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Identification of genes responding to nematode infection in red grouse

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Abstract

The identification of genes involved in a host's response to parasite infection provides both a means for understanding the pathways involved in immune defence and a target for examining host–parasite co-evolution. Most studies rely on a candidate gene approach derived from model systems to identify gene targets of interest, and there have been a dearth of studies geared towards providing a holistic overview of immune response from natural populations. We carried out an experiment in a natural population of red grouse (*Lagopus lagopus scoticus*) to manipulate levels of *Trichostrongylus tenuis* parasite infection. The transcriptomic response of individuals was examined from standard cDNA and suppressive subtractive hybridization (SSH) libraries produced from gut, liver and spleen, enriching for genes expressed in response to *T. tenuis* infection. A total of 2209 and 3716 unique transcript sequences were identified from the cDNA and SSH libraries, respectively. Forty-five of these had Gene Ontology annotation associated with immune response. Some of these genes have previously been reported from laboratory-based studies of model species as important in immune response to gastrointestinal parasite infection; however, multiple novel genes were also identified. These may reveal novel pathways involved in the host response of grouse to *T. tenuis* and provide a resource that can be utilized as candidate genes in other species.

Keywords: candidate genes, immune response, *Lagopus lagopus scoticus*, suppressive subtractive hybridization, *Trichostrongylus tenuis*

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Introduction

A central goal in evolutionary biology is to understand the genetic basis of ecologically meaningful and adaptively important phenotypic traits (Ellegren & Sheldon 2008). Chronic infection by gastrointestinal (GI) nematodes is increasingly recognized as a fundamentally important trait that can affect both ecological and evolutionary processes operating in natural populations. From an evolutionary perspective, antagonistic relationships between parasite and host can generate and maintain genetic variation in natural populations through the continual turnover of co-evolving host and parasite lineages, and antagonistic pleiotropy within hosts (Clayton & Moore 1997). From an ecological perspective, host susceptibility to parasites is a major contributor to variance in fitness (survival and reproductive success) among individuals, and at a population level, parasites can regulate population size and influence population dynamics (Hudson *et al.* 1998; Albon *et al.* 2002; Redpath *et al.* 2006).

Identifying genes that affect variation in parasite load among individuals provides targets for marker-assisted selective breeding for genetically resistant hosts and also identifies those key gene regions that can be examined directly to determine the mode and tempo of selection operating during host–parasite co-evolution. However, determining the genetic basis of host susceptibility to GI nematodes is far from straightforward (Behnke *et al.* 2003). Nematode epidemiology is not described by the classical susceptible–infected–recovered pattern but instead is characterized by persistent chronic infection or re-infection leading to host morbidity throughout life. The impact this has on hosts will be dependent on both the levels of infection and the individual host condition. Parasite burden in natural populations is thus the result

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of a complex interplay between genetic background and nongenetic factors like age, condition, demography and environment (Wilson *et al.* 2002).

Such interactions question the utility of the various laboratory host–parasite models that have been developed to inform our understanding of factors influencing variation in parasite load in natural populations (Beraldi *et al.* 2007; Sieberts & Schadt 2007). The extent to which the genetic architecture of fitness traits in the laboratory reflects that of natural populations is controversial, primarily because in the laboratory, the very environmental factors that may shape or influence trait variation are reduced or controlled for, which will bias results (Calisi & Bentley 2009). It is also unclear the extent to which environmental variation may overwhelm the effects of individual genes of large effect in laboratory experiments (Calisi & Bentley 2009).

One way to identify genes involved in the response to infection in natural populations is to compare the transcriptomic profile of individuals that differ in their parasite burden (Piertney & Webster 2010). Here, we illustrate how such an approach can be useful for identifying components of the genetic basis of immune response of red grouse (*Lagopus lagopus scoticus*) to its major intestinal nematode *Trichostrongylus tenuis*.

The red grouse is an economically important gamebird species that is common in heather-dominated regions of northern and western UK. A considerable body of research has been directed towards understanding the individual- and population-level consequences of infection of red grouse by *T. tenuis*. It has been shown that infection has a major effect on host fitness, reducing female fecundity, breeding success and increasing mortality (Hudson *et al.* 1992). *T. tenuis* burden has also been implicated in driving the cyclic population dynamics that are characteristic of red grouse populations, with high burdens reducing female breeding success to a level that limits the number of young available for recruitment into the breeding population (Hudson *et al.* 1998; Redpath *et al.* 2006).

