms were sequentially dropped from the full model to give a minimum adequate model. The significance of remaining components was determined by Wald F-tests.

RESULTS

S. solidus was found to be the dominant parasite species in both populations, with a prevalence of 54.3% (95% binomial confidence interval 39.0%-69.0%) and an average intensity in infected individuals of 8.6 (SE±1.7) in Bhar and a prevalence of 43.5% (95% binomial confidence interval 28.9%-58.9%) and intensity of 4.5 (SE±1.0) in Host. *G. arcuatus* was also found, with a prevalence of 6.5% (95% binomial confidence interval 1.4%-10.8%) and an intensity on infected individuals of 2 (SE±0.6) in Bhar and a prevalence of 19.6% (95% binomial confidence interval 9.4%-33.9%) and an average intensity of 2.3 (SE±0.3) in Host. No other parasite species were identified in the sampled fish at sufficient rates to include in the analysis.

Variation in Individual Condition

PCA was used to summarise the four different measures that reflect individual condition. Condition PC1 accounted for 41% of variation, with loadings of K = 0.134, HSI = 0.641, SSI = -0.489, and AI = -0.577, whilst PC2 accounted for 24.9% of variance, with loadings of K = 0.973, HSI = -0.119, SSI = 0.186, and AI = -0.062 (Figure 1). Factors relating to condition PC1 and PC2 scores are summarised in Table 1. In both Bhar and Host, individuals investing in attaining reproductive condition had higher PC1 scores (Bhar $F_{(2,39)}$=4.69, p=0.036, Host $F_{(2,39)}$=12.08, p<0.001), and this also varied with sex (Bhar $F_{(2,39)}$=4.89, p=0.033, Host $F_{(2,39)}$=10.53, p<0.001) such that females had higher condition PC1 scores than males for a given reproductive condition score. Infection with *S. solidus* was associated

with a decrease in condition PC1 score (Figure 1) in both Bhar ($F_{(1,39)}$=37.57, p<0.001) and Host ($F_{(1,39)}$=10.594, p=0.002). In both Bhar and Host, larger fish had lower condition PC2 scores (Bhar $F_{(1,44)}$=6.86, p=0.012, Host $F_{(1,40)}$=13.51, p<0.001). In Host, males had lower condition PC2 scores than females ($F_{(1,40)}$=6.49, p=0.015), scores were higher in fish in reproductive condition ($F_{(2,40)}$=6.09, p=0.005) and infection with *S. solidus* was associated with an increase in condition PC2 score ($F_{(1,40)}$=5.01, p=0.031).

Summarising Immune Expression

Fish from Bhar and Host differed significantly in their overall immune profiles (Figure 2; MANOVA $F_{(1,90)}$=10.169, p<0.001). The populations differed in expression of TNFα ($F_{(1,90)}$=13.58, p<0.001), Stat6 ($F_{(1,90)}$=10.29, p=0.002), CMIP ($F_{(1,90)}$=14.30, p<0.001), FoxP3 ($F_{(1,90)}$=1.38, p=0.039) and TGFβ ($F_{(1,90)}$=27.13, p<0.001), but not IL-1β ($F_{(1,90)}$=1.23, p=0.270), Stat4 ($F_{(1,90)}$=0.55, p=0.461) and Tbet ($F_{(1,90)}$=0.69, p=0.409).

Grouped immune gene expression measures were summarised in each population using PCA, with high PC1 values in all groups indicating high expression levels of included genes. In Bhar, PC1 of innate immune measures explained 69.6% of variation in the data, with loadings of IL-1β=0.856 and TNFα=0.516. For the Th1-type measures, PC1 explained 79.7% of variation, with loadings of Stat4=0.267 and Tbet=0.964. The Th2-type measures were found to give PC1 loadings in opposite directions, so were kept separate for subsequent analysis. The regulatory measures gave a PC1 explaining 81.7% of variation, with loadings of FoxP3=0.965 and TGFβ=0.263.

In Host, PC1 of innate measures explained 78.5% of variation, with loadings of IL-1β=0.961 and TNFα=0.277. For the Th1-type measures, PC1 explained 84.8% of variation, with loadings of Stat4=0.439 and Tbet=0.898. PC1 of the Th2-type measures explained 81.4% of variation, with loadings of Stat6=0.222 and CMIP=0.975. The regulatory measures PC1 accounted for 82.2% of variation, with loadings of FoxP3=0.969 and TGFβ=0.245.

Factors Relating to Immune Expression

A range of factors were found to relate to expression levels of different arms of the immune system, as summarised in Table 2. Some factors relating to innate and Th1-type expression levels had the same effects in both populations, whilst others either had opposing effects or were only related to expression levels within a single population.

