Fish & Shellfish Immunology 60 (2017) 275–281

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

No evidence of local adaptation of immune responses to *Gyrodactylus* in three-spined stickleback (*Gasterosteus aculeatus*)

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

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

ARTICLE INFO

Article history:
Received 29 February 2016
Received in revised form
24 November 2016
Accepted 27 November 2016
Available online 29 November 2016

Keywords:
Local adaptation
Coevolution
Gene expression
qPCR
Immunoecology

ABSTRACT

Parasitism represents one of the most widespread lifestyles in the animal kingdom, with the potential to drive coevolutionary dynamics with their host population. Where hosts and parasites evolve together, we may find local adaptation. As one of the main host defences against infection, there is the potential for the immune response to be adapted to local parasites. In this study, we used the three-spined stickleback and its *Gyrodactylus* parasites to examine the extent of local adaptation of parasite infection dynamics and the immune response to infection. We took two geographically isolated host populations infected with two distinct *Gyrodactylus* species and performed a reciprocal cross-infection experiment in controlled laboratory conditions. Parasite burdens were monitored over the course of the infection, and individuals were sampled at multiple time points for immune gene expression analysis. We found large differences in virulence between parasite species, irrespective of host, and maladaptation of parasites to their sympatric host. The immune system responded to infection, with a decrease in expression of innate and Th1-type adaptive response genes in fish infected with the less virulent parasite, representing a marker of a possible resistance mechanism. There was no evidence of local adaptation in immune gene expression levels. Our results add to the growing understanding of the extent of host-parasite local adaptation, and demonstrate a systemic immune response during infection with a common ectoparasite. Further immunological studies using the stickleback-*Gyrodactylus* system can continue to contribute to our understanding of the function of the immune response in natural populations.

1. Introduction

Parasitism is one of the most widespread lifestyles in the animal kingdom [1], with at least one parasite species for every species of host [2]. Parasites have the potential to influence the dynamics of host populations [3], manipulate host behaviour [4] and affect host life history [5,6], and hosts in turn can affect parasite populations [7,8]. However, the majority of host parasite interactions fail to result in successful infection [9], and parasites infecting one host population are generally less likely to establish infections on hosts from other populations [10–12]. Such variation in infectivity between hosts may be the result of the local adaptation of host parasite pairs through a shared evolutionary history [13–15], although this may depend on the specific mode of transmission and parasite lifestyle of a specific host-parasite pair.

The immune system is a major defence of hosts against infection. Immune system genes show elevated levels of selection [16–19], suggesting that parasites may represent a significant selective pressure. The expression levels of resistance genes can be determined by host-parasite genotype x genotype interactions (G_H x G_P), or modulated by the external environment [20]. There is evidence from both vertebrates and invertebrates that variation in the expression levels of immune response genes in a host can determine parasite resistance [21,22], and that expression of resistance genes can vary with both host [23–25] and parasite [26–28] genotype. Modern molecular immunological techniques make it possible to measure the immune response of infected individuals, adding another level at which the possibility of host-parasite local adaptation can be examined.

The three-spined stickleback (*Gasterosteus aculeatus*, hereafter 'stickleback') and its *Gyrodactylus* parasites provide an ideal system in which to perform such work. Stickleback have repeatedly colonised novel freshwater habitats from their ancestral marine form since the end of the last ice age, creating a number of now isolated populations [29]. Adaptations to freshwater have evolved

* Corresponding author.
E-mail address: plsxr3@nottingham.ac.uk (S. Robertson).

http://dx.doi.org/10.1016/j.fsi.2016.11.058

in a short period of time in response to local ecological conditions [30–32]. Parasites may play a role in driving this adaptation; populations show consistent differences in parasite community composition [33–35], and there is growing evidence for within and between population variation in parasite resistance [36,37]. Furthermore, patterns of variation in stickleback immune gene expression levels have been found which correlate with parasite species [25,38–40] and genotype [28]. Parasites may represent a significant selection pressure in stickleback populations, and fish would be expected to evolve to resist their local parasite fauna.

Parasites of the *Gyrodactylus* genus are common monogenean ectoparasites of freshwater and saltwater fish. Infection can result in high rates of host mortality, with *Gyrodactylus salaris* responsible for the destruction of Atlantic salmon (*Salmo salar*) stocks in Norwegian rivers [41]. *Gyrodactylus* infect fish by attaching to the external surfaces and feeding on epithelial cells and mucous, where they can cause significant damage and leave fish susceptible to secondary infections [42,43]. *Gyrodactylus gasterostei* and *Gyrodactylus arcuatus* are frequent, often dominant, parasites of stickleback populations [33,44,45]. Infection with *Gyrodactylus* has fitness consequences for stickleback, with infected individuals having lower growth [36] and a 2.5% mortality rate under laboratory conditions (Mahmud, Robertson and MacColl, unpublished data). *Gyrodactylus* thus have the potential to drive classic evolutionary dynamics and adaptation of the hosts' immune response, although evidence for adaptation is mixed. There is evidence for local adaptation of both hosts [36] and parasites [46], of local adaptation of both hosts and parasites [47], or of no adaptation at all [48]. These studies focus on parasite load as a measure of infection success, but have yet to examine the extent of host-parasite specificity in the immune response in this context.

Here we examine how infection dynamics on, and the immune responses of, naïve hosts from two widely separated populations vary when infected with two closely related parasite species, each of which is sympatric to one of the host populations. Whilst studies in the wild have shown changes in the immune response with *Gyrodactylus* infection [39,40], such patterns are confounded by a wide range of additional external factors. By performing an experiment under controlled laboratory conditions, we can look directly at infection dynamics and the response of the immune system to infection, as well as examining whether the immune response shows adaptation to local parasite strains. We made F1 families of fish from a population in northern Scotland, and a population in the midlands of England, giving offspring with geographically distinct genetic backgrounds and no previous parasite exposure. Parasites were collected from the same locations a year later and used to perform a fully cross-factored reciprocal infection experiment, with the inclusion of uninfected fish acting as a control. The expression levels of a set of immune system genes was measured using real-time quantitative PCR, to allow us to examine the function of the immune response during the course of the infection experiment.

