MOLECULAR ECOLOGY

Molecular Ecology (2010) doi: 10.1111/j.1365-294X.2010.04906.x

# Transcriptomic response of red grouse to gastro-intestinal nematode parasites and testosterone: implications for population dynamics

L. M. I. WEBSTER,* S. PATERSON,† F. MOUGEOT,‡§ J. MARTINEZ-PADILLA* and S. B. PIERTNEY*

*Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK, †School of Biological Sciences, University of Liverpool, Liverpool L69 7ZB, UK, ‡Estación Experimental de Zonas Áridas (EEZA, CSIC), Ctra. de Sacramento s/n La Cañada de San Urbano, 04120 Almería, Spain, §Instituto de Investigación en Recursos Cinegéticos (IREC; CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain

## Abstract

**A central issue in ecology is in understanding the relative influences of intrinsic and extrinsic effects on population regulation. Previous studies on the cyclic population dynamics of red grouse (*Lagopus lagopus scoticus*) have emphasized the destabilizing effects of either nematode parasites or territorial behaviour and aggression. The potential interacting effects of these processes, mediated through density-dependent, environmentally induced alterations of host immunocompetence influencing susceptibility to parasites have not been considered. Male red grouse at high density are more aggressive, associated with increased testosterone, which potentially could lead to reduced immunocompetence at a stage when parasites are most prevalent. This could depress individual condition, breeding performance and survival and thus drive or contribute to overall reductions in population size. Here, we characterize the transcriptomic response of grouse to nematode parasite infection and investigate how this is subsequently affected by testosterone, using a microarray approach contrasting red grouse with high and low parasite load at both high and low testosterone titre. A suite of 52 transcripts showed a significant level of up-regulation to either chronic parasite load or experimental parasite infection. Of these, 51 (98%) showed a reduced level of expression under conditions of high parasite load and high testosterone. The genes up-regulated by parasites and then down-regulated at high testosterone titre were not necessarily associated with immune response, as might be intuitively expected. The results are discussed in relation to the fitness and condition of individual red grouse and factors influencing the regulation of abundance in natural populations.**

*Keywords*: Host–parasite interactions, *Lagopus lagopus scoticus*, transcriptomics, *Trichostrongylus tenuis*

*Received 24 June 2010; revision received 2 September 2010; accepted 13 September 2010*

## Introduction

A major challenge in ecology is to identify the factors that regulate the abundance of animals in natural populations (Turchin 2003). Considerable effort in this area has been invested in species displaying cyclic population dynamics (Krebs *et al.* 1995; Moss & Watson 2001; Berryman 2002) primarily because the processes affecting abundance occur repeatedly at regular intervals and can have large effects over short-time periods.

Mathematical models have shown that population cycles can be generated when there is a delayed or lagged response between population density and the cause of changes in density (Turchin 2003). This can be mediated through extrinsic drivers such as climate,

Correspondence: Dr Stuart B. Piertney, Fax: +44 122 427 2396; E-mail: s.piertney@abdn.ac.uk

ArrayExpress accession number E-MEXP-2913

predators, parasites, food quality or food quantity (e.g. Krebs *et al.* 1995; Berryman 2002; Turchin 2003), or alternatively through self-regulating intrinsic factors such as territorial behaviour, senescence and genetic selection of individuals within the population (e.g. Matthiopoulos *et al.* 2003; Watson & Moss 2008).

In red grouse (*Lagopus lagopus scoticus*), both extrinsic and intrinsic mechanisms have been hypothesized as causing the 4–10 year cycles in abundance typically observed in natural populations (Watson & Moss 2008). The prevailing view was that these cycles are caused by a gastro-intestinal nematode parasite, the caecal threadworm *Trichostrongylus tenuis* (Hudson *et al.* 1992, 1998). The vast majority of grouse in natural populations are infected by *T. tenuis* (prevalence >97%), and with no effective acquired immunity to the parasite, individuals harbour worm burdens through life (Hudson 1986b; Shaw & Moss 1989). The mean adult worm burden within populations is negatively correlated with both population growth rate and female breeding performance (Hudson 1986a) such that population crashes are associated with high parasite load (Hudson *et al.* 1992). Models predict that parasite burden can cause population cycles if the reduction in host fecundity is large in relation to any impact on survival (Dobson & Hudson 1992).

The importance of parasites for driving grouse population cycles was reinforced by population level experiments that purported to show that reducing parasite burdens in grouse prevented cyclic population declines (Hudson *et al.* 1998). This experiment was, however, criticized for several reasons (Lambin *et al.* 1999; Watson & Moss 2008), and a subsequent experiment showed that whilst parasite reductions did influence productivity and density of grouse in autumn, they did not prevent cyclic population declines (Redpath *et al.* 2006). Parasites are therefore not sufficient to explain these cyclic dynamics, and an alternative driver of population cycles must operate (Redpath *et al.* 2006; Watson & Moss 2008).

An alternative explanation posits that red grouse population cycles are caused by delayed density-dependent changes in male aggressiveness influencing recruitment ('the territorial behaviour hypothesis' Moss & Watson 2001; Watson & Moss 2008). These lagged changes in aggressiveness are caused by changes in the kin structuring of male populations and differential aggressive behaviour between kin and nonkin ('the kinship hypothesis' Moss & Watson 2001; Watson & Moss 2008). Male red grouse are territorial and monogamous, so the number of territory holding males determines both male and female population sizes. Males obtain a territory in autumn through direct competition with previously established males and young cocks born in that year. The recruitment of males to the adult breeding population requires a territory, and at low population density, males facilitate the recruitment of related individuals into the population, which results in the formation of kin clusters that subsequently proliferate through positive feedback (MacColl *et al.* 2000). This continues until space becomes limiting, when birds become aggressive and recruitment is depressed to a level that cannot compensate for mortality. At this point, the population goes into decline and will only return to a positive growth rate once the density is sufficiently low to recommence the process of kin-facilitated recruitment (MacColl *et al.* 2000). Experimentally enhancing testosterone in grouse has been shown to increase aggressiveness, reduce recruitment and breeding density, change population trajectories from increasing to declining (Mougeot *et al.* 2003) and break down prevailing kin structure (Mougeot *et al.* 2005c). Moreover, the long-term changes in kin structure that occur in natural populations are sufficient to cause the variation in aggressiveness required to drive cyclic dynamics (Piertney *et al.* 2008). As such, the underlying intrinsic mechanism driving population dynamics is associated with changing levels of testosterone among years.

