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A co-infection with two gastrointestinal nematodes alters host immune responses and only partially parasite dynamics

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SUMMARY

Given their global distribution and abilities to persist in the host, helminths can play a crucial role in affecting risk of infections by increasing individual variation in infection. Helminth co-infections are of particular interest because by altering host immune responses, they can modify host susceptibility and thus intensity and transmission of other parasites/pathogens. The dynamics of co-infection were examined using two helminths of the European rabbit. Individuals were simultaneously challenged with a primary dose of both parasites, and changes in intensity were examined in relation to local and systemic immune responses. Both helminths persisted in co-infected rabbits; however, contrasting dynamics and immune responses were observed. Graphidium strigosum *intensity was high throughout the co-infection, while* Trichostrongylus retortaeformis *intensity decreased but was not completely cleared. A Th2 response was observed against* G. strigosum*, while a mixed Th1/Th2 profile was found to* T. retortaeformis*. A comparison with our previous work on single infections showed that* G. strigosum *intensity was higher in co-infected than single infected hosts, while* T. retortaeformis *showed no significant changes. Differences were also observed in the cytokine profiles, blood cell concentrations and antibody trends. Overall, host variability during helminth co-infections can be generated by significant differences in immune responses and/or parasite dynamics.*

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INTRODUCTION

Parasitic helminths often cause persistent lifelong infections that result in hosts harbouring an infracommunity of parasites that can interact directly, if sharing the same habitat, or indirectly *via* the host immune response and/or parasite excretory–secretory compounds. A frequent observation from field and laboratory studies is that there is large variation in the patterns of co-infection, and associated immune responses such as infection with one helminth can enhance, suppress or have no apparent effect on the abundance and immunity to the second helminth species (1–5). In the majority of cases, interactions appear to be asymmetrical, that is, changes in parasite dynamics and infection severity are more evident for one of the two co-infecting species. While phylogenetic dissimilarity may explain a large amount of this bias, differences in parasite life strategies and immunophysiological properties of the organs infected may also play a critical role.

Infections with helminths generally lead to the polarization of the immune system towards a Th2 response, characterized by elevated production of IL-4, IL-5, IL-13 as well as IgE and eosinophilia (6, 7). A few exceptions have been reported for some tissue-dwelling helminths where a transitory Th1 reaction develops during a specific tissue phase of the infection process (8–11). Despite robust immune responses, helminths can endure in the host as subclinical or asymptomatic infections. Crucially, by altering the immune profile and/or the interactions within and between parasite species, a second parasite can influence the intensity as well as the life-history traits and pathology associated with the first parasite species (12–18) and, from here, infection dynamics and long-term persistence. At the host population level, this

can increase individual variation to infection where co-infected individuals can carry a higher parasite burden or shed more infective particles, than single infected cases, and contribute to a significant portion of the transmission (19). Given their worldwide distribution, understanding patterns and mechanisms of helminth co-infections is fundamental to enable better appreciation of the processes that drive parasite persistence and risk of infection and also to develop effective intervention procedures for disease control.

In the current study, we examined the dynamics of co-infection of two gastrointestinal nematodes, *Graphidium strigosum* and *Trichostrongylus retortaeformis*, in laboratory rabbits (*Oryctolagus cuniculus*). These parasites naturally infect the European rabbit (*Oryctolagus cuniculus*) with high intensity and prevalence and show distinct immuno-epidemiological properties (20–22). In laboratory single infections, *T. retortaeformis* generates a mixed Th1/Th2 response, where the initial Th1-mediated mucosal IFN-γ appears to facilitate parasite establishment/development in the small intestine, while the subsequent Th2-mediated responses contribute to IL-4 expression and rapid increase in antibodies and eosinophils, resulting in reduction but not always complete clearance of the parasite (23, 24). In contrast, the intensity of *G. strigosum* in the stomach remains high despite stimulating a strong mucosal IL-4, high serum IgG and IgA but low mucus antibody responses (23). Field and laboratory co-infections of these two helminths with the systemic myxoma virus or the respiratory bacterium *Bordetella bronchiseptica* confirmed the contrasting immuno-epidemiological properties of the two helminths. *T. retortaeformis* intensity increased in virus-co-infected rabbits, while no significant changes were observed for *G. strigosum* intensity (25). On the contrary, laboratory rabbits co-infected with *B. bronchiseptica* rapidly cleared *T. retortaeformis* but not *G. strigosum* that increased significantly, compared with single worm infections (24, 26). Natural co-infections of rabbits with the two helminths harboured higher numbers of *T. retortaeformis,* whereas no clear changes were observed for *G. strigosum* (21).

To examine in more detail, the dynamics of co-infection of the two helminths and how they differ from single infections, the systemic and local immune response of rabbits challenged with a single mixed dose of *G. strigosum* and *T. retortaeformis* was investigated. Based on our recent findings, we predicted that the presence of the second helminth would lead to higher intensity of *T. retortaeformis* but not *G. strigosum*, relative to single infections. Results are discussed in the context of previous studies on single infections and co-infections with these two helminths.

MATERIALS AND METHODS

Study design and sample collection

Outbred, 2-month-old New Zealand white rabbits (Harlan, Hillcrest, UK) were simultaneously infected with a single oral dose of 5500 *T. retortaeformis*- and 650 *G. strigosum*-infective third-stage larvae (L3) diluted in 3 mL of a mineral water solution. Control, uninfected animals were orally treated with mineral water only. The infection dose, animal husbandry, tissue collection procedures and parasite enumeration were performed following previous experiments of single infection with these two parasites (23). Briefly, six individuals (four infected and two controls) were sampled on 7, 14, 30, 45, 60, 90 and 120 days post-infection (DPI) and the gastrointestinal tract carefully processed. The stomach was divided into two sections, fundus and antrum, while the small intestine was divided into four sections of equal length with the first segment next to the stomach and the fourth segment preceding the large intestine (from the duodenum to the ileum: SI-1, SI-2, SI-3 and SI-4). For every section, along with extraction and enumeration of parasites (i.e. *G. strigosum* from the stomach and *T. retortaeformis* from the small intestine), mucus was collected and stored at −20°C, for the quantification of local antibody responses, and small pieces of tissue were preserved in RNAlater (Sigma-Aldrich, Dorset, UK) at −80°C for expression of cytokine coding genes. Blood samples were collected from every animal once a week to quantify longitudinal antibody production and haematological measures. Complete details of these methodologies are reported in Murphy *et al.* (23). All listed laboratory animal procedures were pre-approved by the University of Glasgow and performed under the UK Animals (Scientific Procedures) Act 1986 (27).