This experimental design was employed to allow us to address two main aims. First, what kind of immune response does *Gyrodactylus* infection produce? By measuring markers of the innate and adaptive immune responses, we can test whether the immune response plays a role during infection, and examine which systems may be involved. Furthermore, we can test whether there is a systemic response to an ectoparasite by measuring expression levels in a central immunological tissue. Second, do we find evidence of local host-parasite associations in infection dynamics and the immune response? We can examine whether there are differences in infection dynamics between the two parasite species, even though their route and mode of infection are very similar. Furthermore, we can examine whether we find an association between parasite species and the immune response to infection, and whether this differs between sympatric and allopatric parasites.

2. Methods

All work involving animals was approved by the University of Nottingham ethics committee, and performed under UK Home Office Licence (PPL-40/3486).

2.1. Study populations and parasites

Parental fish were collected from two geographically separated populations, Loch Ob nan Stearnain ('Uist', 57°36′09″N; 7°10′19″W), a saltwater lagoon on the island of North Uist, Scotland, and Jubilee Lake ('Nott', 52°57′02″N; -1°11′13″W), a freshwater lake on the campus of the University of Nottingham, England. For each population, we produced F1 progeny in May 2014 for use in the controlled infection experiments by making crosses between unrelated breeding adults to create full-sib families, following the procedure of De Roij, Harris [36]. Fertilised eggs were transported to aquarium at the University of Nottingham, with each family placed into a quarter-tank partition of a 100 L tank. After hatching, we split families between multiple partitions to give 8 fish per partition, to ensure all fish were maintained at the same density. After six months, 1 or 2 individuals from a large number of families (>20) were mixed at random into single tanks, at 30 fish per tank to give mixed family groups from a single source population. All fish were kept in a climate controlled room, with a natural temperature regime and photoperiod changing throughout the year.

Fish in Jubilee Lake ('Nott') were infected with *Gyrodactylus gasterostei*, whilst fish in North Uist ('Uist') were infected with *Gyrodactylus arcuatus*, and were expected to have coevolved with these different but closely related parasites species. Two weeks prior to the start of the experimental infection, in May 2015, we collected wild fish to act as parasite donors. Fish were caught in Obse and the Tottle Brook (52°56′06"; -1°11′41″), a small stream running through the University of Nottingham campus. *G. gasterostei* infections were unusually low at the time of sampling in Jubilee Lake, so fish from nearby Tottle Brook were used instead. These donor fish were housed in groups of 20–25 for one week, to encourage growth of parasite populations.

2.2. Experimental design

Overall, 30 12-month-old stickleback were exposed to *G. gasterostei* and 30 to *G. arcuatus,* with 24 uninfected fish kept as controls, assigned to an experimental group at random in a fully cross-factored design (Table 1). Fish from each population were selected at random from a tank containing individuals from a large number of mixed families (>20). All fish were housed individually in 3 L tanks containing 2 L of dechlorinated water, with 25% of the water changed every three days. By housing fish individually, we could track the infection on each individual. Temperature is important for the dynamics of *Gyrodactylus* infections, so fish were kept in a temperature controlled room. The average daytime temperature was 15.2°, dropping to an average of 13.7° overnight, with minimum and maximum temperatures staying constant (±0.5°) over the course of the experiment. The photoperiod was maintained at 16 h light and 8 h dark per day. The number of parasites on each fish was counted at 7, 14, 21, 29, and 36 days post infection (dpi). At 14, 29 and 45 dpi, we selected five fish from each treatment group and four from each control group at random, to be sampled for immunological analysis. By employing this experimental design, we had all fish x *Gyrodactylus* combinations,

Table 1
Outline experimental plan showing sampling time points for immunological measures. Fish were raised in controlled laboratory conditions, with crosses between parents from two sources (Nottingham, 'Nott'; North Uist, 'Uist'). Each fish was infected with *Gyrodactylus* from Nottingham ('Nott') or North Uist ('Uist') to give all sympatric and allopatric infection combinations, along with uninfected control individuals. The number of parasites on each individual was counted at each time point given, with the number of individuals sampled for immunological analysis at a given time point also indicated.

Fish Source	Gyro. Source	Treatment	n	Time (days post infection)					
				7	14	21	29	36	45
Nott	Nott	Sympatric	15	–	5	–	5	–	5
Nott	Uist	Allopatric	15	–	5	–	5	–	5
Nott	–	Control	12	–	4	–	4	–	4
Uist	Nott	Allopatric	15	–	5	–	5	–	5
Uist	Uist	Sympatric	15	–	5	–	5	–	5
Uist	–	Control	12	–	4	–	4	–	4
Total			84		28		28		28

allowing us to look at the extent of local adaptation of host-parasite infection dynamics and of the immune response. The addition of uninfected controls allowed us to examine how the immune system responds to infection.

2.3. Infection protocol and sample collection

Naturally infected fish from Obse and Tottle Brook were used as parasite donor fish. These were euthanized by overdose of MS-222 (400 mg L^{-1}) followed by destruction of the brain, in accordance with UK Home Office regulations. Fish were placed into a petri dish containing a small amount of dechlorinated water, and any tissues with attached *Gyrodactylus* removed under low powered microscopy. Tissues were left for 10 min to allow *Gyrodactylus* worms to detach. We removed *Gyrodactylus* from a number of fish into the same petri dish, to ensure no fish contributed an excessive number of parasites to the overall infection procedure. To infect a fish, it was lightly anaesthetised in MS222 (40 mg L^{-1}), and its caudal fin was held near two unattached *Gyrodactylus* until the worms attached to the fin. All fish receiving Nott parasites were infected first. Fish from each population were infected alternately, to ensure exposure to worms from a single donor fish was as uniform as possible. We anaesthetised and handled all control fish in the same manner as infected fish.

After 7 days, we counted the numbers of parasites on each exposed fish. Previous work has found both these *Gyrodactylus* species to infect the skin and fins in the populations used here, and only very rarely on the gills (SR, ADCM and M. Mahmud, unpublished data; Anna K. Rahn, personal communications). As such, we examined the caudal, anal, dorsal and pectoral fins, as well as the dorsal spined, pelvic girdle, flanks and head for parasites, with fish under light anaesthesia, as described above. Again, we anaesthetised and handled control fish in the same manner as infected fish. This counting procedure was repeated at 14, 21, 30, 36 and 45 dpi on all remaining fish.