The strategy used here was to maximize the difference in parasite load between two groups of grouse sampled from a natural population, either using an anthelmintic to purge an individual's parasite load or controlled infection to enhance it. Biopsies from immunologically important organs were then taken from which mRNA transcript populations are isolated. Suppressive subtractive hybridization (SSH) libraries were generated from low vs. high parasite abundance birds. This involved suppressing common sequences from the high and low parasite treatment groups to enrich for transcripts that were upregulated in parasite-infected individuals and thus involved in the pathway responding to the parasite insult. BLAST and Gene Ontology (GO) analyses were then employed to infer the function of the genes identified, a process facilitated by taxonomic similarity of grouse to chicken for which considerable genomic resources are already available (Hillier *et al.* 2004). Standard cDNA libraries were produced from the same tissues to provide a broader genomic resource for identifying transcripts that may be upregulated in response to parasites.

Such a 'top-down' approach of combining SSH and cDNA libraries for characterizing the response of grouse to *T. tenuis* infection should identify those candidate genes that have previously been linked to immune response to GI nematode infection in other vertebrate groups (Behnke *et al.* 2003; Gause *et al.* 2003; Artis 2006), whilst in addition identifying new candidate genes hitherto unrecognized as important in natural populations.

Methods

Experimental manipulation of Trichostrongylus tenuis parasite burden

During spring 2006, at Edinglassie Estate (57°11.4'N; 3°6.2'W) in NE Scotland, ten male red grouse of reproductive age (>1 year old) were caught at night using standard lamping techniques (Hudson 1986; Mougeot *et al.* 2004). Birds were ringed and fitted with a radio-collar (TW3 necklace radio-tags, BioTrack) to allow subsequent recapture and identification and retained overnight in holding cages. Prior to release the following morning, faecal samples were collected from all individuals for the determination of parasite burden. Birds were randomly assigned to one of two treatment groups. 'Anthelmintic' birds (n = 5) were given a broad-spectrum anthelmintic (levamisole hydrochloride, Nilverm Gold, Schering-Plough Animal Health, Welwyn Garden City, UK), which has been previously shown to be very effective at purging grouse of their *T. tenuis* worms (Hudson 1986; Mougeot *et al.* 2003). 'Infected' birds (n = 5) were given an oral dose of *c.* 5000 *T. tenuis* infective L3 larvae, which had been previously reared in the laboratory according to Shaw (1988). This figure of 5000 larvae was chosen to represent a large infection for the site, but well within the range of infection seen in red grouse (0–30 000 worms per bird, Webster unpublished data).

Birds were recaptured 21 days later and killed by vertebral dislocation under Home Office license (UK). Immediately after death, liver, spleen and caecum biopsies were taken and placed in RNAlater (Ambion). Gut contents were collected and kept at 4 °C to ascertain parasite burden after experimental treatment. A 3-week time period was chosen for the experiment to allow time for both the experimental infection to develop in the 'Infected' birds and for the anthelmintic effects, and chronic response to parasites to have passed in the

'Anthelmintic' birds. We expect the 'Infected' birds to be responding to both a chronic and an emergent infection of *T. tenuis*, whereas the 'Anthelmintic' birds should have a substantially reduced response to these nematode parasites. The initiation of an immune response to the parasite treatment was confirmed by a decreased albumin/globulin ratio in the infected birds (40% decrease in Infected birds, 5% decrease in Anthelmintic-treated birds; data not shown).

Parasite abundance

T. tenuis worm burdens can be reliably estimated from red grouse by counting parasite eggs present in the caecal faeces using a modified McMaster technique (Moss *et al.* 1993; Seivwright *et al.* 2004). Egg counts were performed for each grouse after the first and second capture. Values were validated for post-treatment birds by direct counting of the number of worms present in the gut post-mortem (Seivwright *et al.* 2004).