Traditionally, intrinsic and extrinsic drivers of grouse population cycles have been viewed as being mutually exclusive. More recently, however, the potential for interaction between intrinsic and extrinsic processes affecting red grouse demography and dynamics has begun to be explored. It has been shown that parasite load influences aggression (Mougeot *et al.* 2005b) and behaviour (Fox & Hudson 2001) and that conversely higher testosterone titre leads to greater parasite burdens (Seivwright *et al.* 2005), mediated through physiological and not behavioural processes (Mougeot *et al.* 2005d). However, the extent to which any interaction might influence longer term population dynamical processes has only been considered theoretically (New *et al.* 2009). Indeed, the best models capable of reproducing observed cyclic population dynamics appear to be those that include both aggression and parasites (New *et al.* 2009). In a commentary that was somewhat ahead of its time, Lochmiller (1996) constructed a conceptual framework that combined aspects of the immunocompetence handicap hypothesis (Folstad & Karter 1992), the behavioural–physiological hypothesis (Geller & Christian 1982) and the immunological dysfunction hypothesis (Mihok *et al.* 1985) to explain how density-dependent, environmentally induced alteration of host immunocompetence could be central to population regulation. It was suggested that the stress-induced immunosuppression would occur at high density leading to enhanced susceptibility to opportunistic parasites (Lochmiller 1996). This has been demonstrated in

dasyurid marsupials with humoral-mediated immunosuppression occurring as a consequence of stress at high density, which elevates levels of glucocorticoids and culminates in increased disease-related mortality (McDonald *et al.* 1986; Bradley 1987). Couched in terms of a cycling red grouse system, Lochmiller's concept would translate as increased aggressiveness at high population density leading to immunosuppression, mediated via testosterone directly acting to reduce immunocompetence (Folstad & Karter 1992) or by associated activation of the HPA axis and concomitant release of immunosuppressive glucocorticoids (Bortolotti *et al.* 2009). This would make individuals more susceptible to the effects of *T. tenuis*, which are already at their highest burdens as a result of the enhanced transmission at high host density. In other words, immediately before a population decline, grouse are attempting to combat their greatest immune insult when they are at their most immunocompromised. An inevitable consequence would be a reduction in fitness, which will decrease breeding success and increase mortality, both of which could precipitate or contribute to a population crash.

The primary aim of the current study is to examine the extent to which an individual grouse's response to high parasite load is affected by high testosterone levels associated with high population density and intense territorial contests, and in doing so test the central assumption of the Lochmiller (1996) hypothesis for population regulation: that immunosuppression is a mechanism that can influence fitness to a level that could influence population dynamic process. Hitherto, measures of immunocompetence in natural populations have taken an overly broad-brush approach such as using simple serological measures (e.g. haematocrit; overall gamma-globulin profile) indicative of immune response, or an individual's inflammatory response to a novel antigenic insult such phytohaemagglutinin or sheep red blood cells (Sorci *et al.* 1996; Christe *et al.* 1998; Camplani *et al.* 1999; Bonneaud *et al.* 2003). These have proven useful in some contexts, but have been widely debated as an accurate descriptor of immune response (e.g. Viney *et al.* 2005). Moreover, such assays are unlikely to encompass all arms of the vertebrate immune response sufficient to define overall immunocompetence. Here, we take an alternative, novel strategy to describe immunocompetence and identify immunosuppression through gene expression. We use a microarray-based approach to identify those genes a grouse up-regulates in response to a controlled immune insult from *T. tenuis*, then the extent to which this response is affected when mounted with a prevailing high testosterone level. The microarray utilized is a bespoke grouse array containing genes from suppressive subtracted hybridization (SSH) libraries constructed to identify genes up-regulated in response to high parasite load of *T. tenuis* and cDNA libraries of grouse from both high and low chronic infection of parasites (Webster *et al.* 2010). The mRNA populations used to interrogate this array are from a multifactorial experimental manipulation of grouse from natural populations to yield individuals with high or low parasite burdens under high and low testosterone titres. We predict that (i) grouse will show an up-regulation of immune genes in response to high parasite load, but that (ii) this response will be depressed under conditions of high parasite load and high testosterone levels. A demonstration that this is actually the case will provide a mechanism through which both intrinsic and extrinsic processes can operate in concert to influence population dynamics.

## Materials and methods

### *Field experiment*

A factorial field experiment was carried out on two grouse moors in autumn 2006—Catterick, England (54°20′N 1°52′W) and Edinglassie, Scotland (57°12′N 3°09′W). Specific details of the sites and experiments are given in Mougeot *et al.* (2009). In brief, 32 male red grouse were captured at night, fitted with a radiocollar (TW3 necklace, Biotrack) to allow recapture and housed overnight in pens to obtain caecal faeces for estimating parasite burden (Seivwright *et al.* 2004). All birds were dosed with 1 mL of anthelmintic (Nilverm Gold; Schering-Plough Animal Health, Welwyn Garden City, UK) to purge them of all helminth parasites. Approximately 2 weeks later, birds were re-caught and randomly allocated into one of four treatment groups: (i) Group TA were implanted with silastic tubes under the skin containing testosterone propionate (Sigma Aldrich, UK) to elevate testosterone levels; (ii) Group IA were given control silastic implants containing saline; (iii) Group TP were given testosterone implants and were also infected with approximately 5000 L3 *T. tenuis* larvae; and (iv) Group IP were given control silastic implants and infected with *T. tenuis* larvae (see Table 1). The nomenclature for each group therefore reflects high or low testosterone (T or I, respectively) and high or low parasites (P or A, respectively).

In addition to these four experimental groups, further two groups of birds were sampled: (v) Group TN ($n = 4$ males) were not manipulated for parasites (through either an initial anthelmintic treatment upon first capture or any subsequent experimental infection with L3 larvae) but were given silastic testosterone implants and (vi) Group IN ($n = 4$ males) that were just given

**Table 1** The groups involved in this experiment, defined by parasite and testosterone treatments. In addition, details of the number of birds involved in the field experiment stage, those whose RNA was of sufficient quality for microarray work and the total number array hybridizations involving each group are provided

| Experimental group | Testosterone treatment | Parasite treatment | N Field | N Microarray | Total arrays |
|---|---|---|---|---|---|
| IA | Control | Anthelmintic | 9 | 3 | 9 |
| IP | Control | Experimental infection (acute) | 7 | 6 | 18 |
| IN | Control | Natural infection (chronic) | 4 | 3 | 13 |
| TA | Enhanced | Anthelmintic | 9 | 4 | 17 |
| TP | Enhanced | Experimental infection (acute) | 7 | 7 | 19 |
| TN | Enhanced | Natural infection (chronic) | 4 | 4 | 10 |
| | | Total | 40 | 27 | |

control silastic implants (Table 1). As such, groups TP and IP were designed to reflect an acute, emerging infection, whereas groups TN and IN reflect longer term chronic parasite infection, in contrast to control groups TA and IA with purged parasite loads. Details of all six treatment groups can be found in Table 1.

On final capture (3–4 weeks following initial parasite and testosterone treatments), birds were killed by vertebral dislocation and dissected within 2 min of death to obtain samples of approximately 500 mg caecal tissue (proximal end), which were stored in RNAlater (Ambion, UK). The remainder of the caecum was stored at 4 °C, to be used to determine counts of *T. tenuis* burden for each bird using standard worm counting methods (Seivwright *et al.* 2004).