Parasite quantification

Nematodes established in the stomach (fundus and antrum) and small intestine (sections: SI-1 to SI-4) were quantified at each sampling point following procedures reported previously (23, 24, 26). *T. retortaeformis* extracted from each SI section was stored in 50-mL tubes, and stage and intensity, estimated from five aliquots of 2·5 mL volume each. *G. strigosum* intensity from the stomach was based on the total number in each collection, except at 7 and 14 days post-infection where five aliquots of 2·5 mL were used (23, 26).

Local cytokine gene expression

Expression of cytokine coding genes was determined using qRT-PCR on tissues from gastrointestinal sections showing the highest intensity of infection, specifically, the stomach

fundus for *G. strigosum* and SI-1 for *T. retortaeformis* (23, 24, 26). Tissues were homogenized and RNA was extracted according to the manufacturers instructions (Qiagen ltd, Manchester, UK). Residual DNA was removed with TURBO DNase (Life Technologies, Paisley, UK) and confirmed by performing a minus reverse transcriptase amplification for each sample (28). Pure RNA was converted to cDNA with the high-capacity RNA-to-cDNA kit following manufacturers instructions (Life Technologies). We focused on the expression of IFN-γ, IL-4 and IL-10 based on our previous studies that showed an important role for these cytokines in the dynamics of single infections and co-infections with these helminths (23, 24, 26, 29). Analyses were performed using the TaqMan-based relative quantification method with hypoxanthine phosphoribosyltransferase (HPRT) as the housekeeping calibrator gene. Details on primer and probe sequences are reported in Pathak *et al.* (28), and the full procedure is described in Murphy *et al.* (23).

Systemic and local antibody quantification

Species-specific IgA and IgG antibody responses were quantified weekly in the serum and at specific sampling points in the mucus of each gastrointestinal section. Excretory/secretory (ES) products from the adult stage of the two nematodes were used as the source of antigen. Unlike whole adult worm homogenates, antibodies against ES showed minimal cross-reactivity between the two nematode species but similar response profiles (unpublished results). Detection of IgA and IgG was performed with ELISAs adapted from Murphy *et al.* (23). Table S1 reports the respective dilutions and concentrations used for the ELISAs. Briefly, 384-well ELISA plates (Greiner Bio-One, Monroe, NC, USA) were coated with 25 μL/well of soluble excretory/secretory protein products at a concentration of 0·75 μg/mL (*G. strigosum*) or 0·25 μg/mL (*T. retortaeformis*), diluted in 0·2 M carbonate/bicarbonate coating buffer (pH 9·6) and incubated overnight at 4°C. Rabbit sera and mucus were added to the plate in duplicates, and each plate included the following: (i) positive controls, prepared from a pool of laboratory rabbit sera/mucus with highest reactivity and infection levels, (ii) negative controls, originated from laboratory naïve rabbits, and (iii) background controls with no sera in the well. Samples were serially diluted 2-fold across the plate and bound antibodies detected with either horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL, USA) or goat anti-rabbit IgA (Abcam, Cambridge, MA, USA) using ABTS as the colorimetric detection reagent (26, 28).

Haematological profile

Weekly, blood cell counts from each individual were determined from whole blood collected in EDTA-coated tubes (Sartorius Stedim, Bohemia, NY, USA). Samples were processed through the Advia 120 haematology analyzer with species-specific software calibration (Siemens Healthcare Diagnostics Inc., Surrey, UK) at the Veterinary Clinical Pathology Laboratory of the University of Glasgow (23). Blood smear and manual leucocyte differential count enabled estimation of the absolute concentrations of eosinophils, neutrophils, basophils, lymphocytes, monocytes and haemoglobin.

Data analysis

Cytokine data were initially normalized to the reference gene (C_t = focal cytokine raw value – HPRT raw value) and subsequently used for the statistical analysis. Visual representation of temporal changes in the mRNA gene expression for each cytokine was based on the method by Livak and Schmittgen (30) and represented as $2^{-\Delta\Delta C_t}$ values, where normalized C_t values from infected rabbits were scaled over control individuals (23). Antibodies were analysed and visually presented using data transformed into standardized antibody O.D. indices (23).

Linear mixed effect models (with restricted maximum likelihood, LME-REML) were used unless specified otherwise. Specifically, to identify changes in the intensity of infection and immune responses (cytokines excluded), nematode intensity or immune variables were examined in relation to treatment (infected and control), sampling time (days or weeks post-infection) and location of the infection (fundus and antrum or SI-1 to SI-4) as fixed variables. To take into account the nonindependent sampling of the same individual over time or the examination of subsections of the same organ from the same host, the individual identification number (ID) was included as a random effect and/or as an autoregressive function of order 1 (AR-1). To identify the immune variable or combination of variables that could affect variation in parasite intensity, generalized linear models (GLM with negative binomial error) were applied using parasite intensity as a response variable and predicted scores from a principal component analysis as independent factors (23). Principal component analysis was implemented to investigate patterns of association among immune variables and the amount of variation in the immune response that could be explained by these associations (23). Analysis was performed using the immune variables to both parasites and subsequently repeated focusing on the local immune response to each helminth. The first two principal components of the species-specific immune responses were examined in relation to parasite intensity.

RESULTS

Nematode intensity

G. strigosum

Intensity remained high throughout the infection, and parasites in the fundus were significantly higher than in the antrum, once variation among hosts and the nonindependent sampling of the two stomach sections from the same individual were taken into account (Figure 1, Table 1). The low number of parasites collected in the antrum at 30 days post-infection was probably a technical artefact (i.e. few worms moved with the ingesta in the antrum while handling the stomach) and should not be considered biologically relevant.

T. retortaeformis

Intensity decreased with the progression of the infection, a pattern consistent across the four sections of the small intestine (from SI-1 to SI-4). The infection in SI-1 was significantly higher compared with the remaining three sections with both SI-3 and SI-4 showing to be the least colonized areas (Figure 1b, Table 1).