T. tenuis worm burdens were examined from faecal egg counts by fitting generalized linear mixed models (GLMMIX) within SAS v. 9.1 using a Poisson error distribution and log function. Variance of the dependent variable does not exceed the sample mean (428.7 ± 367.3), which justify the use of Poisson distribution of errors in our analyses. 'Individual' was included as a random effect to account for repeated measures. After including 'recapture' (before vs. after treatment) and 'treatment' (Anthelmintic vs. Infected), and the 'recapture × treatment' interaction as fixed effects, tests for variable differences between treatment groups over time were carried out. When analysing parasite burdens before (estimated worm numbers) or after treatment (estimated worm numbers and direct worm counts, post-mortem), worm numbers per host were fitted to generalized linear models (GENMOD) using a Poisson distribution of errors. Sample sizes and therefore degrees of freedom of the analyses may change because it was not possible to collect faecal samples from all birds. All tests were two-tailed.

RNA extraction and cDNA synthesis

Total RNA was extracted from liver, spleen and caecum (10–20 mg) using Trizol (Invitrogen), followed by RNeasy clean-up (Qiagen) according to manufacturers' protocols and eluted in 30 μL RNase-free water (Ambion) with 1 μL SuperaseIn (Ambion) to inhibit RNase activity.

The concentration of each RNA sample was estimated using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc, DE, USA), and the quality was then checked using a 2100 Bioanalyser (Agilent Technologies, Wokingham, UK). RNA integrity number scores over 8.5 was used as the primary criterion that quality was sufficient for subsequent library construction. Synthesis of cDNA and amplification was performed using the SMART™ PCR cDNA synthesis kit (Clontech), using 1 μg of total RNA for each reaction, following the manufacturer's instructions.

SSH library construction

Suppressive subtractive hybridization libraries were produced for liver, spleen and caecum separately, using the PCR Select cDNA subtraction kit (Clontech) according to the manufacturer's protocol. For each tissue, SMART™ cDNA pooled from the 'Anthelmintic' grouse ($n = 5$) was used as the driver and cDNA pooled from the 'Infected' grouse ($n = 5$) was used as the tester. The procedure involved subtracting the cDNA transcripts that were common to both treatment groups from the pool, leaving an enriched library of transcripts that were upregulated in the tester group (Infected) compared to the driver (Anthelmintic). These sample sizes are comparable with other studies using the SSH approach for gene discovery in non-model systems (Matejusova *et al.* 2006; Chen *et al.* 2009). The libraries were amplified for storage using the TA Cloning® kit (Invitrogen). The ligation used 2 μL of secondary PCR product from the subtraction procedure with the pCR® 2.1 cloning vector and followed the manufacturer's protocol to transform into One Shot® TOP TEN cells. The libraries were grown overnight at 37 °C on LB agar plates supplemented with 100 μg/mL ampicillin and 80 μg/mL X-gal to enable blue-white screening.

cDNA library construction

Standard cDNA libraries for liver, spleen and caecum from pooled samples of both infected and uninfected birds ($n = 10$) were constructed using the SMART™ cDNA library kit (Clontech) following the manufacturer's protocol. Once the library had been amplified, the manufacturer's mass conversion protocol was used to convert the phagemids (λTriplEx) into plasmids (pTriplEx) for library storage. The converted clones were grown at 37 °C overnight. Individual colonies from both library types were hand-picked into 384-well plates containing 50 μL LB glycerol (10%), 100 μg/mL ampicillin. These were grown overnight at 37 °C, replicated and then stored at −80 °C.

Library amplification and sequencing

Libraries were amplified from their 384-well format using PCR in four 96-well plates. Each individual PCR included 10× Tricine buffer (500 mM KCl, 300 mM Tricine pH8, 20 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 μM of each

primer and 1.5 units of Biotaq (Bioline) in a total reaction volume of 70 μL. For the SSH libraries, the primers M13F (5′-GTAAAACGACGGCCAGTG-3′) and M13R (5′-GGA AACAGCTATGACCATG-3′) were used. For cDNA libraries, the primers 5′LD (5′-CTCGGGAAGCGCGC CATTGTGTTGGT-3′) and 3′LD (5′-ATACGACTCAC TATAGGGCGAATTGGCC-3′) were used. The PCR protocol was as follows: 95 °C for 2 min, followed by 44 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 150 s with a final extension step of 72 °C for 10 min. A 10 μL aliquot of each PCR was cleaned up using a standard ExoSAP procedure. All products were sequenced using Big Dye Chemistry in one direction only on an ABI 3730 Automated DNA sequencer (NERC Molecular Genetics Facility, University of Edinburgh) using the primers T7 (5′-TAATACGACTCACTATAGGG-3′) and PR2F3 (5′-CTCGGGAAGCGCGCCATTGT-3′) for the SSH and cDNA libraries, respectively. A total of six libraries were created; one SSH and one cDNA library each from liver, spleen and caecum tissues, respectively. A total of 1536 clones were picked from both the SSH and cDNA libraries for liver and spleen tissues, 2304 clones were picked from the caecum SSH library and 1152 from the caecum cDNA library, producing a total of 9600 clones (Table 1). Insert size ranged from 400–1500 bp for the SSH clones and from 400–3000 bp for standard cDNA libraries.