### *Microarray construction*

A bespoke red grouse microarray was constructed based on 9600 clones from suppressive subtractive hybridization (SSH) and standard cDNA libraries (Webster *et al*. 2010). Polymerase chain reaction (PCR) amplicons were derived from each clone under the following reaction conditions: 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, London, UK) with 1 μL of suspended cells in a total reaction volume of 70 μL. The primers M13F (5′-GTAAAACGACGGCCAGTG-3′) and M13R (5′-GGAAACAGCTATGACCATG-3′) and 5′LD (5′-CTCGGGAAGCGCGCCATTGTGTTGGT-3′) and 3′LD (5′-ATACGACTCACTATAGGGCGAATTGGCC-3′) were used for the SSH and cDNA libraries, respectively. 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 product was dried and resuspended in 12 μL spotting buffer (50% glycerol, 0.05% Triton X) prior to printing, in duplicate, onto GAPS II-coated glass slides (Corning Inc, NY, USA) using an AJ100 Inkjet Microarray Spotter (Arrayjet Limited, Dalkeith, Scotland).

The 9600 clones represented on the array were clustered into a subset of 5925 unique transcripts, hereafter termed 'contigs' (Webster *et al*. 2010). A contig can be defined as a unique transcript that represents either a single clone sequence or the consensus sequence from a set of overlapping sequences from the clone libraries.

### *Microarray hybridization*

For each experimental grouse from groups 1–6, total RNA was extracted from the caecum (10–20 mg) using the Trizol-PLUS kit (Invitrogen, Paisley, UK), according to manufacturers' protocols and eluted in 30 μL RNase-free water with 1 μL SuperaseIn (Ambion, Warrington, UK) 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 then checked using a 2100 Bioanalyser (Agilent Technologies, Wokingham, UK). Two hundred nanograms of RNA from samples with RIN > 8.5 were then amplified using the TargetAmp 1-Round aminoallyl-aRNA amplification Kit (Epicentre Biotechnologies, WI, USA) according to manufacturer's protocols, and re-quantified on the 2100 Bioanalyser.

Prior to microarray hybridization, up to 5 μg of aRNA was labelled with either Cy3 or Cy5 monoreactive dyes (GE Healthcare, Little Chalfont, UK). Unincorporated dyes were removed using RNeasy minelute spin columns (Qiagen, Crawley, UK). One microgram of Cy3 aRNA was combined with 1 μg Cy5 aRNA in a loop design to incorporate dye-swapping, whilst minimizing the number of arrays required and maximizing the power of treatment comparisons (Kerr 2003; Wit *et al*. 2005). The samples were fragmented by incubation at 70 °C for 15 min with fragmentation buffer (Ambion, Warrington, UK). Twenty-five microlitres of hybridization buffer (3× SSC, 0.1% SDS, 0.1 mg/mL BSA) was then added, and this mixture was denatured

at 95 °C for 1 min, then applied to the surface of the microarrays and hybridized in chambers (Genetix, New Milton, UK) at 50 °C for 18 h.

Following hybridization, the microarrays were washed once with 2× SSC, 0.1% SDS (50 °C) for 5 min, twice with 0.1× SSC, 0.1% SDS (50 °C) for 5 min, five times in 0.1× SSC (50 °C) for 1 min, and then once in 0.01× SSC (50 °C) for 1 min. Hybridized microarrays were scanned using a Scan Array Express (Perkin Elmer, Cambridge, UK) at 532 and 635 nm for Cy3 and Cy5, respectively. The BlueFuse program (BlueGnome, Cambridge, UK) was then used to extract the data from each spot on the array prior to data analysis.

### *Microarray data analysis*

Spot intensities for each cDNA clone were normalized within arrays by the print tip loess method and between arrays by the aquantile method in the limma package in R/Bioconducor (Gentleman *et al.* 2004; Smyth 2005). The magnitude and significance of treatment effects for each spot intensity were estimated using linear models. False discovery rates (FDRs) were estimated using the Benjamini–Hochberg method (Wit & McClure 2004). As in some cases multiple cDNA clones corresponded to a single contig, data for the 9600 cDNA clones were condensed into data for 5925 contigs by calculating the mean log fold change and the median *P*-value and FDR for each contig.

Contigs of interest were interrogated using BLAST against the Swissprot database (as of March 2010). The top candidate (cutoff E-10) was used for putative gene identification. Gene ontology (GO) analysis was performed within the BLAST2GO framework (Conesa *et al.* 2005), using Swissprot identifiers to establish GO terms for candidate genes.

### *Microarray validation by real-time PCR*

To assess the sensitivity of the microarray approach to detect differential expression, five target genes were selected for comparison with real-time PCR (Table 2). These genes included transcripts that, by microarray analysis, exhibited both strong differential expression (FTH1, HSP90A, CD74) and weak differential expression (LCP2, NOS2). Thus, these genes were selected to be representative of fold changes exhibited by transcripts across the microarray. Four reference genes were selected for normalization, two of which were strongly expressed on the array (EF1α, GAPDH), and two of which were weakly expressed (SDHA, TBP, Table 2). Several reference genes were used in order that a more accurate normalization factor could be calculated (Vandesompele *et al.* 2002) and also so that any gene affected by experimental treatments could be identified and eliminated (Dheda *et al.* 2005).

New RNA extracts were prepared from the caecum samples as previously described. Following extraction,

**Table 2** Details of genes and primers used for real-time quantitative PCR

| Symbol | Name | Amplicon length (bp) | Primers (5′-3′) |
|---|---|---|---|
| Targets for validation | | | |
| FTH1 | Ferritin, heavy polypeptide 1 | 64 | Forward -AAAAGAATGACCCACACCTGTGT |
| | | | Reverse -GGCTTTCACCTGCTCATCCA |
| HSP90A | Heat shock protein 90kDa alpha (cytosolic), class A member 1 | 63 | Forward -CACGTCTACTACATTACTGGTGAGACAA |
| | | | Reverse -GGCGCTCCACAAAAGCA |
| CD74 | Major histocompatibility complex, class II invariant chain | 79 | Forward -ATGGCGATGTCCATGATGAA |
| | | | Reverse -TCCATGGGCGTGTTACCAA |
| NOS2 | Nitric oxide synthase 2, inducible | 79 | Forward -CAATATGTTCTCCAGGCAGGTA |
| | | | Reverse -TCCAGTCGTGTTGCCATTCTAG |
| LCP2 | Lymphocyte cytosolic protein 2 | 87 | Forward -AAGAGGATTTTTCTTCTGTAGCACACA |
| | | | Reverse -CTTGAACCCCTGTCTTTTCCAT |
| Reference genes | | | |
| EF1A | Elongation factor 1 alpha | 62 | Forward -GATTCCTGGCAAACCCATGT |
| | | | Reverse -CAAAACGACCCAGAGGAGGAT |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | 63 | Forward -CCCCCAACGTCTCTGTTGTT |
| | | | Reverse -TGTCATCATATTTGGCTGGTTTCT |
| SDHA | Succinate dehydrogenase complex, subunit A | 59 | Forward -CTGCAAGCCTGGAGAACCA |
| | | | Reverse -CAGCTGATTCTTCACCAGCATT |
| TBP | TATA box binding protein | 62 | Forward -TGCAACACCGGCATCTGA |
| | | | Reverse -CCGTGGACACAATATTCTGTAGCT |

DNase treatment was performed using TurboDNAse (Ambion, Warrington, UK) and the RNA was precipitated and re-suspended in 20 μL nuclease-free water. For each sample, 2 μg of RNA was reverse transcribed to cDNA using Superscript III reverse transcriptase (Invitrogen, Paisley, UK) and 50 ng of random primers, following the manufacturers' protocol.