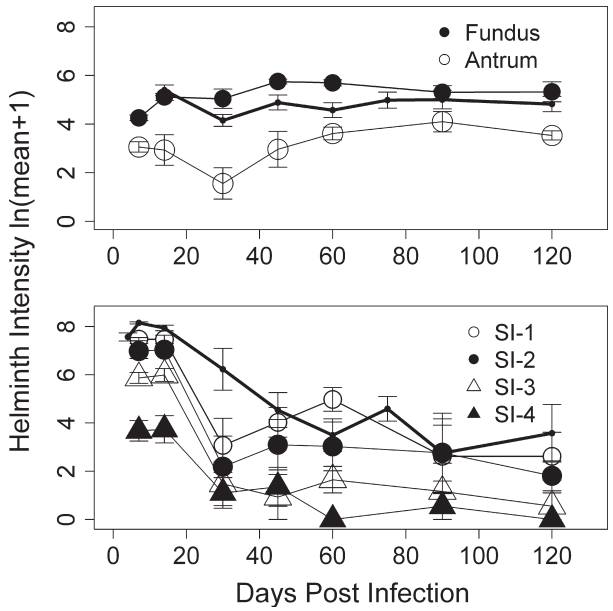

Figure 1 Parasite intensity (mean ± SE) over the course of *G. strigosum* infection in the stomach (fundus and antrum) (top panel) and of *T. retortaeformis* infection in the small intestine (SI-1 to SI-4, that is, from the duodenum to ileum, respectively) (bottom panel). To facilitate comparison with the single infections of the two nematodes [Murphy *et al.* (23)], we have included the intensity (mean ± SE) of *G. strigosum* in the fundus and *T. retortaeformis* in the SI-1 at each sampling point (thick black line).

Table 1 Linear mixed effect model (LME) between parasite intensity (either *G. strigosum* or *T. rertortaeformis*) and day post-infection (DPI) and organ section (stomach fundus and antrum or small intestine section SI-1 to SI-4) as fixed variables. The standard deviation for the intercept (random effect) and the autocorrelation value for using different sections of the same organ from the same host (AR-1) are reported. The two-way interaction organ section DPI was not significant for both parasite species. For *T. retortaeformis*, intensity in SI-1 is automatically coded as the reference category for the intensity in the remaining sections

	Coefficient, SE, d.f.	*P*
G. strigosum		
Intercept	4·76 ± 0·23, 27	<0·0001
Organ section (antrum)	−2·11 ± 0·24, 27	<0·0001
DPI	0·008 ± 0·003, 26	0·008
Random effect, Intercept SD: host ID	0·26	
Autocorrelation (AR-1)	−0·15	
AIC	166·70	
T. retortaeformis		
Intercept	6·57 ± 0·52, 681	<0·0001
SI-2	−0·76 ± 0·28, 81	0·009
SI-3	−2·09 ± 0·36, 81	<0·0001
SI-4	−3·12 ± 0·40, 81	<0·0001
DPI	−0·04 ± 0·01, 26	<0·0001
Random effect, Intercept SD: host ID	0·70	
Autocorrelation (AR-1)	0·62	
AIC	428·01	

Based on the parasite counts in the fundus and SI-1 at each sampling point, we found that the two infections were negatively correlated; in other words, *T. retortaeformis* intensity decreased with the course of the infection, while *G. strigosum* remained relatively constant (Pearsons product-moment: $r = -0{\cdot}53$, d.f. = 26, $P < 0{\cdot}01$). To highlight whether these intensities were comparable with single infections, parasite counts from this study were compared with our previous work based on a similar experimental design and infection doses (23). *G. strigosum* intensity in the fundic region, where most of the worms are concentrated, was consistently higher in co-infected than single infected rabbits, and this pattern was consistent throughout the infection (Figure 1, single vs. dual infection mean ± SE: 150·25 ± 16·61 vs. 218·21 ± 22·02, GLM with negative binomial error, coefficient ± SE: 0·37 ± 0·16, d.f. = 55, $P < 0{\cdot}05$). In contrast, no significant differences were observed for *T. retortaeformis* intensity in the SI-1 section – or combined SI-1 and SI-2 sections – where the majority of worms are found (Figure 1, mean ± SE: 1010·45 ± 208·90 vs. 586·86 ± 154·27). This lack of a significant pattern appeared to be caused by variation in worm intensity within and between sampling points.

Local cytokine expression

G. strigosum

The normalized C_t values of the three cytokines in the stomach fundus were significantly higher in infected individuals relative to the controls (GLM with Poisson error distribution, coefficient ± SE: IFN-γ = −0·22 ± 0·09, IL-10 = −0·27 ± 0·12, IL-4 = −0·51 ± 0·11, for all: d.f. = 40, $P < 0·05$; and for IL-4 $P < 0·0001$). Based on the $2^{-\Delta\Delta C_t}$ visual representation of the data (i.e. normalized values from infected hosts scaled over the controls), IL-4 exhibited the highest expression with a clear peak at 45 days post-infection (Figure 2). IFN-γ expression was at the highest at 14 days post-infection and decreased thereafter, while IL-10 expression was generally low for the duration of the infection (Figure 2). Cytokines showed positive pairwise correlations where high C_t values of one cytokine were related to high C_t values of the second cytokine and vice versa (Pearsons product-moment, r range: 0·76–0·88, for all d.f. = 40, $P < 0·0001$).

T. retortaeformis

Normalized C_t cytokine values in the small intestine SI-1 were significantly higher in infected than in control individuals (GLM, coefficient ± SE: IFN-γ = −0·42 ± 0·15, $P < 0·01$; IL-10 = −0·32 ± 0·15, $P < 0·05$; IL-4 = −0·66 ± 0·20, $P < -0·001$, for all: d.f. = 40). The $2^{-\Delta\Delta C_t}$ representation of these data showed a strong peak in IFN-γ at 7 days post-infection, although values quickly declined and remained low for the rest of the infection (Figure 2). IL-4 and IL-10 levels remained relatively low over the course of the trial (Figure 2). Pairwise comparisons between cytokines were positive: the lowest correlation was found between IFN-γ and IL-10 ($r = 0·39$, d.f. = 40, $P < 0·05$), while similar values were observed for the remaining pair combinations (IFN-γ–IL-4, $r = 0·76$ and IL-4–IL-10 $r = 0·70$, for both: d.f. = 40, $P < 0·0001$).

Local and systemic antibody responses

G. strigosum

Mucus IgA and IgG responses against E/S products were higher in infected compared with control rabbits, although values remained relatively low for IgG and in part for IgA (Figure 3, Table 2). Higher O.D. indices were found in the mucus from the fundus, and values increased with the progression of the experiment. Serum IgA and IgG responses were consistent with this general local pattern: antibody levels were higher in infected than in control individuals and increased with time but reached an asymptote around 9–11 weeks post-infection. IgA levels showed a tendency to decrease in the last few weeks of the trial (Figure 4, Table 2).

