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Explaining patterns of infection in free-living populations using laboratory immune experiments

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SUMMARY

The host response to different helminth species can vary and have different consequences for helminth persistence. Often these differences are generated by changes in the dynamics and intensity of the immune components against parasites with distinct life history strategies. We examined the immune response of rabbits to primary infections of the gastrointestinal nematodes Trichostrongylus retortaeformis *and* Graphidium strigosum *under controlled conditions for 120 days post-challenge. Results showed that rabbits developed a robust and effective immune response against* T. retortaeformis *and abundance quickly decreased in the duodenum and was completely cleared in the remaining sections of the small intestine within 4 months. Infected individuals exhibited an initial strong inflammatory response (IFN-γ), IL-4 expression also increased and was coupled to a rapid serum and mucus IgG and IgA and eosinophilia. Strong IL-4, serum IgA and IgG responses and eosinophilia were also observed against* G. strigosum*. However, parasite abundance remained consistently high throughout the infection, and this was associated with relatively low mucus antibodies. These findings suggest that immunity plays a key role in affecting the abundance of these nematodes, and different immune mechanisms are involved in regulating the dynamics of each infection and their long-term persistence in free-living host populations.*

Keywords Graphidium strigosum*, local and systemic acquired immunity, rabbit, small intestine, stomach,* Trichostrongylus retortaeformis

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INTRODUCTION

The host immune response represents one of the most powerful lines of defence against helminth infections, and, not surprisingly, hosts have developed a large variety of immune components and functions to recognize and target different parasite life stages and their products (e.g. eggs and excretory/secretory compounds). The immune system can control the initial establishment of infective larvae, regulate their development and influence the survival, fecundity and clearance of the mature stages (1–9). Yet, the immune response to different helminth species is highly variable such that it may appear rapid and effective against one parasite species and slow to develop and inadequate for protection against another species. To gain a better appreciation of the strategies adopted by both parties and how they optimize their conditions, i.e. a healthy host and a parasite with high fitness, we need to understand the immunological processes that affect host–parasite interactions and see if they equally explain parasite dynamics in free-living animal populations and laboratory systems.

We have been investigating the epidemiology of infection of two gastrointestinal nematodes, *Trichostrongylus retortaeformis* and *Graphidium strigosum*, in a free-living population of European rabbits (*Oryctolagus cuniculus*) by examining the relationship between host age and parasite intensity (10,11). Based on the shape of this association (linear, logistic or convex), it is possible to infer the fundamental mechanisms of host–parasite regulation (10,12,13). We proposed a parsimonious hypothesis for the dynamics of the rabbit–nematode system where the seasonal dynamics of *T. retortaeformis* were driven primarily by the host acquired immune response affecting helminth development and fecundity (10,14,15), while *G. strigosum* was not constrained by immunity, so that parasite abundance increased exponentially with host age (11). Previous studies supported the hypothesis of an immune-regulated

T. retortaeformis infection and noted that third-stage larvae may enter arrested development under adverse immunological conditions (16). The tendency to arrest the development in the mucosa and the evidence of intestinal pathology were more recently confirmed in laboratory experiments (17,18). Laboratory infections of rabbits with *G. strigosum* showed a clear increase in serum IgG but this was not sufficient to clear the infection, and high intensities were still observed 3 months after the initial challenge (19). No clinical symptoms but chronic asthenic gastritis were also reported in rabbits exposed to different infection doses (20). Overall, these studies indicate that rabbits develop different immune responses against *T. retortaeformis* and *G. strigosum*, which can explain the different patterns of infection observed in free-living rabbit populations.

The identification of the processes affecting host–parasite interactions can be challenging in natural animal systems if more than one mechanism is taking place and, even more, when there are confounding variables that cannot be ruled out (10,21). Motivated by our epidemiological work and to gain a better understanding of the immuno-parasitological mechanisms influencing the interaction between the host and its parasites, we undertook a comprehensive study to quantify changes in the rabbit's immunological components and associated helminth intensities, during a primary infection of *T. retortaeformis* and *G. strigosum*. Laboratory infections were performed, wherein rabbits were challenged with third-stage larvae (L3) and the dynamics of the systemic and local immune response quantified for 120 days post-challenge. Our prediction was that the immune response to the two helminths differed fundamentally in the intensity but not the type of components activated, so that *T. retortaeformis* would elicit a stronger response than *G. strigosum*, and this would lead to the clearance of the first but not the second nematode. The ultimate goal of this study was twofold: first, to identify the most common immunological processes and essential components affecting the epidemiology of these gastrointestinal infections and second, to highlight the immunological differences between these helminths and discuss how they can explain the epidemiology of infection in free-living rabbit populations.

MATERIALS AND METHODS

The parasites

Trichostrongylus retortaeformis and *G. strigosum* are ubiquitous gastrointestinal nematodes of the European rabbit and closely associated with this host during their evolution (22). Both nematodes have a direct life cycle, and infection occurs by ingestion of free-living infective third-stage larvae (L3); *T. retortaeformis* colonizes the small intestine, and *G. strigosum* inhabits the stomach. In the host, nematodes develop into adults and reproduce sexually, and eggs are shed through the rabbit faeces; the prepatent period is about 11 days for *T. retortaeformis* and 42 days for *G. strigosum* (23–26). For our laboratory infections, third-stage infective larvae of *T. retortaeformis* were kindly provided by Dr Dominique Kerboeuf (INRA, France), while *G. strigosum* larvae were extracted by culturing faeces from rabbits initially infected with adult parasites collected from our free-living population of rabbits in Tayside, Scotland (10).

Experimental design and sample collection

The laboratory experiments were designed as primary monospecific infections of rabbits with 5500 *T. retortaeformis* or 650 *G. strigosum* third-stage larvae (L3). The infection doses (force of infection) were estimated following Cattadori *et al.* (27) and based on the intensity of adult nematodes in a free-living rabbit population monitored from 1977 to 2003. Outbred, 60-day-old New Zealand White male rabbits, free of helminths and other parasites or pathogens (Harlan, Hillcrest, UK), were housed in individual cages with food and water *ad libitum* and a 12-h light cycle. Following a 1-week acclimation period, the individuals were orally challenged by gavage with a mineral water solution (5 mL) of L3 nematodes or mineral water for the controls. Groups of six individuals (four infected and two controls, eight infected and four controls at day 60) were euthanized with Euthatal™ (Merial, Harlow, UK), and post-mortem analysis carried out at days 4, 7, 14, 30, 45, 60, 75, 90 and 120 post-infection (DPI); for *G. strigosum*, the first two sampling points (day 4 and 7) were not collected. These points were chosen to quantify the immune response at time intervals that correspond to the different developmental stages of these helminths, L3, L4, immature and adults (25,26) but also to closely follow changes in the immune response during the infection period.

For *T. retortaeformis* single infection, the small intestine (SI) was divided into four equal sections, SI-1 to SI-4 from the duodenum to the ileum. Each section was further divided into four equal segments; segments 1 and 3 were stored in PBS (pH 7·4), for nematode counts, and segments 2 and 4 were processed. To quantify mucosal cytokine expression, five pieces of tissue (5×5 cm) were collected from segment 2 and stored in RNAlater (Sigma, St Louis, MO, USA) at −80°C. We selected the mucosa tissue because we were interested in

a cytokine response at the site of infection and how this was related to nematode abundance. Here, we focus on SI-1, where most of the parasites were found. To quantify the localized antibody response, mucus was gently scraped from the remaining tissue, collected into a 5-mL tube and diluted with 3 mL of PBS supplemented with a protease inhibitor cocktail (Roche, Burgess Hill, West Sussex, UK). Samples were mixed at 4°C overnight, spun at 25 000 g at 4°C for 30 min, and the supernatant collected and stored at −80°C. A sample of tissue (3 × 3 cm) was removed from the first section (SI-1) and fixed in 10% neutral buffered formalin for histological analysis. These general procedures were repeated for the *G. strigosum* single infection. Specifically, the stomach was divided in two equal longitudinal sections; the right section with the food content and the wash from the left section were stored in PBS for nematode counts, while the left section was cut below the oesophagus connection in two parts, the fundus and the antrum (i.e. top and bottom). RNAlater samples and mucus were collected from the top and bottom parts as previously described; a small sample of the top section was also removed and fixed in 10% neutral buffered formalin.