Bioinformatic analysis

The cDNA and SSH libraries were analysed separately. DNA sequence base-calling was performed using Phred 1.7, and vector sequences were masked by Cross_match (http://www.phrap.org/phredphrapconsed.html). Reads containing fewer than 40 high-quality bases after trimming vector segments were considered as low-quality reads and discarded. Any 5′ and 3′ regions with bases with a quality score <15 were discarded as low-quality regions of the reads. ESTs were defined as bad repeat ESTs if the content of any base is >80% of the sequence. A two-step clustering procedure was carried out using the sequence alignment program CAP3 (Huang & Madan 1999) to assemble ESTs first into groups with weak sequence similarity, and subsequently into putative unique sequences. The first round clustered ESTs with >40 bps overlap and 85% identity together, while in the second round, the sequences were subjected to more stringent clustering (>40 bps overlap and 90% identity) to yield the final group of sequences. Library redundancy scores were calculated as the number of original sequences divided by the total number of unique ESTs after clustering.

Table 1 Total number of clones picked from each of the suppressive subtractive hybridization (SSH) and standard cDNA libraries, and the number of unique contig sequences found after quality control and clustering

Library type tissue	SSH	cDNA	Total
Liver	1536	1536	3072
Spleen	1536	1536	3072
Caecum	2304	1152	3456
Total	5376	4424	9600
Unique ESTs	3716	2209	5925

DNA sequences were then interrogated via a BLAST priority search against a series of databases (in search order): UniProtKB/Swiss-Prot, unip_sw_53.2; *Gallus* gallus protein; *Gallus gallus* BBSCR-EST; nonredundant (nr); 5′UTR vertebrates; 3′UTR vertebrates and CDD (Altschul *et al*. 1997; Marchler-Bauer *et al*. 2002). The e-value cut-off of e^{-10} was used for all the searches, except those made against the UTR databases, where a cut-off of e^{-5} was applied. Gene Ontology (GO) annotation was assigned using the Blast hits against the Swissprot and *Gallus* peptide databases. Identifiers from these hits were used to retrieve GO annotation from the Uniprot (Consortium 2009) and BioMART websites (Haider *et al*. 2009). These annotations were then imported to an R-environment (http://www.r-project.org) where GO parental and ancestor terms were added to existing annotation for all the three hierarchies: molecular function, biological process and cellular component. To identify genes that could potentially be involved in grouse immune response to *T. tenuis*, the annotation was screened for the term GO:0006955 (immune response).

Results

Parasite burden

Before treatment, *Trichostrongylus tenuis* prevalence in the ten males was 80% with an abundance of 429 ± 366 SE worms. Parasite abundance was similar in each group prior to treatment (59.7 ± 40.3 and 663.4 ± 159.3 estimated worms for birds treated with anthelminthic and infected, respectively, GENMOD, $\chi^2_7 = 1.73$, $P = 0.103$). However, changes over time (between capture and recapture) in parasite abundance significantly differed between treatment groups (GLMMIX, interaction recapture*treatment, $F_{1,20} = 27.16$, $P < 0.001$). Post-treatment, the birds that had been infected had 781.5 ± 345.4 estimated worms, while birds treated with anthelmintic had 0 estimated worms. Specifically, in 'Anthelmintic' birds, estimated worm counts from caecal samples decreased significantly (GLMMIX, $F_{1,8} = 7.19$, $P = 0.027$). Estimated worm counts from caecal samples in 'Infected' birds were similar over time (GLMMIX, $F_{1,11} = 0.05$,

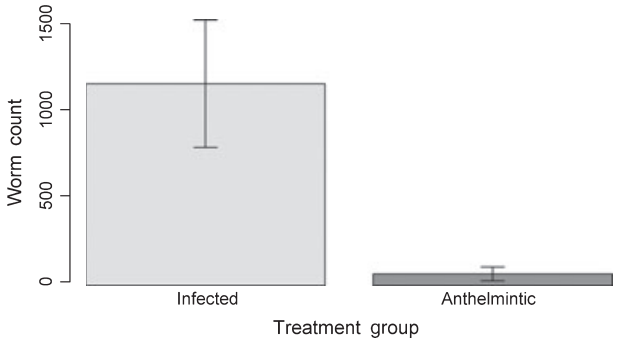

Fig. 1 Mean (±S.E.) worm burden at the end of the experiment for each treatment group.

