International Journal for Parasitology 45 (2015) 455–463

Contents lists available at ScienceDirect

International Journal for Parasitology

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

Resource limitation alters the consequences of co-infection for both hosts and parasites

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ARTICLE INFO

Article history:
Received 17 September 2014
Received in revised form 3 February 2015
Accepted 8 February 2015
Available online 24 March 2015

Keywords:
Parasite interaction
Immune trade-off
Disease ecology
Trophic framework
Tolerance
Resistance
Super shedder

ABSTRACT

Most animals are concurrently infected with multiple parasite species and live in environments with fluctuating resource availability. Resource limitation can influence host immune responses and the degree of competition between co-infecting parasites, yet its effects on individual health and pathogen transmission have not been studied for co-infected hosts. To test how resource limitation affects immune trade-offs and co-infection outcomes, we conducted a factorial experiment using laboratory mice. Mice were given a standard or low protein diet, dosed with two species of helminths (alone and in combination), and then challenged with a microparasite. Using a community ecology trophic framework, we found that co-infection influenced parasite survival and reproduction via host immunity, but the magnitude and direction of responses depended on resources and the combination of co-infecting parasites. Our findings highlight that resources and their consequence for host defenses are a key context that shapes the magnitude and direction of parasite interactions.

1. Introduction

Most free-living animals are infected with multiple parasite species simultaneously, with co-infection being the norm rather than the exception (Petney and Andrews, 1998). Co-infection can affect host susceptibility to future infections (Telfer et al., 2010), parasite virulence (May and Nowak, 1995), and a number of other host and parasite traits. In addition to being challenged by multiple parasites, hosts often live in environments where resource availability varies spatially and temporally. Resource limitation can affect host immune defenses against parasites (Koski and Scott, 2001), and many co-infecting parasites interact indirectly via the host immune system (Cox, 2001). Interactions between parasites within hosts may also be mediated by competition for shared resources (Graham, 2008). Yet, despite the considerable potential for resources to influence both immune- and resource-mediated interactions among co-occurring parasites, the effects of host resources on host and parasite performance (e.g. growth, fecundity, etc.) during co-infection are largely undescribed.

Ecological theory offers a mechanistic framework for understanding the potential network of direct and indirect interactions that can occur among hosts and parasites (Pedersen and Fenton, 2007). When a trophic framework is applied to parasites, the host's immune defenses are analogous to top-down predation pressure, whereas host resources exert bottom-up effects by limiting critical nutrients. Indirect interactions between parasites and host immunity also arise because immune responses often depend on resource availability (French et al., 2009). The effects of resource augmentation on the fitness of any single parasite can be positive or negative, depending on whether added resources are used by parasites for replication or by hosts for immune defense (Cressler et al., 2014). As such, the consequences of added resources for the outcome of co-infections are challenging to predict because positive and negative effects can arise depending on whether co-occurring parasites compete for the same resources, and whether the effects of immune interactions are antagonistic or facilitative.

Protein is a host resource that has been tightly linked to host-parasite interactions. Protein limitation is strongly associated with increased susceptibility to many parasites and pathogens, while protein supplementation is often associated with higher levels of immune mediators (Coop and Kyriazakis, 2001; Koski and Scott, 2001). Yet, it remains unclear how protein limitation will affect

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http://dx.doi.org/10.1016/j.ijpara.2015.02.005
0020-7519/

co-infecting parasites that may interact via the host's immune system and compete for protein resources. To investigate how protein limitation and immunity influence interactions among co-occurring parasites, we conducted a co-infection experiment using laboratory mice (*Mus musculus*). Mice were fed either a standard protein (SP) or low protein (LP) food, dosed with one or two species of parasitic nematodes, *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus bakeri* and then challenged with an intracellular microparasite, *Mycobacterium bovis*. We selected these helminth species because previous studies suggest that protein limitation reduces host immunity (e.g. eosinophils, serum antibodies) to both *H. p. bakeri* and *N. brasiliensis* infection, resulting in longer infection durations, higher adult worm loads and increased egg shedding (Ing et al., 2000; Coltherd et al., 2009, 2011; Jones et al., 2009). Furthermore, *M. bovis* infection is associated with and may exacerbate LP status (van Lettow et al., 2003).

All three of our focal parasites can potentially interact via the host immune system. Adult *N. brasiliensis* and *H. polygyrus* sensu lato (s.l.) (Cable et al., 2006) worms live in the small intestine, although *N. brasiliensis* larvae first migrate through the lungs. *Nippostrongylus brasiliensis* stimulates a predominantly T-helper type 2 (Th2) immune response, whereas *H. polygyrus* s.l. principally triggers a regulatory T cell (Treg) response (Maizels et al., 2012). T-helper cells coordinate immune responses by secreting chemical messengers (cytokines) to direct the action of other immune cells. The microparasite, *M. bovis*, occurs in the lungs and host responses to primary infection are characterised by a T-helper type 1 (Th1) immune response (Flynn and Chan, 2001). Th1 and Th2 immune responses are mutually inhibitory, which can lead to facilitative interactions between helminths and intracellular microparasites (Maizels et al., 2012). Moreover, the Treg response stimulated by *H. polygyrus* s.l. suppresses both Th1 and Th2 immunity, which can lead to facilitative interactions with a wide range of other parasites (Maizels et al., 2012). Resource competition between the two worms, *H. p. bakeri* and *N. brasiliensis*, is also possible because both consume similar nutrients (e.g. protein, carbohydrates, micronutrients) in the host intestine.

We tested a series of predictions about how resource availability and immunity combine to influence parasite interactions. In standard protein treatment, we expected that the Treg response stimulated by *H. p. bakeri* infection would reduce Th2 responses to *N. brasiliensis* and positively affect *N. brasiliensis* egg shedding. We also predicted microparasite infection to stimulate a strong Th1 response, reduce immune defense to the helminths, and increase egg shedding. Further, we predicted that protein limitation might reduce immune responses and relax the Treg-Th2 facilitation of *H. p. bakeri* on *N. brasiliensis*, with a net negative effect on *N. brasiliensis* but no effect on *H. p. bakeri*. Alternatively, protein limitation might intensify resource competition, with net negative effects on *N. brasiliensis* or *H. p. bakeri*. In terms of interactions between the microparasite and helminths, protein limitation might relax or intensify either the Th1–Th2 facilitation of *M. bovis* on *N. brasiliensis* and/or Th1-Treg facilitation of *M. bovis* on *H. p. bakeri*. Thus, the outcome could cause a net positive or negative effect on *N. brasiliensis*, with a lesser effect on *H. p. bakeri*. Finally, we expected that limited host resources and any interactions that increased parasite fitness would ultimately decrease host performance. To fully understand this complex network of interactions, we combined structural equation models (SEMs) with more traditional analyses to quantify the direction and strength of connections among parasites, resources and immunity.