Primer Express™ (Applied Biosystems, Warrington, UK) was used to design oligonucleotides for real-time PCR (Table 2). All reactions were run in duplicate, and all reactions for the same target/reference were run on the same plate, including a fivefold dilution series for estimating primer efficiency. For transcripts with strong expression on the microarray, a 1:100 dilution of the cDNA was used as template, whereas for weakly expressed transcripts a 1:10 dilution was used. Each 20 μL reaction contained 10 μL FastStart SYBR Green Master (Roche, Welwyn Garden City, UK), 0.05 μM of each primer and 5 μL of cDNA, and on a Lightcycler 480 (Roche, Welwyn Garden City, UK) using the following program: 10 min at 95 °C, followed by 55 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s. The fluorescence produced by the SYBR green was recorded for each cycle at the extension step, and these raw values were exported from the Lightcycler software and analysed using the MoBPA package (Alvarez *et al.* 2007) within the R environment (R, Development, Core & Team 2007). MoBPA uses a model-based approach to estimate sample-specific amplification efficiency ($E$) values, thereby allowing for variation in efficiency among samples to calculate estimated starting template quantity ($T_o$). These $T_o$ values were then used to identify the best set of reference genes for normalization using geNORM (Vandesompele *et al.* 2002). From this, GAPDH and TBP were found to be unsuitable, so were subsequently excluded. Using the remaining two housekeeping genes (EF1A and SDHA), normalization factor (NF) was then calculated and applied to each $T_o$ value ($T_o$/NF), and all values are then re-scaled to make 1 the lowest expression value for any sample.

The patterns of expression for the target genes are in the same direction for both the microarray and real-time analyses (Fig. 1), with the exception of two discrepancies in levels of expression for CD74. For the IN versus IA contrast, microarray analysis indicated down-regulation, whereas quantitative PCR indicated up-regulation. Similarly, for the TN versus IA contrast, the microarray indicated down-regulation, whereas quantitative PCR detected no differential expression. Given that these CD74 fold change values were not significantly different from zero on the microarray analysis, these discrepancies may simply reflect the lower accuracy of estimates given the lower sensitivity of microarrays.

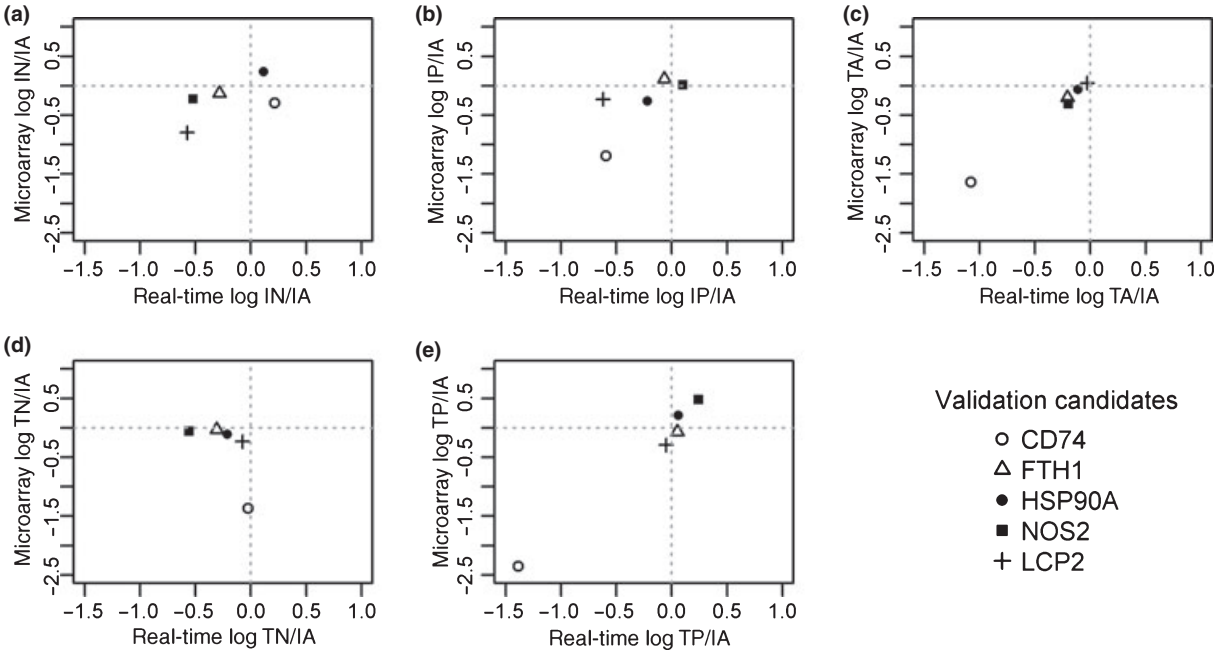


**Fig. 1** Comparison between Log2 fold changes for (a) IN versus IA, (b) IP versus IA, (c) TA versus IA, (d) TN versus IA and (e) TP versus IA comparisons as measured by microarray and real-time quantitative PCR. Symbols indicate the different target genes used for validation.

## Results

### *Experimental manipulations*

As has been demonstrated previously (see Mougeot *et al.* 2005a, 2009), implanting red grouse with silastic tubing containing testosterone propionate increases circulating testosterone titre. For the 20 birds used across the treatment groups where testosterone was experimentally enhanced with a silastic implant, testosterone levels changed from 0.85 (±0.29, SE) to 3.34 ng/mL (±0.34, SE) from before treatment to death. In spite of the increase, these values are well within the natural range of circulating testosterone observed in red grouse (Mougeot *et al.* 2005a, 2009). This is in contrast to the control implanted birds whose testosterone levels changed from 0.84 (±0.31, SE) to 1.00 ng/mL (±0.35, SE) over the same period.

Similarly, experimental parasite manipulation affected parasite levels (see Mougeot *et al.* 2009). Initial anthelmintic treatment of birds from the IP, IA, TP and TA groups reduced parasites loads from 599 worms (±131.7, SE) to 24 worms (±128.2, SE). Subsequent experimental reinfection of TP and IP birds increased parasite levels to 147 worms (±42.3, SE). The parasite levels of birds that were not given a parasite challenge (IA and TA) remained low at 29 worms (±6.9, SE).

### *Microarray analysis*

The microarray analysis was based on 27 grouse that yielded caecal RNA of sufficient quality and quantity (Table 1). As the arrays were hybridized in a loop design, this resulted in a total of 43 array hybridizations (Table 1; full details of all contrasts are available as Table S1, Supporting information).