Innate Expression. In Bhar, larger fish had lower innate expression levels (Figure 3, $F_{(1,40)}$=14.11, p<0.001). An increase in condition PC2 was associated with an increase in innate expression levels ($F_{(1,40)}$=5.48, p=0.024), with the effect being less pronounced as fish get bigger ($F_{(1,40)}$=5.30, p=0.027). Individuals infected with *S. solidus* had lower innate expression levels at a given condition PC2 score ($F_{(1,40)}$=6.85, p=0.012). Larger fish in Host also had lower innate expression levels (Figure 3, $F_{(1,38)}$=8.20, p=0.007), but this also varied by sex ($F_{(1,38)}$=5.84, p=0.021), with males showing a larger decrease in expression with increasing size. An increase in condition PC1 score was associated with an increase in innate expression ($F_{(1,38)}$=12.94, p<0.001), whilst an increase in condition PC2 was associated with a decrease ($F_{(1,38)}$=11.32, p=0.002). In contrast to Bhar, infection with both *G. arcuatus* ($F_{(1,38)}$=5.19, p=0.028) and *S. solidus* ($F_{(1,38)}$=6.59, p=0.014) were associated with an increase in innate expression levels.

Th1-type Expression. In Host, fish infected with *S. solidus* had lower Th1-type expression levels (Figure 4, $F_{(1,39)}$=11.07, p=0.002), with the difference increasing as fish got larger ($F_{(1,39)}$=5.70, p=0.022). Individuals in reproductive condition had higher Th1-type expression levels for a given condition PC1 score ($F_{(1,39)}$=5.15, p=0.029). In Bhar, fish infected with *S. solidus* also had lower Th1-type expression than uninfected fish (Figure 4, $F_{(1,37)}$=13.51, p<0.001). Males had lower expression levels than females ($F_{(1,37)}$=10.59, p=0.002), and this effect varied with reproductive status ($F_{(1,37)}$=5.90, p=0.020), with the difference between sexes much greater in non-reproductive individuals. Sex also altered the relationship between condition PC2 score and Th1-type expression ($F_{(1,37)}$=7.71, p=0.009), with the difference between sexes decreasing as condition PC2 score increases. As in Host, the effect of condition PC1 score also varied with reproductive status in Bhar ($F_{(1,37)}$=8.87,

297 p=0.005), with reproductive individuals having lower Th1-type expression at a given
298 condition PC1 score.

299 *Th2-type Expression.* No significant factors were found in the models for CMIP expression in
300 Bhar or Host. In Bhar, males showed lower Stat6 expression levels than females
301 ($F_{(1,36)}$=4.77, p=0.036). Sex influenced the relationship between condition PC1 and Stat6
302 expression ($F_{(1,36)}$=8.35, p=0.007) and condition PC2 and Stat6 expression ($F_{(1,36)}$=4.91,
303 p=0.033), with males having lower expression than females for a given condition PC score.
304 The effect of condition PC1 score on Stat6 expression also varied with length ($F_{(1,36)}$=4.43,
305 p=0.042), with larger fish having lower Stat6 expression levels at a given condition PC1
306 score. The effect of condition PC1 score on Stat6 expression also varied with *S. solidus*
307 infection ($F_{(1,36)}$=6.31, p=0.017), with infected fish having higher Stat6 expression levels than
308 uninfected fish at a given condition PC1 score. In Host, larger individuals had higher Stat6
309 expression levels ($F_{(1,40)}$=7.61, p=0.009), as did individuals in reproductive condition
310 ($F_{(1,40)}$=11.87, p=0.001). The effect of length also varied with reproduction ($F_{(1,40)}$=14.28,
311 p<0.001), with the difference between reproductive and non-reproductive individuals
312 decreasing with increasing length. Individuals in reproductive condition had higher Stat6
313 expression levels than non-reproductive individuals at a given condition PC1 score
314 ($F_{(1,40)}$=5.95, p=0.019).

315 *Regulatory Expression.* In Host, larger fish had lower regulatory gene expression levels
316 ($F_{(1,43)}$=6.20, p=0.017), whilst an increase in condition PC2 score was associated with a
317 decrease in expression levels ($F_{(1,43)}$=6.06, p=0.018). In Bhar, individuals infected with *S.*
318 *solidus* had higher regulatory gene expression levels than uninfected individuals
319 ($F_{(1,33)}$=6.33, p=0.016), but this difference decreased with increasing fish length ($F_{(1,33)}$=5.91,
320 p=0.020). The difference in regulatory gene expression levels between *S. solidus* infected
321 and uninfected individuals decreased with increasing condition PC1 score ($F_{(1,33)}$=6.33,
322 p=0.016), and with increasing condition PC2 score ($F_{(1,33)}$=7.11, p=0.011).

DISCUSSION

In the present study we see differences in immune gene expression levels between populations, and identify a range of factors which contribute to shaping this expression. Data collected from wild populations is inherently noisy, yet we are able to detect variation in immune gene expression levels that are associated with infection with two common parasite species, *S. solidus* and *G. arcuatus,* as well as associations with a range of other factors. Overall gene expression profiles differed between fish from Host and Bhar, primarily driven by higher expression levels of Th2-type and regulatory genes in Bhar. Previous work has shown that underlying gene expression levels differ between populations (Robertson *et al.*, 2015), and an individual's ability to respond has some genetic basis (Rauch *et al.*, 2006; Robertson *et al.*, 2015), thus the measured variation in expression here could represent underlying differences in responsiveness between populations, or differences in the challenges being faced in each population.