$P = 0.892$). Worm counts post-mortem confirmed that worm burden was significantly higher in 'Infected' birds than in the 'Anthelmintic' group (Anthelmintic-treated birds: 1148 ± 364; dosed birds: 66 ± 40; GENMOD, $\chi^2_8 = 15.95$, $P < 0.001$, Fig. 1). As such, whilst infected birds did not have a significantly enhanced parasite burden as a consequence of manipulation, the experiment resulted in significantly different worm burdens between the infected and anthelmintic groups (Fig. 1).

SSH and cDNA library sequence analysis

From the 9600 clones picked, the final number of unique transcript sequences (hereafter termed contigs) following the 2-step clustering procedure was 3716 (515 with more than one overlapping EST and 3201 singletons) and 2209 (374 with more than one overlapping EST and 1835 singletons) for the SSH and cDNA libraries, respectively (Table 1). Library redundancy (ratio of number of clones to the number of nonredundant sequences) was low at 1.71 and 1.37 for the cDNA and SSH libraries, respectively. The high proportion of singletons found in both types of library suggests a high rate of discovery of novel genes. However, short sequences are common in the SSH libraries, and this may prevent the assembly of fragments from the same transcript, potentially contributing to the high number of singletons seen. BLAST searches assigned a total of 54% (1997 unique contigs) and 66% (1464 unique contigs) of the SSH and cDNA sequences, respectively, to annotated sequences.

Functional classification of library sequences based on Gene Ontology analysis

Of the unique set of contigs from the SSH and cDNA libraries, a total of 864 and 895 sequences respectively were found to have GO annotation based on scans against the NCBI nr database. As the distribution of GO terms was qualitatively similar among the libraries, these results were pooled (Fig. 2a–c). In the 'biological process' category, the largest proportions were assigned to cellular (31%) and metabolic processes (23%), and 0.9% of assignments with annotation were assigned to immune system process (Fig. 2a). Within 'molecular function', the category for binding predominated (48%), and a reasonably large proportion of assignments were made to antioxidant activity (7%). Cell (30%) and cell part (30%) dominated the 'cellular component' assignments (Fig. 2c).

A total of 106 contigs had GO annotation linked to the immune response term (GO:0006955), representing 45 different entries of the Swissprot database (Table 2). Of these, 18 were only found among SSH libraries, 23 in cDNA libraries and 4 in both (Table 2). A table

