Lello, J., McClure, S. J., Tyrrell, K., & Viney, M. E. (2018). Predicting the effects of parasite co-infection across species boundaries. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1874), [20172610]. https://doi.org/10.1098/rspb.2017.2610

Title: Predicting the Effects of Parasite Coinfection Across Species Boundaries.

Short title: Predicting Coinfection

Authors: Joanne Lello[a,b,c,1], Susan J. McClure[c], Kerri Tyrrell[c] and Mark E. Viney[d],

Affiliations:

a. School of Biosciences , Cardiff University, Cardiff, CF10 3AX, UK
b. Department of Biodiversity and Molecular Ecology, Research and Innovation Centre, Fondazione Edmund Mach, S. Michele all'Adige (TN), 38010, Italy
c. Division of Animal, Food and Health Sciences, CSIRO, Armidale, NSW, 2350, Australia
d. School of Biological Sciences, University of Bristol, Bristol, BS8 1TQ, UK

[1] Corresponding author:

Joanne Lello: School of Biosciences, Cardiff University, Cardiff, CF10 3AX, UK

Email: lello@cardiff.ac.uk

Phone: +44 (0)2920 875885,

Keywords: Coinfection, population dynamics, immune response, immunomodulation, nematode, helminths

Abstract

It is normal for hosts to be coinfected by parasites. Interactions among coinfecting species can have profound consequences, including changing parasite transmission dynamics, altering disease severity, and confounding attempts at parasite control. Despite the importance of coinfection, there is currently no way to predict how different parasite species may interact with one another, nor the consequences of those interactions. Here we demonstrate a method that enables such prediction by identifying two nematode parasite groups based on taxonomy and characteristics of parasitological niche. From an understanding of the interactions between the two defined groups in one host system (wild rabbits), we predict how two different nematode species, from the same defined groups, will interact in coinfections in a different host system (sheep), and then we test this experimentally. We show that as predicted, in coinfections, the blood-feeding nematode *Haemonchus contortus* suppresses aspects of the sheep immune response, thereby facilitating the establishment and / or survival of the nematode *Trichostrongylus colubriformis*; and that the *T. colubriformis*-induced immune response negatively affects *H. contortus*. This work is the first to use empirical data from one host system to successfully predict the specific outcome of a different coinfection in a second host species. The study therefore takes the first step in defining a practical framework for predicting interspecific parasite interactions in other animal systems.

Introduction

Coinfecting parasite species can interact with one another potentially altering both within-host infection dynamics [1-3] and between-host transmission (*e.g.* by increasing or decreasing parasite reproductive output or by altering host susceptibility) [2, 4-7]. In turn, changes in infection dynamics within hosts can alter host disease severity and / or duration [8-10] and may directly or indirectly confound attempts to control parasite infection [3, 11, 12]. In most cases, whether or not particular parasite species interact, and the nature of such interactions, are unknown. Despite the important consequences of coinfection, the potential interactions among parasites are, therefore, rarely considered in either clinical settings or during the design of infection control programmes. One possible solution to this problem would be to discover and define rules that determine when and how parasites interact. Such a concept has been explored at a broad-scale for macroparasite-microparasite interactions using a meta-analysis of different infection combinations in mice [13]. This meta-analysis demonstrated that macroparasite-microparasite coinfection would normally result in increased numbers of microparasites due to helminth-induced impairment of the anti-microparasite immune response, but that such effects would be moderated where resource competition was also present. This was a seminal contribution to the field of coinfection biology, highlighting the potential to predict coinfection using easily obtained parasite traits. However, due to the necessarily broad categorisations in this analysis, and the focus on a single model host system, application of these findings in a clinical or public health setting is difficult. Two key questions therefore follow logically from this meta-analysis: i) can predictions also be made at a species-specific scale appropriate for use in clinical and public heath settings? and ii) can patterns of parasite interspecific interaction be robustly predicted across different host species?