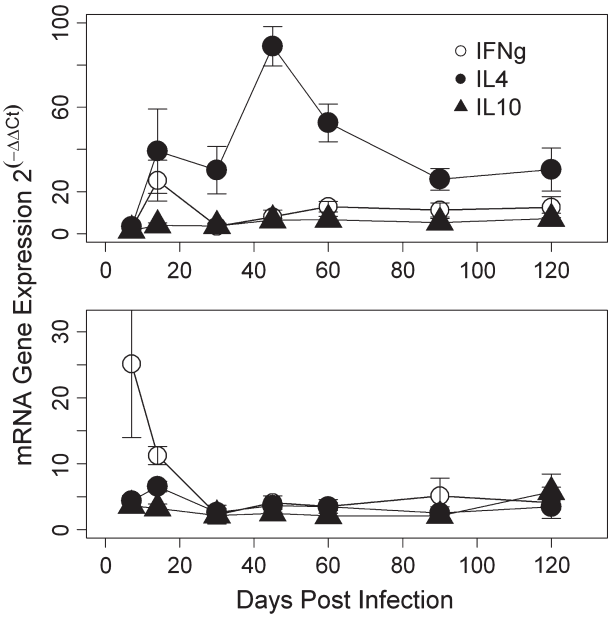

Figure 2 Cytokine expression (Mean ± SE) over the course of the infection in the stomach fundus for *G. strigosum* (top panel) and in the small intestine SI-1 for *T. retortaeformis* (bottom panel). Data are presented as $2^{-\Delta\Delta C_t}$ (i.e. normalized C_t values of infected hosts scaled over the control hosts), while analysis in Table 2 is based on normalized C_t data only (i.e. scaled over the housekeeping gene, HPRT).

T. retortaeformis

Mucus IgA response was consistently low in infected individuals; however, values were significantly higher in infected than in control hosts, especially in the SI-1 (Figure 3, Table 3). Mucus IgG was higher in infected animals and increased with the course of the infection (Figure 3, Table 3). No significant differences were found in the O.D. indices across the four sections of the small intestine. Overall, both serum IgA and IgG values were relatively low, although significantly higher than in control individuals; levels increased and remained high from 5 weeks post-infection (Figure 4, Table 3). We found some variability in the antibody response from the serum of control individuals; *a posteriori* examination indicated that this was mainly driven by the sensitivity of the ELISAs to a relatively low antibody response.

Haematological profiles

The cellular and haemoglobin responses in the rabbits blood were not parasite species specific and should be regarded as a cumulative reaction to the co-infection

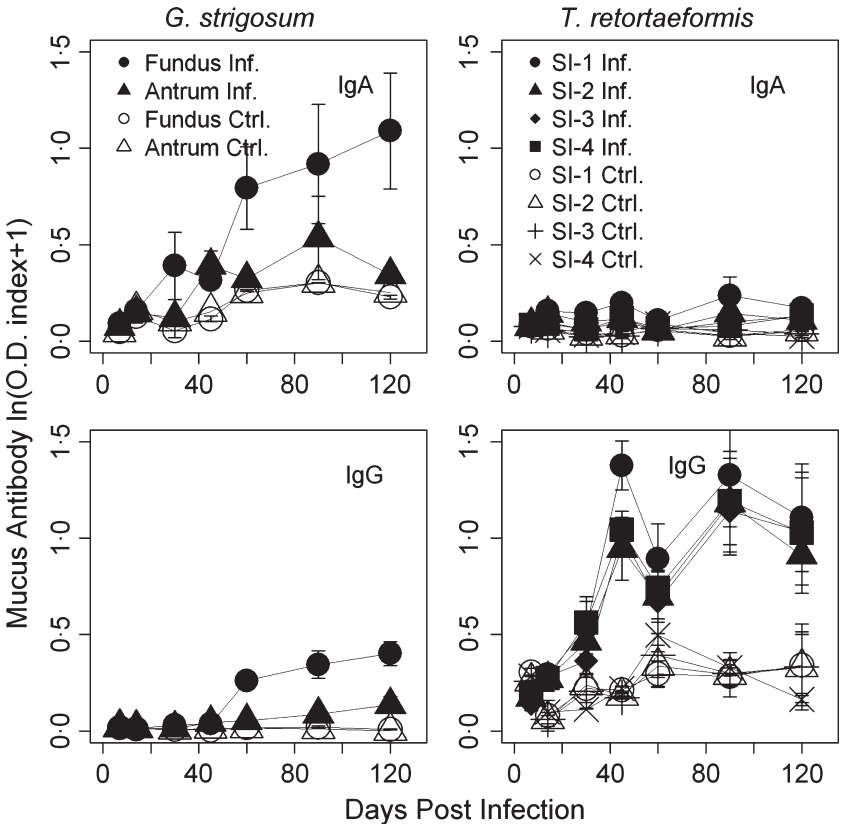

Figure 3 Mucus IgA and IgG antibody responses (mean OD absorbance index ± SE) to the E/S products of both *G. strigosum* (left panel) and *T. retortaeformis* (right panel) in infected and control individuals by different sections of the stomach and small intestine over the course of the infection.

(Figure 5). Eosinophils and monocytes were significantly higher in infected than in control individuals (LME coefficient ± SE: 0·08 ± 0·02, d.f. = 40, $P < 0·001$ and 0·03 ± 0·01, d.f. = 40, $P < 0·05$, respectively); values peaked around 2–4 weeks post-infection and, again, later on in the infection for the eosinophils. Similar trends were observed for neutrophils and lymphocytes when covarying with sampling time (LME two-way interaction blood variable-sampling week, coefficient ± SE: −0·02 ± 0·01, d.f. = 244, $P < 0·01$ and 0·01 ± 0·005, d.f. = 244, $P < 0·02$, respectively); neutrophils levels remained high in the first 3 weeks post-infection. Basophils and haemoglobin were indistinguishable between treatments over time.

Relationship between nematode intensity and immune components

To identify associations between immune variables and how they could explain variability in the immune response, principal component analysis was performed on different combinations of immune components. Initially, the full immune scenario was considered where the multivariate relationship among local variables (cytokines and mucus antibodies), and the systemic blood cell response was examined. The first two principal components explained 46% of the total variation observed (proportion of variance ± SD: PC-1 = 0·29 ± 2·07 and PC-2 = 0·17 ± 1·62) (Figure 6a). Particularly, IFN-γ (0·41) and IL-4 (0·41) against *T. retortaeformis* followed by IgA (0·36) to *G. strigosum* mainly explained the first component, while IFN-γ (0·47), IL-4 (0·41) and IL-10 (0·38) to *G. strigosum* and systemic eosinophils (0·38) mostly accounted for the second principal component. Graphically, three main clusters were identified, variables describing the first and second principal component and a third group combining antibody to *T. retortaeformis*, neutrophils and basophils (Figure 6a). This general pattern is consistent with the development of a species-specific immune response and differences in the timing of maximum peak of some of these variables. Analyses were then repeated focusing on the local immune response to each parasite and how it related to parasite intensity. Specifically, we examined changes in