A range of factors were found to contribute to shaping expression of each response type, with factors relating to different arms of the immune system in different ways. Some explanatory factors were found to have common affects in both populations, including size, sex and condition, whilst others had opposing affects or only related to expression levels in a single population. Interactions between the factors also varied. Recent thinking in immunology suggests that controlled lab-based studies of the immune system lack complexity when compared to a natural setting (Bradley, 2015; Pedersen & Babayan, 2011). The broad set of factors found here which relate to gene expression levels, and the interactions between them, confirm that immune function in the wild is complex, and shows that studies in immunology should consider multiple factors simultaneously.

Both sex and reproductive status correlated with an individual's immune expression profile. Previous studies in vertebrates have found that there are general differences in immune function between the sexes (Hawley & Altizer, 2011; Restif & Amos, 2010), and we expected

males to have lower expression levels in general than females. Sex correlated with innate expression levels in Host, and with Th1-type, Th2-type and Stat6 expression levels in Bhar; in all these cases, males had lower expression levels than females. Investing in reproduction can also influence immune response ability (Downs *et al.*, 2014). Individuals investing in reproduction had lower Th1-type gene expression levels in both Bhar and Host, and had higher Stat6 expression levels in Host. During the breeding season, the immune response can be affected by circulating hormone levels (Cuesta *et al.*, 2007; Maule *et al.*, 1996), and other factors, such as sex specific behavioural changes, may also play a role. Further studies at different times of year could indicate whether such sex differences are transient, and longitudinal studies could show whether different investment strategies affect breeding success, and ultimately fitness.

Infection with *Schistocephalus solidus* was associated with variation in expression of all immune response types, but some of these effects differed between the studied populations. The immune response of individuals has been shown to change with *S. solidus* infection in experimental studies (Scharsack *et al.*, 2004; Scharsack *et al.*, 2007b), with growing evidence of parasite driven manipulation of the host immune system (For review, see Barber & Scharsack, 2010). The role of Th1 / Th2 differentiation during *S. solidus* infection is not well understood due to a previous lack of appropriate assays. Here we see a decrease in Th1-type expression with infection in both populations, as previously observed in wild populations on North Uist (Robertson *et al.*, 2015), and an increase in Th2-type and regulatory expression in Bhar only. Work in mice on Schistosomes, a digenean trematode residing in the blood stream, has shown a shift towards an increased Th1-type response in order to avoid the potentially harmful Th2 or humoral type responses (Herve *et al.*, 2003), an approach that may be employed by *S. solidus* (Barber & Scharsack, 2010). Our data, however, shows the opposite change in Th1-type response. Our results support the involvement of the adaptive response during infection, but a better understanding of the

Th1/Th2 mechanism in teleost fish is required to shed light on the role of *S. solidus* in manipulating the host's immune system.

Condition measures were found to relate to expression levels of all response types in both populations, although the effect and interactions of the condition factors varied between populations. The condition PC1 axis represents opposing changes in liver size against spleen size and adipose fat tissue, and was associated with differences between sexes, in reproductive condition, and *S. solidus* infection levels. Changes in liver, spleen and adipose tissue were expected to correspond with *S. solidus* infection (Arme & Owen, 1967). The condition PC2 axis represents general body condition, measured as Fulton's condition factor, and generally decreases with increasing length. In turn the condition PC1 and PC2 scores interact with a number of factors in the models for both Bhar and Host, again including sex, reproduction and *S. solidus* infection. There appears to be a complex interplay between factors, where the direction of causality in the relationship between factors determining condition, condition itself, and immune gene expression levels is an area open to further research. Condition may affect an individual's ability to respond, or response may determine condition. Furthermore, factors such as reproductive investment and *S. solidus* infection may have direct effects on immune gene expression levels whilst also acting indirectly through condition.

CONCLUSIONS

Studying the immune expression profiles of individuals from two wild populations of stickleback has provided new insights into the function of the immune system in a natural setting. We demonstrate that two populations differ in their immune gene expression profiles, and that a complex interplay of multiple factors correlates to individual immune expression levels within each population. There appear to be important roles of sex, reproductive status, and individual condition. Infection with a parasite with fitness consequences to the host, *Schistocephalus solidus*, relates to the innate and adaptive responses, and may represent

parasite manipulation of the host. Whilst some factors have the same pattern in both populations, others only relate to gene expression levels in a single population. The present study adds to our understanding of the immune response in a natural setting, and shows that studying the effect of single factors in isolation may overlook important and complex interactions in the wild. Whilst the patterns observed are correlative, they identify a range of areas for further research.

ACKNOWLEDGMENTS

We would like to thank all the members of the MacColl lab at the University of Nottingham for their assistance in sampling in the field, and Ann Lowe for her help in the laboratory. This work was funded by a Natural Environment Research Council (NERC) studentship awarded to S.R..

REFERENCES

Arme, C. & Owen, R.W. 1967. Infections of the three-spined stickleback, *Gasterosteus aculeatus* L., with the plerocercoid larvae of *Schistocephalus solidus* (Muller, 1776), with special reference to pathological effects. *Parasitology* **57**: 301-14.

Barber, I. 2013. Sticklebacks as model hosts in ecological and evolutionary parasitology. *Trends in Parasitology* **29**: 556-566.

Barber, I. & Scharsack, J.P. 2010. The three-spined stickleback-*Schistocephalus solidus* system: an experimental model for investigating host-parasite interactions in fish. *Parasitology* **137**: 411-424.