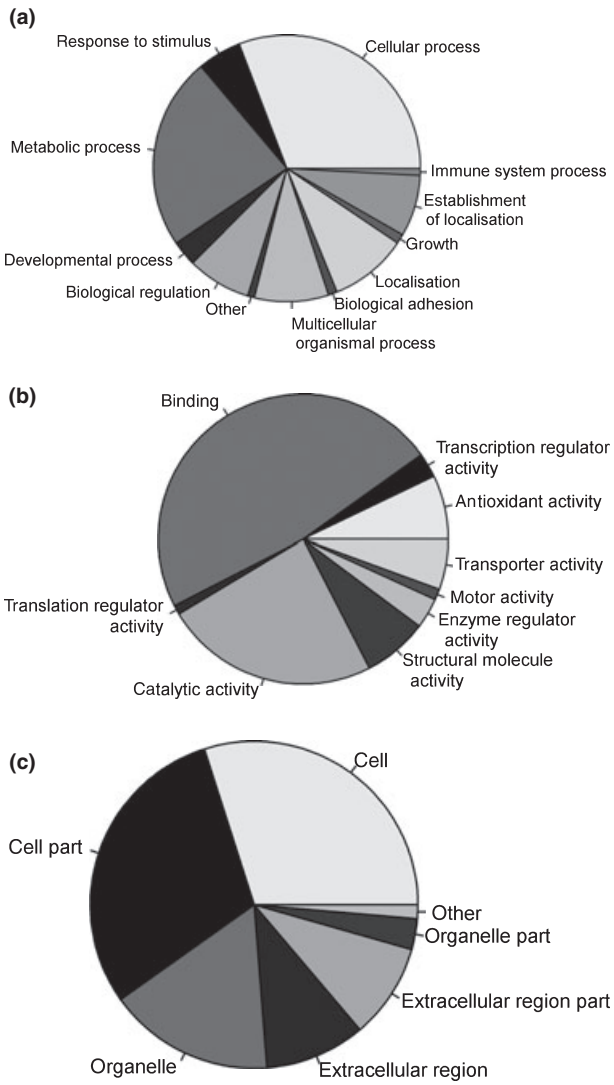

Fig. 2 Distribution of GO terms combined across libraries, from level 2 categories (limited to those with >0.8% contigs assigned) according to: (a) Biological Process, (b) Molecular Function and (c) Cellular Component. Note that individual contigs can have multiple GO annotations.

Table 2 Contigs that BLAST to Swissprot database sequences (cutoff e^{-10}), which have GO annotation that maps back to GO:0006955: immune response. Representing 106 ESTs, indicating the tissue (C = caecum, S = spleen, L = liver) and type of library

Description	Swissprot accession	Tissue	Library type	Number of ESTs
MHC class II antigen gamma chain; CD74	P04233	C, S, L	cDNA	10
Ferritin heavy chain; ferritin H subunit	P08267	C, S, L	Both	28
Annexin A11; Calcyclin-associated annexin-50	P27214	C, S	Both	3
Heme oxygenase 1; HO-1	P14791	S, L	cDNA	6
Complement C1r subcomponent precursor	Q5R1W3	S, L	Both	2
Chemokine receptor-like 1;	Q99788	S, L	SSH	2
TNF ligand superfamily member 13B; CD257antigen	Q9Y275	S, L	cDNA	2
TNF receptor superfamily member 11B precursor	O00300	C	SSH	1
IkappaB kinase complex-associated protein	O95163	C	SSH	1
Immunoglobulin J chain precursor	P01592	C	SSH	4
DNA mismatch repair protein MSH6; MutS-alpha 160	P54276	C	SSH	1
Angiomotin	Q4VCS5	C	cDNA	1
Lymphocyte antigen 75 precursor; CD205 antigen;	Q60767	C	SSH	2
Protein kinase C delta type; nPKC-delta	Q05655	C	SSH	1
Myoferlin; Fer-1-like protein 3	Q69ZN7	C	SSH	1
Zinc finger and BTB domain-containing protein 21	Q9ULJ3	C	cDNA	1
Cystatin-F precursor; Cystatin-7; Leukocystatin	O89098	S	cDNA	2
Ig lambda chain V-1 region precursor	P04210	S	cDNA	2
Complement factor H precursor; H factor 1	P08603	S	SSH	1
Complement recept type 2 precursor; CD21 antigen	P19070	S	cDNA	1
T-cell-specific surface glycoprotein CD28 precursor	P31043	S	cDNA	1
EBV-induced G-protein coupled receptor 2; EBI2	P32249	S	cDNA	1
Ras-related protein Rab-27; GTP-binding protein	P51159	S	SSH	1
60S ribosomal protein L24; L30	P83732	S	SSH	1
Lymphocyte cytosolic protein 2; SLP-76	Q13094	S	cDNA	1
CD59 glycoprotein precursor;	Q28216	S	cDNA	2
YTH domain family protein 2	Q4R5D9	S	cDNA	1
Ubiquitin-conjugating enzyme E2 N	Q5R7J6	S	cDNA	1
Complement component C7 precursor	Q5RAD0	S	cDNA	2
CD180 antigen precursor; Lymphocyte antigen 78	Q62192	S	cDNA	1
C-X-C chemokine receptor type 4; CD184 antigen;	Q7YS92	S	Both	2
C-C motif chemokine 19 precursor	Q99731	S	cDNA	4
Caspase recruitment domain-containing protein 11;	Q9BXL7	S	cDNA	1
Programmed cell death 1 ligand 1; CD274 antigen	Q9NZQ7	S	SSH	1
Complement factor I precursor	Q61129	L	cDNA	1
Complement factor H-related protein 5 precursor	Q9BXR6	L	SSH	1
Complement factor H precursor	P06909	L	SSH	1
Complement C5 precursor	P01031	L	cDNA	1
Complement C4 precursor	P08649	L	SSH	1
complement protein C1s precursor	P15156	L	SSH	1
Ovotransferrin precursor; Allergen Gal d 3	P02789	L	SSH	1
Ovoinhibitor precursor	P10184	L	cDNA	1
60S acidic ribosomal protein P0; L10E	P47826	L	cDNA	1
C4b-binding protein alpha chain precursor	Q63514	L	SSH	3
Beta-galactoside alpha-2,6-sialyltransferase 1;	Q92182	L	cDNA	2