In earlier work we demonstrated, using previously published data, that if parasites were grouped according to both the immune responses they stimulate and those which affect them [14], it was possible to predict the result of a coinfection. This approach was limited, however, by the necessity for detailed immunological data for each of the coinfecting parasites. Here we develop and extend this approach by using taxonomic and parasite niche traits (*i.e.* resource use, site of infection) to assign parasite species to groups, making the assumption that organisms assigned to these groups will interact with the host immune system in a similar fashion to one another. Subsequently, we infer what the immune interaction of each parasite group will be with its host, and hence the likely immune relationship between the groups, based on a known example of a coinfection interaction between representative species from those groups.

In a previous study of the parasite community of wild rabbits (*Oryctolagus cuniculus*), we described a range of interspecific interactions, including the interaction between two gut nematodes; the blood-feeding stomach worm *Graphidium strigosum* and the intestinal worm *Trichostrongylus retortaeformis*, a mucosal-browser [3]. We showed that an increasing abundance of *G. strigosum* was associated with increased infection intensity of *T. retortaeformis* but, conversely, that the presence of *T. retortaeformis* was associated with a reduced intensity of *G. strigosum*. We further proposed that these effects occurred because (i) *G. strigosum* down-regulated anti-worm immune response in the host, and *T. retortaeformis* was given an advantage by this suppression, while (ii) *T. retortaeformis* induced an immune response which, though reduced in coinfection, acted against *G. strigosum* [3]. In sheep, there are parasite species that are taxonomically and functionally equivalent to the parasite groups found in the rabbit; specifically, the nematode *Haemonchus contortus*, which lives in the abomasum (stomach) of the sheep and feeds on host blood, and *Trichostrongylus colubriformis*, which lives down-stream in the small

intestine and feeds on the host mucosa. We predict that these two parasites of sheep will interact with the same pattern, and by the same process, as the functionally equivalent parasite species in the rabbit. This is the first empirical attempt to predict the consequences of a hitherto untested interspecific interaction and to do so using data from different host and parasite species.

Not all parasitic nematodes are equal in the immune responses that they stimulate, or that which affects them [15, 16]. While the immune control of the majority of gut nematodes is associated with a T-helper cell 2 (Th2) immune response [17, 18], many nematodes are able to subvert this response to varying degrees. Such immunomodulation may be particularly important for blood-feeding species. These nematodes are usually very harmful to their host, causing both tissue damage and anaemia, with heavy infections sometimes proving fatal [19]. In addition, blood-feeding nematodes are frequently found at a high prevalence in their host populations [20, 21]. Therefore, it would be reasonable to expect hosts to evolve strong immune responses against blood-feeding nematodes. Yet age-prevalence and age-intensity curves for these parasites show that they cause chronic infections and / or repeatedly re-infect the host [20], suggesting that immune responses are functionally unsuccessful against them. Further, many blood-feeding nematode species have been shown to have wide-ranging immunomodulatory capacities (e.g. *Ancylostoma duodenale*, *A. caninum, Necator americanus*, *Angiostrongylus cantonensis, H. contortus* [22-26]). While these species do induce a strong Th2 response [23, 27], many simultaneously subvert that response through a range of mechanisms [28]. These immunomodulatory effects may have consequences for other coinfecting parasite species. In contrast to blood-feeding nematode species, *Trichostrongylus* spp. browses on intestinal mucosa and bacteria, and shows limited invasion and penetration into host tissues [29]. These nematodes tend to produce shorter lived infections than those of

blood-feeding species, being more rapidly and effectively controlled by the host [30, 31]. While there is evidence that *Trichostrongylus* spp. may have some immunomodulatory capacity, it does not appear to be as immunologically broad ranging as that observed among the blood-feeding species [16, 32]. Further evidence of the different immune responses to these parasite groups is seen in rabbits, where the temporal pattern of natural and laboratory infections suggests that *T. retortaeformis* is effectively removed by the host while *G. strigosum* is not [3, 33]. In summary, we therefore propose that how these two parasite groups interact with their hosts' immune responses will result in predictable interspecific interactions.