Barber, I., Wright, H.A., Arnott, S.A. & Wootton, R.J. 2008. Growth and energetics in the stickleback-Schistocephalus host-parasite system: a review of experimental infection studies. *Behaviour* **145**: 647-668.

Bradley, J.E. 2015. Wild immunology. *Parasite Immunology* **37**: 217-219.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. & Wittwer, C.T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**: 611-622.

Cuesta, A., Vargas-Chacoff, L., Garcia-Lopez, A., Arjona, F.J., Martinez-Rodriguez, G., Meseguer, J., Mancera, J.M. & Esteban, M.A. 2007. Effect of sex-steroid hormones,

testosterone and estradiol, on humoral immune parameters of gilthead seabream. *Fish and Shellfish Immunology* **23**: 693-700.

De Roij, J., Harris, P.D. & MacColl, A.D.C. 2011. Divergent resistance to a monogenean flatworm among three-spined stickleback populations. *Funct. Ecol.* **25**: 217-226.

De Roij, J. & MacColl, A.D.C. 2012. Consistent differences in macroparasite community composition among populations of three-spined sticklebacks, *Gasterosteus aculeatus* L. *Parasitology* **11**: 1478-1491.

Dheda, K., Huggett, J.F., Chang, J.S., Kim, L.U., Bustin, S.A., Johnson, M.A., Rook, G.A. & Zumla, A. 2005. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. *Analytical biochemistry* **344**: 141-3.

Dittmar, J., Janssen, H., Kuske, A., Kurtz, J. & Scharsack, J.P. 2014. Heat and immunity: an experimental heat wave alters immune functions in three-spined sticklebacks (*Gasterosteus aculeatus*). *J. Anim. Ecol.* **83**: 744-757.

Downs, C.J., Adelman, J.S. & Demas, G.E. 2014. Mechanisms and methods in ecoimmunology: integrating within-organism and between-organism processes. *Integrative and Comparative Biology* **54**: 340-352.

Eizaguirre, C., Lenz, T.L., Sommerfeld, R., Harrod, C., Kalbe, M. & Milinski, M. 2011. Parasite diversity, patterns of MHC II variation and olfactory based mate choice in diverging three-spined stickleback ecotypes. *Evol Ecol* **25**: 605-622.

El Nagar, A. 2014. PhD Thesis - The role of parasites in the ecological speciation of three-spined stickleback *Gasterosteus aculeatus*: University of Nottingham.

Fassbinder-Orth, C.A. 2014. Methods for quantifying gene expression in ecoimmunology: from qPCR to RNA-Seq. *Integrative and Comparative Biology* **54**: 396-406.

Giles, N. 1983. The possible role of environmental calcium levels during the evolution of phenotypic diversity in Outer-Hebridean populations of the 3-Spined Stickleback, *Gasterosteus aculeatus*. *J Zool* **199**: 535-544.

Haase, D., Rieger, J.K., Witten, A., Stoll, M., Bornberg-Bauer, E., Kalbe, M. & Reusch, T.B.H. 2014. Specific gene expression responses to parasite genotypes reveal redundancy of innate immunity in vertebrates. *PLoS ONE* **9**: e108001.

Hawley, D.M. & Altizer, S.M. 2011. Disease ecology meets ecological immunology: understanding the links between organismal immunity and infection dynamics in natural populations. *Funct. Ecol.* **25**: 48-60.

Hendry, A.P., Peichel, C.L., Matthews, B., Boughman, J.W. & Nosil, P. 2013. Stickleback research: the now and the next. *Evol Ecol Res* **15**: 111-141.

Herve, M., Angeli, V., Pinzar, E., Wintjens, R., Faveeuw, C., Narumiya, S., Capron, A., Urade, Y., Capron, M., Riveau, G. & Trottein, F. 2003. Pivotal roles of the parasite PGD2 synthase and of the host D prostanoid receptor 1 in schistosome immune evasion. *Eur J Immunol* **33**: 2764-2772.

Hibbeler, S., Scharsack, J.P. & Becker, S. 2008. Housekeeping genes for quantitative expression studies in the three-spined stickleback *Gasterosteus aculeatus*. *Bmc Mol Biol* **9**: 18-28.

Jackson, J.A., Begon, M., Birtles, R., Paterson, S., Friberg, I.M., Hall, A.M.Y., Lowe, A.N.N., Ralli, C., Turner, A., Zawadzka, M. & Bradley, J.E. 2011. The analysis of immunological profiles in wild animals: a case study on immunodynamics in the field vole, *Microtus agrestis*. *Molecular Ecology* **20**: 893-909.

Jackson, J.A., Friberg, I.M., Bolch, L., Lowe, A., Ralli, C., Harris, P.D., Behnke, J.M. & Bradley, J.E. 2009. Immunomodulatory parasites and toll-like receptor-mediated tumour necrosis factor alpha responsiveness in wild mammals. *Bmc Biol* **7**.

Jackson, J.A., Hall, A.J., Friberg, I.M., Ralli, C., Lowe, A., Zawadzka, M., Turner, A.K., Stewart, A., Birtles, R.J., Paterson, S., Bradley, J.E. & Begon, M. 2014. An immunological marker of tolerance to infection in wild rodents. *PLoS Biology* **12**: e1001901.