summarizing EST sequences contributing to these candidates and a FASTA file of contig sequences can be found in Tables S1 and S2.

Discussion

The experimental manipulation of *Trichostrongylus tenuis* burden in the anthelmintic and infected treatment groups maximized differences in parasite burden among individuals such that a parasite-derived signature of transcriptomic response could be examined. A total of 8702 ESTs were submitted to GenBank (GW698221-GW706922) from the SSH and coding DNA libraries of red grouse spleen, liver and caecum. This represents a wealth of genomic information for a species where there were previously only 51 entries on the NCBI dbEST. A

total of 106 of our contigs had GO annotation that linked them to immune response (Table 2). As predicted, some of these have been associated with resistance or susceptibility to GI nematode infection in other species (e.g. Behnke *et al.* 2003), but many novel genes have also been identified.

Four candidates (Table 2) were identified from both SSH and cDNA libraries: (i) C-X-C chemokine receptor type 4 is involved in orchestrating the migration of lymphocytes (Stein & Nombela-Arrieta 2005). It has been shown to be upregulated in sheep resistant to mixed infections of GI nematodes (Diez-Tascon *et al.* 2005); (ii) Ferritin plays a protective role during periods of oxidative stress (Cairo *et al.* 1995; Koorts & Viljoen 2007). It has been shown that *T. tenuis* infection leads to increased oxidative stress in red grouse (Mougeot *et al.* 2009), which would suggest that candidates such as ferritin that may ameliorate these effects would be critical to protect from oxidative damage during helminth infection. In this case, ferritin was the most abundant candidate identified, as measured by the number of clones returned from the SSH and cDNA libraries and was recorded in all three tissues types examined; (iii) Annexin 11 belongs to a family of cellular proteins whose association with immune system is primarily via the inhibition of the inflammatory response (Rescher & Gerke 2004). (iv) Complement C1R is a precursor to C1, the first component of the classical pathway for complement—one of the cornerstones of the innate immune response. Finding these four candidates in both library types suggests that they are not only upregulated in response to *T. tenuis* in red grouse but also expressed at a high level to be found in the non-enriched cDNA libraries. A lack of further overlap in the libraries may be artefactual due the shorter sequences produced by the SSH process and subsequent reduced potential for gene identification for these libraries (Table 1). The immune response genes found solely in cDNA libraries, where samples from both treatments were pooled, may represent other responses common to both experimental groups. Although no other helminth parasites were recorded post-mortem, coccidian parasites were present in both groups. The immune response genes found only in cDNA libraries therefore may include genes involved in very different pathways than response to helminths.

Aside from ferritin, the only other candidate to be found in all three tissue types, although only from the cDNA libraries, was the major histocompatibility complex (MHC) class II antigen gamma chain, also known as CD74. CD74 has been shown to be upregulated in mice susceptible to *Trichuris muris* infection, relative to resistant strains (Datta *et al.* 2005). Moreover, it has also been found to respond to inflammation of the GI tract (Beswick & Reyes 2009). The occurrence of MHC in our libraries is not particularly surprising, given the central role of MHC in the recognition of nonself antigens. Indeed, multiple studies from across a broad range of taxa are describing relationships between specific MHC alelles and/or heterozygosity with nematode parasite burden (Oliver *et al.* 2009). That said, the target region examined in most of these studies is the peptide-binding site, variation at which dictates the range of pathogenic epitopes that can be processed within an individual. Genotypic variation at the CD74 site has rarely been investigated, however, and its absence from the SSH libraries may indicate that it is not upregulated specifically in response to *T. tenuis* infection.