Here we test our hypothesis in sheep experimentally coinfected with *H. contortus* and *T. colubriformis* (comparing them to sheep mono-infected with each species, and with uninfected controls), by measuring nematode intensity and the host immune response. We specifically predict that in coinfections (i) the blood-feeding *H. contortus* will suppress aspects of the host immune response, thereby facilitating the establishment and / or survival of *T. colubriformis* and (ii) the *T.* colubriformis-induced immune response will negatively affect *H. contortus*.

Materials and Methods

Pre-infection Protocol

Following approval by the FD McMaster Laboratory, Chiswick, Animal Ethics Committee, at weaning, 132 Merino wethers (castrated rams) were brought into CSIRO Livestock Industries animal house where faecal samples were analysed using a modified McMaster technique (as in [34]) to diagnose any helminth infection. Animals were then treated with a mixture of Abamectin and Praziquantel, Levamisole, and Benzimidazole, using the manufacturers' recommended doses. Twelve days later a second faecal screen for

helminth infection was performed to confirm that animals were helminth free. All animals were blood sampled via jugular venepuncture to provide a pre-infection baseline immune and health status measure. Animals were then assigned to one of four treatment groups using a stratified random assignment (where groups were balanced for body mass, body condition and original faecal egg count). The four treatment groups were: (i) control, uninfected (n=12), (ii) *H. contortus* mono-infected (n=40), (iii) *T. colubriformis* mono-infected (n=40), or (iv) *H. contortus* and *T. colubriformis* coinfected (n=40) (Fig. 1).

Infections and Sampling

An overview of the experimental protocol is shown in Fig. 1. Animals in the coinfected and mono-infected groups were each infected twice weekly for ten weeks with 300 larvae of *H. contortus* and / or 1500 larvae of *T. colubriformis*. For animals in the coinfection groups, doses of both parasite species were given simultaneously as an additive dose. Differential dosing was used because of the different size and pathogenicity of the two helminth species, *T. colubriformis* being considerably smaller and less pathogenic than *H. contortus* [35]. Animals in the control, uninfected group were handled in the same manner as other animals. Throughout the experiment animals where maintained on raised slatted floors to prevent self-reinfection, provided fresh water *ad libitum*, and fed daily with a ration of 700 g of standard pellets consisting of lucerne (500 g/kg), wheat (100 g/kg), pollard (200g/kg), bran (160 g/kg), salt (20 g/kg) and ammonium chloride (20 g/kg), the quantity of which was set for normal growth.

At weeks 6, 10, 14 and 18 post initial infection (where initial infection indicates the first day of larval dosing) all animals were blood sampled, as above, and body mass and body condition (assessed using industry standard scale of 0 to 5, www.lifetimewool.com.au/conditionscore.aspx) were recorded. At each of these four

sample points a sub-set of animals (10 for each infection group, and 3 for the control, uninfected group) were humanely slaughtered and tissue collected, and processed as described below.

Worm Counts

From killed animals the abomasum and small intestine were sampled in sections, placed into separate dissecting trays, the tissue opened and the contents gently washed into collecting jars to remove all adult nematodes. The number of worms in sub-samples was then counted to determine the total number of worms of each species infecting each animal. Samples of abomasal and jejunal tissues (4 cm^2 squares) were fixed in Bouin's solution for later histological analysis. *H. contortus* larvae can developmentally arrest within the host at the L4 stage, a form of diapause known as hypobiosis. Hypobiosis does not occur in the strain of *T. colubriformis* used in our study. Remaining abomasal tissue was, therefore, digested in phosphate buffered saline containing 10% v/v HCl to release any arrested *H. contortus* fourth stage larvae, which were then counted.