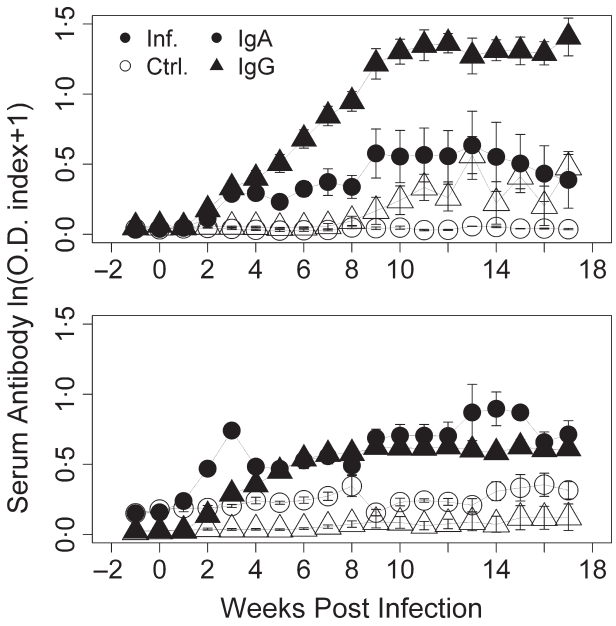

Figure 4 Serum IgA and IgG antibody responses (mean OD absorbance index ± SE) to the E/S products of *G. strigosum* (top panel) and *T. retortaeformis* (bottom panel) in infected and control individuals over the course of the infection. Antibody values in the sampling week before 0 represent the responses from animals bled prior to infection.

parasite counts to the host immune response, and not vice versa, because we were interested in describing how the host could control the worms following a primary infection.

G. strigosum

The linear combination of cytokine expression and mucus antibodies in the stomach fundus accounted for 85% of variation in the first two principal components (PC-1 = 0·61 ± 1·74 and PC-2 = 0·24 ± 1·09) (Figure 6b). IL-10 (−0·52), IL-4 (−0·46) and IFN-γ (−0·44) explained most of the first component, whereas antibodies (IgA = −0·58 and IgG = −0·56) described the second component. The relationship between these two axes and worm intensity was examined, and a significant association was found between the first component and intensity (GLM with negative binomial error: coefficient ± SE: 0·21 ± 0·05, d.f. = 25, $P < 0·0001$). The positive association suggests that high *G. strigosum* infection stimulates a high cytokine response following single dose infection (*Ct* values are inversely related to cytokine expression) (Figure 7a). This also suggests that cytokines have a stronger effect on *G. strigosum* than other immune variables. No significant relationship was observed with the second component.

Table 2 Linear mixed effect model (LME) between local and systemic antibody response to *G. strigosum* E/S and treatment (infected, control), sampling time (day post-infection, DPI; or week post-infection, WPI) and organ section (stomach fundus and antrum) as fixed variables. Additional model explanations are reported in Table 1

	Coefficient, SE, d.f.	*P*		Coefficient, SE, d.f.	*P*
Mucus IgA			Mucus IgG		
Intercept	−0·24 ± 0·18, 39	n.s.	Intercept	0·01 ± 0·05, 38	n.s.
Treatment (inf., cntrl)	0·51 ± 0·22, 38	<0·05	Treatment (inf., cntrl)	0·11 ± 0·06, 38	n.s.
Organ section	0·03 ± 0·10, 39	n.s.	Organ section	−0·001 ± 0·03, 38	n.s.
Days post-infection (DPI)	0·002 ± 0·002, 38	n.s.	DPI	−0·00004 ± 0·0003, 38	n.s.
Treatment *organ section	−0·33 ± 0·12, 39	<0·05	Treatment *organ section	−0·10 ± 0·04, 38	<0·05
Treatment *DPI	0·005 ± 0·002, 38	<0·05	Treatment *DPI	0·002 ± 0·0004, 38	<0·0001
Random effect, intercept SD: host ID	0·03		Random effect, intercept SD: host ID	0·04	
Autocorrelation (AR-1)	0·23		Autocorrelation (AR-1)	−0·94	
AIC	81·73		AIC	−136·33	
Serum IgA			Serum IgG		
Intercept	−0·01 ± 0·05, 339	n.s.	Intercept	−0·02 ± 0·04, 340	n.s.
Treatment (inf., cntrl)	0·04 ± 0·06, 40	n.s.	Treatment (inf., cntrl)	0·07 ± 0·05, 40	n.s.
Week post-infection (WPI)	−0·0001 ± 0·007, 339	n.s.	Week post-infection (WPI)	0·02 ± 0·01, 340	<0·01
Treatment*WPI	0·03 ± 0·01, 339	<0·0001	Treatment*WPI	0·07 ± 0·01, 340	<0·0001
Random effect, intercept SD: host ID	0·00004		Random effect, intercept SD: host ID	0·00002	
Autocorrelation (AR-1)	0·81		Autocorrelation (AR-1)	0·78	
AIC	−423·10		AIC	−463·14	

Inf., infected; cntrl, control; n.s., not significant.

Table 3 Linear mixed effect model (LME) between local and systemic antibody response to *T. retortaeformis* E/S and treatment (infected, control), sampling time (day post-infection, DPI; or week post-infection, WPI) and organ section (small intestine SI-1 to SI-4) as fixed variables. Additional model explanations are reported in Table 1

	Coefficient, SE, d.f.	*P*		Coefficient., SE, d.f.	*P*
Mucus IgA			Mucus IgG		
Intercept	0·005 ± 0·02, 122	n.s.	Intercept	−0·15 ± 0·16, 124	n.s.
Treatment (inf., cntrl)	0·13 ± 0·03, 40	<0·0001	Treatment (inf., cntrl)	0·14 ± 0·20, 38	n.s.
Organ section	0·001 ± 0·01, 122	n.s.	Organ section (antrum)	0·001 ± 0·002, 38	n.s.
Treatment *organ section	−0·02 ± 0·01, 122	<0·01	Treatment *organ section	0·009 ± 0·003, 38	<0·01
Treatment *DPI			Treatment *DPI		
Random effect, intercept SD: host ID	0·00001		Random effect, intercept SD: host ID	0·34	
Autocorrelation (AR-1)	0·80		Autocorrelation (AR-1)	0·46	
AIC	−511·94		AIC	8·67	
Serum IgA			Serum IgG		
Intercept	−0·21 ± 0·04, 339	<0·0001	Intercept	−0·01 ± 0·03, 339	n.s.
Treatment (inf., cntrl)	0·10 ± 0·05, 339	0·059	Treatment (inf., cntrl)	0·07 ± 0·03, 40	0·059
Week post-infection (WPI)	0·019 ± 0·01, 339	<0·01	Week post-infection (WPI)	0·005 ± 0·004, 339	n.s.
Treatment*WPI	0·03 ± 0·01, 339	<0·001	Treatment*WPI	0·04 ± 0·005, 339	<0·0001
Random effect, intercept SD: host ID	0·02		Random effect, intercept SD: host ID	0·000003	
Autocorrelation (AR-1)	0·54		Autocorrelation (AR-1)	0·85	
AIC	−100·27		AIC	−937·03	

Inf., infected; cntrl, control; n.s., not significant.