Kalbe, M. & Kurtz, J. 2006. Local differences in immunocompetence reflect resistance of sticklebacks against the eye fluke Diplostomum pseudospathaceum. *Parasitology* **132**: 105-116.

Konijnendijk, N., Raeymaekers, J., Vandeuren, S., Jacquemin, L. & Volckaert, F.A. 2013. Testing for local adaptation in the *Gasterosteus-Gyrodactylus* host-parasite system. *Evol Ecol Res* **15**: 489-502.

Kurtz, J., Kalbe, M., Aeschlimann, P.B., Haberli, M.A., Wegner, K.M., Reusch, T.B.H. & Milinski, M. 2004. Major histocompatibility complex diversity influences parasite resistance and innate immunity in sticklebacks. *P Roy Soc B-Biol Sci* **271**: 197-204.

Lazzaro, B.P. & Little, T.J. 2009. Immunity in a variable world. *Philosophical Transactions of the Royal Society B-Biological Sciences* **364**: 15-26.

Lenz, T.L., Eizaguirre, C., Rotter, B., Kalbe, M. & Milinski, M. 2013. Exploring local immunological adaptation of two stickleback ecotypes by experimental infection and transcriptome-wide digital gene expression analysis. *Molecular Ecology* **22**: 774-86.

MacColl, A.D.C. 2009. Parasite burdens differ between sympatric three-spined stickleback species. *Ecography* **32**: 153-160.

MacColl, A.D.C., El Nagar, A. & de Roij, J. 2013. The evolutionary ecology of dwarfism in three-spined sticklebacks. *J. Anim. Ecol.* **82**: 642-652.

Maizels, R.M. & Nussey, D.H. 2013. Into the wild: digging at immunology's evolutionary roots. *Nat Immunol* **14**: 879-83.

Martin, L.B., Hawley, D.M. & Ardia, D.R. 2011. An introduction to ecological immunology. *Funct. Ecol.* **25**: 1-4.

Maule, A.G., Schrock, R., Slater, C., Fitzpatrick, M.S. & Schreck, C.B. 1996. Immune and

endocrine responses of adult chinook salmon during freshwater immigration and sexual maturation. *Fish Shellfish Immun* **6**: 221-233.

Nash, R.D., Valencia, A.H. & Geffen, A.J. 2006. The origin of Fulton's condition factor—setting the record straight. *Fisheries* **31**: 236-238.

Oko, L., Aduddell-Swope, B., Willis, D., Hamor, R., Coons, T.A., Hjelle, B. & Schountz, T. 2006. Profiling helper T cell subset gene expression in deer mice. *Bmc Immunol* **7**: 18.

Pedersen, A.B. & Babayan, S.A. 2011. Wild immunology. *Molecular Ecology* **20**: 872-880.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research* **29**: e45.

R Core Team. 2014. R: a language and environment for statistical computing, ed^. Vienna, Austria: R Foundation for Statistical Programming, .

Rauch, G., Kalbe, M. & Reusch, T.B.H. 2006. Relative importance of MHC and genetic background for parasite load in a field experiment. *Evol Ecol Res* **8**: 373-386.

Restif, O. & Amos, W. 2010. The evolution of sex-specific immune defences. *Proceedings of the Royal Society of London B: Biological Sciences* **277**: 2247-2255.

Robertson, S., Bradley, J.E. & MacColl, A.D.C. 2015. Measuring the immune system of the three-spined stickleback - investigating natural variation by quantifying immune expression in the laboratory and the wild. *Molecular Ecology Resources*.

Scharsack, J.P., Kalbe, M., Derner, R., Kurtz, J. & Milinski, M. 2004. Modulation of granulocyte responses in three-spined sticklebacks *Gasterosteus aculeatus* infected with the tapeworm *Schistocephalus solidus*. *Dis Aquat Organ* **59**: 141-150.

Scharsack, J.P., Kalbe, M., Harrod, C. & Rauch, G. 2007a. Habitat-specific adaptation of immune responses of stickleback (G*asterosteus aculeatus*) lake and river ecotypes. *P Roy Soc B-Biol Sci* **274**: 1523-1532.

Scharsack, J.P., Koch, K. & Hammerschmidt, K. 2007b. Who is in control of the stickleback immune system: interactions between *Schistocephalus solidu*s and its specific vertebrate host. *P Roy Soc B-Biol Sci* **274**: 3151-3158.

Schountz, T., Prescott, J., Cogswell, A.C., Oko, L., Mirowsky-Garcia, K., Galvez, A.P. & Hjelle, B. 2007. Regulatory T cell-like responses in deer mice persistently infected with Sin Nombre virus. *P Natl Acad Sci USA* **104**: 15496-15501.

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M. & Nguyen, M. 2010. A practical approach to RT-qPCR-publishing data that conform to the MIQE guidelines. *Methods (San Diego, Calif.)* **50**: S1-5.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. & Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* **3**: RESEARCH0034.