Blood samples were collected twice weekly from the marginal ear vein of every animal, and a small aliquot (0·2 mL) was stored into EDTA-coated tubes (Sartorius, Goettingen, Germany) for blood cell count and the remaining (0·8 mL) spun down at 12 000g for 10 min; thereafter, serum was extracted and stored at −80°C for antibody detection. Individual body mass was recorded weekly, and animals were monitored routinely for health status. All listed animal procedures were approved by the University of Glasgow and carried out under the authority of the UK Animals Act 1986 by the Home Office.

Parasitology

To quantify the number of nematodes established in the small intestine (sections SI-1 to SI-4) or stomach (top and bottom) at each sampling point (DPI), the samples stored in PBS were washed over a sieve (100 μM) with tap water. Nematodes and the remaining gut contents were then collected into conical flasks, allowed to settle at room temperature overnight; the excess supernatant carefully removed and the remainder stored in 50-mL tubes. For *T. retortaeformis*, five 2·5 mL aliquots were counted and the average number scaled to the length of every section; developmental stages (L4, immature or adult) and sex (adult parasites) were also determined. This procedure was repeated for fourth-stage larvae and immature *G. strigosum*, while for the adults the total number of parasites was counted in each tube.

Cytokine expression

RNA isolation and reverse transcription

Cytokine gene expression in the duodenum (SI-1) and fundic (top) mucosa was determined using a Q-RT-PCR approach. Initially, RNA was extracted from small intestine or stomach samples using the Qiagen RNeasy Lipid Tissue kit following tissue disruption in Qiazol lysis reagent and using a Tissueruptor homogeniser for 40 s (Qiagen, Hilden, Germany). The RNA was then treated with TURBO DNase (Ambion, Austin, TX, USA) to remove any contaminating DNA, and the quality assessed using a 2100 Bioanalyser (Agilent, Santa Clara, CA, USA). RNA concentration was determined by measuring absorbance at 260 nm (A260) with a BioTek Spectrophotometer (Winooski, VT, USA) and adjusted to 250 ng/μL with RNAse-free water (Sigma). Two micrograms of RNA was then reverse transcribed with High Capacity RNA-to-cDNA kit following manufacturers' instructions (Applied Biosystems, Foster City, CA, USA). Complementary DNA samples (cDNA) were then diluted 1 : 5 in RNAse-free water and stored at −20°C for further use.

Real-time RT-PCR

The expression level of IL-4, IL-10 and IFN-γ was determined by relative quantification using Taqman Q-RT-PCR. Hypoxanthine phosphoribosyl transferase (HPRT) was included as a housekeeping gene and custom-designed by Applied Biosystems based on sequences obtained from Genbank for IL-4, IFN-γ and HPRT (Accession numbers AF169170, D84216 and M31642, respectively), while for rabbit IL-10, a predesigned assay from Applied Biosystems was used (Oc03396942_m1). Primer-probe pairs sequence for the three cytokines, and the house keeping gene are reported in Pathak *et al.* (28). Reactions were performed in MicroAmp® Optical 96-well plates using 1× Taqman Gene Expression Master Mix, 1× expression assay and 100 ng cDNA in a 25 μL reaction. PCRs were performed on a 7500 Real Time PCR system using the default cycling conditions: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s for 40 cycles, 60°C for 1 min (Applied Biosystems). Real-time data were expressed as C_t (cycle threshold) values. C_t values for IL-4, IL-10 and IFN-γ were normalized to the HPRT to control for variability in cDNA amount and reaction efficiencies.

Systemic and local antibody detection

To quantify local (mucus) and systemic (serum) changes in the IgA and IgG response to the establishment (L3) and

survival (adults) of both nematodes, an enzyme-linked immunosorbent assay (ELISA) was performed. As a source of antigen, we used L3 larvae extracted from a culture of faeces harvested from rabbits infected with the same batch of nematode larvae used in these experiments, while adult nematodes were collected from our wild rabbit population. Nematodes from wild rabbits showed less antibody background noise at the ELISA than the adults extracted from the laboratory infected rabbits (results not showed). Nematodes were washed in PBS and protease inhibitors and subsequently homogenized in a Hybaid ribolyser (2 mm steel balls, twelve 30 s pulses). The extract was spun at 13 000 rpm for 5 min, the soluble extract removed, and the protein concentration determined using the Bradford assay (Sigma, Dorset, UK) and then stored at −20°C.

The ELISA design was similar for serum and mucus samples of both infections. Antigen concentrations and antibody dilutions were optimized using a checkerboard titration and the optimal dilutions selected at the inflection point from the resulting dilution curves. The dilutions established for the antigen, mucus and secondary antibodies to *T. retortaeformis* and *G. strigosum* are reported in Table 1. Antigens were coated onto 96-well high binding plates in 0·05 M sodium bicarbonate buffer pH 9·6 for 16 h at 4°C, then washed in TBS/0·05% Tween-20 (TBS-T), and the wells blocked with 5% dried milk in TBS-T for 2 h at 37°C. Plates were washed again, samples diluted in TBS-T then incubated for 1 h at 37°C. Goat-anti-rabbit IgA or IgG (ab2759/ab6721; Abcam, Cambridge, UK) was added to each well, plates incubated for a further hour, washed and 100 μL of TMB substrate (Insight Biotechnology, Wembley, UK) added to each well; plates were finally incubated for 10 min at room temperature before the reaction was stopped by adding an equal volume of 1 M H_3PO_4. The optical density of each plate was read at 450 nm using a FLUOstar Optima plate reader (BMG labtech, Aylesbury, UK). Positive and negative controls, made from pools of high responders and control animals, respectively, were included on each plate along with a no-serum or no-mucus control to determine the background reading. Serum and mucus samples as well as high, low and background controls were run in duplicate on each plate.

Table 1 Concentrations used in the ELISA reagents for the somatic antigen detection in serum and mucus. Secondary antibodies are from goat-anti-rabbit IgA/IgG

	Serum		Mucus	
Isotypes	IgA	IgG	IgA	IgG
Trichostrongylus retortaeformis				
Antigen concentration (μg/mL)				
L3	5	5	5	5
Adult	1	1	1	1
Serum or mucus dilution				
L3	1 : 10	1 : 200	1 : 20	1 : 10
Adult	1 : 20	1 150	1 : 10	1 : 10
Conjugated secondary antibody dilution				
L3	1 : 5000	1 : 20 000	1 : 5000	1 : 5000
Adult	1 : 10 000	1 : 20 000	1 : 5000	1 : 5000
Graphidium strigosum				
Antigen concentration (μg/mL)				
L3	1	1	1	1
Adult	1	1	1	1
Serum or mucus dilution				
L3	1 : 10	1 : 75	1 : 10	1 : 10
Adult	1 : 10	1 : 75	1 : 10	1 : 10
Conjugated secondary antibody dilution				
L3	1 : 5000	1 : 10 000	1 : 5000	1 : 5000
Adult	1 : 5000	1 : 10 000	1 : 5000	1 : 5000

Haematology

Weekly individual blood samples anticoagulated with EDTA were analysed using an Advia 120 haematology analyser with species-specific software (Siemens Healthcare Diagnostics Inc., Surrey, UK) at the Glasgow University Veterinary Clinical Pathology Laboratory (Glasgow, UK). Analytes measured included: erythrocyte concentration (RBC), haemoglobin concentration (Hb) and leucocyte concentration (WBC). Blood smears were stained using May-Grünwald and Giemsa and examined for platelet clumps and morphological abnormalities. A manual leucocyte differential count was performed on 200 cells, and the absolute concentration for each leucocyte type (eosinophils, basophils, neutrophils, lymphocytes) was calculated by multiplying the percentage of each leucocyte type present by the WBC. Platelet indices were not reported for samples containing platelet clumps.