T. retortaeformis

The multivariate linear combination of cytokine and mucus antibodies in the small intestine SI-1 explained 62% of variance of the first two principal components (PC-1 = 0·42 ± 1·45 and PC-2: 0·20 ± 1·01) (Figure 6c). The first component was mostly described by IL-4 (0·64) and IFN-γ (0·54), whereas the second component, by IgA (0·67) and IL-10 (0·52). These two components were examined in relation to parasite intensity, and a negative association was found between the first principal component and *T. retortaeformis* intensity (GLM with negative binomial error: coefficient ± SE: −1·05 ± 0·19, d.f. = 25, $P < 0·0001$) (Figure 7b). As previously observed, this indicates that a primary infection of worms activates a strong cytokine response. No significant intensity–second principal component relationship was found.

DISCUSSION

A primary, concurrent infection of rabbits with two gastrointestinal nematodes leads to persistent infection of *G. strigosum* in the stomach but a reduction, although not complete removal, of *T. retortaeformis* in the small intestine. A comparison with single infections, performed following a similar experimental design, showed that the presence of the second parasite increased the intensity but did not change the overall dynamics of *G. strigosum,* while it reduced, although not significantly, *T. retortaeformis* load. These contrasting responses were associated with distinct immune profiles. *G. strigosum* stimulated a strong, albeit delayed mucosal IL-4 expression, elicited slow production of mucus IgA and IgG to E/S antigen and contributed to higher eosinophil and neutrophil concentration in the blood. In contrast, *T. retortaeformis* led to a strong, early mucosal IFN-γ response and a generally low IL-4 expression and promoted the prompt production of mucus IgG but not IgA along with playing a part in blood cell recruitment. Overall, the rabbit immune system lacked the ability to synergistically boost full parasite clearance; instead, we found an asymmetrical increase in *G. strigosum* but no apparent changes in *T. retortaeformis* intensity.

For both helminths, the general immune profiles to single infection and co-infection were comparable; however, a few important differences in the timing of the responses were observed. In the stomach, the main IL-4 peak was delayed by 30 days, while IFN-γ peak was anticipated by 30 days in co-infected than single infected hosts [for comparison: Figure 2 in Murphy *et al.* (23)]. This cytokine swapping, in addition to the slow development of antibodies, may have facilitated *G. strigosum* larval establishment and adult survival and the higher infection intensity observed. Higher *G. strigosum* numbers were also reported in rabbits co-infected with the respiratory bacterium

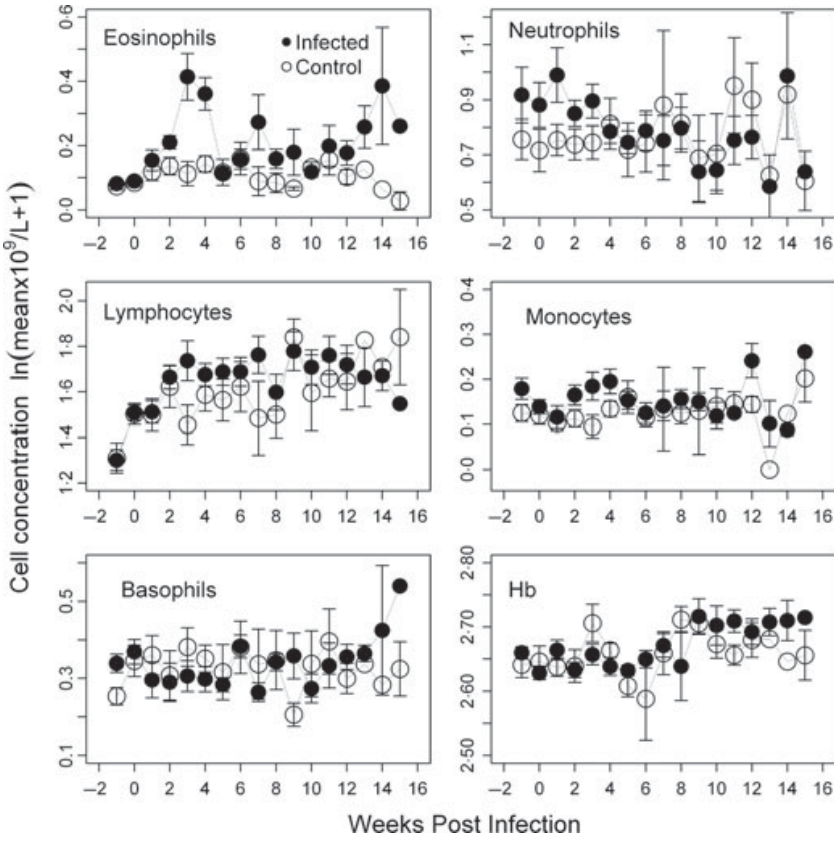

Figure 5 Haematological profiles from infected and control animals over the course of the infection. Data represent longitudinal sampling of individuals. Concentration values in the sampling week before 0 represent the responses from animals bled prior to infection. Hb = haemoglobin.

B. bronchiseptica (26). In this case, the strong and early peak in IL-10, rather than IFN-γ, could have contributed to the increased parasite load. These findings support the hypothesis that for *G. strigosum,* the presence of a second parasite/pathogen can lead to similar dynamics of infection, but different immune pathways – under the same experimental design – and more attention should be given to the interpretation of these dynamics in hosts co-infected with different infectious agents, particularly in natural settings.

Consistent with our previous work on single infections and co-infections, we found that *T. retortaeformis* generated an early and robust IFN-γ peak (23, 24). We have previously suggested that this is an inflammatory response to bacterial infiltration into the small intestine mucosa following brief movements of larvae into the tissue (23, 24, 29, 31). The short-lived peak observed in the current study probably contributed to the initial, albeit not statistically significant, lower intensities than single infections, suggesting that IFN-γ might interfere with *T. retortaeformis* early regulation. We recently showed that neutrophils, in combination with IL-4, antibodies and eosinophils are important for the rapid clearance of *T. retortaeformis* in *B. bronchiseptica*-co-infected rabbits and that the lack of significant neutrophil recruitment in the single infection may have reduced worm expulsion (24). In the current study, we found early neutrophil accumulation followed by antibodies and eosinophils, suggesting that while they contributed to *T. retortaeformis* reduction, their involvement was not notably different from the single infection. Together, these findings indicate that the immune profile to *T. retortaeformis* is adjusted to whether the hosts are co-infected with another helminth or a bacterium, and the outcome can lead to major changes in parasite dynamics.