589
590 Wegner, K.M., Kalbe, M., Rauch, G., Kurtz, J., Schaschl, H. & Reusch, T.B.H. 2006. Genetic
591 variation in MHC class II expression and interactions with MHC sequence polymorphism in
592 three-spined sticklebacks. *Molecular Ecology* **15**: 1153-1164.
593
594 Zapata, A., Diez, B., Cejalvo, T., Gutierrez-de Frias, C. & Cortes, A. 2006. Ontogeny of the
595 immune system of fish. *Fish and Shellfish Immunology* **20**: 126-36.
596
597

Table 1: Summaries of minimum adequate models fitted to condition data from wild fish from two lakes ('Bhar' and 'Host') on North Uist, Scotland. Condition PC1 represents variation in hepato-somatic index (HSI), spleen somatic index (SSI) and adipose index (AI), whilst condition PC2 represents variation in Fulton's condition factor (K). Terms that were not included in the final minimum model for a population, but which were retained in the minimum model for the other population, are marked 'n/a'.

		Bhar			Host		
	Factor	**F**	**df**	**p**	**F**	**df**	**p**
Condition PC1	Sex	4.50	1,39	0.040	0.05	1,39	0.826
	Reproduction	4.69	1,39	0.036	12.08	1,39	<0.001
	S. solidus presence	37.57	1,39	<0.001	10.59	1,39	<0.001
	Sex : Reproduction	4.89	1,39	0.033	10.53	1,39	<0.001
Condition PC2	Length	6.86	1,44	0.012	13.51	1,40	<0.001
	Sex		n/a		6.49	1,40	0.005
	Reproduction		n/a		6.09	1,40	0.015
	S. solidus presence		n/a		5.01	1,40	0.031

Table 2: Summaries of minimum adequate models fitted to expression data from wild fish from two lakes ('Bhar' and 'Host') on North Uist, Scotland. 'Innate', 'Th1', and 'Regulatory' response variables are first principal components of the following pairs of genes respectively: IL-1β and TNFα; Stat4 and Tbet; FoxP3 and TGFβ. Significant p values ($P<0.05$) are marked in bold. The direction of the effect (+ or -) of each factor or interaction term is shown (M relative to F for 'Sex', reproductive relative to non-reproductive for 'Reproduction'). Terms that were not included in the final minimum model for a population, but which were retained in the minimum model for the other population, are marked 'n/a'.

		Bhar				Host			
	Factor	**F**	**df**	**p**	**Effect**	**F**	**df**	**p**	**Effect**
Innate	Length	14.11	1,40	**<0.001**	-	8.20	1,38	**0.007**	-
	Sex			n/a		4.84	1,38	**0.034**	-
	Condition PC1			n/a		12.94	1,38	**<0.001**	+
	Condition PC2	5.48	1,40	**0.024**	+	11.32	1,38	**0.002**	-
	G. arcuatus presence			n/a		5.19	1,38	**0.028**	+
	S. solidus presence	0.39	1,40	0.534	-	6.59	1,38	**0.014**	+
	Length : Sex			n/a		5.84	1,38	**0.021**	+
	Length : Condition PC2	5.30	1,40	**0.027**	-			n/a	
	Condition PC2 : *S. solidus* presence	6.85	1,40	**0.012**	-			n/a	
Th1	Length			n/a		3.89	1,39	**0.056**	-
	Sex	10.59	1,37	**0.002**	-			n/a	
	Reproduction	0.14	1,37	0.714	-	0.46	1,39	0.540	-
	Condition PC1	9.03	1,37	**0.005**	-	3.69	1,39	0.062	-
	Condition PC2	0.15	1,37	0.697	+			n/a	
	S. solidus presence	13.51	1,37	**<0.001**	-	11.07	1,39	**0.002**	-
	Length : *S. solidus* presence			n/a		5.70	1,39	**0.022**	+
	Sex : Reproduction	5.90	1,37	**0.020**	+			n/a	
	Sex : Condition PC2	7.71	1,37	**0.009**	-			n/a	
	Reproduction : Condition PC1	8.87	1,37	**0.005**	+	5.15	1,39	**0.029**	+
Stat6	Length	0.52	1,36	0.476	+	7.61	1,40	**0.009**	+
	Sex	4.77	1,36	**0.036**	-			n/a	
	Reproduction			n/a		11.87	1,40	**0.001**	+
	Condition PC1	3.42	1,36	0.072	+	2.60	1,40	0.115	-
	Condition PC2	0.07	1,36	0.793	+			n/a	
	S. solidus presence	0.87	1,36	0.358	+			n/a	
	Length : Reproduction			n/a		14.28	1,40	**<0.001**	-
	Length : Condition PC1	4.43	1,36	**0.042**	-			n/a	
	Sex : Condition PC1	8.35	1,36	**0.007**	-			n/a	
	Sex : Condition PC2	4.91	1,36	**0.033**	-			n/a	
	Reproduction : Condition PC1			n/a		5.95	1,40	**0.019**	+
	Condition PC1 : *S. solidus* presence	6.31	1,36	**0.017**	+			n/a	
Regulatory	Length	2.29	1,38	0.139	+	6.20	1,43	**0.017**	-
	Condition PC1	2.53	1,38	0.120	-			n/a	
	Condition PC2	3.79	1,38	0.060	+	6.06	1,43	**0.018**	-
	S. solidus presence	6.33	1,38	**0.016**	+			n/a	
	Length : *S. solidus* presence	5.91	1,38	**0.020**	-			n/a	
	Condition PC1 : *S. solidus* presence	6.33	1,38	**0.016**	+			n/a	
	Condition PC2 : *S. solidus* presence	7.11	1,38	**0.011**	-			n/a	

Figure 1: a) Individual scores of the first and second principal components of condition measures (with % variation explained) on fish from two lake populations (Bhar and Host) on North Uist, Scotland. PC1 and PC2 summarise variation in Fulton's condition factor (K), adipose index (AI), spleen-somatic index (SSI) and hepato-somatic index (HSI). b) Fish infected with *S. solidus* ('Infected') had lower condition PC1 scores than uninfected fish ('Uninf.') in both study populations (Bhar = ●, Host = ▲). A high PC1 indicates a high hepato-somatic index, with a low spleen-somatic index and adipose index.