Measures of Immune Response

We measured the number of immune cells in the fixed abomasal and jejunal tissue, which, following standard sectioning, were stained with haematoxylin and eosin, and toluidine blue [36]. For both tissue samples, cell counts and scores were estimated *per* villus-crypt unit (*i.e.* from the tip of one villus to the next). For the abomasal tissue, we determined the number of globule leukocytes, mast cells, eosinophils, and scores for lymphocyte infiltration (0 = no infiltration, to 4 = heavy infiltration). For jejunal tissue the same cell counts and scores were made, but in addition the number of goblet cells, and a score of the proportion of goblet cells containing granules (0 = no cells contained granules, to 5 =

most cells contained granules) were also recorded, together with a score of the thickness of the smooth muscle layer (0 = very thin, to 4 = thick).

We determined the concentration of IgG1 antibodies against *H. contortus* and against *T. colubriformis* L3 antigens using previously described ELISAs [36, 37].

Statistical analyses

One animal was removed from the control group prior to infection due to ill health, leaving a control group sample size of 11 animals. One animal was also removed from each of the coinfection and *H. contortus* mono-infection groups prior to the 6 week sample point, due to ill health unrelated to the helminth infections, leaving a sample size of 39 sheep for each of these two groups. A small number of other sampling losses, due to processing problems are detailed in S1, which provides an overview of sample size by sample point for all analyses.

Analyses were conducted in R v3.1.2 [38]. The effect of infection treatment group on the number of adult *T. colubriformis* worms, the number of adult *H. contortus* worms, and the number of *H. contortus* arrested larvae were assessed in three general linear models (GLMs). Infection group (mono- or coinfected), days post initial infection (*i.e.* cull day; included as a categorical variable) and their interaction were included as independent variables. In addition, the faecal egg count pre-anthelminthic treatment, and animals' total gain in mass were also accounted for by inclusion as independent terms. Following preliminary model assessments, the number of arrested larvae of *H. contortus* was square root transformed (sqrt(x+1)) to normalize the residuals of that GLM. Neither Poisson nor negative binomial error distributions provided better model fits for any model (S2).

We used two steps to determine how treatment group affected the measures of immune responses in the abomasum and jejunum. First, two principal components analyses were conducted separately on the abomasal and jejunal measures of immune responses, using a singular value decomposition of the centered and scaled data matrix [39]. All scores where treated as numeric data and scaling was applied. The measures of the abomasal immune responses were compared between the *H. contortus* mono- and coinfection groups; and measures of the jejunal immune responses were compared between *T. colubriformis* mono- and coinfection groups; in both cases this separation reflects the location of these species within the animals. The principal component (PC) explaining the majority of the variation in each analysis was then used as the dependent variable in a GLM where treatment group, time of sampling, and their interaction, were the explanatory variables. Models were refined in a stepwise manner by evaluating the F statistics (terms were rejected when $P > 0.05$). Where the GLM analyses showed significant differences in PC values between treatment groups, the second step in the analysis was undertaken. In these second analyses the bootstrapped mean value was calculated for each individual measure of immune response, to qualitatively explore the effect of treatment group on these individual measures. For the treatment groups, bootstrapped mean values were calculated for each time of sampling. For the uninfected control animals, the data were pooled across sample points due to the smaller sample size in this group.

The effect of treatment group on anti-*H. contortus* and anti-*T. colubriformis* IgG1 titres were assessed in two general linear mixed models (GLMMs using the R package ASReml-R v3.0 [7]) in which each animals' individual identification number was included as a random term to control for pseudoreplication. The titres of IgG1 were transformed to normalize residuals in the model, as $((x+1)^{0.12})$ for anti-*T. colubriformis* and $((x+1)^{0.18})$ for anti-*H. contortus* responses. Results shown here are back-transformed. In these models,

treatment group, time of sampling (included as a categorical variable), and their interaction were included as fixed effects. This fixed effect model was refined in a stepwise manner using the Wald test and evaluation of the conditional F statistics (terms were rejected when $P > 0.05$). Where treatment group was found to be a significant effect, differences between treatment groups were assessed by within-model contrasts.