More generally, this co-infection study confirmed the fundamentally distinct immune profiles of the two helminths and the observations that stomach and small intestine, while connected and part of the more complex digestive tract, have distinct immunophysiological properties and parasites, adapt their life histories and strategies to these diverse habitats.

Long-term monitoring of a rabbit population naturally co-infected with these helminths supported only partially the laboratory findings (20, 21). In the field and consistent

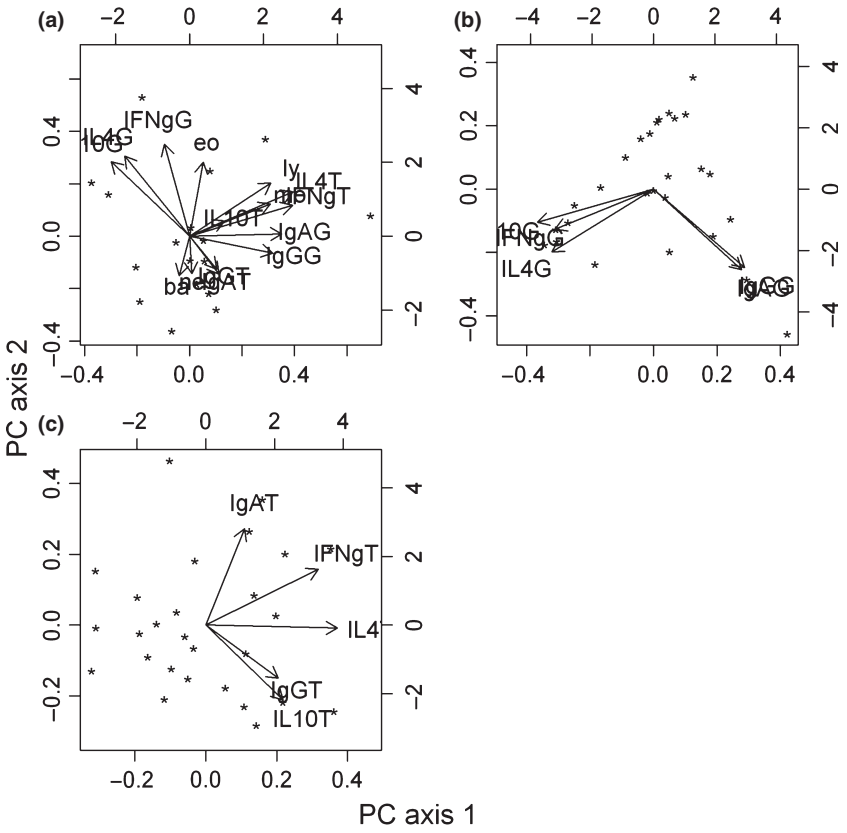

Figure 6 Relationship between the first two principal components from a principal component analysis on the local and systemic immune response of rabbits to co-infection with two gastrointestinal parasites. (a) Systemic and local immune response to both helminths; (b) local immune response to *G. strigosum* and (c) local immune response to *T. retortaeformis*. Variables ending with 'G' represent *G. strigosum* and 'T' represent *T. retortaeformis*. ba: basophils, eo: eosinophils, ly: lymphocytes and ne: neutrophils.

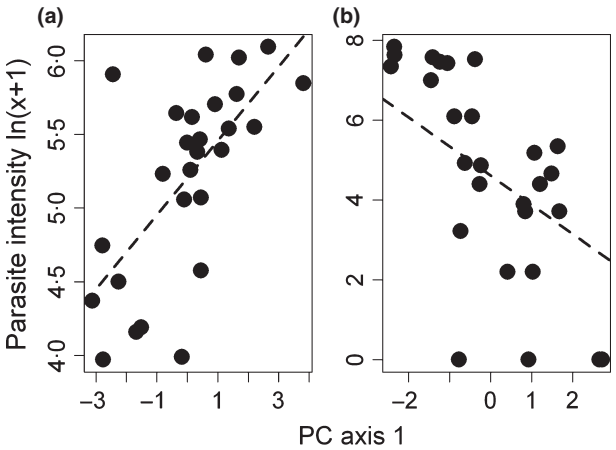

Figure 7 Relationship between parasite intensity and principal component one (PCA axis 1) for (a) *G. strigosum* (most representative immune variables in PCA axis 1: IL-10, IL-4 and IFN-γ) and (b) *T. retortaeformis* (most representative immune variables in PCA axis 1: IL-4 and IFN-γ). It should be noted that normalized C_t values are inversely related to gene expression. For example, a negative relationship between parasite intensity and PC-1, which is mainly explained by a positive association with cytokines, means that high worm load stimulates high cytokine expression.

with the current study, both parasites persist in co-infected rabbits, although immunity has better control of *T. retortaeformis* than of *G. strigosum* (20–22, 26). Contrary to the laboratory work, individuals naturally co-infected with the second parasite exhibited higher *T. retortaeformis* intensity, particularly in older individuals, but lower numbers or no apparent changes in *G. strigosum* load (20, 21). Serum IgA and IgG to *T. retortaeformis* E/S antigen were higher in co-infected than single infected hosts, but the opposite was observed for *G. strigosum* (unpub. results). The contrasting outcomes between field and laboratory are not contradictory, once the intrinsic immuno-epidemiology of the system is considered. In other words, wild rabbits are constantly re-infected with parasites that accumulate in the hosts and repeatedly challenge the immune system as well as density-dependent parasite constraints (20–22). The laboratory trial should be interpreted as a snapshot from the field where a single parasite dose – representative of an average infection in mature wild rabbits – stimulates similar immune responses but without re-infections and immune boosts or the additional contribution of intensity-dependent parasite constraints.

This study provides further evidence that helminth co-infections are an important source of host heterogeneity to infections. This variability can be generated by significant changes in the immune responses and/or parasite dynamics. Importantly, by altering host susceptibility, co-infections can enhance the risk of infection to other infectious agents of welfare and economic concern. Yet, more work is necessary to understand the immuno-epidemiology of persistent helminth co-infections. Laboratory trials on model systems have been instrumental in clarifying some of the critical mechanisms taking place; there is now a need to confirm these patterns in natural settings where these infections commonly occur.