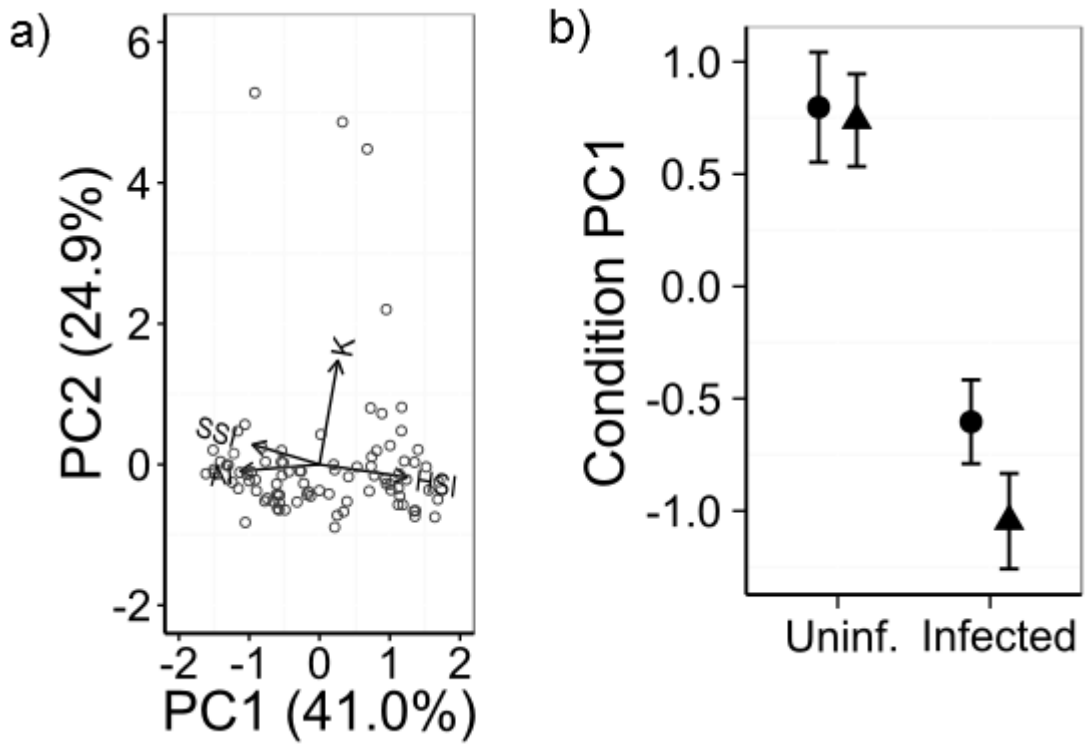

Figure 2: Relative gene expression levels (Mean ± SE) of two lake populations (Bhar = ●, Host = ▲) on North Uist, Scotland. Genes with significant expression differences are indicated (*), with expression being lower in Host in all cases. IL-1β and TNFα represent the innate immune response, Stat4 and Tbet the Th1-type adaptive response, Stat6 and CMIP the Th2-type adaptive response, and FoxP3 and TGFβ the regulatory response.