2. Materials and methods

2.1. Animal and protein treatment protocols

We used a factorial experiment with two protein treatments (SP versus LP), four helminth treatments (no nematodes (CTL), *H. p. bakeri* only (HB), *N. brasiliensis* only (NB), and both nematodes (COINF)), and two *M. bovis* treatments (no *M. bovis* (MB−) or *M. bovis* (MB+)) to investigate the consequences of co-infection. We randomly assigned eight mice to each treatment combination and sampling time point, and mice were housed four per cage. We also incorporated three sampling time points to examine the effects on eosinophils over time: day (D)0, protein ($n = 16$); D8, protein × helminths ($n = 64$); D22, protein × helminths × *M. bovis*, $n = 128$; Total, $n = 208$ mice). We selected a genetic line of mice (BALB/c) with generally robust Th2 responses, but both Treg and Th2 responses to *H. polygyrus* s.l. (Filbey et al., 2014), to test how co-infection and resources influenced top-down pressure on helminth reproduction. All mice were female and 6–7 weeks old at the beginning of the experiment. Prior to the start of the experiment, mice were fed a SP rodent diet (LabDiet® 5002, 21% protein), and at the start of the experiment (D(−6)), half of the mice were switched to a LP diet treatment (LabDiet® 5CR4, 14% protein). Both feeds have nearly identical caloric content and micronutrient composition. Mice were fed ad libitum and weighed to the nearest 0.1 g at D(−6), 1 week after initiation of the protein treatments and every second day thereafter. Eight mice per protein treatment were culled prior to helminth infection to assess the effects of protein limitation on eosinophils.

2.2. Parasite infection and immune assays

Mice received helminth treatments 1 week after the start of the protein limitation treatment (D0), a period sufficient to establish protein-based differences in a single-infection study (Tu et al., 2007). Mice assigned to the HB and COINF treatments were intubated orally with 200 infective *H. p. bakeri* larvae. COINF and NB mice received 200 infective *N. brasiliensis* larvae via s.c. injection. CTL mice with no nematodes received equal volumes of sterile PBS via oral gavage and s.c. injection. Eight days post-helminth infection (D8), eight mice per treatment (64 individuals) were euthanised to examine host eosinophil responses. D8 is a key time-point because it falls after complete development of both helminths but prior to clearance of *N. brasiliensis*. Also on D8, half of the remaining mice (e.g., eight mice per helminth and protein treatment, $n = 64$) were infected intratracheally with a low dose of *M. bovis* H37Rv (60 colony forming units; (Serbina and Flynn, 2001; Botha and Ryffel, 2003; Kang et al., 2014)), while the others remained *M. bovis*-negative controls ($n = 64$). Mice were euthanised 2 weeks after *M. bovis* infection (D22) to examine effects of acute infection on eosinophil abundance as a measure of immune defense to helminth infection. The entire experiment was first run for the SP treatment and then repeated for the LP treatment. Within each protein treatment replicate, we staggered the start day of each helminth treatment over the course of 4 days.

To quantify helminth egg shedding, individual mice were isolated in separate cages for 30–120 min every second day from D(−6) to D22 for faecal sample collection. The number of helminth eggs per g of faeces was counted using a modified McMaster egg faecal counting protocol (Ministry of Agriculture and Food, 1980). Based on preliminary single infection trials, the eggs of the two helminth species were distinguished based on size and colour (Supplementary Fig. S1). Because intestines had to be processed for flow cytometry immediately after mice were culled (see below) adult worm and worm fecundity counts were not performed.

To assess immune function relevant to helminth infection, we quantified eosinophil responses. Eosinophils are upregulated during Th2 responses, act as a white blood cell defence against helminth infections, and contribute to host resistance to *N. brasiliensis* and *H. p. bakeri* during primary and subsequent infections (Janeway, 2008; Knott et al., 2009). We quantified eosinophils by flow cytometry, which provides a quantitative estimate of the proportion of cells in the tissue that are eosinophils. Cells were isolated from homogenised small intestine and lung tissues. Isolated cells were stained with fluorescent markers for viability (LIVE/DEAD® Fixable Violet Stain Kit, Invitrogen, USA) and eosinophil surface proteins (APC anti-MHCII, PE anti-Siglec-F and FITC anti-CD11), then fixed in formalin. Fluorescence was measured using a CyAn ADP Analyzer™ (Beckman Coulter, USA), and eosinophils were quantified by calculating the percentage of single, live cells that were MHCII negative, Siglec-F positive and CD11 intermediate (Stevens et al., 2007).

2.3. Statistical analysis

We used ANOVA to test the effects of protein limitation, helminth infection, *M. bovis* infection and their two-way interactions on total helminth egg shedding for both helminth species. For each helminth species, the total egg shedding by each mouse from D0 through D22 was calculated by integrating under the faecal egg count (FEC) versus time curve of each mouse. Tukey's post-hoc tests were used to make comparisons between levels of each factor when effects were significant. *Nippostrongylus brasiliensis* infection duration was analysed using a similar procedure. *Heligmosomoides polygyrus bakeri* infection duration was not examined since most mice remained infected at the end of the experiment. To normalise residuals, data were box-cox transformed when necessary (i.e. *N. brasiliensis* infection duration, total *N. brasiliensis* egg shedding by D22). Separate ANOVAs for *N. brasiliensis*-infected and *H. p. bakeri*-infected mice were used to test the effects of protein, individual total helminth egg shedding and their interaction on total weight gain (D−6 to D22). We used Chi-square tests to determine whether there were more helminth super shedders (i.e. individuals that contribute disproportionately to transmission) than expected by chance due to protein limitation, helminth co-infection or *M. bovis* infection. Super shedders were classified using the distribution of total egg shedding for each helminth species with individuals falling in the upper 20% identified as super shedders. This estimate of super shedders was based on the based on the "20/80 rule", the general phenomenon that 20% of host populations typically contribute to 80% of parasite transmission (Woolhouse et al., 1997). For each helminth species, we used a Chi square test to determine whether there were more super shedders than expected due to protein limitation, helminth co-infection or *M. bovis* infection.