Histology

Tissue samples from the first section (SI-1) of the small intestine and the top section of the stomach fixed in formalin were dehydrated in graded ethanol solutions and embedded in paraffin wax. Histological sections (4 μm thick) were then stained with haematoxylin and eosin. Slides were examined and scored for a range of nematode-associated pathological criteria including: recruitment of eosinophils, lymphocytes, villous atrophy, crypt hyperplasia, focal glandular destruction and epithelial de-differentiation (analysis kindly performed by Dr A. Philbey, University of Glasgow, UK).

Data transformation and analysis

Initially, the normalized cytokine C_t values were averaged between the two replicates, for each individual at every sampling point. Data were visually presented following the comparative $2^{-\Delta\Delta Ct}$ method (29) where C_t values of infected rabbits at every sampling point (DPI) were scaled over the average C_t of the whole controls and the mean and standard error of the scaled C_ts from the infected hosts calculated at each sampling point. For analytical purposes, the normalized mean C_t values, from infected and controls, were used.

To confirm uniformity of the ELISA among plates, the relationship between corrected optical density high controls (OD high control – OD background control) and corrected optical density low controls (OD low control – OD background control) was examined; plates were repeated when results were reported as outliers, and the high/low ratio among all plates was not consistent with a linear relationship and a Pearson's correlation coefficient (r) above 0·65. The antibody optical density values, background corrected, were then transformed and standardized into optical density indexes as: Xi = (OD test sample – OD negative control)/(OD positive control-OD negative control) where Xi represents a replicate for each individual at every sampling point (30,31). The average of the two replicates was then calculated for each individual at every sampling point, and the new standardized mean optical density indexes used for the statistical analyses.

Linear mixed effect models (with restricted maximum likelihood, LME-REML) were used unless otherwise specified. To highlight differences in the dynamics of infection compared to the controls, nematode abundance or immune variables (cytokines, blood cells, systemic and local antibodies), as response variable, were examined in relation to treatment (infected and control), time (days or weeks post-infection, DPI or WPI) or location of the infection (SI-1 to SI-4 or stomach top & bottom) as independent variables. The individual identification code (ID) was included as a random effect or/and as an autoregressive function of order 1 (AR-1) to take into account the nonindependent sampling of the same individual through time or the monitoring of different parts of the same organ from the same individual. To identify the combination of immunological variables that mainly affected parasite abundance, this analysis was repeated using parasite abundance as a response variable and immune variables as independent factors.

The immune variables were initially selected through a principal component analysis (PCA singular value decomposition) based on the infected individuals. Specifically, the multivariate association of different combinations of variables was examined, and the predictions from the combinations that explained most of the variance of the first and second principal components were then used for the linear mixed effect models. These analyses were performed for both *T. retortaeformis* and *G. strigosum* infections.

RESULTS

Nematode abundance

Infection of rabbits with *T. retortaeformis* or *G. strigosum* led to the successful establishment of infective larvae (82% for *T. retortaeformis* at seven DPI and 44% for *G. strigosum* at 15 DPI) and subsequent development into adults (Figure 1).

Trichostrongylus retortaeformis: Abundance significantly decreased with time of infection (DPI), and among sections, the highest intensity was recorded in the duodenum

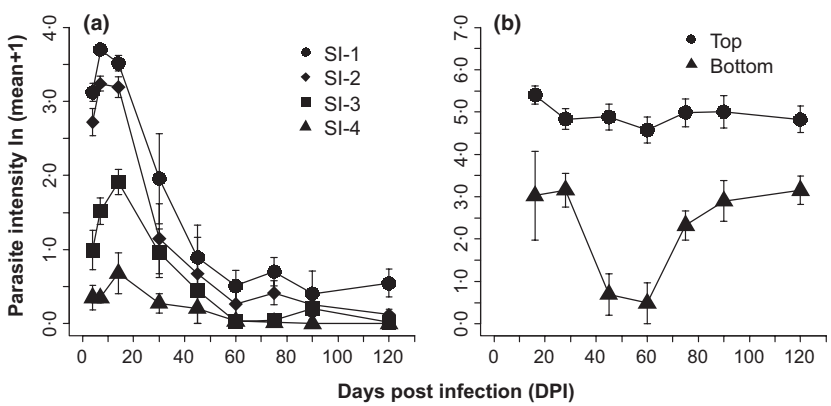

Figure 1 Mean parasite intensity (± standard errors) in infected rabbits by days post-infection (DPI): (a) scaled by the length of the small intestine (SI-1 to SI-4, from duodenum to ileum, respectively) for *Trichostrongylus retortaeformis* and (b) stomach section (top and bottom, from fundus to antrum, respectively) for *Graphidium strigosum*.

Table 2 Summary of the linear mixed effect model (LME) between parasite intensity, as response, and day post-infection (DPI) and small intestine location (SI-1 to SI-4), or stomach (top and bottom), as independent variables. The standard deviation for the intercept (random effect) and the autocorrelation effect of using different sections of the same organ from the same host (AR-1) are also reported

	Coeff, SE, d.f.	*P*
Trichostrongylus retortaeformis		
Intercept	3·133 ± 0·181, 114	<0·00001
DPI	−0·031 ± 0·003, 38	<0·00001
SI-2	−0·440 ± 0·114, 114	<0·001
SI-3	−1·770 ± 0·153, 114	<0·00001
SI-4	−2·699 ± 0·179, 114	<0·00001
DPI:SI-2	0·002 ± 0·002, 114	n.s.
DPI:SI-3	0·016 ± 0·002, 114	<0·00001
DPI:SI-4	0·026 ± 0·003, 114	<0·00001
AIC	270·871	
Random effects (inter. SD)	6·1607e-05	
AR(1)	0·801	
Graphidium strigosum		
Intercept	4·930 ± 0·213, 27	<0·00001
Bottom	−2·681 ± 0·263, 27	<0·00001
AIC	181·1091	
Random effects (inter. SD)	0·1001765	
AR(1)	0·229	

(SI-1) and the lowest in the ileum (SI-4), once differences among individuals and the nonindependent sampling of the four sections from the same organ of each individual were taken into account (Figure 1a and Table 2). The increase in larvae from day 3 to 7 days post-challenge was probably due to the gradual migration of L3 from the stomach to the different sections of the small intestine (24,25). Individuals never completely cleared the infection, and nematodes were still present, although with very low numbers, in the first section at 120 days post-challenge.

Graphidium strigosum: Abundance was consistently higher in the fundus compared to the antrum, and no temporal changes were observed between sampling points (or the interaction between sampling point and organ section), when differences among individuals and the nonindependent sampling of the two parts of the stomach from the same individual were considered (Figure 1b, Table 2). All infected individuals maintained a constant number of nematodes up to 120 days post-infection. The drop in parasite number in the antrum at day 40 and 60 post-challenge was caused by a sampling procedure and should not be considered biologically relevant.

These results were consistent with our long-term observations on the intensity of infection of these nematodes in free-living rabbits of different age, specifically, rabbits can reduce or clear *T. retortaeformis* but not *G. strigosum*.