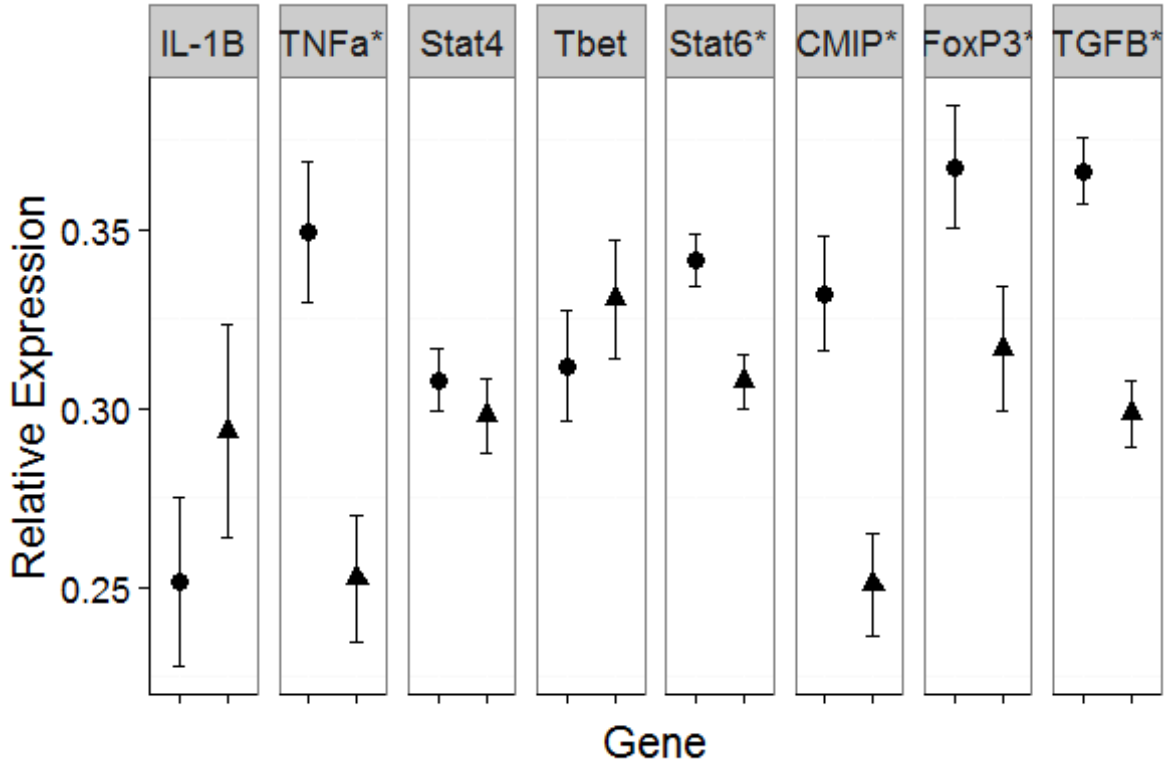

Figure 3: Larger fish had lower innate gene expression levels (Mean ± SE) in two lake populations, Bhar and Host, on North Uist, Scotland (including linear best fit lines). The innate response variable is the first principal component scores of IL-1β and TNFα gene expression levels, with PCA performed separately on fish from each population. A high PC1 score indicates high expression levels of both genes.

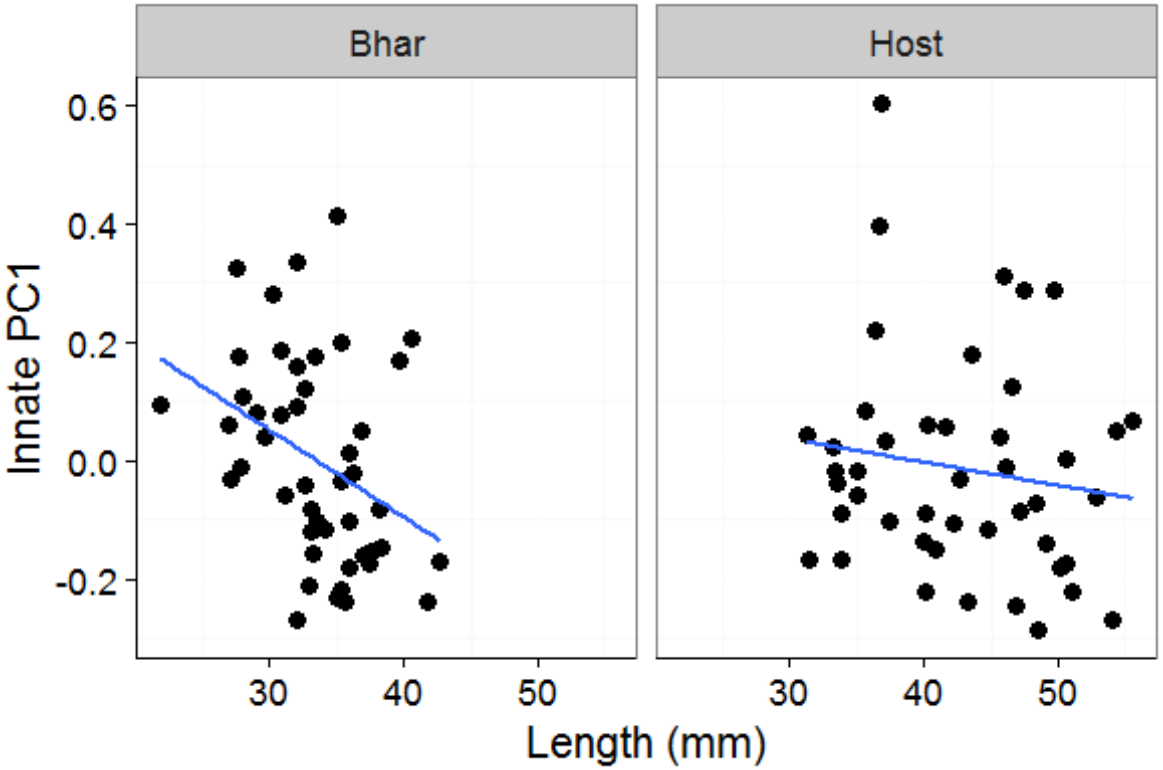

Figure 4: Fish infected with *S. solidus* had lower Th1-type gene expression levels (Mean ± SE) in two lake populations (Bhar = ●, Host = ▲) on North Uist, Scotland. The Th1 response variable is the first principal component scores of Stat4 and Tbet gene expression levels, with PCA performed separately on fish from each population. A high PC1 score indicates high expression levels of both genes.