A total of 52 contigs on the array were significantly up-regulated (>1-fold increase; FDR < 0.05), and 11 significantly down-regulated (>1-fold decrease, FDR < 0.05) by *T. tenuis* infection (groups IN or IP) when compared to controls that were purged of parasites (group IA). Of those up-regulated, 47 were significantly up-regulated in birds with chronic infection (group IN; mean fold increase = 1.87), 13 were significantly up-regulated in birds with experimental infection (group IP; mean fold increase = 1.29) and eight contigs were up-regulated in both IN and IP groups. These represent the genes expressed in response to chronic (IN) and emerging (IP) parasite infection (Table 3). To examine the effect of testosterone treatments on these up-regulated

**Table 3** Genes up-regulated by *Trichostrongylus tenuis* infection, either chronic (IN) or experimental (IP), compared to controls (IA) (bold values in the fold change columns are significant). A further 32 contigs were up-regulated by *T. tenuis* infection (6 in IN and IP, 23 in IN only, 3 in IP only) but with no significant BLAST hits for annotation

| Copies on array | Genbank ID* | Group Up-regulated | IN fold increase | IP fold increase | Description of top hit from Swissprot | Top hit Accession |
|---|---|---|---|---|---|---|
| 15 | GW705610 | IN and IP | **1.3012** | **1.4373** | Dynein light chain roadblock-type 1 | Q9NP97 |
| 4 | GW704812 | IN and IP | **1.0346** | **1.2273** | Ubiquitin | P63049 |
| 2 | GW704191 | IN | **1.1003** | 0.4188 | Beta-galactoside alpha- sialyltransferase 1 | Q92182 |
| 1 | GW703787 | IN | **2.4594** | 1.0593 | Ef-hand domain-containing family member a1 | Q8IYU8 |
| 11 | GW703383 | IN | **1.6218** | 0.2598 | Fibrinogen beta chain contains | Q02020 |
| 20 | GW703527 | IN | **1.2090** | 0.2414 | Fibrinogen gamma chain flags | P02680 |
| 1 | GW704306 | IN | **2.7734** | 1.1261 | Haloacid dehalogenase-like hydrolase domain-containing protein 1a | Q08623 |
| 1 | GW703599 | IN | **1.1903** | 0.3556 | Heat repeat-containing protein 1 | Q9H583 |
| 1 | GW706441 | IN | **1.6780** | −1.0648 | Interferon-induced transmembrane protein 2 | Q01629 |
| 1 | GW700690 | IN | **1.2254** | 0.6270 | Probable methionyl-trna synthetase | Q9SVN5 |
| 290 | GW703964 | IN | **1.9593** | 0.6548 | Serum albumin | P19121 |
| 1 | GW704102 | IN | **1.1836** | 0.1848 | Serum albumin | P19121 |
| 1 | GW704256 | IN | **2.1899** | 0.6748 | Serum albumin | P19121 |
| 1 | GW704099 | IN | **1.0550** | 0.2292 | Serum albumin | P19121 |
| 1 | GW704383 | IN | **2.4513** | 0.7314 | Serum albumin | P19121 |
| 1 | GW703310 | IN | **3.1362** | 1.2099 | Serum albumin | P19121 |
| 1 | GW703301 | IN | **1.7390** | 0.6887 | wd repeat and socs box-containing protein 1 | Q9Y6I7 |
| 1 | GW703611 | IN | **1.3707** | 0.2708 | n-acyl phosphatidylethanolamine phospholipase d | Q769K2 |
| 1 | GW706626 | IP | −0.0614 | **1.3266** | Haemoglobin subunit beta | P02113 |
| 1 | GW704704 | IP | 0.7779 | **1.1168** | Nucleolar complex protein 3 homologue | Q8VI84 |

*Where more than one clone was represented, an example single sequence ID is provided

genes, their level of expression was also determined in the corresponding testosterone + parasite treatments (TN or TP, Fig. 2). The mean fold change was 0.96 for TN birds and −0.46 for the TP birds. None of the 52 contigs showed a significant level of up-regulation in the relevant testosterone treatment (FDR $P > 0.05$). This demonstrates that genes up-regulated by *T. tenuis* infection are then down-regulated by testosterone (Fig. 2). At the individual gene level, all but one gene (accession GW702355) has a lower fold change in birds treated with testosterone under the same parasite conditions. The eleven contigs exhibiting down-regulation in response to parasite treatments (IN or IP) compared to controls (IA) exhibit similar down-regulation in testosterone-treated birds (TN or TP, data not shown).

Of the 52 contigs that were significantly up-regulated in response to parasite infection, 20 returned a BLAST hit to the Swissprot database (<E-10) to indicate gene orthology (Table 3). Several of the contigs returned the same putative gene, re-affirming that up-regulation was not spurious but reflected a true response to the treatments. Gene ontology (GO) analysis placed these genes into a range of biological process, cellular component and molecular function categories (Table S2, Supporting information). The proportions of GO Biological Process Level 2 descriptors for these 20 identified contigs are given in Fig. 3.

There were 14 contigs that showed a significant up- or down-regulation in the TA group compared to IA group (Table 4). These genes represent the response of grouse to high testosterone in the absence of *T. tenuis*. The mean fold change difference (compared to the IA group) of up- and down-regulated genes was 1.03 and −0.96, respectively. The gene identities of these contigs as ascertained by BLAST analysis are given in Table 4, and associated GO descriptors are given in Table S3, Supporting information.

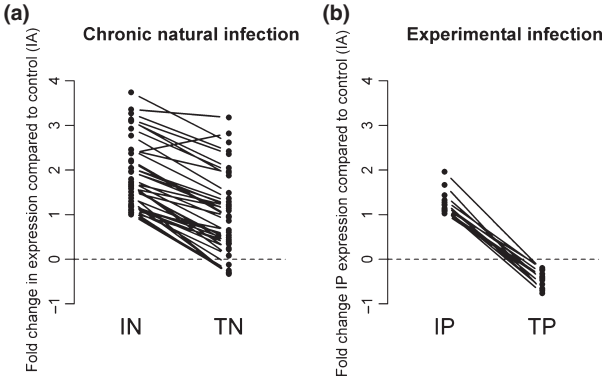


**Fig. 2** Fold change in levels of expression for transcripts that are significantly up-regulated in response to parasites, alongside the response of the same transcripts to parasite plus testosterone treatments. (a) Chronic, natural infection of *Trichostrongylus tenuis* (IN) and with elevated testosterone (TN), and (b) experimental infection with *T. tenuis* (IP) and with elevated testosterone (TP). All fold change estimates are in relation to grouse with no parasites and normal testosterone levels (IA).

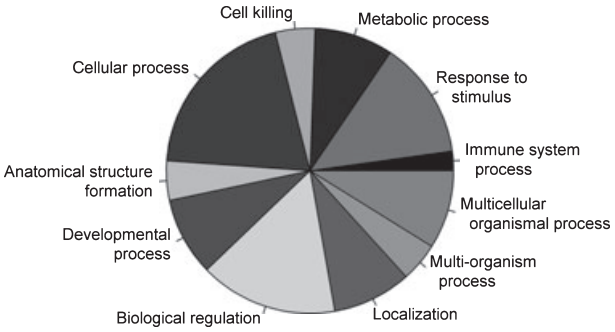


**Fig. 3** Pie diagram showing the proportions of Gene Ontology (GO) Biological Process Level 2 descriptors identified for the genes up-regulated in response to high *Trichostrongylus tenuis* burden (either IP or IN versus IA contrasts).