Cytokine expression

Trichostrongylus retortaeformis: A strong IFN-γ expression in the first section of the small intestine of infected rabbits was observed during the first 30 days post-challenge; thereafter, no dominant pattern was observed (Figure 2a). Analysis based on the normalized C_t values (that differs from the $2^{-\Delta\Delta Ct}$ transformation in Figure 2) found that changes in IFN-γ and IL-4 significantly differed between treatments (infected and controls) and time post-infection (DPI): IFN-γ decreased while IL-4 increased in transcription with the infection course, IL-10 exhibited constant expression over time although was significantly higher in infected compared to controls (Table 3).

Graphidium strigosum: A robust IL-4 expression was observed in the top section of the stomach of infected rabbits; however, the between-individual variability was high as highlighted by the large standard error bars

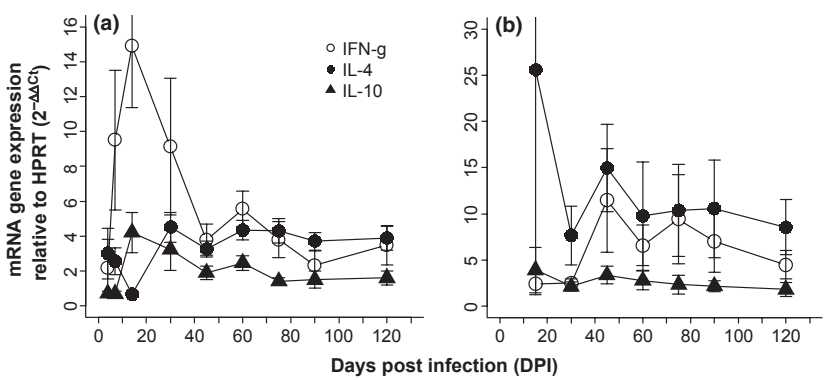

Figure 2 Mean cytokine expression (± standard errors) by day post-infection (DPI) in: (a) first section of the small intestine for *Trichostrongylus retortaeformis* and (b) stomach top section for *Graphidium strigosum*. C_t data have been normalized to the housekeeping gene, HPRT, and corrected for the total controls mean value.

Table 3 Summary of the linear mixed effect model (LME) between cytokine expression, as response, and day post-infection (DPI) and treatment (infected and controls), as independent variables. Additional explanations are reported in Table 2

Trichostrongylus retortaeformis			*Graphidium strigosum*		
	Coeff, SE, d.f.	*P*		Coeff, SE, d.f.	*P*
INF-g			INF-g		
Intercept	1·774 ± 0·089, 56	<0·0001	Intercept	2·700 ± 0·027, 40	<0·0001
Sampling time (DPI)	0·002 ± 0·001, 56	<0·05	–	–	–
Treatment (inf. vs. ctrls)	−0·329 ± 0·083, 56	<0·001	Treatment (inf. vs. ctrls)	−0·189 ± 0·033, 40	<0·0001
AIC	51·29473		AIC	−55·31513	
Random effects (inter. SD)	0·2792725		Random effects (inter. SD)	0·09529224	
IL-4			IL-4		
Intercept	1·822 ± 0·071, 56	<0·0001	Intercept	2·415 ± 0·053, 40	<0·0001
Sampling time (DPI)	−0·002 ± 0·001, 56	<0·01	–	–	–
Treatment (inf. vs. ctrls)	−0·302 ± 0·065, 56	<0·0001	Treatment (inf. vs. ctrls)	−0·364 ± 0·065, 40	<0·0001
AIC	24·12647		AIC	−1·981936	
Random effects (inter. SD)	0·2191188		Random effects (inter. SD)	0·1856037	
IL-10			IL-10		
Intercept	1·861 ± 0·039, 57	<0·0001	Intercept	2·170 ± 0·041, 40	<0·0001
Treatment (inf. vs. ctrls)	−0·115 ± 0·047, 57	<0·05	Treatment (inf. vs. ctrls)	−0·164 ± 0·050, 40	<0·01
AIC	−26·57442		AIC	−23·25842	
Random effects (inter. SD)	0·1578360		Random effects (inter. SD)	0·1422602	

(Figure 2b). Based on the C_t values, the expression of the three cytokines was higher in the infected compared to the controls but no significant changes were recorded during the course of the infection (Table 3).

The two infections clearly showed different cytokine profiles, which imply differences in the effectors and timing of their activation as well as their dynamical consequences.

Antibody detection, systemic and local response

The somatic antibody response of infected rabbits to L3 and adult stage was similar both for IgA and IgG against the two nematodes supporting the hypothesis that the two parasite stages cross-react at the antibody level. As such, we only present the results for the adult stage (Figures 3 and 4) and summarize in the supplement the findings for the L3 stage (Figures S1 and S2).

Trichostrongylus retortaeformis: A strong systemic and localized IgA and IgG response to adult nematodes (as well as L3, S1 in Supporting Information) was observed in infected hosts compared to the controls. At the systemic level, serum IgA peaked around 3 weeks post-infection (WPI) and decreased thereafter (Figure 3). Serum IgG quickly increased to a asymptote around three WPI and remained consistently high throughout the infection (Figure 3). Changes in serum IgA and IgG were significantly different between treatment (infected and controls) and the interaction between treatment and WPI, when the analysis was corrected for the random effect of the host and the nonindependence of sampling the same individual over time (Table 4). Mucus IgA and IgG patterns exhibited similar trends: values were significantly higher in the infected, compared to controls, and decreased from section 1 to section 4 of the small intestine; mucus IgG also increased with sampling time in infected rabbits (Table 4).

Graphidium strigosum: Infected rabbits mounted a strong somatic IgA and IgG response at the systemic level but the local antibody response was relatively low to both adult and L3 stages (Figures 4 and S2). Specifically, serum IgA and IgG significantly differed between treatments and increased with infection time in infected individuals (Table 5). Mucus IgA and IgG were higher in the infected compared to the controls, and for the infected, values increased with the course of infection showing stronger response in the fundus compared to the antrum section of the stomach (Table 5).

Together these findings suggest that rabbits develop an effective systemic and local antibody response to *T. retortaeformis* but an inefficient mucosal response to *G. strigosum*.

Haematological profile

Trichostrongylus retortaeformis: Total white blood cell and lymphocyte counts were significantly higher in infected hosts compared to the controls and consistently increased over the course of the infection (Figure 5). No significant trend was recorded for eosinophils and neutrophils, corrected for the random effect of the host and the