Results

Coinfection Affects *T. colubriformis* and *H. contortus*

T. colubriformis was a more successful parasite of sheep when it was in a coinfection with *H. contortus* (the number of adult *T. colubriformis* differed between the coinfection and mono-infection groups through time post initial infection $F_{3,69} = 3.38$, $P = 0.023$; Fig. 2). There were more adult *T. colubriformis* worms in coinfected sheep than in *T. colubriformis*-only infections at 14 and 18 weeks post initial infection (t = -2.08, df = 69, $P = 0.041$; t = -3.96, df = 69, $P < 0.001$, respectively). A total of 30,000 *T. colubriformis* infective larvae were given to each sheep, which by week 14 could all have developed into adult worms. In the coinfected animals a mean of 23,380 adults were present (78%), whereas only 16,761 (56%) were found in the *T. colubriformis*-only infections (see S3 for mean and SD of raw counts through time).

H. contortus was also affected by coinfection, but differently compared with *T. colubriformis*. To assess the *H.* contortus infection we analysed both the number of arrested L4 stage larvae in the host tissues along with adult worms (see S3 for mean and SD of raw counts through time). There were fewer *H. contortus* arrested larvae in coinfections, compared with *H. contortus*-only infections ($F_{1,71} = 4.15$, $P = 0.045$; Fig. 2); the number of these larvae was also affected by the time post initial infection ($F_{3,71} = 9.79$, $P < 0.001$; S4). In contrast, the number of adult *H. contortus* was not affected by

coinfection, though numbers did vary through time post initial infection ($F_{3,72}$ = 14.73, *P* <0.001; S5). As the number of adults show no evidence of being bolstered by larvae leaving the arrested state in the coinfection group, together these data mean that in coinfections there are overall fewer *H. contortus* worms.

Coinfection Affects Host Cellular Immune Responses

T. colubriformis infects the jejunum and to measure the immune responses in this site we used a principal components analysis of jejunal immune measures. All immune measures positively loaded onto principal component axis 1 (PC1), which explained 49% of the variance in these components (S6). PC1 was subsequently used in the GLM analysis and transformed (Ln(PC1+3)) resulting in a normal distribution of the model residuals, the results shown in the figures are back-transformed. The PC1 scores significantly differed between the coinfection and mono-infection groups, through time post initial infection (GLM analysis of PC1 scores $F_{3,71}$ = 3.84, *P* = 0.013; Fig. 3). The PC1 scores for the coinfected group did not vary with time post initial infection, whereas those of the mono-infected group increased through time. The predicted PC1 values in the coinfected animals were significantly lower than in the *T. colubriformis*-only infection group (significant difference between coinfected and mono-infected group at weeks 14 and 18 post initial infection t = 2.32, df = 71, *P* = 0.023, t = 4.50, df = 71, *P* < 0.001). Together, this means that the jejunal immune response induced by *T. colubriformis* was suppressed in coinfected animals. Analysis of the individual cell types in the jejunum also showed that the greatest responses were in the *T. colubriformis*-only infection group and lower in the coinfected animals, presumably due to the immunosuppressive effect of *H. contortus* (Fig. 4, S7). In animals mono-infected with *H. contortus*, the jejunal immune responses were often as low as those in the control (uninfected) animals, which is unsurprising given that *H. contortus* is not present in the jejunum. *H. contortus* infects the abomasum, and

abomasal immune measures loaded positively onto PC1 explaining 62% of the variance (S8). PC1 was subsequently used in the GLM analysis and transformed (Ln(PC1+3)) resulting in a normal distribution of the model residuals, the results shown in the figures are back-transformed. GLM analyses of the abomasal PC1 scores showed that they did not differ significantly between the coinfected and mono-infected animals, nor did they vary through time post initial infection.