## Discussion

The experimental manipulations of parasite burden and testosterone in grouse from natural populations successfully maximized differences among the treatment groups (see also Bortolotti *et al.* 2009; Mougeot *et al.* 2009) and thus allowed the characterization of the transcriptomic response to chronic and emerging parasite burdens, high testosterone titre and a combination of the two. Congruent levels of gene expression of targeted candidate genes quantified using parallel microarray and real-time quantitative PCR indicates that the microarrays provide an accurate description of patterns of up- and down-regulation of genes to the treatments imposed on the birds. The salient finding of the microarray analysis was that the transcriptomic response a grouse would raise to an immune challenge by *T. tenuis* is not achieved under conditions of high testosterone titre.

A total of 52 contigs were significantly up-regulated in the IN versus IA (natural chronic infection levels versus parasite purged) contrast or the IP versus IA (parasite challenged versus parasite purged) contrast. The only differences between IN and IP treatment birds with IA individuals is higher parasite burden, either via natural infection or imposed infection through experimental infection with L3 *T. tenuis* larvae. Therefore, the up-regulation of these contigs must be in response to *T. tenuis* parasites. As such, this up-regulation represents the response a grouse mounts to deal with an enhanced parasite load. Of these contigs, 51/52 had a suppressed level of gene expression if the response to

**Table 4.** Gene expression in response to testosterone in the absence of *Trichostrongylus tenuis* parasites—Comparison of treatment group TA with control group IA.

| Number of clones | Genbank ID* | Fold Change | Swissprot | Swissprot accession |
|---|---|---|---|---|
| 1 | GW701906 | 1.144284 | No hit | |
| 2 | GW702715 | 1.143336 | Lipid phosphate phosphohydrolase 1 | O88956 |
| 1 | GW703224 | 0.794072 | No hit | |
| 1 | GW706822 | −0.66665 | Ectonucleoside triphosphate diphosphohydrolase 8 | O93295 |
| 1 | GW704564 | −0.68355 | Nicotinamide riboside kinase 2 | Q9D7C9 |
| 1 | GW704643 | −0.79934 | Glyceraldehyde-3-phosphate dehydrogenase | O57479 |
| 1 | GW706821 | −0.86138 | No hit | |
| 1 | GW704838 | −0.90208 | No hit | |
| 1 | GW704508 | −0.93687 | Growth hormone-inducible transmembrane protein | Q9H3K2 |
| 1 | GW706318 | −1.02652 | No hit | |
| 1 | GW705755 | −1.03495 | Glyoxalase domain-containing protein 4 | Q9CPV4 |
| 1 | GW706384 | −1.13901 | Polymeric immunoglobulin receptor | O70570 |
| 5 | GW706415 | −1.16247 | Polymeric immunoglobulin receptor | P01833 |
| 1 | GW700914 | −1.33732 | Polymeric immunoglobulin receptor | O70570 |

*Where more than one clone was represented, an example single sequence ID is provided

parasite infection was attempted at high testosterone level (TP or TN relative to IA groups).

These findings provide important insight into how individual fitness of grouse may be influenced by prevailing levels of parasite load and aggressiveness in natural populations and a mechanism that could influence population dynamics through the interacting effects of intrinsic and extrinsic population processes. Central to both the parasite (Hudson *et al.* 1992, 1998) and territorial behaviour (Watson & Moss 2008), hypotheses for explaining cyclic population dynamics is a mechanism that reduces recruitment into the adult population at peak population density. The parasite hypothesis suggests this is mediated through delayed density-dependent reductions in female fecundity associated with high parasite intensity, whereas the territorial behaviour hypothesis posits it is caused by delayed density-dependent changes in aggressiveness that limit the recruitment of young males into territorial populations (Mougeot *et al.* 2003; Piertney *et al.* 2008). Here, we have shown how these two processes could operate interactively. At low density, males will have low parasite burdens and an ability to mount a required response to cope with the associated immune challenge because at low density there is also little aggression and low testosterone titre. However, following peak density, males will have inherently greater parasite intensities (Hudson *et al.* 1992; Redpath *et al.* 2006) and will be individually less capable of raising the necessary response to the parasite challenge owing to the high levels of testosterone associated with elevated aggression (Moss & Watson 2001; Piertney *et al.* 2008). This will have direct effects, negatively impacting male fitness and positively affecting male mortality, but also indirect effects by further increasing parasite burdens, as seen in previous experiments where elevated testosterone increased susceptibility to *T. tenuis* infective larvae (Mougeot *et al.* 2005b; Seivwright *et al.* 2005). Given that transmission of parasites among grouse in natural populations is sufficiently effective to couple parasite burdens between the male and female populations, an increased parasite load in males will result in an increased parasite load in females that will have a negative effect on fecundity.

At first glance, a demonstration of a dampened transcriptomic response to parasites during periods of high testosterone titre is consistent with the conceptual models of Lochmiller (1996) that were developed to explain cyclic dynamics in vertebrates. In these models, elevated stress at high density would suppress the immune system to a level that would increase disease-associated mortality to parasites, forcing the population into decline. However, in the case of grouse, the genes up-regulated in response to parasites and suppressed by testosterone are not necessarily associated primarily with immune function. That is not to say that there is no immunosuppressive effect. The top three transcripts that are down-regulated by testosterone in the absence of *T. tenuis* represent the same gene—polymeric immunoglobulin receptor (PIGR), which has GO terms associated with immune response. PIGR is responsible for transporting secretory IgA antibodies across intestinal epithelial cells (Bruno *et al.* 2010). Increased expression of this gene has been recorded in response to enteric pathogens (Davids *et al.* 2006) as well as in cows with higher resistance to nematode infection (Li *et al.* 2007).

However, in this experiment, the PIGR transcripts on the array were not significantly up-regulated in response to *T. tenuis* infection. The most common candidate up-regulated by *T. tenuis* is serum albumin (Table 3), which is mainly produced in the liver and is the predominant protein of plasma. Leakage of serum albumin from the host into the gastrointestinal tract has been reported in sheep infected with gastrointestinal nematodes (Vaughan *et al.* 2006). This leakage is believed to be a result of intestinal pathology caused by the parasite rather than some consequence of immune response (Vaughan *et al.* 2006), although other studies have suggested a possible role for serum albumin in innate defence (Shamay *et al.* 2005). The up-regulation of serum albumin in caecal tissue seen in IN birds here may be in response to leakage, or as part of an innate response, but it is not seen in birds with increased testosterone (Fig. 2a). Other candidates in Table 3 may also be up-regulated in response to intestinal pathology caused by *T. tenuis* (e.g. fibrinogen, haemoglobin subunit). As such, the main response to *T. tenuis* in the caecal tissue may be to ameliorate the effects of gut damage by the parasite and tolerate the infection (Roy & Kirchner 2000; Boots *et al.* 2009) rather than mount an expensive immune response. Therefore, testosterone may not suppress an immune response to *T. tenuis*, but it may suppress a response to tolerate the parasite. Overall, the results presented here do not agree with our first prediction—that parasites would lead to an increase in expression of immune response genes—however, they do agree with our second prediction, as the response to parasites is almost universally suppressed by testosterone.