We tested the effects of protein limitation, helminth infection, *M. bovis* infection and their two-way interactions on mouse weight and eosinophil counts (both intestine and lung) using single and multi-factor ANOVAs for mice culled at D0, D8 and D22. Tukey's post-hoc tests were used to make comparisons between levels of each factor when effects were significant. Lung and intestinal eosinophils on D22 were box cox transformed to meet normality assumptions. Tukey's post-hoc tests were applied when effects were significant. In the one case where the data could not be transformed to meet normality (D8, intestinal eosinophil levels), we used a Kruskal–Wallis test to test for effects of protein limitation and helminth treatments.

In addition to the traditional analyses above, we used SEMs to examine the relative importance of direct and indirect effects on helminth egg shedding (see Supplementary Data S1 for further detail). A SEM provides a means of simultaneously evaluating the relative importance of multiple directional paths (Grace, 2006). Guided by previous research and our own results, we hypothesised causal linkages for models of *N. brasiliensis* and *H. p. bakeri* egg shedding (Supplementary Fig. S2). We explicitly tested whether: (i) co-infecting parasites influence both immunity and resources; (ii) protein limitation influences immunity and resources; (iii) immunity influences resources, or conversely, resources influence immunity; and (iv) egg shedding influences and is influenced by immunity and resources. $P < 0.05$ was considered significant.

3. Results

3.1. Protein limitation

The helminth species showed distinct patterns of egg shedding and protein limitation had opposing effects on these patterns. *Nippostrongylus brasiliensis* egg shedding began between 4 and 6 days p.i. (D4–D6; Fig. 1A), whereas *H. p. bakeri* egg shedding began 9–10 days p.i. (D9–D10) and remained high throughout the duration of the experiment (Fig. 1B). Protein limitation did not affect the duration of *N. brasiliensis* egg shedding (Table 1; Fig. 1A). However, over the course of the experiment, mice fed the SP food shed more *N. brasiliensis* eggs than those on the LP treatment (Table 1; Fig. 1A). Interestingly, mice in the SP treatment were more likely to be *N. brasiliensis* super shedders than mice in the LP treatment (Chi square test: SP = 31%, LP = 9.4%, $\chi^2 = 4.73$, df = 1, $P = 0.03$, $n = 64$). In contrast, mice in the SP treatment shed slightly fewer *H. p. bakeri* eggs than those in the LP treatment (Table 1; Fig. 1B). Protein limitation did not influence *H. p. bakeri* super shedding (Chi square test: $\chi^2 = 2.41$, df = 1, $P = 0.12$, $n = 64$).

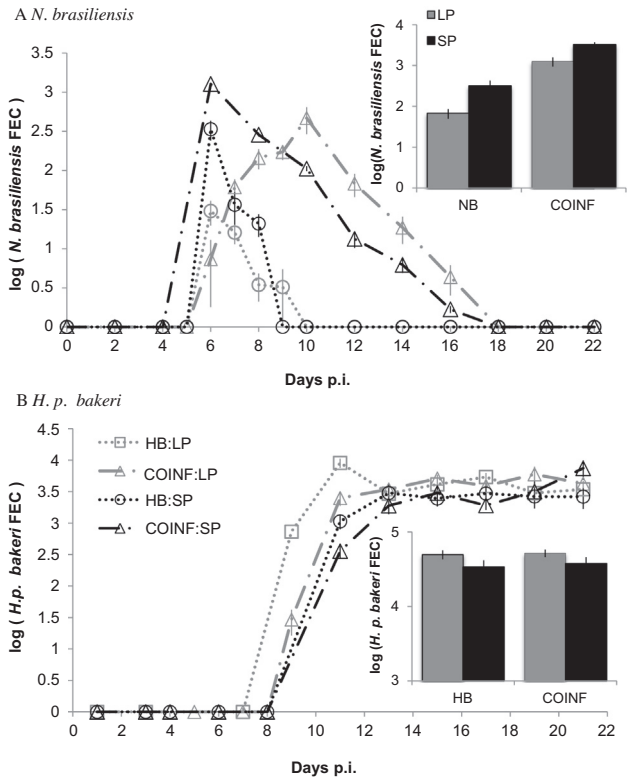

Fig. 1. Helminth egg shedding (faecal egg count (FEC) ± 1 S.E.) of mice infected with only *Nippostrongylus brasiliensis* (NB), *Heligmosomoides polygyrus bakeri* (HB) or both helminths (COINF) and assigned to either a standard protein (SP) or low protein (LP) treatment. Mice were infected with helminths on day zero (D0) and *Mycobacterium bovis* on day 8 p.i. (D8) The inset graphs show mean (±1 S.E.) total egg production for each helminth species.

Protein limitation also had effects on the host. Prior to helminth infection (D(−6)–D0), mice in the SP treatment gained more than twice as much weight as those in the LP treatment (LP = 0.069 ± 0.022 g/day, SP = 0.152 ± 0.030; ANOVA: $F_{1,14} = 5.0$, $P = 0.041$). Neither protein limitation nor the interaction between protein limitation and helminth treatment had detectable effects on mouse weight gain for the remainder of the experiment (Table 2). However, when the number of helminth eggs shed by a mouse was substituted for helminth treatment, protein limitation interacted with total *N. brasiliensis* egg shedding to influence the amount of weight gained by mice over the entire course of the experiment (D(−6) to D22; Table 3). LP mice gained less weight for a given *N. brasiliensis* egg burden, while there was no association between egg shedding and weight gain for standard protein treatment mice (Table 3; Fig. 2A). Protein limitation did not influence the relationship between *H. p. bakeri* infection and weight gain (Table 3; Fig. 2B).

Eosinophils were affected by protein limitation across multiple time points. On D0, there was no effect of protein limitation on eosinophil levels in the intestine (LP: 0.22 ± 0.10, SP: 0.26 ± 0.13;

Table 1
Effects of protein limitation and *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus bakeri* and *Mycobacterium bovis* co-infection on outcomes of helminth infection. Data were analysed using ANOVAs and, when significant, post-hoc tests were used to distinguish between factor levels and are reported in Sections 3.2 and 3.3.