At 14, 30 and 45 dpi, a subset of fish were removed and sampled. Fish were euthanized in a random order. Their spleens, an immunologically important tissue in fish [49], were removed and immediately placed in RNAlater (Life Technologies). Spleen samples were kept at 4 °C for 24 h, then at −20 °C until RNA extraction. We again counted the number of parasites infecting each fish.

Of the 84 fish used in the experiment, three were euthanized prior to their pre-determined sample point due to deteriorating health, with one fish each coming from the Nott Fish/Uist parasite group, one from the Nott Fish/Nott parasite group, and one from the Uist fish control group. We did not use these fish for gene expression analysis, as the cause of their ill health could not be determined, giving a total of 81 spleen samples for use in the gene expression analysis.

2.4. Gene expression quantification

We measured the expression levels of eight genes of interest, along with two reference genes. Genes of interest were *IL-1β*, *TNFα*, *Stat4*, *Tbet*, *Stat6*, *CMIP*, *FoxP3a*, and *TGFβ*. These genes were chosen to give an overall measure of the function of the immune response at the time of sampling, by measuring key genes from different immune response pathways: *IL-1β* and *TNFα* represent the innate pro-inflammatory response; *Stat4* and *Tbet* the Th1-type response against intracellular pathogens; *Stat6* and *CMIP* the Th2-type response against extracellular metazoan parasites; whilst *FoxP3a* and *TGFβ* have broad immunosuppressive roles [For full details, see 39]. A reference sample was made by pooling cDNA from each experimental sample, to control for between plate variation. A total of 81 cDNA samples were split randomly between two plates, with reactions performed in duplicate for each sample, and each plate also contained the reference sample and negative controls.

RNA extractions, reverse transcription and qPCR reactions were performed as described in Ref. [39]. 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 [50]. To select the most appropriate normalization strategy, we performed a geNorm analysis 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 expression was performed in qbase+ (Biogazelle), which identified *RPL13A* and *HPRT1* as the most stable combination of reference genes for this study.

Relative expression values were calculated using the ΔΔCq method [51], adjusted for the amplification efficiencies of each primer pair and standardized against the geometric mean Cq of the two reference genes for each sample [52].

2.5. Data analysis

All relative expression data were $\log_{10}(x+1)$ transformed prior to analysis, due to the inherently skewed distribution of such data. All data analysis was performed in R v.3.1.2 [53].

2.5.1. Infection dynamics

The magnitude of infection was summarised in two ways. Peak abundance was defined as the highest number of parasites found during any count on an individual. This included individuals sampled at 14 days even though all counts for these individuals preceded the peak for fish infected with Nott parasites, as some of the early counts represent peak infection for Uist parasite infected fish. Mean abundance was calculated as the total parasite burden from all counts on an individual divided by the infection length, determined by the day at which an individual was sampled.

To examine whether infection dynamics differ between hosts or parasites, we fitted general linear models (glms) with peak abundance or mean abundance as the response. Host origin (Nott or Uist), parasite origin (Nott or Uist) and the host by parasite interaction term were included as explanatory factors. Due to the skewed distribution of parasite count data, a quasipoisson error function and log link was included in the model of mean abundance, with significance calculated using Wald F tests. For the peak abundance model, a Poisson error function and log link were used, with significance calculated using χ^2 likelihood ratio tests. Non-

significant terms were sequentially dropped to give the minimum adequate model.

To estimate the effect size of local adaptation (E) of both peak and mean abundance, we used the approach developed by Ref. [54] and used in a number of studies to investigate parasite local adaptation [For example, see 48, 55]. This was calculated as the natural log ratio of 'X_S/X_A', where 'X_S' is the mean measure of the parasites on their sympatric hosts and 'X_A' is the mean measure of the parasites on their allopatric hosts. A positive E value indicates parasite adaptation to its local host, whilst a negative E value indicates parasite maladaptation to the local host (or adaptation of the host to its local parasite).

2.5.2. Control vs. exposed immune response

We first compared multivariate immune gene expression profiles between control and infected individuals, to see whether we could detect a response to infection, whether the response differed with parasite species, and whether the immune response changed over the course of the experiment. We performed a multivariate analysis of variance (MANOVA) with expression values as the response and fish origin (Nott or Uist), parasite treatment (infected vs control), and sample day (15, 30 or 45) as the explanatory variables, fitted sequentially in this order, along with their interaction terms. Overall differences were calculated using the Pillai method and F statistic. This was followed by examination of expression levels of each immune gene separately, using the false discovery rate (fdr) to control for multiple comparisons. For significant single gene ANOVAs between treatment groups, we tested all possible pairwise comparisons using post-hoc Tukey's HSD tests.

2.5.3. Local adaptation of immune measures

To test whether there was local adaptation of immune gene expression levels, we fitted a MANOVA with the gene expression levels of infected fish (control fish were excluded from this analysis) as the response, and fish origin (Uist or Nott), parasite origin (Nott or Uist) and their interaction term as the explanatory variables. Evidence of local adaptation would be seen as a significant interaction term, with the exact pattern depending on the direction of the interaction. Overall differences were calculated using the Pillai and F statistic, followed by the separate examination of each gene, with fdr applied to control for multiple comparisons.

3. Results

3.1. Infection dynamics

Average parasite burdens over the course of the experiment are shown in Fig. 1. Mean abundance differed between parasite species ($F_{1,45} = 24.14$, $p < 0.001$), with a mean burden of 0.26 (SE ± 0.04) Uist parasites and 1.06 (SE ± 0.18) Nott parasites. There was no difference in mean abundance between host origins ($F_{1,44} = 0.29$, $p = 0.590$), and no host by parasite interaction ($F_{1,43} = 0.71$, $p = 0.405$). Peak parasite abundance differed between parasite species ($LRT_{1,44} = 93.29$, $p < 0.001$), with an average peak of 5.24 (SE ± 1.16) Uist parasites and 22.37 (SE ± 5.10) Nott parasites. Peak parasite abundance also differed between host origins ($LRT_{1,44} = 14.17$, $p < 0.001$), with an average peak parasite abundance of 15.26 (SE ± 5.44) on Nott fish, and 14.52 (SE ± 3.49) on Uist fish. For peak parasite abundance there was also a parasite origin by host origin interaction (Fig. 2, $LRT_{1,44} = 12.31$, $p < 0.001$), with no difference in Nott parasite peak abundance between hosts, but lower numbers of Uist parasites on Uist fish.