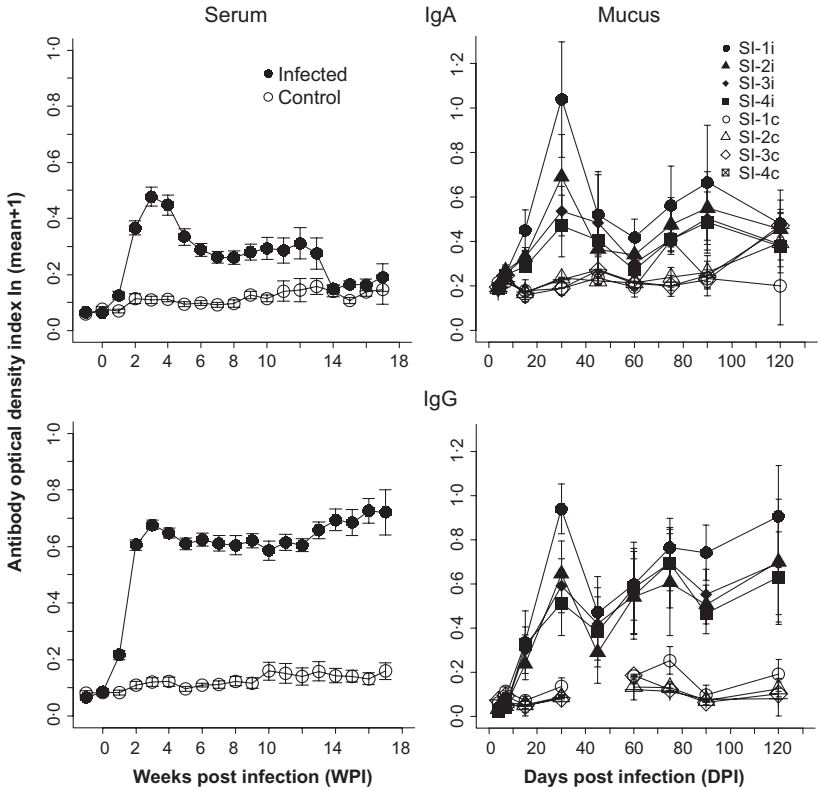

Figure 3 Mean absorbance (OD index ± standard errors) of systemic (serum) and local (mucus) antibody response against somatic *Trichostrongylus retortaeformis* adult stage by: treatment (infected and controls), sampling time [weeks post-infection (WPI) or days post-infection (DPI)] and small intestine location (from SI-1 to SI-4) for mucus. Week-1: sampling was performed the week antecedent the infection.

Table 4 Summary of the linear mixed effect model between systemic and localized antibody level (OD index) to *Trichostrongylus retortaeformis*, as response, and weeks post-infection (WPI), treatment (infected and controls) and organ location (for the mucus samples) as independent variables. Additional explanations are reported in Table 2

Trichostrongylus retortaeformis

	Coeff, SE, d.f.	*P*		Coeff, SE, d.f.	*P*
Serum IgA			Serum IgG		
Intercept	0·077 ± 0·028, 949	<0·01	Intercept	0·093 ± 0·042, 887	<0·01
Sampling time (WPI)	0·004 ± 0·004, 949	n.s.	Sampling time (WPI)	0·005 ± 0·006, 887	n.s.
Treatment (inf. vs. ctrls)	0·085 ± 0·034, 57	<0·05	Treatment (inf. vs. ctrls)	0·136 ± 0·050, 55	<0·05
Treatment:WPI	0·011 ± 0·005, 949	<0·05	Treatment:WPI	0·037 ± 0·007, 887	<0·05
AIC	−1747·323		AIC	−1500·372	
Random effects (inter. SD)	1·107786e-05		Random effects (inter. SD)	1·710009e-05	
AR(1)	0·814		AR(1)	0·874	
Mucus IgA			Mucus IgG		
Intercept	0·289 ± 0·050, 174	<0·001	Intercept	−0·020 ± 0·085, 150	n.s.
SI-2	−0·062 ± 0·016, 174	<0·001	SI-2	−0·115 ± 0·019, 150	<0·0001
SI-3	−0·082 ± 0·022, 174	<0·001	SI-3	−0·097 ± 0·019, 150	<0·0001
SI-4	−0·103 ± 0·026, 174	<0·001	SI-4	−0·109 ± 0·019, 150	<0·0001
Treatment (inf. vs. ctrls)	0·181 ± 0·058, 57	<0·01	Treatment (inf. vs. ctrls)	0·355 ± 0·080, 48	<0·001
			Sampling time (DPI)	0·004 ± 0·001, 48	<0·001
AIC	−211·8638		AIC	−153·5671	
Random effects (inter. SD)	5·718914e-05		Random effects (inter. SD)	0·2544637	
AR(1)	0·856		AR(1)	0·069	

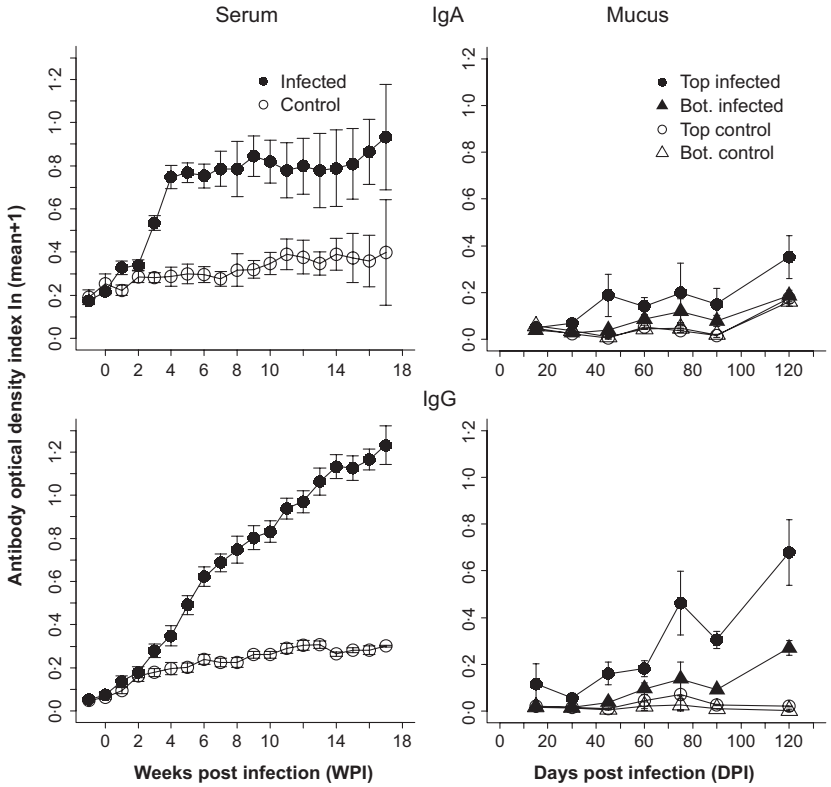

Figure 4 Mean absorbance (OD index ± standard errors) of systemic (serum) and local (mucus) antibody response against somatic *Graphidium strigosum* adult stage by: treatment (infected and controls), sampling time [weeks post-infection (WPI) or days post-infection (DPI)] and stomach location (top and bottom) for mucus. Week-1: sampling was performed the week antecedent the infection.

Table 5 Summary of the linear mixed effect model between systemic and localized antibody level (OD index) to *Graphidium strigosum*, as response, and weeks post-infection (WPI), treatment (infected and controls) and organ location (for the mucus samples) as independent variables. Additional explanations are reported in Table 2

Graphidium strigosum					
	Coeff, SE, d.f.	*P*		Coeff, SE, d.f.	*P*
Serum IgA			Serum IgG		
Intercept	0·115 ± 0·064, 754	n.s.	Intercept	−0·003 ± 0·051, 732	n.s.
Sampling time (WPI)	0·037 ± 0·005, 754	<0·0001	Sampling time (WPI)	0·043 ± 0·003, 732	<0·0001
Treatment (inf. vs. ctrls)	0·254 ± 0·075, 40	<0·01	Treatment (inf. vs. ctrls)	0·227 ± 0·061, 40	<0·001
AIC	−841·0541		AIC	−2004·927	
Random effects (inter. SD)	6·33025e-05		Random effects (inter. SD)	1·428634e-05	
AR(1)	0·9043314		AR(1)	0·960	
Mucus IgA			Mucus IgG		
Intercept	−0·01 ± 0·032, 39	n.s.	Intercept	−0·074 ± 0·046, 41	n.s
Treatment (inf. vs. ctrls)	0·069 ± 0·026, 39	<0·01	Treatment (inf. vs. ctrls)	0·165 ± 0·037, 39	<0·001
Bottom	−0·051 ± 0·016, 39	<0·01	Bottom	−0·128 ± 0·026, 41	<0·0001
Sampling time (DPI)	0·002 ± 0·001, 39	<0·001	Sampling time (DPI)	0·003 ± 0·001, 39	<0·0001
AIC	−122·4270		AIC	−54·19339	
Random effects (inter. SD)	0·007380853		Random effects (inter. SD)	0·00046	
AR(1)	0·421		AR(1)	0·279	