The use of microarray-based assays to define a response of grouse to some environmental perturbation and infer potential downstream ecological effects clearly offers many opportunities, but also raises several challenges. First, an obvious assumption of this approach is that levels of mRNA from tissue extracts reflect some meaningful measure of phenotypic response of the individual to the treatments. There are several reasons why mRNA levels might not provide an accurate measure of overall response. Factors such as differences in transcription versus translation kinetics, post-translational modification and accumulation of inactive mature protein within tissues may all act to skew levels of mRNA relative to active gene product (e.g. Brockmann *et al.* 2007). However, such a potential bias is more likely in analysis of individual genes, as opposed to a more holistic response identified from a microarray, as in this experiment. Second, attempting to distill a complex transcriptomic response into a more simplistic phenotypic effect is difficult given the range of functions of the different genes. However, in this case, an obvious up-regulation of multiple genes to parasites, both from chronic and experimental infection, and down-regulation of the same genes to testosterone implicate a major phenotypic response that can have clear individual and knock-on population level consequences. Third, demonstrating a suppression of response by grouse to high parasite load during elevated testosterone in the experimental manipulations does not indicate that this drives population dynamics. Testing this would require a population level experiment that manipulates parasites and testosterone and relates changes in transcriptomic suppression to reproductive success and population growth rate. In addition, a survey of transcriptomic changes through a naturally cycling population would indicate whether transcriptomic suppression predicts population decline. Each of these steps requires nondestructive monitoring of transcriptomic responses, which will prove problematic but could be achieved through blood-based assays.

In spite of these challenges, this study has demonstrated the utility of broad scale postgenomics technologies such as microarrays in nonmodel species living in natural populations to gain insight into ecological and evolutionary aspects of host–parasite interaction: the former through highlighting individual and population level consequences of host–parasite load under different environmental conditions, and the latter by identifying key genes associated with host–parasite interaction that can be used as candidates to examine aspects such as signatures of selection across space and time.

## Acknowledgements

We thank D. Calder and T. Helps for access to study sites; G. Murray-Dickson and M. Oliver for help with fieldwork; and H. Evans, M. Hughes and L. Mello for laboratory and bioinformatic support. This project was funded via NERC's postgenomics and proteomics theme (NE/D000602/1). FM was funded by a NERC advanced fellowship and the Ministerio de Educacion y Ciencia of Spain (project CGL-2006-11823).

We held the necessary UK Home Office licences for conducting the procedures described in the study.

## References

Alvarez MJ, Vila-Ortiz GJ, Salibe MC, Podhajcer OL, Pitossi FJ (2007) Model based analysis of real-time PCR data from DNA binding dye protocols. *BMC Bioinformatics*, **8**, 85.

Berryman AA (2002) *Population Cycles: The Case for Trophic Interactions*. Oxford University Press, Oxford.

Bonneaud C, Mazuc J, Gonzalez G *et al.* (2003) Assessing the cost of mounting an immune response. *The American Naturalist*, **161**, 367–379.

Boots M, Best A, Miller MR, White A (2009) The role of ecological feedbacks in the evolution of host defence: what does theory tell us? *Philosophical Transactions of the Royal Society B: Biological Sciences*, **364**, 27–36.

Bortolotti GR, Mougeot F, Martinez-Padilla J, Webster LMI, Piertney SB (2009) Physiological stress mediates the honesty of social signals. *PLoS ONE*, **4**, e4983.

Bradley AJ (1987) Stress and Mortality in the Red-Tailed Phascogale, Phascogale-Calura (Marsupialia, Dasyuridae). *General and Comparative Endocrinology*, **67**, 85–100.

Brockmann R, Beyer A, Heinisch JJ, Wilhelm T (2007) Posttranscriptional expression regulation: what determines translation rates? *PloS Computational Biology*, **3**, 531–539.

Bruno MEC, Rogier EW, Frantz AL, Stefka AT, Thompson SN, Kaetzel CS (2010) Regulation of the polymeric immunoglobulin receptor in intestinal epithelial cells by Enterobacteriaceae: implications for mucosal homeostasis. *Immunological investigations*, **39**, 356–382.

Camplani A, Saino N, Moller AP (1999) Carotenoids, sexual signals and immune function in barn swallows from Chernobyl. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **266**, 1111–1116.

Christe P, Moller AP, de Lope F (1998) Immunocompetence and nestling survival in the house martin: the tasty chick hypothesis. *Oikos*, **83**, 175–179.

Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, **21**, 3674–3676.

Davids BJ, Palm JED, Housley MP *et al.* (2006) Polymeric immunoglobulin receptor in intestinal immune defense against the lumen-dwelling protozoan parasite Giardia. *Journal of Immunology*, **177**, 6281–6290.

Dheda K, Huggett JF, Chang JS *et al.* (2005) The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. *Analytical Biochemistry*, **344**, 141–143.

Dobson AP, Hudson PJ (1992) Regulation and stability of a free-living host-parasite system: *Trichostrongylus tenuis* in red grouse 2. Population models. *Journal of Animal Ecology*, **61**, 487–498.

Folstad I, Karter AJ (1992) Parasites, Bright Males, and the Immunocompetence Handicap. *The American Naturalist*, **139**, 603–622.

Fox A, Hudson PJ (2001) Parasites reduce territorial behaviour in red grouse (Lagopus lagopus scoticus). *Ecology Letters*, **4**, 139–143.

Geller MD, Christian JJ (1982) Population-Dynamics, Adrenocortical Function, and Pathology in Microtus-Pennsylvanicus. *Journal of Mammalogy*, **63**, 85–95.

Gentleman RC, Carey VJ, Bates DM *et al.* (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology*, **5**, R80.

Hudson PJ (1986a) The Effect of a Parasitic Nematode on the Breeding Production of Red Grouse. *Journal of Animal Ecology*, **55**, 85–92.

Hudson PJ (1986b) *Red Grouse, the Biology and Management of a Wild Gamebird*. The Game Conservancy Trust, Fordingbridge.

Hudson PJ, Newborn D, Dobson AP (1992) Regulation and stability of a free-living host-parasite system: *Trichostrongylus tenuis* in red grouse. 1. Monitoring and parasite reduction experiments. *Journal of Animal Ecology*, **61**, 477–486.

Hudson PJ, Dobson AP, Newborn D (1998) Prevention of population cycles by parasite removal. *Science*, **282**, 2256–2258.

Kerr MK (2003) Design considerations for efficient and effective microarray studies. *Biometrics*, **59**, 822–828.

Krebs CJ, Boutin S, Boonstra R *et al.* (1995) Impact of food and predation on the snowshoe hare cycle. *Science*, **269**, 1112–1115.

Lambin X, Krebs CJ, Moss R, Stenseth NC, Yoccoz NG (1999) Population cycles and parasitism. *Science*, **286**, 2425.