Both Uist and Nottingham parasites had negative values of E for both mean abundance (Uist parasite E = −0.602, Nottingham parasite E = −0.043) and peak abundance (Uist parasite E = −0.708, Nottingham parasite E = −0.094), indicating that parasites are maladapted to their local hosts, or hosts are adapted to resist infection with their local parasites.

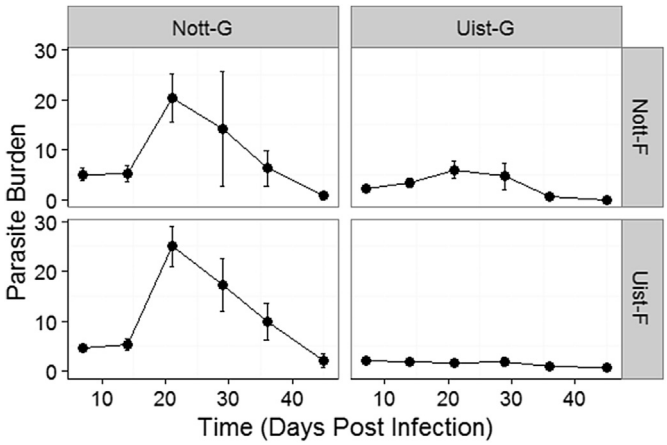

Fig. 1. Mean parasite burden (±SE) over the course of the infection experiment for each host by parasite combination in a reciprocal artificial infection experiment. Infections with *G. gasterostei* from Nottingham ('Nott-G') are in the left column, and infections with *G. arcuatus* from North Uist ('Uist-G') are in the right column. Infections on fish from Nottingham ('Nott-F') are shown in the top row and infections on fish from North Uist ('Uist-F') in the bottom row.

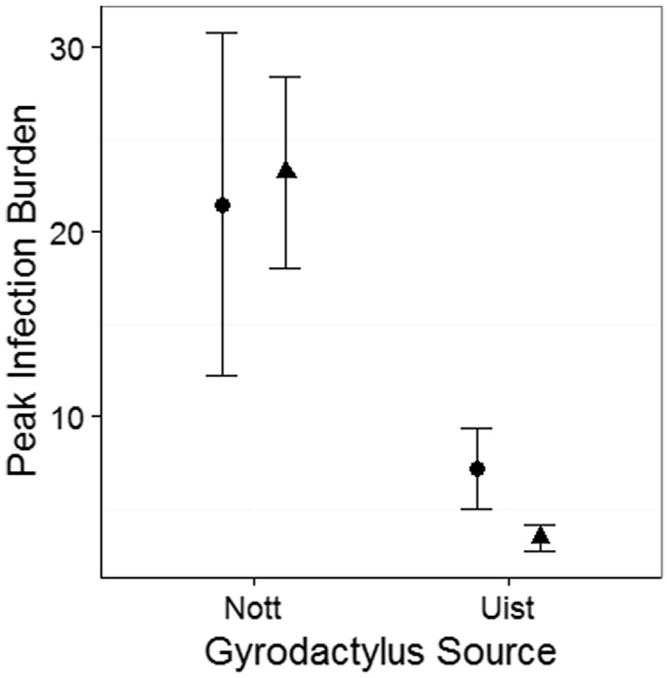

Fig. 2. Peak parasite burden (Mean ± SE) varies with parasite species ('Uist' *G. arcuatus* and 'Nott' *G. gasterostei*) on hosts from Nottingham (●) and North Uist (▲). Peak infection burdens of Uist parasites were lower on Uist fish than those from Nottingham, but there was no difference in peak burden between fish source for Nottingham parasites.

3.2. Control vs exposed immune response

Overall immune expression profiles differed between fish from different source populations (MANOVA $F_{1,77} = 9.27$, $p < 0.001$), with fish from Obse having higher expression levels of *IL-1β* ($F_{1,77} = 37.23$, $p < 0.001$), *TNFα* ($F_{1,77} = 4.80$, $p = 0.049$), *Stat4*

($F_{1,77}$ = 9.66, p = 0.006), *Stat6* ($F_{1,77}$ = 22.57, p < 0.001), *CMIP* ($F_{1,77}$ = 4.50, p = 0.049), and *TGFβ* ($F_{1,77}$ = 30.02, p < 0.001), with no difference in the expression of *Tbet* ($F_{1,77}$ = 0.64, p = 0.428) or *FoxP3* ($F_{1,77}$ = 3.89, p = 0.059).

Overall immune response levels differed between control and infected individuals (MANOVA $F_{2,77}$ = 1.87, p = 0.028), with infected individuals showing a general decrease in immune gene expression levels. When examining individual genes, expression levels of *TNFα* ($F_{2,77}$ = 8.19, p = 0.005), *Stat4* ($F_{2,77}$ = 4.48, p = 0.039), *Stat6* ($F_{2,77}$ = 3.91, p = 0.048) and *TGFβ* ($F_{2,77}$ = 6.02, p = 0.015) differed between control and infected individuals (Fig. 3), whilst expression levels of *IL-1β* ($F_{2,77}$ = 1.33, p = 0.291), *Tbet* ($F_{2,77}$ = 2.64, p = 0.124), *CMIP* ($F_{2,77}$ = 1.25, p = 0.291) and *FoxP3* ($F_{2,77}$ = 1.92, p = 0.205) did not. Expression levels of *TNFα* where lower in Uist parasite (Tukey p < 0.001) and Nott parasite (Tukey p = 0.036) infected fish than in controls, but did not differ between the two infection types (Tukey p = 0.253). For *Stat4* expression, Uist parasite infected fish had lower expression than Nott infected (Tukey p = 0.026) or control fish (Tukey p = 0.038), but there was no difference between Nott infected and controls (Tukey p = 0.999). Uist parasite infected fish having lower expression levels of *Stat6* than Nott infected (Tukey p = 0.039) or control (Tukey p = 0.008) fish, but there was no difference between Nott infected and control fish (Tukey p = 0.731). Fish infected with Uist parasites had lower TGFβ expression levels than Nott infected (Tukey p = 0.039) or control (Tukey p = 0.008) fish, but there was no difference between Nott infected and control fish (Tukey p = 0.731).