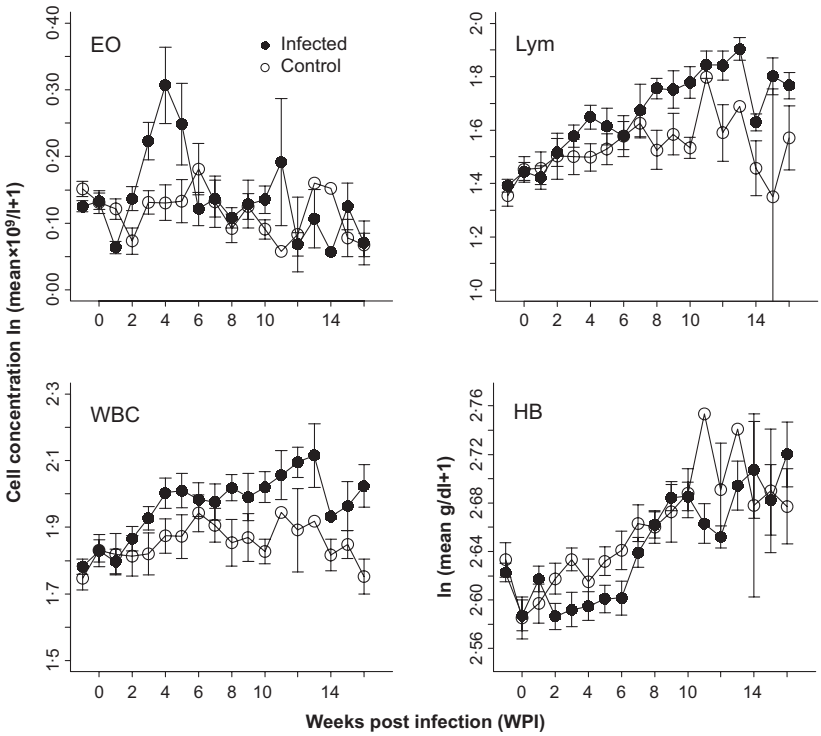

Figure 5 Mean blood cell concentration (± standard errors) in *Trichostrongylus retortaeformis* experiment by weeks post-infection (WPI) and treatment (infected and controls). EO, eosinophils; LY, lymphocytes; WBC, while blood cells; HB, haemoglobin.

dependence of sampling the same individual over time (Figure 5). However, a more detailed analysis showed that during the second-to-fifth WPI, coinciding with the peak in antibody response, a strong eosinophilia, anaemia (haemoglobin) and high total white blood cells were recorded in infected compared to control rabbits (Table 6).

Graphidium strigosum: A consistent increase in the concentration of eosinophils, lymphocytes, total white blood cells and haemoglobin was observed with the progression of the experiment but no significant differences were recorded between the infected and the controls (Figure 6, Table 6). In line with *T. retortaeformis* infection high eosinophilia, neutrophilia and total white blood cell concentrations were found during the second-to-the fourth WPI in infected compared to the controls; no significant development of anaemia was observed during this infection.

These trends support the evidence of a strong peripheral leucocyte-mediated immune response to both nematodes albeit short and mainly concentrated in the first few WPI.

Histology

Trichostrongylus retortaeformis: The establishment, development and survival of nematodes in the small intestine caused significant villous atrophy, increased crypt hyperplasia, reduction in the height-depth villus-crypt ratio and local recruitment of plasma cells, eosinophils and lymphocytes, compared to the controls (For all Fisher's exact test: $P < 0{\cdot}05$). No significant changes in the intensity of the damage were observed with the course of the infection.

Graphidium strigosum: Consistent focal glandular destruction, epithelial dedifferentiation and higher recruitment of eosinophils, lymphocytes and plasma cells were observed in the stomach tissue of infected compared to control individuals (For all Fisher's exact test: $P < 0{\cdot}01$).

Overall, both nematodes appeared to cause pathological damage by altering mucosa structure and recruitment of leucocytes to the site of infection.

Relationship between immunity and parasite abundance

Trichostrongylus retortaeformis: The linear combination of IFN-γ, IL-4, IgA, IgG, eosinophils and lymphocytes, measured at the local site of infection (i.e. mucosa tissue or mucus of the SI-1 section), explained a large proportion of variation in the immune response to *T. retortaeformis*. The multivariate combination of these variables accounted for 64% of total variation in the first two principal components of a PCA (proportion of variance ± SD:

Table 6 Summary of the linear mixed effect model between blood cells, as response, and weeks post-infection (WPI) and treatment (infected and controls) as independent variables. Analysis based on 2nd-to-5th (or to 4th for *Graphidium strigosum*) WPI. Additional explanations are reported in Table 2

Trichostrongylus retortaeformis			*Graphidium strigosum*		
	Coeff, SE, d.f.	*P*		Coeff, SE, d.f.	*P*
Eosinophils			Eosinophils		
Intercept	−0·015 ± 0·057, 92	n.s.	Intercept	0·077 ± 0·013, 59	<0·0001
Treatment (inf. vs. ctrls)	0·113 ± 0·032, 38	<0·01	Treatment (inf. vs. ctrls)	0·071 ± 0·016, 39	<0·001
Sampling time (WPI)	0·038 ± 0·014, 92	<0·01			
AIC	−36·16184		AIC	235·4936	
Random effects (inter. SD)	5·492562e-06		Random effects (inter. SD)	1·498456e-06	
AR(1)	−0·185		AR(1)	0·191	
White blood cells			White blood cells		
Intercept	1·715 ± 0·066, 92	<0001	Intercept	1·724 ± 0·048, 59	<0·0001
Treatment (inf. vs. ctrls)	0·114 ± 0·055, 38	<0·05	Treatment (inf. vs. ctrls)	0·176 ± 0·059, 39	<0·01
Sampling time (WPI)	0·036 ± 0·013, 92	<0·01			
AIC	−47·16216		AIC	−58·05324	
Random effects (inter. SD)	0·1177207		Random effects (inter. SD)	0·1526094	
AR(1)	0·223		AR(1)	0·111	
Haemoglobin			Neutrophils		
Intercept	2·627 ± 0·012, 93	<0·0001	Intercept	0·780 ± 0·087, 58	<0·0001
Treatment (inf. vs. ctrls)	−0·031 ± 0·015, 38	<0·05	Treatment (inf. vs. ctrls)	0·150 ± 0·068, 39	<0·05
			Sampling time (WPI)	−0·056 ± 0·024, 58	<0·05
AIC	−404·1618		AIC	7·499853	
Random effects (inter. SD)	0·03621779		Random effects (inter. SD)	0·1613055	
AR(1)	−0·023		AR(1)	0·032	