Li RW, Sonstegard TS, Van Tassell CP, Gasbarre LC (2007) Local inflammation as a possible mechanism of resistance to gastrointestinal nematodes in Angus heifers. *Veterinary Parasitology*, **145**, 100–107.

Lochmiller RL (1996) Immunocompetence and animal population regulation. *Oikos*, **76**, 594–602.

MacColl ADC, Piertney SB, Moss R, Lambin X (2000) Spatial arrangement of kin affects recruitment success in young male red grouse. *Oikos*, **90**, 261–270.

Matthiopoulos J, Moss R, Mougeot F, Lambin X, Redpath SM (2003) Territorial behaviour and population dynamics in red grouse Lagopus lagopus scoticus. II. Population models. *Journal of Animal Ecology*, **72**, 1083–1096.

McDonald IR, Lee AK, Than KA, Martin RW (1986) Failure of Glucocorticoid Feedback in Males of a Population of Small Marsupials (*Antechinus-Swainsonii*) During the Period of Mating. *Journal of Endocrinology*, **108**, 63–68.

Mihok S, Turner BN, Iverson SL (1985) The Characterization of Vole Population-Dynamics. *Ecological Monographs*, **55**, 399–420.

Moss R, Watson A (2001) Population cycles in birds of the grouse family (Tetraonidae). *Advances in Ecological Research*, **32**, 53–111.

Mougeot F, Redpath SM, Leckie F, Hudson PJ (2003) The effect of aggressiveness on the population dynamics of a territorial bird. *Nature*, **421**, 737–739.

Mougeot F, Dawson A, Redpath SM, Leckie F (2005a) Testosterone and autumn territorial behavior in male red grouse *Lagopus lagopus scoticus*. *Hormones and Behavior*, **47**, 576–584.

Mougeot F, Evans SA, Redpath SM (2005b) Interactions between population processes in a cyclic species: parasites reduce autumn territorial behaviour of male red grouse. *Oecologia*, **144**, 289–298.

Mougeot F, Piertney SB, Leckie F *et al.* (2005c) Experimentally increased aggressiveness reduces population kin structure and subsequent recruitment in red grouse *Lagopus lagopus scoticus*. *Journal of Animal Ecology*, **74**, 488–497.

Mougeot F, Redpath SM, Piertney SB, Hudson PJ (2005d) Separating behavioral and physiological mechanisms in testosterone-mediated trade-offs. *The American Naturalist*, **166**, 158–168.

Mougeot F, Martinez-Padilla J, Webster LMI, Blount JD, Perez-Rodriguez L, Piertney SB (2009) Honest sexual signalling mediated by parasite and testosterone effects on oxidative balance. *Proceedings of the Royal Society Biological Sciences Series B*, **276**, 1093–1100.

New LF, Matthiopoulos J, Redpath S, Buckland ST (2009) Fitting Models of Multiple Hypotheses to Partial Population Data: Investigating the Causes of Cycles in Red Grouse. *The American Naturalist*, **174**, 399–412.

Piertney SB, Lambin X, MacColl ADC *et al.* (2008) Temporal changes in kin structure through a population cycle in a territorial bird, the red grouse *Lagopus lagopus scoticus*. *Molecular Ecology*, **17**, 2544–2551.

R, Development, Core & Team (2007) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Redpath SM, Mougeot F, Leckie FM, Elston DA, Hudson PJ (2006) Testing the role of parasites in driving the cyclic population dynamics of a gamebird. *Ecology Letters*, **9**, 410–418.

Roy BA, Kirchner JW (2000) Evolutionary dynamics of pathogen resistance and tolerance. *Evolution*, **54**, 51–63.

Seivwright LJ, Redpath SM, Mougeot F, Watt L, Hudson PJ (2004) Faecal egg counts provide a reliable measure of *Trichostrongylus tenuis* intensities in free-living red grouse *Lagopus lagopus scoticus*. *Journal of Helminthology*, **78**, 69–76.

Seivwright LJ, Redpath SM, Mougeot F, Leckie F, Hudson PJ (2005) Interactions between intrinsic and extrinsic mechanisms in a cyclic species: testosterone increases parasite infection in red grouse. *Proceedings of the Royal Society B-Biological Sciences*, **272**, 2299–2304.

Shamay A, Homans R, Fuerman Y *et al.* (2005) Expression of Albumin in Nonhepatic Tissues and its Synthesis by the Bovine Mammary Gland. *Journal of Dairy Science*, **88**, 569–576.

Shaw JL, Moss R (1989) Factors affecting the establishment of the caecal threadworm *Trichostrongylus tenuis* in red grouse (*Lagopus lagopus scoticus*). *Parasitology*, **99**, 259–264.

Smyth GK (2005) Limma: linear models for microarray data. In: *Bioinformatics and Computational Biology Solutions Using R and Bioconductor* (eds Gentleman RC, Carey VJ, Dudoit S, Irizarry R, Huber W), pp. 397–420. Springer, New York.

Sorci G, Clobert J, Michalakis Y (1996) Cost of reproduction and cost of parasitism in the common lizard, *Lacerta vivipara*. *Oikos*, **76**, 121–130.

Turchin P (2003). *Complex Population Dynamics. A Theoretical/Empirical Synthesis*. Princeton University Press, Princeton.

Vandesompele J, De Preter K, Pattyn F *et al.* (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, **3**, research0034.

Vaughan AL, Greer AW, McCanulty RW, Sykes AR (2006) Plasma protein loss in lambs during a mixed infection of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*—a consequence of the immune response? *Proceedings of the New Zealand Society of Animal Production*, **66**, 83–87.

Viney ME, Riley EM, Buchanan KL (2005) Optimal immune responses: immunocompetence revisited. *Trends in Ecology & Evolution*, **20**, 665–669.

Watson A, Moss R (2008) *Grouse*. Harper Collins, London.

Webster LMI, Paterson S, Mello LV, Mougeot F, Martinez-Padilla J, Piertney SB (2010) Identification of genes responding to nematode infection in red grouse. *Molecular Ecology Resources*, doi: 10.1111/j.1755-0998.2010.02912.x.

Wit E, McClure J (2004) *Statistics for Microarrays: Design, Analysis and Inference*. John Wiley and Sons, Chichester.

Wit E, Nobile A, Khanin R (2005) Near-optimal designs for dual channel microarray studies. *Journal of the Royal Statistical Society Series C-Applied Statistics*, **54**, 817–830.

---

This study is part of a collaboration linking evolutionary biology (SBP, LMIW and SP) and population ecology (FM and JM-P) to understand the interactions between population and gene dynamics in natural systems.

---

## Supporting information

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

**Table S1** Table of contrasts for microarray experiment

**Table S2** Gene Ontology (GO) descriptors associated with up-regulated by parasites (Group IP or IN versus IA contrast). P = biological process; C = cellular component; F = molecular function

**Table S3** Gene Ontology (GO) descriptors associated with genes up- or down-regulated regulated by testosterone in the absence of parasites (TA versus IA contrast)

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.