No significant interaction terms were found in the MANOVA of overall expression levels, but the effect of treatment on *TNFα* expression levels varied between fish origin (Fig. 4, $F_{2,63}$ = 3.17, p = 0.048), and there was also an effect of treatment on *Tbet* expression levels that varied between fish and with sample day (Fig. 5, $F_{4,63}$ = 2.57, p = 0.046).

3.3. Local adaptation of immune measures

There was no significant overall interaction between fish origin and parasite origin ($F_{1,54}$ = 0.57, p = 7.99) in the multivariate analysis of overall immune expression, and the interaction was not significant for any of the single gene comparisons, indicating that there is no evidence for local adaptation in the host immune response. There were overall expression differences between fish from Uist and Nott ($F_{1,54}$ = 5.48, p < 0.001), and as observed in the previous comparison, Obse fish had higher expression levels of *IL-1β*, *Stat4*, *Stat6* and *TGFβ*. There was no overall expression difference with parasite treatment ($F_{1,54}$ = 2.00, p = 0.067), although the expression levels of *Stat4* ($F_{1,54}$ = 6.81, p = 0.048) and *TGFβ* ($F_{1,54}$ = 6.84, p = 0.048) were higher in fish infected with Nott parasites when each gene was examined separately (For full results of single gene comparisons, see supplementary results).

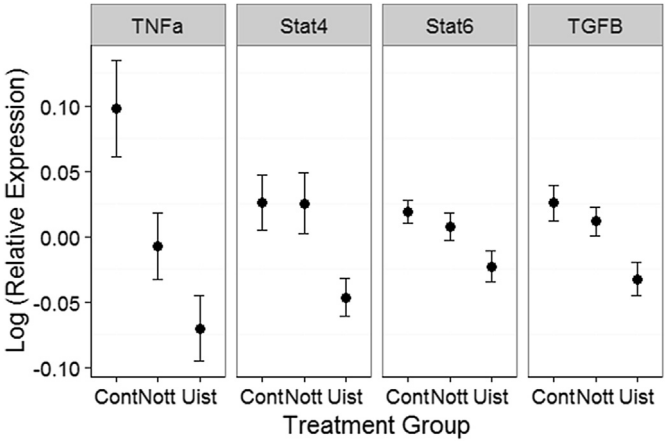

Fig. 3. Relative gene expression levels (Mean ± SE) of TNFα, Stat4, Stat6 and TGFβ differ between treatment groups in a controlled infection experiment. Expression values have been standardized against the mean of each fish source population for display, to control for underlying expression differences. Individual fish were either uninfected controls ('Cont'), infected with *G. gasterostei* from Nottingham ('Nott') or infected with *G. arcuatus* from North Uist ('Uist').

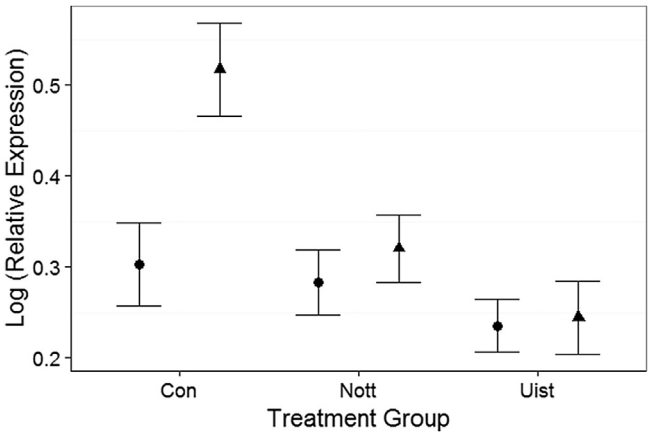

Fig. 4. The effect of treatment on *TNFα* relative expression levels varies between source fish population in a controlled infection experiment. Individual fish from Nottingham (●) or North Uist (▲) were either uninfected controls ('Con'), infected with *G. gasterostei* from Nottingham ('Nott') or infected with *G. arcuatus* from North Uist ('Uist'). Control fish show underlying differences in gene expression levels, whilst Uist fish show a decrease in *TNFα* expression in response to infection and Nott fish do not.

4. Discussion

In this study, we performed a reciprocal cross infection experiment with two host-parasite pairs to examine the type of immune response *Gyrodactylus* infection elicits in stickleback, and to quantify local adaptation of infection dynamics and the immune

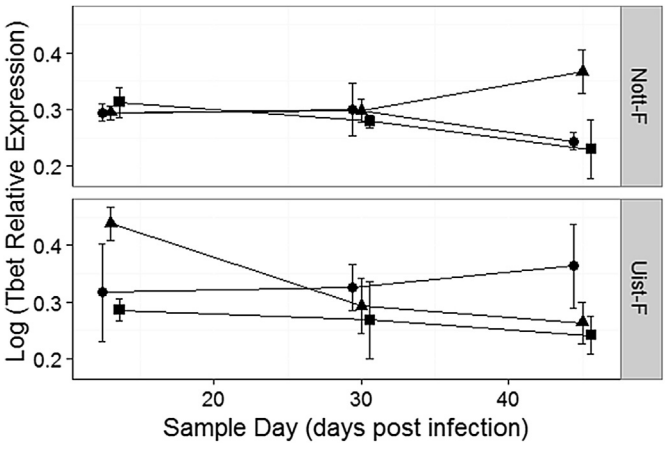

Fig. 5. The effect of treatment groups on Tbet relative expression levels varies with fish source and across sample days. The response of fish from Nottingham ('Nott-F') is shown in the top graph, whilst fish from North Uist ('Uist-F') are in the bottom graph. Individual fish were left as untreated controls (●), infected with *G. gasterostei* from Nottingham (▲), or infected with *G. arcuatus* from North Uist (■). Fish were sampled at 14, 30 and 45 days post infection (dpi). Uist fish have higher Tbet expression when infected with Nottingham parasites at 14 dpi, and lower expression when infected with either parasite at 45 dpi. In Nottingham fish, we see higher expression in Nottingham parasite infections at 45 dpi.