Response	Factor	df	Est	Est S.E.	*F*	*P*
N. brasiliensis infection duration	Protein	1, 57	0.02	0.09	0.047	0.83
	Helminth	1, 57	−0.46	0.09	146	<0.0001[a]
	Protein * Helminths	1, 57	−0.31	0.10	8.6	0.0049[a]
	M. bovis	1, 57	−0.06	0.09	1.5	0.23
	Helminths * *M. bovis*	1, 57	−0.03	0.10	0.10	0.75
	Protein * *M. bovis*	1, 57	0.28	0.10	7.2	0.010[b]
N. brasiliensis total egg shedding	Protein	1, 57	−0.90	0.18	21	<0.0001[a]
	Helminths	1, 57	−1.05	0.18	108	<0.0001[a]
	Protein * Helminths	1, 57	0.14	0.21	0.43	0.51
	M. bovis	1, 57	−0.10	0.18	1.4	0.25
	Helminths * *M. bovis*	1, 57	−0.24	0.21	11	0.0018[a]
	Protein * *M. bovis*	1, 57	0.69	0.21	−1.1	0.27
H. p. bakeri total egg shedding	Protein	1, 57	18.3	7.11	20	<0.0001[a]
	Helminths	1, 57	−3.00	7.11	0.34	0.57
	Protein * Helminths	1, 57	3.75	8.21	0.21	0.65
	M. bovis	1, 57	10.3	7.11	3.1	0.083
	Helminths * *M. bovis*	1, 57	−2.50	8.21	0.093	0.76
	Protein * *M. bovis*	1, 57	−3.50	8.21	0.18	0.67

df, degrees of freedom; Est, estimate.
[a] $P < 0.001$.
[b] $P < 0.01$.

Table 2
Effects of protein limitation and helminth infection treatments (i.e., no nematodes, *Nippostrongylus brasiliensis* only, *Heligmosomoides polygyrus bakeri* only, and both nematodes) on individual weight gain prior to *Mycobacterium bovis* infection from 6 days prior to infection (D(−6)) to 8 days p.i. (D8) and after *M. bovis* infection and from 8 to 22 days p.i. (D8–D22). Data were analysed using separate ANOVAs for mice culled on D8 ($n = 64$) and D22 ($n = 128$). When significant, post-hoc tests were used to distinguish between factor levels and are reported in Sections 3.2 and 3.3.

Response	Factor	df	Est	Est S.E.	*F*	*P*
Weight gain from D(−6) to D8	Protein	1, 56	−0.013	0.329	0.013	0.91
	Helminths	3, 56			4.9	0.0041[a]
	Protein * Helminths	3, 56			0.16	0.92
Weight gain from D8 to D22	Protein	1, 115	0.25	0.23	0.17	0.68
	Helminths	3, 115			5.6	0.0013[a]
	Protein * Helminths	3, 115			0.52	0.67
	M. bovis	1, 115	−0.94	0.23	14	0.0004[a]
	Helminths * *M. bovis*	3, 115			5.4	0.0017[a]
	Protein * *M. bovis*	1, 115	−0.17	0.20	0.67	0.42

df, degrees of freedom; Est, estimate.
[a] $P < 0.001$.

Table 3
Effects of protein limitation and the total number of *Nippostrongylus brasiliensis* or *Heligmosomoides polygyrus bakeri* eggs shed on individual weight gain over the entire course of the experiment. Data were analysed using ANOVAs.

Response	Factor	df	Est	Est S.E.	*F*	*P*
Weight gain in *N. brasiliensis*-infected mice	Protein	1, 60	−0.018	0.33	3.1	0.085
	N. brasiliensis eggs	1, 60	1.2×10^{-4}	9×10^{-5}	0.019	0.89
	Protein * *N. brasiliensis* eggs	1, 60	-4.7×10^{-4}	2×10^{-4}	5.7	0.020[a]
Weight gain in *H. p. bakeri*-infected mice	Protein	1, 60	0.31	0.49	1.42	0.24
	H. bakeri eggs	1, 60	1.0×10^{-6}	6×10^{-6}	0.81	0.37
	Protein * *H. bakeri* eggs	1, 60	-1.2×10^{-5}	9×10^{-6}	1.82	0.18

df, degrees of freedom; Est, estimate.
[a] $P < 0.05$.

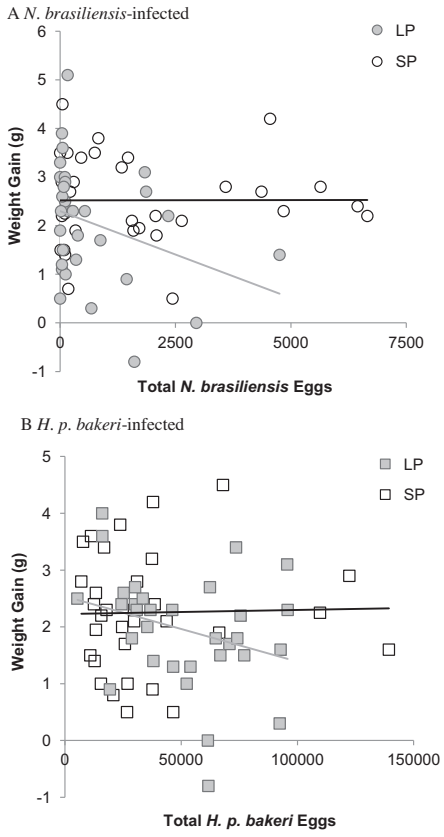

Fig. 2. Protein limitation (SP, standard protein; LP, low protein) interacted with (A) total *Nippostrongylus brasiliensis* egg shedding to influence overall host weight gain throughout the experiment, but did not effect the relationship between (B) total *Heligmosomoides polygyrus bakeri* egg shedding and host weight gain.

$F_{1,14} = 0.02$, $P = 0.88$) or the lungs (LP: 4.95 ± 1.22, SP: 5.58 ± 0.45; $F_{1,14} = 0.24$, $P = 0.63$). However, by D8 and D22, mice in the LP treatment had higher percentages of eosinophils in the intestine and lung than those in the SP treatment (Table 4; Supplementary Fig. S4).

3.2. Helminth co-infection

Co-infection had strong, asymmetrical effects on the two helminths. For *N. brasiliensis*, the duration of egg shedding, the total number of eggs shed, and variability in shedding were all altered by co-infection. COINF mice shed *N. brasiliensis* eggs for an average of 12.1 days p.i. compared with 7.4 days for NB mice, and this difference in infection duration was significant (Tukey contrast: $P < 0.0001$; Table 1; Fig. 1A). Protein limitation had significant but opposing effects on *N. brasiliensis* infection duration in single and co-infected hosts; the LP treatment led to an 1.8 day longer *N. brasiliensis* infection duration for COINF mice (Tukey contrast: $P < 0.0001$), but a 0.7 day shorter duration for NB mice (Table 1) compared to standard protein mice (Tukey contrast: $P < 0.0001$). With respect to total egg shedding, COINF mice shed almost sevenfold more *N. brasiliensis* eggs than did NB mice (Table 1; Fig. 1A). Notably, COINF mice were also more likely to be *N. brasiliensis* super shedders compared with singly infected NB mice (Chi square test: COINF: 68%, NB: 0%; $\chi^2 = 16.3$, df = 1, $P < 0.0001$, $n = 64$). Co-infection did not influence total number of *H. p. bakeri* eggs shed or variability in *H. p. bakeri* egg shedding. Total *H. p. bakeri* egg shedding did not differ between HB and COINF mice and there was no interaction with protein limitation (Table 1; Fig 1B). Likewise, there was no effect of co-infection on the occurrence of *H. p. bakeri* super shedding (Chi square test: HB: 33%; COINF: 18.5%; $\chi^2 = 0.873$, df = 1, $P = 0.35$, $n = 64$).