Coinfection Increases anti-*H. contortus* Larval Immune Responses

The concentration of anti-*H. contortus* IgG1 was significantly different between coinfected and *H. contortus*-only infected animals (effect of treatment group excluding the *T. colubriformis* mono-infection group $F_{8,300} = 3.31$, $P = 0.001$; Fig. 3). The response was significantly greater in the coinfected animals, compared with the *H. contortus*-only infected and control animals, which did not differ from one another (Fig. 3). In the coinfected animals at 18 weeks post initial infection the IgG1 response was reduced, coinciding with a reduced number of arrested *H. contortus* larvae (S4).

The concentration of anti-*T. colubriformis* IgG1 was significantly affected by treatment group (effect of treatment group, excluding the *H. contortus* mono-infection group, $F_{8,300} = 3.09$, $P = 0.002$, S9). Specifically, these responses were significantly higher in the coinfected and *T. colubriformis*-only infection groups compared with the control, uninfected group. The coinfected and *T. colubriformis*-only infection groups were not significantly different from one another (S9).

Discussion

We hypothesised that, by defining parasite groups using taxonomy and parasite traits, we could infer the host response to those groups and hence the expected interaction among

coinfecting parasites. Our hypothesis was supported. Specifically we demonstrate that immune suppression by the blood feeder *H. contortus* had a positive effect upon the numbers of mucosal browser *T. colubriformis*, while the immune response promoted by the mucosal browser negatively affected the numbers of the blood feeder.

Effect of the Blood Feeder on the Mucosal Browser

The presence of *H. contortus* resulted in comparatively more *T. colubriformis* adult worms in coinfected sheep. The trajectory of adult worm numbers in the *T. colubriformis* mono-infected sheep shows a classic convex age-intensity curve, indicative of host immune responses removing adult worms [30, 40, 41]. In the coinfection treatment group the number of worms reached an asymptote suggesting that adult worms were not being removed by the host immune response. There was, however, some evidence of a reduction in larval establishment in this coinfection group (though less than in the mono-infected group), likely indicating that anti-*T. colubriformis* response was beginning to develop. This is consistent with previous studies that have shown the anti-*T. colubriformis* immune response acts first against incoming larvae [42].

As we hypothesised, the difference in the number of *T. colubriformis* adults between coinfection and mono-infection groups appears to be immune mediated. Our data demonstrate that there was a reduced immune response in the jejunum in the coinfected animals, compared to the *T. colubriformis* mono-infected animals, and most pronounced in the latter time points (weeks 14 and 18 post initial infection) (Fig. 3, 4 & S7). This differentiation between the infection groups suggests that the immune suppression we observe is dependent on the adult *H. contortus* (since by week 14 all larvae would have developed to adulthood or arrested their development). We use the presented immune measures as general indicators of anti-helminth immune responses, rather than

implicating individual immune components. Nevertheless, all these immune components have been associated with the immune response against helminths in sheep [43-45].

Effect of the Mucosal Browser on the Blood Feeder

There was no evidence of an effect of coinfection on the number of *H. contortus* adults, nor on the abomasal cellular immune response. However, the significantly fewer arrested larvae in the coinfected animals demonstrates that coinfection still has a negative effect on *H. contortus* (Fig. 2). In natural infections, arrested larvae resume development to adulthood during periods of host stress [36]. There are significantly less arrested larvae in the coinfection group but no more adults. These missing larvae must, therefore, (i) be lost to the system entirely, or (ii) have replaced adults that have been lost. Thus, these larvae either (a) never established in the arrested state in the first place, (b) were destroyed in, or expelled from, the tissues, or (c) following a period in the arrested state, resumed their development and either replaced lost adults, or failed to establish as adults. The difference in the number of larvae found in the arrested state between singly and coinfected groups of sheep is relatively small, approximately 40 larvae, and is thus unlikely to be of clinical significance in these sheep. We highlight, however, that this study is not focused upon clinical significance per se, but upon the ability of our predictive framework to establish the form and direction of the parasite interactions, which we have achieved. Nevethereless, even these few larvae, as adults, could contribute substantially to the potential infectious burden on pasture under natural conditions. Assuming an average daily fecundity of 4,700 eggs per female (see [46]) and a sex ratio of 1:1, twenty adult female worms could be adding >94,000 eggs per day to pasture.