response. Previous studies of stickleback and *Gyrodactylus* have found mixed evidence of the occurrence of local adaptation [36,46–48]. Here, we find large differences in virulence between the two species, irrespective of whether they infect a sympatric or allopatric host type, and even though both parasite species have very similar modes of infection. *G. gasterostei* from Nottingham had significantly higher peak and mean abundance than *G. arcuatus* from North Uist. Burdens of *G. arcuatus* were lower on the sympatric host, suggesting that Uist fish have resistance to their local parasite strain. This was reflected in the effect size of local adaptation (E) values, which shows the greater degree of maladaptation of North Uist parasites to North Uist fish than Nottingham parasites to Nottingham fish (or an adaptation of hosts to local parasites). This reflects the general pattern seen in guppies, where parasite virulence varies with strain and resistance varies between host populations, but without extensive host-parasite local adaptation [56,57]. As evidence for local adaptation is still mixed, the reciprocal cross-infection approach employed here could be extended to include a larger number of host and parasite populations, giving a clearer understanding of the generality of host-parasite adaptation in this system.

There was no evidence of local adaptation in the immune response, as there was no significant interaction between host and parasite origin in immune gene expression levels of infected fish. Whilst immune gene expression levels may not change with host:parasite combination, we did detect changes in expression levels in response to infection. There were large underlying differences in immune gene expression levels between fish derived from different populations, supporting previous work showing population level differences in underlying immune function [39]. Above these underlying differences we found that infection with either parasite caused a decrease in *TNFα* expression levels. Closer examination indicated this was the result of a decrease in expression levels in fish from North Uist not seen in fish from Nottingham. Infection with *G. arcuatus* caused additional decreases in expression levels of *Stat4*, *Stat6* and *TGFβ* that were not seen during infection with *G. gasterostei*. So whilst we did not find an overall pattern of local adaptation in the immune response, we can see that both host and parasite origins drive differing immune response patterns in the host.

The patterns of expression observed differ from those seen in other fish species during *Gyrodactylus* infection, suggesting that different resistance mechanisms may be acting in stickleback. Infection studies in guppies found evidence for both innate and acquired responses to infection [58], although this study did not measure the immune response directly. Here we find changes in markers of the innate, Th1-type adaptive, Th2-type adaptive and regulatory response pathways. Expression levels of IL-1β and TNFα increase in the skin of *Gyrodactylus* infected rainbow trout [59,60] and Atlantic salmon [61]. Here, a decrease in TNFα expression occurred during infection with both parasites, and in *Stat4*, *Stat6* and *TGFβ* levels in fish infected with the less virulent parasite. Although a decrease in immune gene expression levels with infection is counterintuitive, they correspond to an apparently high level of resistance in this instance.

Past studies of the immune response to *Gyrodactylus* infection have concentrated primarily on measuring the immune response in the skin at the site of infection. Here we show that systemic responses to infection are detectable in the spleen, a central immunological tissue in fish. A decrease in expression levels in a major immunological tissue could correspond to expression levels increasing in other immunological tissues, or at the site of infection. Fish immune systems are relatively complex, and responses often compartmentalised, thus the decrease in expression observed here in the spleens of infected fish could indicate the diversion of immune resources to other immunological tissues or to the site of infection. Whilst we chose to focus on a single immune tissue in this study, sampling multiple tissue types during infection is required to better understand the changes seen here.

In studies in wild three-spined stickleback using the same set of immune assays, infection with *Gyrodactylus* tends to correlate with increases in innate expression and decreases in regulatory gene expression levels [39]. In the wild, individuals are likely to be faced by multiple challenges, and trade-offs between costly immune function and other necessary activities will be required [62,63]. Artificial infection experiments, where individuals are kept in benign conditions, struggle to replicate the variation associated with natural conditions [62], but do allow us to isolate the factor in which we are interested. Whilst the changes observed here can be directly attributed to infection, the difference in pattern seen when compared to data from wild individuals may represent the difference between healthy individuals able to cope with infection and individuals facing multiple challenges and a wide range of energetic demands. Furthermore, the direction of causality of infection is not clear in wild individuals, as changes in immune function could be a response to infection, or may themselves have made an individual more susceptible to infection. Infection with *Gyrodactylus* can also increase the chance of secondary infections [42], possibly as a result of changes in immune system function. Controlled infection studies involving multiple parasite species are possible, and represent a next step to better understand how changes in response to one infection affect the ability of individuals to respond to subsequent challenge.

5. Conclusions

We found large differences in the virulence of two closely related parasite species, *G. gasterostei* and *G. arcuatus*. Infection with both parasite elicited changes in the innate immune response, whilst infection with *G. arcuatus* also elicited changes in the adaptive immune response. As *G. arcuatus* was the less virulent species, this may represent the marker of a possible resistance mechanism. There was evidence of differential expression of the innate and Th1-type adaptive response, dependent upon host, parasite and time, which may represent local adaptation of the immune response. Differences between patterns of expression observed in the wild and the laboratory demonstrate the importance of combining both approaches. The stickleback-*Gyrodactylus* system represents an ideal system in which to advance our understanding of host-parasite local adaptation and the function of the immune response in a natural setting.

Author contributions

SR, JEB and ADCM designed the study and contributed to this manuscript. SR performed the infection experiment, laboratory work and data analysis.