Infection with individual helminth species had demonstrable effects on host weight (Table 2, Supplementary Fig. S3), and co-infected mice experienced similar reductions in weight gain. Between D(−6) and D8 when mice were shedding *N. brasiliensis*, but not *H. p. bakeri* eggs, both NB and COINF mice gained 70% less weight than HB mice, driving a significant difference in weight gain among these groups (Table 2, Supplementary Fig. S3A). NB and COINF mice also gained 51% less weight than CTL mice, although this difference was not significant (Supplementary Fig. S3A). Between D8 and D22, HB mice gained significantly less weight than CTL and NB mice (Table 2, Supplementary Fig. S3B), which corresponds to the time period when mice were shedding *H. p. bakeri* eggs.

Overall, helminth infection had significant effects on intestinal and lung eosinophilia (Table 4), but once again, there was little

Table 4
Effects of protein limitation, helminths, and *Mycobacterium bovis* infection on the relative abundance of eosinophils at 8 and 22 days p.i. (D8 and D22, respectively) were analysed using ANOVAs, except D8 intestine where a Kruskal–Wallis test was applied due to non-normality. When significant, post-hoc tests were used to distinguish between factor levels and are reported in Sections 3.2 and 3.3.

Response	Factor	df	Est	Est S.E.	*F*	*P*
D8 intestinal eosinophils	Protein	1	4.06[a]	0.044[c]		
	Helminths	3	26.6[a]	<0.0001[b]		
D22 intestinal eosinophils	Protein	1, 112	−0.45	0.07	31	<0.0001[b]
	Helminths	3, 112			11	<0.0001[b]
	Protein * Helminths	3, 112			3.3	0.024[c]
	M. bovis	1, 112	−0.21	0.07	0.77	0.38
	Helminths * *M. bovis*	3, 112			3.1	0.031[c]
	Protein * *M. bovis*	1, 112	0.24	0.07	13	0.0005[b]
D8 lung eosinophils	Protein	1, 56	10.7	2.1	6.6	0.013[c]
	Helminths	3, 56			6.7	0.0006[b]
	Protein * Helminths	3, 56			8.5	<0.0001[b]
D22 lung eosinophils	Protein	1, 115	1.09	0.37	26	<0.0001[b]
	Helminths	3, 115			4.3	0.0069[b]
	Protein * Helminths	3, 115			1.7	0.18
	M. bovis	1, 115	0.85	0.37	45	<0.0001[b]
	Helminths * *M. bovis*	3, 115			0.58	0.63
	Protein * *M. bovis*	1, 115	0.03	0.34	0.006	0.94

df, degrees of freedom; Est, estimate.
[a] χ^2 test statistic.
[b] $P < 0.001$.
[c] $P < 0.05$.

evidence of an added co-infection effect. With respect to intestinal eosinophils, CTL mice consistently had lower eosinophil responses than helminth-infected mice, and co-infected mice never had higher responses than singly infected mice. On D8, NB and COINF mice had higher intestinal eosinophil levels than CTL mice (Tukey contrasts: $P < 0.05$; Supplementary Fig. S4B). Similarly, on D22, eosinophil levels remained elevated in the intestines of all helminth-infected mice compared with controls (Tukey contrasts: $P < 0.03$; Supplementary Fig. S4C). Interestingly, COINF mice had higher intestinal eosinophil levels than NB mice on D22 (Tukey contrast: $P = 0.045$; Supplementary Fig. S4C). Helminth infection also influenced eosinophil levels in the lungs under certain conditions. On D8, CTL mice had lower lung eosinophil levels than NB, HB and COINF mice, but only when protein was limited (Tukey contrasts: $P < 0.01$; Supplementary Fig. S4B), accounting for a significant interaction effect of protein limitation and helminth infection on lung eosinophils (Table 4). On D22, eosinophil levels were elevated in the lungs of HB mice compared with CTL and NB mice (Tukey contrasts: $P < 0.02$; Supplementary Fig. S4D).

3.3. Helminth-microparasite co-infection

Similar to helminth co-infection, helminth-*M. bovis* co-infection had clear effects on parasites, and these effects were strongly asymmetrical and dependent on protein treatment. *Mycobacterium bovis* infection had no main effect on *N. brasiliensis* infection duration (Table 1). For mice in the LP treatment, *M. bovis* infection increased the duration of *N. brasiliensis* infection (Tukey contrast: $P = 0.04$), but no effect of *M. bovis* on *N. brasiliensis* was seen among the mice on the SP treatment (Tukey contrast: $P = 0.73$; Table 1). Similarly, there was no main effect of *M. bovis* infection on total *N. brasiliensis* egg shedding, but protein limitation interacted with *M. bovis* such that MB+ mice in the LP treatment had 3.5-fold higher egg shedding than MB− mice (Table 1, Tukey contrast: $P = 0.014$; Fig. 3). For mice in the standard protein treatment, there was no difference in egg shedding between MB+ and MB− mice (Table 1, Tukey contrast: $P = 0.46$, Fig. 3). Thus, *M. bovis* infection had a positive effect on *N. brasiliensis* but only in protein-limited mice. *Mycobacterium bovis* infection did not affect the likelihood of being a *N. brasiliensis* super shedder (Chi square test: MB–: 23%, MB+: 28%; $\chi^2 = 0.10$, df = 1, $P = 0.75$, $n = 64$). *Mycobacterium bovis* infection also did not influence total *H. p. bakeri* egg shedding (Table 1) or the likelihood of being a *H. p. bakeri* super shedder (Chi square test: MB−: 18.5%, MB+: 33%; $\chi^2 = 0.87$, df = 1, $P = 0.35$, $n = 64$).