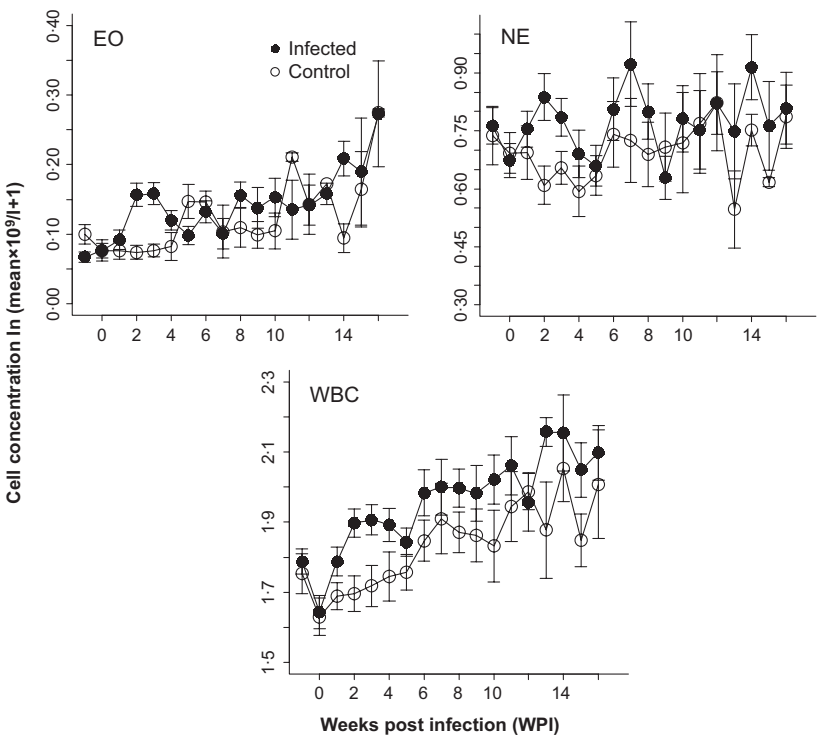

Figure 6 Mean blood cell concentration (± standard errors) in *Graphidium strigosum* experiment by weeks post-infection (WPI) and treatment (infected and controls). EO, eosinophils; NE, neutrophils; WBC, while blood cells.

PC-1 = 0·35 ± 1·44 and PC-2 = 0·29 ± 1·325). The first principal component was mainly driven by the effect of eosinophils (coeff. = 0·525), lymphocytes (0·562) and the opposite effect of IFN-γ (−0·500). The second principal component was affected by mucus IgA (−0·570) and IgG (−0·567) and the opposite contribution of IL-4 (0·456). C_t values are inversely related to cytokine expression, so that, high values -or a positive correlation- represent low cytokine expression and vice-versa. Changes in *T. retortaeformis* abundance were examined in relation to the estimated principal components, and a significant positive relationship was found with the second principal component (coeff. ± SE = 0·601 ± 0·274, d.f = 38, $P < 0·05$, Figure 7a), indicating that a decrease in parasite abundance was associated with an increase in IL-4 and antibody responses. No significant association was observed with the first principal component. Nematode abundance was tested against the variables selected in the PCA, and the results confirmed that *T. retortaeformis* infection was negatively associated with IL-4 (coeff. ± SE: 0·718 ± 0·348, $P < 0·05$) and positively associated with IFN-γ (−0·569 ± 0·247, $P < 0·05$). A negative relationship was also found with mucus IgG and mucosa eosinophils and lymphocytes (second-order interaction with time, for all $P < 0·05$, IgG: $P = 0·056$), while a positive association was observed with mucus IgA alone ($P < 0·01$).

Graphidium strigosum: The PCA based on the immune variables quantified in the fundus (stomach top) showed that the linear combination of IFN-γ, IL-4, mucus IgA and IgG and mucosa eosinophils and lymphocytes also explained a large percentage of variation in the immune response to *G. strigosum*. The first 2 principal components accounted for 67% of total variation in the immune variables (proportion of variance ± SD: PC-1 = 0·44 ± 1·63 and PC-2 = 0·23 ± 1·164). The first component was equally explained by eosinophils (coeff. = −0·47), lymphocytes (−0·48), mucus IgA (−0·43) and IgG (−0·41), while the second component was driven by IFN-γ (−0·71) and IL-4 (−0·56). Unexpected was the positive association between IFN-γ and IL-4 (also supported by the significant correlation of their C_t values, Pearson's $r = 59\%$ $n = 28$, $P < 0·01$). *Graphidium strigosum* abundance was negatively related to the first principal component (coeff. ± SE = −0·238 ± 0·064, $P < 0·01$, Figure 7b), indicating a positive association with antibodies and peripheral leucocytes. No significant relationship was observed with the second principal component. The analysis between helminth abundance and the immune variables selected in the PCA confirmed the positive correlation of the nematodes with IL-4, eosinophil and lymphocyte (coeff. ± SE: −0·145 ± 0·061, 0·380 ± 0·118 and 0·321 ± 0·135, respectively, for all $P < 0·05$), once corrected for the random effect of the host code (ID). No significant relationship was observed with IFN-γ or antibodies.

These general findings suggest that cytokines, leucocytes and antibodies modulate the dynamics of parasite infection; however, antibodies or leucocytes alone are not sufficient for parasite clearance.

DISCUSSION

We used a controlled experimental approach to explore the dynamics of primary infections and the immune response of rabbits with the gastrointestinal nematodes *T. retortaeformis* and *G. strigosum* over a period of 120 days. Rabbits mounted a robust local and systemic immune response to *T. retortaeformis* that resulted in the almost complete clearance of the nematode by the end of the trial. In contrast, *G. strigosum* persisted at high abundance throughout the infection, and this pattern was

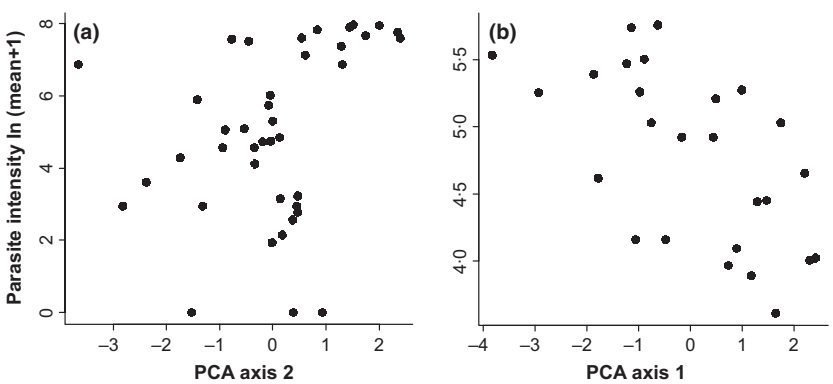

Figure 7 Relationship between principal component axis (PCA) and parasite intensity. (a) *Trichostrongylus retortaeformis* vs. PCA axis 2 (LME: coeff. ± standard error = 0·601 ± 0·274, PCA most representative variables for axis 2: mucus IgA, IgG and IL-4); (b) *Graphidium strigosum* vs. PCA axis 1 (LME: coeff. ± standard error = −0·238 ± 0·0641, PCA most representative variables for axis 1: eosinophils, lymphocytes, mucus IgA and IgG).

associated with relatively high serum but low mucus antibodies. Overall, the dynamics of infection of these nematodes were consistent with the age–intensity relationships we observed in our free-living rabbit population. Rabbits immuno-regulate the abundance of *T. retortaeformis*, and this results in the turnover of the age–intensity curve with a decrease in adult parasites in older rabbits (10). In contrast, immunity is not effective in removing *G. strigosum*, and intensities increased as a function of accumulated exposure to the parasite (11). The current study confirmed that the dynamics of infection in these two species can be explained by differences in the intensity and kinetics of the immune profile towards these parasites. We shall now discuss these results for each parasite, highlighting their major differences and relate them to the general immunoparasitology of helminth infections.