As predicted, the loss of *H. contortus* arrested larvae appears to be immune mediated. Although the abomasal immune components do not differ among infection groups, the

concentration of anti-*H. contortus* IgG1 was significantly higher in coinfected animals (Fig. 3). *H. contortus* larvae were the source antigen for the IgG1 assay and it is likely that this antibody response reduces larval development, as has been previously been reported [47].

Is the Observed Interaction Robust?

The host immune response to *T. colubriformis* and *H. contortus* in mono-infections is well documented [47-50]. A feature of these responses is that they differ in strength depending on host species (sheep or goat), breed [17, 51-53], age [30, 54], and diet [55], although the same immune components are implicated in helminth control amongst these host groups. An important consideration, then, is whether the interactions we have described between the coinfecting parasite groups, would be robust to such host differences. Since the immune components involved in the host response are the same, we suggest that while there may be quantitative differences in intensity of infection due to variation in the strength of the immune response, the qualitative result (i.e. positive consequences for a mucosal-browsing nematode and negative for the blood-feeding group) will likely persist. This view is further supported by the identical pattern of interaction seen in the rabbit-coinfection system between its blood feeding and mucosal browsing nematode parasites. It should be noted that one laboratory study of coinfection with the same rabbit helminths did not find this pattern of interaction during coinfection [33]. That laboratory study, however, used a single, high dose infection (rather than the trickle infections we used), which can dramatically alter the form of the elicited immune response [56], in turn altering the nature of the interspecific interactions.

Our hypothesis for the interaction between the sheep nematodes was based on data from a different host and different parasite species, where we defined parasite groups based

on their taxonomic and parasitological (i.e. resource use, site of infection) traits. We suggest that this novel approach can be more generally applied to other host and parasite systems. While we have successfully applied this approach here, we acknowledge that this is a single test and that further work is required to confirm that the approach could be applied beyond our defined parasite groupings. However, we note that our predictive ability crossed host species (rabbits and sheep) that are distinct taxonomically, behaviourally and physiologically, suggesting that host similarity does not underlie our successful prediction. Regarding the parasites, we also emphasize that our hypothesis of how the sheep parasites would interact came solely from our predictive framework. Specifically, despite extensive prior study of these parasites in sheep, the interactions we correctly predicted had never previously been hypothesised. Together this suggests that our predictive framework is neither host nor parasite species specific. Future exploration of this topic could include a meta-analysis to determine whether parasite traits can represent patterns of immune function across multiple host types and different forms of parasite (i.e. beyond helminths).

Notably the parasite species in our study all belong to the superfamily Trichostrongyloidea and it is possible that the interaction observed would be restricted to species within this superfamily – though this would still be an important result. Nevertheless, we have described here the common immunomodulatory features of several blood-feeding nematode species, which further supports this parasite grouping and also proposes a mechanism (i.e. suppression of the intestinal cellular immune response), for this groups' potential interaction with other parasite groups. There is less information available to support the grouping of mucosal browsing nematodes, as the host immunological response to this group has been less well studied. Even if we narrow this group to mucosal browsing *Trichostrongylus* spp. the only immune function studies conducted appear to be

on *T. colubriformis* and *T. retortaeformis*, the species involved in our studies. It will therefore be interesting to determine whether other members of the group also stimulate, and are controlled by, a classic Th2 response, which underlies the mechanism of their interaction with the blood feeders and, further, whether the group could be expanded to other helminth species displaying similarly low levels of tissue invasion, i.e. browsing nematodes beyond *Trichostrongylus* spp.