3.4. Mycobacterium bovis

Co-infection effects were strongly manifest in the host, but these effects depended on protein treatment. Overall, MB− mice gained more weight than MB+ mice (Table 2), and this difference was significant for COINF mice, but not the control or single-infection treatments (Table 2, Supplementary Fig. S3). *Mycobacterium bovis* infection affected both lung and intestinal eosinophils, and these effects once again depended on protein treatment. Among mice in the LP treatment, MB+ mice had lower levels of intestinal eosinophils than MB− mice on D22 (Supplementary Fig. S4C). *Mycobacterium bovis* infection interacted with helminth infection such that among MB+ mice, intestinal eosinophils were higher in HB mice than CTL mice (Tukey contrast: $P < 0.01$), and in COINF mice compared with NB mice (Tukey contrast: $P < 0.025$) and CTL mice (Tukey contrast: $P < 0.001$). Among MB- mice, no differences were detected between helminth treatments. Lung eosinophil levels were higher in MB+ mice compared with MB− mice (Table 4; Supplementary Fig. S4D).

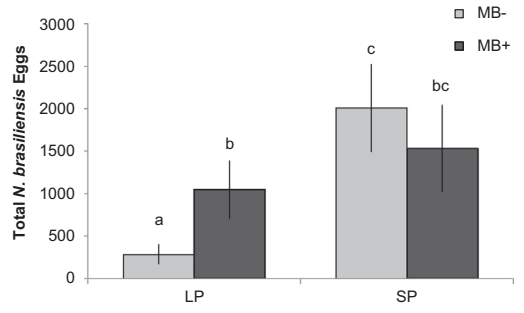

Fig. 3. The facilitative effect of *Mycobacterium bovis* infection (MB−, uninfected; MB+, infected) on *Nippostrongylus brasiliensis* egg shedding (±1 S.E.) was apparent in the low protein (LP) treatment but not the standard protein (SP) treatment. Lowercase letters denote significant differences among treatments.

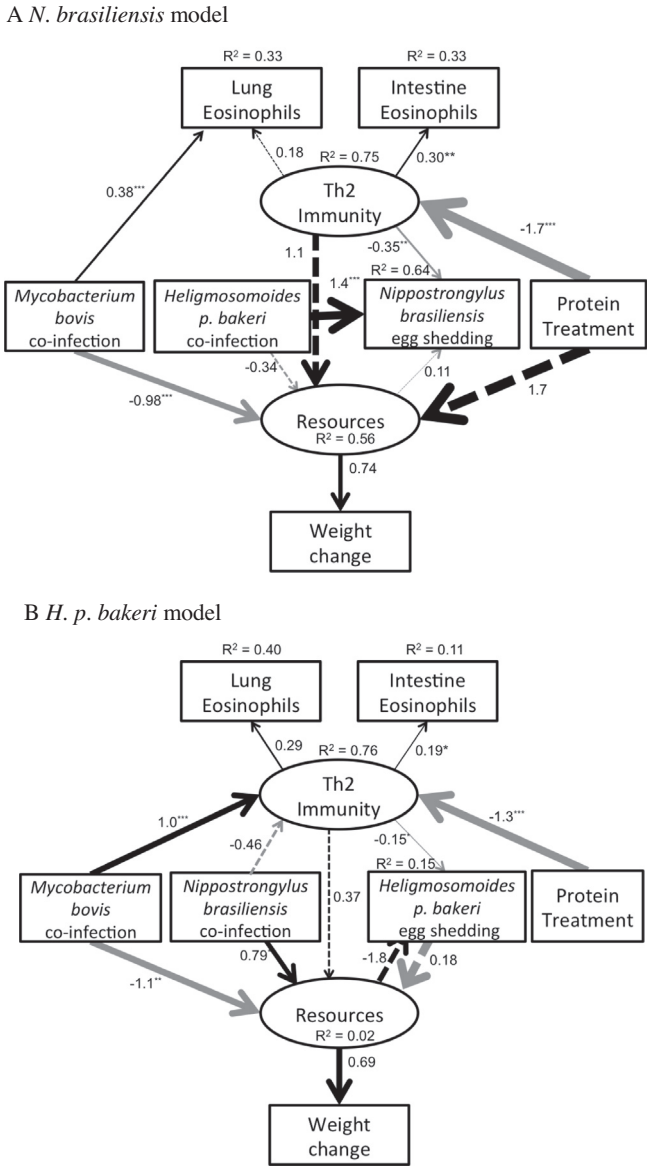

Fig. 4. The final structural equation models (SEM) show weighted relationships among (A) *Nippostrongylus brasiliensis* and (B) *Heligmosomoides polygyrus bakeri* egg shedding for mice given a standard or low protein treatment and infected with *Mycobacterium bovis*. Standardized path coefficients are noted beside connections and asterisks indicate the level of significance. Non-significant effects are indicated by dashed lines. Black lines indicate positive effects and grey lines indicate negative effects. The explanatory power of the model (R^2) is noted above the response variables. Significance is indicated by: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.5. Structural equation models

SEMs evaluated how treatments (co-infection and protein) influenced immunity, resources and helminth egg shedding. The lack of significant differences between the models and egg shedding data indicate good model fits (Chi square test: *N. brasiliensis*: $\chi^2 = 7.56$, df = 7, $P = 0.37$; *H. p. bakeri*: $\chi^2 = 7.49$, df = 7, $P = 0.38$). The effects of protein treatment on egg shedding were likely manifested via host immunity rather than host resources (Fig. 4A, B). The SEMs found support for negative effects of immunity on *N. brasiliensis* and *H. p. bakeri* egg shedding, as well as negative effects of protein treatment and positive effects of *M. bovis* co-infection on immunity. Together, these significant links support indirect, immune-mediated pathways for protein and *M. bovis* to affect helminth egg shedding (Fig. 4A, B). Unlike the traditional analyses, the SEMs also detected a direct, positive link between *H. p. bakeri* co-infection and *N. brasiliensis* egg shedding (Fig. 4A). No significant resource-mediated direct or indirect effects on egg shedding or immunity were detected (Fig. 4A, B).

4. Discussion

The goals of this study were to examine how host resource limitation influences the outcomes of co-infection and to explore potential underlying mechanisms for these outcomes. During protein limitation, immune-mediated facilitation between the two helminths had a weaker effect on helminth reproductive output but lengthened infection duration. Conversely, during protein limitation, immune-mediated facilitation between a microparasite (*M. bovis*) and a helminth (*N. brasiliensis)* was apparent, increasing helminth egg shedding and lengthening infection duration. Using SEMs, we found support for the hypothesis that resources affected immunity and that immunity was the stronger driver of variability in parasite fitness. There was no support for resource-mediated interactions among parasites. For hosts, parasite infection affected weight gain but protein limitation only influenced weight gain from days 0 to 6. Hosts gained less weight during periods of peak helminth egg shedding and hosts infected with all three parasites gained the least weight.