A single dose of 5500 *T. retortaeformis* infective larvae generated a strong inflammatory response as shown by an early increase in IFN-γ and tissue damage in the duodenum of infected rabbits. At 3 days post-infection, IL-4 expression probably contributed to the production of serum and mucus IgA and IgG, and facilitated parasite removal from the four sections of the small intestine. The mechanisms involved in the early IFN-γ activation are still unknown. One possibility is that the nematode up-regulated the expression of a Th1 phenotype to avoid the rapid expulsion. Alternatively, IFN-γ is produced by the host as a response to tissue damage and the possible bacterial/micro-flora infiltration into the mucosa tissue. In this respect, a pilot analysis of cytokine expression (IFN-γ, IL-4 and IL-10) in nonre-stimulated spleen of infected rabbits at 7 days post-infection found no evidence of increased IFN-γ expression, supporting the hypothesis of a host-driven response to tissue damage. The relatively rapid activation of a Th2 phenotype in the presence of IFN-γ indicates that both immune phenotypes can operate and target different components of the infection process, namely, nematode expulsion and tissue repair. Antibodies quickly developed and remained relatively high throughout the infection for IgG but not IgA, suggesting long-term persistence of both systemic and local IgG and some level of protection to reinfections. We found evidence of antibody cross-reactivity to the somatic products of adult and L3 stages. However, the significant increase in serum antibody in infected hosts at 1 week post-infection was clearly a response to the larval stage L3 and probably L4, as adults are present by 10 days post-challenge (25). A strong but short-lived systemic eosinophilia and blood cells recruitment to the site of infection appeared to develop as a response to the infection dose and contributed to nematode reduction, as observed in other studies of gastrointestinal helminth infections (32). Parasites were consistently eliminated from the relatively less colonized third and fourth sections of the small intestine, supporting the hypothesis that worm clearance was mainly driven by immune-mediated processes rather than parasite density-dependent mechanisms. As a consequence of the *T. retortaeformis* infection, rabbits developed anaemia but regularly gained body mass with the *ad lib* food regime.

Our findings on the spatio-temporal distribution of *T. retortaeformis* along the small intestine and the evidence of tissue damage and cells infiltration were consistent with previous studies of rabbits infected with different numbers of larvae (17,24). Our results were also in line with a prompt Th2 immune response to a gastrointestinal helminth infection as highlighted by the relatively rapid IgA, IgG and eosinophil recruitment, probably IL-4 and IL-5 mediated. Finally, this experiment confirmed the analytical and modelling work on the epidemiology of *T. retortaeformis* in a free-living rabbit population (10,14). Host acquired immunity is the major driver of the seasonal dynamics of this nematode, where immunity develops in response to the force of infection, which depends on the current and history of previous exposure.

Contrary to our expectations, a single inoculum of 650 *G. strigosum* infective larvae elicited a robust and persistent expression of IL-4 at the stomach mucosa and a clear systemic IgA and IgG response against adult and L3 somatic extracts compared to control individuals. Serum IgA increased and reached constant values around 4 weeks post-challenge, while IgG steadily increased throughout the infection suggesting, as proposed for *T. retortaeformis*, a possible long-term antibody protection to reinfection. Nevertheless, mucus IgA was relatively low compared to the controls, and IgG slowly developed, and together they appeared to facilitate the persistence of *G. strigosum* throughout the experiment. The lack of parasite clearance was also observed in our field studies that recorded an exponential increase in *G. strigosum* intensity with host's age, a pattern consistent with cohorts of rabbits born in different months of the year (11). We found a negative association between parasite abundance and the principal component axis described by the variation in mucus-specific antibodies, eosinophils and lymphocytes. These findings indicate that, although an immune response and some degree of protection were developed against *G. strigosum*, they were not sufficient to remove the infection within 4 months post-challenge, and parasites persisted without causing host's anaemia or loss in body mass.

The systemic antibody response, leucocytes recruitment and tissue pathology observed were in line with recent studies based on rabbits challenged with higher L3 doses, suggesting that our findings are not just dose dependent but a characteristic of this host–parasite system (19,20).

Overall, the contrasting findings of an immune response but the lack of parasite expulsion indicates that either rabbits can tolerate *G. strigosum*, for example, by reducing antibody-mediated clearance in the stomach or the parasite can manipulate the immune effectors to enhance host's tolerance or, besides, that the immune response successfully removes the infection at much later time.

An increasing number of studies found that antibodies (IgA, IgG and IgE) and eosinophils are necessary but not sufficient to clear nematode infections (33–40). Antibodies have also been shown to have a negative impact on parasite development and fecundity both during primary and secondary infections (5,6,36,41–43). A possible mechanism for parasite clearance has been suggested, wherein antibody-dependent and cell-mediated cytotoxicity (eosinophils, alternative activated macrophages) can directly affect parasite survival and its functions, for instance, development and fecundity (44). A combination of polyclonal and specific antibody effectors has been recently shown to alter *Heligmosomoides polygyrus* abundance and fitness during primary and secondary infections of mice (45), and a number of intestinal epithelial cells have been proposed to be involved in the nematode expulsion (46). While the mechanisms that control *T. retortaeformis* and *G. strigosum* abundance remain obscure, our findings support the hypothesis of a Th2-mediated antibody and eosinophil clearance of primary infections to the former species but not the latter nematode (47–50). Our recent modelling of the immune response network to *T. retortaeformis*, based on this study, was consistent with a Th2-mediated antibody/eosinophil clearance and an IL-4 anti-inflammatory protection against this nematode (Takar *et al.,* in preparation). However, additional experiments are necessary to confirm these conclusions. In this respect, the evidence that IL-4 can induce Foxp3-expressing Treg and the potential for parasite tolerance (51) raises the question of whether the persistence of *G. strigosum* in the presence of high IL-4 mucosa expression involves some tolerance mechanisms activated by the rabbit or whether this is an intrinsic property of the stomach to avoid immuno-pathology.

Closely related helminth infections of other herbivores such as sheep and cattle have highlighted the less effective immune response to the abomasal parasites *Teladorsagia circumcincta*, *Haemonchus contortus*, *Ostertagia ostertagi* and *Haemonchus placei*, compared to the more efficient response against the intestinal nematodes *Trichostrongylus colubriformis*, *Trichostrongylus vitrinus* and *Cooperia* spp. Our study is consistent with these general findings, specifically stomach and small intestine are distinct environments with different immune properties (52) and colonized by helminths with contrasting life history traits (53,54). Based on these systems, helminths in the stomach/abomasal, such as *G. strigosum*, tend to have larger body size, slower development and higher fecundity. They also appear to stimulate an immune response either that is slow to develop or has higher tolerance to infections, or can be more easily immuno-suppressed by the helminth. Helminths in the small intestine, e.g. *T. retortaeformis*, have the opposite of these life history features, probably as a response to a more effective immune response. The co-evolution of the host immune system and the helminth life history traits in the stomach and small intestine appear to have followed different strategies. Nevertheless, in our rabbit–nematode system, the outcome has been equally successful as these parasites cause persistent chronic infections.

In conclusion, we have shown that *T. retortaeformis* and *G. strigosum* exhibited different immuno-parasitological characteristics during primary infections of naïve rabbits. These nematodes appear to elicit an unequivocal Th-2-biased immune response. However, more detailed work is necessary to identify the molecular processes involved with the clearance of *T. retortaeformis* and the persistence of *G. strigosum*.

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SUPPORTING INFORMATION

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

Figure S1. Mean absorbance (OD index ± standard errors) of systemic (serum) and local (mucus) antibody response against somatic *Trichostrongylus retortaeformis* third larval stage by: treatment (infected and controls), sampling time [weeks post-infection (WPI) or days post-infection (DPI)] and small intestine location (from SI-1 to SI-4) for mucus. Week-1: sampling was performed the week antecedent the infection.

Figure S2. Mean absorbance (OD index ± standard errors) of systemic (serum) and localized (mucus) antibody response against whole third larval stage of *Graphidium strigosum* by: treatment (infected and controls), sampling time [weeks post-infection (WPI) or days post-infection (DPI)] and stomach location (top and bottom) for stomach. Week-1: sampling was performed the week antecedent the infection.

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