Many of the candidates were found in only one or two of the tissue types assayed, illustrating the potential for tissue- and site-specific responses and highlighting that analysis of multiple tissue types is required to provide a more holistic view of the response to GI parasites. The immune candidates emerging from both the SSH and cDNA libraries from the liver are dominated by complement-related genes (Table 2) primarily from the classical pathway (e.g. C4b-binding protein alpha chain precursor, C1R), but regulators of the alternative pathway are also present (e.g. complement factor H). The alternative complement pathway has been shown to play an important role in the recognition of the helminth parasite *Nippostrongylus brasiliensis* in mice and recruitment of leucocytes during early infection, however the duration of this role is short-lived as the parasite larvae soon become resistant to the effects of the complement C3 (Giacomin *et al.* 2008).

In red grouse, previous studies have shown a positive correlation between spleen mass and *T. tenuis* infection (Mougeot & Redpath 2004), which suggests a role for the spleen in immune response to this ubiquitous parasite. Candidates identified from the spleen libraries are associated with T-cell response (e.g. CD surface molecules, Table 2) and B-cell proliferation (e.g. TNF ligand superfamily member 13B, Table 2), as well as chemokines (e.g. CXCR4) and immunoglobulins (e.g. immunoglobulin lambda chain V-1 region). TNF13B has been associated with susceptibility to helminth infection in humans (Williams-Blangero *et al.* 2002), and genotypic variants of this gene are correlated with specific antibody production to *Ascaris lumbricoides* (Acevedo *et al.* 2009). This may, therefore, be a promising candidate in the search for genotypic differences underlying phenotypic variation in susceptibility to *T. tenuis*.

The caecum was chosen as it is the site of *T. tenuis* infection and thus where any expulsion mechanisms are effected. The immune response candidates from the caecum provide evidence of both a type 2 cytokine response and also a type 1 inflammatory response. For example, the TNF receptor from the 11B superfamily (synonymous with osteoprotegerin) is known to be

upregulated in endothelial cells by IL13 and IL4 (Stein *et al.* 2008) and is thought to be an important immunoregulatory factor (Vidal *et al.* 2004). Both IL13 and IL4 are vital for a nematode-resistant type 2 cytokine response in model systems (Gause *et al.* 2003). However, the presence of immunoglobulins and a key dendritic cell receptor (CD205) suggest that a type 1 cytokine inflammatory response may also be underway in the caecum (Soares *et al.* 2007). As tissue from several grouse were mixed to create the libraries, it cannot be ascertained whether these type 1 and type 2 responses occurred in concert, or separately among individuals with variable resistance to infection. Nevertheless, the genes discovered here provide us with informative candidates with which to assess individual-level responses to *T. tenuis* infection.

The prevailing view has been that red grouse do not develop acquired immunity to *T. tenuis* (Shaw & Moss 1989). This is because red grouse do not appear capable of purging *T. tenuis* infection (Shaw & Moss 1989). Here, we have identified that the transcriptomic response of red grouse involves upregulation of genes from both the innate and the adaptive arms of the immune system, and as such whilst acquired immunity to parasites is not reached, an acquired response does occur. Other limiting factors such as nutrition may prevent grouse from purging *T. tenuis* after first exposure, and indeed, the cost of mounting an immune response to expel an infection has to be balanced with the needs for overall maintenance. It may be more common for natural selection to favour individuals that can tolerate infection and reduce the negative impact of GI parasites.

An underlying rationale of this study was to examine the extent to which the candidate genes identified from laboratory model systems are useful in predicting the genes involved in response to a chronic infection in a natural population of a nonmodel system (Behnke *et al.* 2003; Artis 2006). Some of the genes identified in model systems do play a role in this nonmodel system, reinforcing the utility of candidate gene approaches involving a 'bottom-up' strategy (Piertney & Webster 2010). Conversely, however, multiple genes previously not reported as important components of the immune response to GI nematodes have been identified, and these can now be added to the portfolio of candidate genes from which to examine the signatures of selection associated with host–parasite interaction and co-evolution. This type of approach is likely to become more popular for nonmodel species with the advent of parallel sequencing approaches that rapidly allow direct comparisons to be made between mRNA populations derived from individuals at high vs. low parasite burden and as such circumvent the need for library construction as detailed here.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 GenBank accession numbers for clones contributing to the immune response candidates in Table 2.

Table S2 FASTA file of contigs detailed in S1.

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