The effects of helminth co-infection were strongly asymmetrical, with higher *N. brasiliensis* fitness (persistence and egg shedding) in co-infected hosts but no effects of co-infection on *H. p. bakeri*. Specifically, co-infected hosts took an average of 5 days longer to clear *N. brasiliensis* infections and shed 7-fold more eggs than singly infected hosts. While positive, immune-mediated effects of *H. p. bakeri* co-infection on Th2-stimulating macroparasites including *N. brasiliensis* have been previously documented (Behnke et al., 2005; Maizels et al., 2012), our study adds to this past work by examining the context-dependency of such facilitations and by investigating the generation of super shedders. While co-infection has been suggested as a factor that can potentially generate super shedders (Stein, 2011), only a handful of studies have documented such effects (Sherertz et al., 1996; Cattadori et al., 2008; Lass et al., 2013). We found that the likelihood of being a *N. brasiliensis* super shedder was higher in co-infected mice compared with *N. brasiliensis* singly infected mice. Our results lend support to the idea that co-infection can generate variability in parasite shedding, either via increased adult survival or fecundity, with potentially important consequences for transmission dynamics.

The positive effects of *H. p. bakeri* on *N. brasiliensis* egg shedding are consistent with indirect, immune-mediated facilitation, a mechanism that is supported by our immunological data and previous studies. *Heligmosomoides polygyrus* s.l. is known to stimulate Treg cells, which dampen Th2 responses (Maizels et al., 2012). Co-infected mice had lower lung eosinophil levels at D8 and intestine eosinophil levels at D22 compared with mice infected with only *N. brasiliensis*. These tissue- and time-specific responses follow the migration of *N. brasiliensis* larvae in the lung and development into adults in the intestine. Although we did not measure T-cell subsets directly, since eosinophil infiltration is triggered by cytokines produced by Th2 cells (Janeway, 2008), the observed pattern of lower eosinophil recruitment in helminth co-infected mice supports the idea that the mechanism underlying the facilitative interaction between *H. p. bakeri* and *N. brasiliensis* was an *H. p. bakeri*-driven reduction of the Th2 immune response. While traditional analysis supported indirect immune-mediated interactions between the helminths, the SEM revealed a direct, positive link between *H. p. bakeri* co-infection and *N. brasiliensis* egg shedding, and no evidence for indirect effects mediated by immunity or resources. This discrepancy could arise because the SEM analysis only included immunological data from D22, rather than D8, when patterns of immune-mediated facilitation were detected using traditional analysis. Alternatively, the SEM could be capturing a biological interaction between the helminth species that was not evident from the traditional analyses.

Although co-infection with *H. p. bakeri* facilitated *N. brasiliensis* infection among mice in the SP and LP treatments, this effect was stronger in protein-limited hosts compared with those in the SP treatment. Specifically, co-infection with *H. p. bakeri* resulted in 6.4-fold higher *N. brasiliensis* egg shedding in hosts in the SP treatment than hosts in the LP treatment. This difference was evident despite the fact that *N. brasiliensis* infection duration was longer in co-infected hosts in the LP treatment compared with the SP treatment. The observed effect of protein limitation on total *N. brasiliensis* egg shedding may be immune-mediated. *Nippostrongylus brasiliensis* single- and co-infected mice in LP treatment had nearly identical levels of eosinophils, whereas co-infected mice in the SP treatment had lower eosinophil levels than *N. brasiliensis* singly infected mice on the same protein treatment. Although this immunological effect was not significant (Tukey contrast: $P = 0.20$), these data support the prediction that resource limitation dampened the immune-mediated facilitation apparent within co-infected mice in the SP treatment. Interestingly, the SEM analysis did not detect the context-dependent interaction between protein and co-infection because it provides only one estimate of the causal relationship among terms, rather than separate tests for mice in the LP and SP treatments.

Our data on helminth-*M. bovis* co-infection suggest that protein limitation may strongly affect the outcome of interactions between helminths and microparasites. *Mycobacterium bovis* infection had a positive effect on *N. brasiliensis* but only when hosts were protein-limited. Specifically, *M. bovis* co-infection caused several fold increases in *N. brasiliensis* egg shedding and prolonged infection durations during protein limitation. The resource dependency of microparasite-macroparasite interactions has not previously been demonstrated experimentally, although a supporting pattern has been observed in a cross-sectional study of African buffalo where a tradeoff in Th1and Th2 immune defenses was detectable only during the resource-limited dry season (Ezenwa and Jolles, 2011). Together, these data -suggest that co-infection outcomes may change when hosts are forced to allocate differing resource pools to competing physiological demands. In contrast, there was no effect of *M. bovis* on *H. p. bakeri* under SP or LP conditions. *Heligmosomoides polygyrus* s.l. induces a relatively weak Th2 response during primary infection (Maizels et al., 2012), so the lack of immune-mediated interaction between a Th1-inducing microparasite and *H. p. bakeri* is not surprising. Our results demonstrate that effects of *M. bovis* on helminth egg shedding are possible, but not universal, consequences of helminth-microparasite co-infection and that the resources available to hosts may strongly influence co-infection outcomes.

The dependence of the *M. bovis–N. brasiliensis* interaction on protein limitation was corroborated by our immunological data. During protein limitation, lung eosinophil levels were lower in *M. bovis*-infected mice compared with uninfected mice, suggesting a trade-off between the Th1 response to *M. bovis* infection and the Th2 response to helminth infection under low resource conditions. Furthermore, the Th1–Th2 trade-off was not localised to the site of *M. bovis* infection; MB+ mice also had lower intestinal eosinophil levels than MB− mice. The lower eosinophil responses following *M. bovis* co-infection are consistent with the higher *N. brasiliensis* egg shedding and infection duration noted in mice in the LP treatment. Interestingly, mice in the SP treatment had higher lung and intestinal eosinophil levels when co-infected with *M. bovis*, yet these differences did not translate into lower helminth egg shedding or shortened infection duration compared with MB- mice. Together, these data support the potential systemic, rather than site-specific, nature of some co-infection-induced immune interactions.

Both the *N. brasiliensis* and *H. p. bakeri* SEMs support causal pathways by which *M. bovis* co-infection could indirectly influence helminth egg shedding. *Mycobacterium bovis* co-infection was associated with higher lung eosinophil levels and higher immune function. Both *N. brasiliensis* and *H. p. bakeri* egg shedding were negatively influenced by immunity in the SEMs. Consequently, via immunity, *M. bovis* co-infection could have indirectly led to lower egg shedding for both helminth species. However, this is in opposition to the Th1-Th2 paradigm and the traditional analyses. Namely, *M. bovis* co-infection was associated with higher *N. brasiliensis* egg shedding during resource limitation. These contradictory results may indicate a weakness of the SEM framework, which could not incorporate the resource-dependent, immune-mediated interaction between *M. bovis* and *N. brasiliensis* detected using traditional analysis. Despite these differences, both the SEM and traditional analyses point to strong immune-mediated interactions between *M. bovis* and helminths that could have the potential to influence helminth transmission.