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REFERENCES

1 Behnke JM, Bajer A, Sinski E & Wakelin D. Interactions involving intestinal nematodes of rodents: experimental and field studies. *Parasitology* 2001; **122**: S39–S49.
2 Cox FE. Concomitant infections, parasites and immune responses. *Parasitology* 2001; **122**: S23–S38.
3 Poulin R. Interactions between species and the structure of helminth communities. *Parasitology* 2001; **122**: S3–S11.
4 Stelekati E & Wherry EJ. Chronic bystander infections and immunity to unrelated antigens. *Cell Host Microbe* 2012; **12**: 458–469.
5 Griffiths EC, Pedersen AB, Fenton A & Petchey OL. The nature and consequences of co-infection in humans. *J Infect* 2011; **63**: 200–206.
6 Anthony RM, Rutitzky LI, Urban JF Jr, Stadecker MJ & Gause WC. Protective immune mechanisms in helminth infection. *Nat Rev Immunol* 2007; **7**: 975–987.
7 Allen JE & Maizels RM. Diversity and dialogue in immunity to helminths. *Nat Rev Immunol* 2011; **11**: 375–388.
8 Mulcahy G, ONeill S, Fanning J, McCarthy E & Sekiya M. Tissue migration by parasitic helminths - an immunoevasive strategy? *Trends Parasitol* 2005; **21**: 273–277.
9 Turaga PSD, Tierney TJ, Bennett KE, *et al.* Immunity to onchocerciasis: cells from putatively immune individuals produce enhanced levels of interleukin-5, gamma interferon, and granulocyte-macrophage colony-stimulating factor in response to *Onchocerca volvulus* larval and male worm antigens. *Infect Immun* 2000; **68**: 1905–1911.
10 Zhang WY, Moreau E, Hope JC, Howard CJ, Huang WY & Chauvin A. *Fasciola hepatica* and *Fasciola gigantica*: comparison of cellular response to experimental infection in sheep. *Exp Parasitol* 2005; **111**: 154–159.
11 Fabre V, Beiting DP, Bliss SK, *et al.* Eosinophil deficiency compromises parasite survival in chronic nematode infection. *J Immunol* 2009; **182**: 1577–1583.
12 Chamone M, Marques CA, Atuncar GS, Pereira AL & Pereira LH. Are there interactions between schistosomes and intestinal nematodes? *Trans R Soc Trop Med Hyg* 1990; **84**: 557–558.
13 Robertson LJ, Crompton DW, Sanjur D & Nesheim MC. Haemoglobin concentrations and concomitant infections of hookworm and *Trichuris trichiura* in Panamanian primary schoolchildren. *Trans R Soc Trop Med Hyg* 1992; **86**: 654–656.
14 Curry AJ, Else KJ, Jones F, Bancroft A, Grencis RK & Dunne DW. Evidence that cytokine-mediated immune interactions induced by *Schistosoma mansoni* alter disease outcome in mice concurrently infected with *Trichuris muris*. *J Exp Med* 1995; **181**: 769–774.
15 Lagrue C & Poulin R. Intra- and interspecific competition among helminth parasites: effects on *Coitocaecum parvum* life history strategy, size and fecundity. *Int J Parasitol* 2008; **38**: 1435–1444.
16 Turner JD, Jackson JA, Faulkner H, *et al.* Intensity of intestinal infection with multiple worm species is related to regulatory cytokine output and immune hyporesponsiveness. *J Infect Dis* 2008; **197**: 1204–1212.
17 Geiger SM, Alexander NDE, Fujiwara RT, *et al. Necator americanus* and Helminth Co-Infections: Further Down-Modulation of Hookworm-Specific Type 1 Immune Responses. *PLoS Negl Trop Dis* 2011; **5**: e1280.
18 Lass S, Hudson PJ, Thakar J, *et al.* Generating super-shedders: co-infection increases bacterial load and egg production of a gastrointestinal helminth. *J R Soc Interface* 2013; **10**: 20120588.
19 Graham A, Cattadori IM, Lloyd-Smith J, Ferrari M & Bjornstad ON. Transmission consequences of co-infection: cytokines writ large? *Trends Parasitol* 2007; **6**: 284–291.
20 Cattadori IM, Boag B, Bjørnstad ON, Cornell SJ & Hudson PJ. Immuno-epidemiology and peak shift in a seasonal host-nematode system. *Proc R Soc B* 2005; **272**: 1163–1169.
21 Cattadori IM, Boag B & Hudson PJ. Parasite co-infection and interaction as drivers of host heterogeneity. *Int J Parasitol* 2008; **38**: 371–380.
22 Cornell S, Bjørnstad ON, Cattadori IM, Boag B & Hudson PJ. Seasonality, cohort-dependence and the development of immunity in a natural host-nematode system. *Proc R Soc B* 2008; **275**: 473–591.
23 Murphy L, Nalpas N, Stear MJ & Cattadori IM. Explaining patterns of infection in free living populations using laboratory immune experiments. *Parasite Immunol* 2011; **33**: 287–302.
24 Thakar J, Pathak AK, Murphy L, Albert R & Cattadori IM. Network model of immune responses reveals key effectors to single and co-infection kinetics by a respiratory bacterium and a gastrointestinal helminth. *PLoS Comput Biol* 2012; **8**: e1002345.
25 Cattadori IM, Albert R & Boag B. Variation in host susceptibility and infectiousness generated by co-infection: the myxoma-*Trichostrongylus retortaeformis* case in wild rabbits. *J R Soc Interface* 2007; **4**: 831–840.
26 Pathak AK, Pelensky C, Boag B & Cattadori IM. Immuno-epidemiology of chronic bacteria-helminth co-infections: observations from the field and evidences from the laboratory. *Int J Parasitol* 2012; **42**: 647–655.
27 Guidance on the Operation of the Animals (Scientific Procedures) Act 1986. http://www.archive.official-documents.co.uk/document/hoc/321/321.htm
28 Pathak AK, Creppage KE, Werner JR & Cattadori IM. Immune regulation of a chronic bacterial infection and consequences for pathogen transmission. *BMC Microbiol* 2010; **10**: 226.
29 Pathak AK, Biarnes MC, Murphy L & Cattadori IM. Snapshot of spatio-temporal cytokine responses to single and

co-infections with helminths and bacteria. *Results Immunol* 2011; **1**: 95–102.
30 Livak KJ & Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods* 2001; **25**: 402–408.
31 Audebert F, Vuong PN & Durette-Desset MC. Intestinal migrations of *Trichostrongylus retortaeformis* (Trichostrongylina, Trichostrongylidae) in the rabbit. *Vet Parasitol* 2003; **112**: 131–146.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Antibody dilutions for ELISAs against E/S antigen of *G. strigosum* and *T. retortaeformis*.

