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

Sarah A. Budischak [a,*], Kaori Sakamoto [b], Lindsey C. Megow [c], Kelly R. Cummings [b], Joseph F. Urban Jr. [d], Vanessa O. Ezenwa [a,b]

[a] Odum School of Ecology, University of Georgia, Athens, GA 30602, USA
[b] College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA
[c] College of Public Health, University of Georgia, Athens, GA 30602, USA
[d] United States Department of Agriculture, Agricultural Research Service, Beltsville Human Nutrition Research Center, Beltsville, MD 20705, USA

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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

* Corresponding author at: 140 E. Green St., Athens, GA 30602, USA. Tel.: +1 610 507 0532; fax: +1 706 542 4819.
E-mail address: sabudischak@gmail.com (S.A. Budischak).

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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.