Protein limitation and co-infection had strong effects on the parasites, but more variable effects on the host. Mice in the SP treatment gained a higher percentage of their initial body weight compared with mice in the LP treatment during the first week of the experiment, potentially due to altered feeding behaviour, but protein treatment had no effect on subsequent weight gain. The two protein treatment feeds had nearly identical caloric content (SP: 4.09 kcal/g, LP: 4.11 kcal/g gross energy), so it is not surprising that weight gain over the course of the experiment was similar. Conversely, parasite infection had strong effects on weight gain. Mice gained less weight during the periods of peak egg shedding for each helminth species. *Mycobacterium bovis* infection led to similar magnitude decreases in weight gain. While the consequences of single infections for the host were clear, parasite co-infections showed little effect. The notable exception was that co-infection with all three parasites resulted in a threefold reduction in weight gain compared with infection with the two helminths. Weight gain may reflect an integrated cost of infection that includes energy lost directly to the parasite, tissue repair, altered nutrient absorption efficiency or immune responses. In wild mice, female weight is strongly correlated with lifetime reproductive success, so weight gain may be a meaningful indicator of the fitness consequences of infection (Ribble, 1992). As such, our results strongly suggest that certain combinations of co-infections may have significant fitness consequences for the host that go beyond the negative effects of a single infection.

Intriguingly, resource-limited mice had stronger immune responses and lower parasite burdens than standard resource mice. Protein supplementation is often associated with higher levels of eosinophils and other immune mediators (Coop and Kyriazakis, 2001; Koski and Scott, 2001). Despite the positive effects of protein limitation on adult survival and egg shedding in several single infection studies of both *N. brasiliensis* (Clarke, 1968; Jones et al., 2009), and *H. p. bakeri* (Boulay et al., 1998; Ing et al., 2000; Tu et al., 2007), the effects of protein limitation on eosinophils were less consistent across previous mouse experiments. For example, many studies detected no difference in eosinophils among treatments with differing protein content (Ing et al., 2000; Tu et al., 2007; Jones et al., 2009), while others found that eosinophil levels were highest at an intermediate protein level (7% protein) compared with low (3%) and high (24%) protein treatments (Boulay et al., 1998). As such, we expected mice in the SP treatment to have lower parasite burdens, but lower or equivalent eosinophil levels. Instead, we observed higher eosinophils and lower *N. brasiliensis* egg shedding during protein limitation, suggesting an inverse relationship between protein and immune responsiveness. The observed difference in immune function and egg shedding during standard and protein-limited conditions may reflect outcomes of tolerance and resistance strategies, respectively. The higher eosinophil levels and lower egg shedding levels among mice in the LP treatment fit predictions of a resistance strategy, where hosts expend energy to control infections. By contrast, the lower eosinophil levels and higher egg shedding among mice in the SP treatment fit predictions of a tolerance strategy, where hosts incur the energetic costs of infection rather than expend resources to control the infection. In support, it is well known that protein supplementation enables sheep and goats to tolerate helminth infections without declines in weight or milk production (Coop and Kyriazakis, 2001). Similarly, SP mice showed no decline in weight gain associated with increased egg shedding, suggesting tolerance. By contrast, weight gain declined precipitously with increasing egg shedding in LP mice. However, on average, LP and SP mice had equivalent weight gains throughout the experiment, suggesting that these strategies were energetically equivalent.

By influencing the propagation of parasites within hosts, the relationship between nutrient limitation and parasite defense strategy could have serious implications for disease transmission in humans, domestic animals and wildlife. If hosts adopt a tolerance strategy under low resource conditions or are simply unable to mount an effective immune response, resource supplementation could increase individual resistance and reduce parasite transmission. However, if hosts adopt a tolerance strategy under high resource conditions, as observed in this study, resource supplementation may unwittingly increase parasite transmission if hosts relax immunological control of the parasite. Additional research into the effects of host nutrition on immunity and infection is warranted because the ecological and management implications of an interaction between resource availability and resistance/tolerance strategy are sizable.

While separate immune- and resource-mediated effects on co-infecting parasites have been detected in previous studies (Graham, 2008), we believe this is the first experiment to explicitly test their relative importance for parasite fitness. SEM analysis allowed us to determine that indirect, immune-mediated interactions had the strongest effects on helminth egg shedding in our study. Interestingly, a recent meta-analysis found that resource-mediated interactions are most common in human co-infections, but the strength of different interactions could not be accounted for due to a lack of requisite data (Griffiths et al., 2014). We also found that parasite fitness and host weight strongly depended on resources and the combination of co-infecting parasites, but we also recognise the need for further studies with different infection orders and dosing regimes. Immune-mediated facilitation between micro- and macroparasite infection was also stronger during resource limitation. Our findings highlight the point that

laboratory studies in which animals receive ad libitum food may not effectively capture the consequences of co-infection that occur in wild systems where resources are often highly variable or limited. Moreover, resource scarcity and helminth infection frequently co-occur in both wildlife and human populations, and our data suggest these populations may also be more vulnerable to microparasite infection. Overall, our study shows that the outcomes of co-infection are context-dependent for both parasites and hosts, and that resources are a key context that shapes the magnitude and direction of parasite interactions.

Acknowledgments

We thank L. Sellers, J. Alagappan, A. Zehner, O. Kamau-Nataki, B. Bringuel, N. Patel, S. Helms, K. Fontleroy, A. Harding, R. Trench, L. Mclean, A. Maryniak, E. Rich, K. Yi and M. Martin for assistance in the laboratory. R. Lankau, S. Altizer, J. Byers, J. Maron, members of the Ezenwa laboratory and two anonymous reviewers provided helpful comments on earlier drafts. Animal protocols were approved by the University of Georgia, USA, Institutional Animal Care and Use Committee. This work was supported by a United States National Science Foundation (NSF), Doctoral Dissertation Improvement Grant to SAB and VOE (DEB-1110459), a U.S. National Center for Research Resources, training fellowship to KC (9T35OD010433-06), a U.S. Department of Agriculture/Agricultural Research Service (USDA/ARS) Project Plan to JFU (8042-32000-094-00D), and a U.S. NSF Ecology of Infectious Diseases Grant, to VOE (DEB-1102493).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2015.02.005.

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