Acknowledgments

We thank the MacColl lab group for their assistance in collecting fish in the field, Ann Lowe and Alan Crampton for fish husbandry, and Muayad Mahmud for assistance in performing the infection experiment. This work was funded by a NERC studentship (NE/K501311/1) awarded to SR.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2016.11.058

References

[1] P.W. Price, Evolutionary Biology of Parasites, Princeton University Press, Princeton, NJ, 1980.
[2] R. Poulin, S. Morand, The diversity of parasites, Q. Rev. Biol. 75 (2000) 277–293.
[3] P.J. Hudson, A.P. Dobson, D. Newborn, Prevention of population cycles by parasite removal, Science 282 (1998) 2256–2258.
[4] F. Thomas, S. Adamo, J. Moore, Parasitic manipulation: where are we and where should we go? Behav. Process. 68 (2005) 185–199.
[5] P. Agnew, J.C. Koella, Y. Michalakis, Host life history responses to parasitism, Microbes Infect. 2 (2000) 891–896.
[6] N. Perrin, P. Christe, H. Richner, On host life-history response to parasitism, Oikos 75 (1996) 317–320.
[7] R. Anderson, D. Gordon, Processes influencing the distribution of parasite numbers within host populations with special emphasis on parasite-induced host mortalities, Parasitology 85 (1982) 373–398.
[8] R.M. Anderson, R.M. May, Regulation and stability of host-parasite population interactions: I. regulatory processes, J. animal Ecol. 45 (1978) 219–247.
[9] J. Antonovics, M. Boots, D. Ebert, B. Koskella, M. Poss, B.M. Sadd, The origin of specificity by means of natural selection: evolved and nonhost resistance in host–pathogen interactions, Evolution 67 (2013) 1–9.
[10] C.M. Lively, Adaptation by a parasitic trematode to local populations of its snail host, Evolution 43 (1989) 1663–1671.
[11] S. Ward, Assessing functional explanations of host-specificity, Am. Nat. 139 (1992) 883–891.
[12] D. Ebert, Virulence and local adaptation of a horizontally transmitted parasite, Science 265 (1994) 1084.
[13] M.F. Dybdahl, A. Storfer, Parasite local adaptation: red queen versus suicide king, Trends Ecol. Evol. 18 (2003) 523–530.
[14] M.A. Greischar, B. Koskella, A synthesis of experimental work on parasite local adaptation, Ecol. Lett. 10 (2007) 418–434.
[15] O. Kaltz, J.A. Shykoff, Local adaptation in host–parasite systems, Heredity 81 (1998) 361–370.
[16] L. Viljakainen, J.D. Evans, M. Hasselmann, O. Rueppell, S. Tingek, P. Pamilo, Rapid evolution of immune proteins in social insects, Mol. Biol. Evol. 26 (2009) 1791–1801.
[17] B. Lazzaro, A. Clark, Rapid Evolution of Innate Immune Response Genes, Oxford University Press, Oxford, 2012.
[18] P. Tiffin, D.A. Moeller, Molecular evolution of plant immune system genes, Trends Genet. 22 (2006) 662–670.
[19] S.J. McTaggart, D.J. Obbard, C. Conlon, T.J. Little, Immune genes undergo more adaptive evolution than non-immune system genes in *Daphnia pulex*, BMC Evol. Biol. 12 (2012) 63.
[20] B.P. Lazzaro, T.J. Little, Immunity in a variable world, Philosophical Trans. R. Soc. B-Biological Sci. 364 (2009) 15–26.
[21] S.M. Barribeau, B.M. Sadd, L. Du Plessis, P. Schmid-Hempel, Gene expression differences underlying genotype-by-genotype specificity in a host–parasite system, Proc. Natl. Acad. Sci. 111 (2014) 3496–3501.
[22] C. Bonneaud, S.L. Balenger, A.F. Russell, J. Zhang, G.E. Hill, S.V. Edwards, Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird, Proc. Natl. Acad. Sci. 108 (2011) 7866–7871.
[23] T.B. Sackton, B.P. Lazzaro, A.G. Clark, Genotype and gene expression associations with immune function in Drosophila, PLoS Genet. 6 (2010) e1000797.
[24] F.S. Brunner, P. Schmid-Hempel, S.M. Barribeau, Immune gene expression in *Bombus terrestris*: signatures of infection despite strong variation among populations, colonies, and sister workers, PloS one 8 (2013) e68181.
[25] K.M. Wegner, M. Kalbe, G. Rauch, J. Kurtz, H. Schaschl, T.B.H. Reusch, Genetic variation in MHC class II expression and interactions with MHC sequence polymorphism in three-spined sticklebacks, Mol. Ecol. 15 (2006) 1153–1164.
[26] S.M. Barribeau, P. Schmid-Hempel, Qualitatively different immune response of the bumblebee host, *Bombus terrestris*, to infection by different genotypes of the trypanosome gut parasite, *Crithidia bombi*, Infect. Genet. Evol. 20 (2013) 249–256.
[27] C. Riddell, S. Adams, P. Schmid-Hempel, E.B. Mallon, Differential expression of immune defences is associated with specific host-parasite interactions in insects, PLoS One 4 (2009) e7621.
[28] D. Haase, J.K. Rieger, A. Witten, M. Stoll, E. Bornberg-Bauer, M. Kalbe, et al., Specific gene expression responses to parasite genotypes reveal redundancy of innate immunity in vertebrates, PLoS ONE 9 (2014) e108001.
[29] A.M. Bell, S.A. Foster, The Evolutionary Ecology of the Threespine Stickleback, Oxford University Press, Oxford, 1994.
[30] J.S. McKinnon, S. Mori, B.K. Blackman, L. David, D.M. Kingsley, L. Jamieson, et al., Evidence for ecology's role in speciation, Nature 429 (2004) 294–298.
[31] D. Schluter, The Ecology of Adaptive Radiation, Oxford University Press, Oxford, 2000.
[32] J.S. McKinnon, H.D. Rundle, Speciation in nature: the threespine stickleback model systems, Trends Ecol. Evol. 17 (2002) 480–488.
[33] J. De Roij, A.D.C. MacColl, Consistent differences in macroparasite community composition among populations of three-spined sticklebacks, *Gasterosteus aculeatus* L, Parasitology 11 (2012) 1478–1491.
[34] J.P. Scharsack, M. Kalbe, C. Harrod, G. Rauch, Habitat-specific adaptation of immune responses of stickleback (*Gasterosteus aculeatus*) lake and river ecotypes, P Roy. Soc. B-Biol Sci. 274 (2007) 1523–1532.
[35] R.E. Young, A.D.C. Maccoll, Spatial and temporal variation in macroparasite communities of three-spined stickleback, Parasitology (2016) 1–14.
[36] J. De Roij, P.D. Harris, A.D.C. MacColl, Divergent resistance to a monogenean flatworm among three-spined stickleback populations, Funct. Ecol. 25 (2011) 217–226.
[37] M. Kalbe, J. Kurtz, Local differences in immunocompetence reflect resistance of sticklebacks against the eye fluke *Diplostomum pseudospathaceum*, Parasitology 132 (2006) 105–116.
[38] T.L. Lenz, C. Eizaguirre, B. Rotter, M. Kalbe, M. Milinski, Exploring local immunological adaptation of two stickleback ecotypes by experimental infection and transcriptome-wide digital gene expression analysis, Mol. Ecol. 22 (2013) 774–786.
[39] S. Robertson, J.E. Bradley, A.D.C. MacColl, Measuring the immune system of the three-spined stickleback - investigating natural variation by quantifying immune expression in the laboratory and the wild, Mol. Ecol. Resour. 16 (2016) 701–713.
[40] S. Robertson, J.E. Bradley, A.D.C. MacColl, Parallelism and divergence in immune responses: a comparison of expression levels in two lakes, Evol. Ecol. Res. 17 (2016) 1–16.
[41] B.O. Johnsen, A.J. Jenser, The *Gyrodactylus* story in Norway, Aquaculture 98 (1991) 289–302.
[42] D. Cone, P. Odense, Pathology of five species of Gyrodactylus nordmann, 1832 (monogenea), Can. J. Zoology 62 (1984) 1084–1088.
[43] E. Sterud, P. Harris, T. Bakke, The influence of *Gyrodactylus salaris* Malmberg, 1957 (Monogenea) on the epidermis of Atlantic salmon, *Salmo salar* L., and brook trout, *Salvelinus fontinalis* (Mitchill): experimental studies, J. Fish. Dis. 21 (1998) 257–263.
[44] P. Harris, Species of *Gyrodactylus* von Nordmann, 1832 (Monogenea: gyrodactylidae) from freshwater fishes in southern England, with a description of *Gyrodactylus rogatensis* sp. nov. from the bullhead *Cottus gobio* L, J. Nat. Hist. 19 (1985) 791–809.
[45] J. Raeymaekers, T. Huyse, H. Maelfait, B. Hellemans, F. Volckaert, Community structure, population structure and topographical specialisation of *Gyrodactylus* (Monogenea) ectoparasites living on sympatric stickleback species, Folia Parasit. 55 (2008) 187–196.
[46] J.A.M. Raeymaekers, M. Wegner, T. Huyse, F.A.M. Volckaert, Infection dynamics of the monogenean parasite *Gyrodactylus gasterostei* on sympatric and allopatric populations of the three-spined stickleback *Gasterosteus aculeatus*, Folia Parasit. 58 (2011) 27–34.
[47] M.A. Mahmud, PhD Thesis - Evolutionary Ecology of Virulence in a Fish Parasite, University of Nottingham, 2016.
[48] N. Konijnendijk, J. Raeymaekers, S. Vandeuren, L. Jacquemin, F.A. Volckaert, Testing for local adaptation in the *Gasterosteus-Gyrodactylus* host-parasite system, Evol. Ecol. Res. 15 (2013) 489–502.
[49] A. Zapata, B. Diez, T. Cejalvo, C. Gutierrez-de Frias, A. Cortes, Ontogeny of the immune system of fish, Fish Shellfish Immunol. 20 (2006) 126–136.
[50] K. Dheda, J.F. Huggett, J.S. Chang, L.U. Kim, S.A. Bustin, M.A. Johnson, et al., The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization, Anal. Biochem. 344 (2005) 141–143.
[51] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic acids Res. 29 (2001) e45.
[52] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, et al., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002). RESEARCH0034.
[53] R. Core Team, R: a Language and Environment for Statistical Computing, R Foundation for Statistical Programming, Vienna, Austria, 2014.
[54] M.S. Rosenberg, D.C. Adams, J. Gurevitch, MetaWin: Statistical Software for Meta-analysis: Sinauer Associates Sunderland, 2000.
[55] J.D. Hoeksema, S.E. Forde, A meta-analysis of factors affecting local adaptation between interacting species, Am. Nat. 171 (2008) 275–290.
[56] F. Pérez-Jvostov, A.P. Hendry, G.F. Fussmann, M.E. Scott, Are host–parasite interactions influenced by adaptation to predators? A test with guppies and *Gyrodactylus* in experimental stream channels, Oecologia 170 (2012) 77–88.
[57] F. Pérez-Jvostov, A.P. Hendry, G.F. Fussmann, M.E. Scott, Testing for local host–parasite adaptation: an experiment with *Gyrodactylus* ectoparasites and guppy hosts, Int. J. Parasitol. 45 (2015) 409–417.
[58] J. Cable, C. Van Oosterhout, The role of innate and acquired resistance in two natural populations of guppies (*Poecilia reticulata*) infected with the ectoparasite *Gyrodactylus turnbulli*, Biol. J. Linn. Soc. 90 (2007) 647–655.
[59] T. Lindenstrom, K. Buchmann, C.J. Secombes, *Gyrodactylus derjavini* infection elicits IL-1 beta expression in rainbow trout skin, Fish. Shellfish Immun. 15 (2003) 107–115.
[60] T. Lindenstrom, C.J. Secombes, K. Buchmann, Expression of immune response genes in rainbow trout skin induced by *Gyrodactylus derjavini* infections, Vet. Immunol. Immunop 97 (2004) 137–148.
[61] T. Lindenstrøm, J. Sigh, M. Dalgaard, K. Buchmann, Skin expression of IL-1β in East Atlantic salmon, *Salmo salar* L., highly susceptible to *Gyrodactylus salaris* infection is enhanced compared to a low susceptibility Baltic stock, J. Fish. Dis. 29 (2006) 123–128.
[62] A.B. Pedersen, S.A. Babayan, Wild immunology, Mol. Ecol. 20 (2011) 872–880.
[63] H. Schulenburg, J. Kurtz, Y. Moret, M.T. Siva-Jothy, Introduction. ecological immunology, Philosophical Trans. R. Soc. B Biol. Sci. 364 (2009) 3–14.