We propose that the form of acquisition of a given resource is likely to be an important indicator of how the host will respond to any parasite. For example, while nematodes and malaria both use the host blood as a resource, they acquire that resource in a different way. We suggest that taxonomically more related parasites are also more likely to evolve related mechanisms of resource acquisition and therefore that a combined grouping strategy involving location, resource use and parasite taxonomy may be a good indicator of host immune response, the ultimate mechanism of the interspecific parasite interaction in our study. Our classification mechanism requires that the resource use of the parasite is known. For some species this will not be the case. However, using physical location in conjunction with taxonomic similarity to other known species will often be a suitable proxy.

Implications for Parasite Control and Economic Losses

H. contortus and *T. colubriformis* are both economically important parasites, causing substantial production losses in both sheep and goats [48]. Production losses due to *T. colubriformis* are likely to be greater in sheep coinfected with *H. contortus*, due to the higher worm burdens and prolonged infection in such coinfections. Notably, the condition and mass of coinfected animals did not significantly differ from the other treatment groups. However, pasture-reared sheep, not provisioned with the high-quality maintenance diet provided in our experiment, would likely experience more severe effects during

coinfection. Transmission of *T. colubriformis* in coinfected sheep could be substantially higher due to the higher worm burdens and prolonged infection during *H. contortus* coinfection, meaning potentially higher worm burdens at a population level, requiring the use of anthelmintics. However, density-dependent reduction in *per capita* worm fecundity has been observed for *T. colubriformis* [49], which may ameliorate such effects. Nevertheless, host immune response appears to play a role in this density dependent restriction of fecundity [17], and thus such immune effects may be reduced during *H. contortus* coinfection. A change in *H. contortus*-induced production losses during coinfection are unlikely, as adult worm burdens of this species were not affected by the coinfection. The economic implications of this coinfection are, therefore, principally a consequence of the altered dynamics of the *T. colubriformis* infection.

Conclusion

This work represents a first experimental proof-of-principle that groups of parasite can be identified and thereafter used to predict the outcome of a previously unexplored interspecific parasite interaction in a different host species. Given the ubiquity and multiplicity of coinfection in nature it is important that we derive such grouping mechanisms. In previous work, we suggested grouping parasites by an immunological profile [14]. A problem with this idea is that immune profiling is complex, expensive and reagents may not be available for novel or lesser-studied hosts. However, the current study offers an alternative mechanism for classification by using taxonomy and more easily identified parasitological traits, to act as a proxy for the immune traits. Further, we have demonstrated that we can successfully use these traits to predict the immunologically-based interaction of two parasite groups. This work therefore proposes a general framework for predicting the relationships between other parasite groups, and next steps should be to determine how widely applicable such a framework can be.

Competing Interests

The authors declare that they have no competing interests

Funding

This work was funded primarily by a Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia, Complex Systems Science grant with match funding from CSIRO Livestock Industries. JL SM & KT where employees of Livestock Industries but there was no intervention by the wider organisation in either analysis or interpretation of data nor in writing the manuscript. A proportion of the time JL spent developing the manuscript was funded by a Marie Skłodowska-Curie Fellowship at the Fondazione E. Mach, Italy (H2020-MSCA-IF-2014; grant no. 661690).

Author Contributions

JL devised and managed the project, conducted the statistical analyses and in conjunction with MEV wrote the manuscript. MEV also was involved in the initial experimental design and interpretation of the data. SJM advised on and supervised all immunological aspects of the project and undertook the abomasal and jejunal immune component analysis, with the exception of the ELISAs which were carried out by KT. KT was also lead technical officer for the project and both supervised and took part in all animal handling and dosing, sampling and tissue preparation. KT also undertook all worm enumeration.

Data Accessibility

All the data described in this study are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.1802hj0.

Ethics Statement

Our work using Merino sheep followed Australian Government guidelines with ethical approval obtained from the FD McMaster Laboratory, Chiswick, Animal Ethics. Approval number AEC0373.

Acknowledgements

We would like to thank the animal house support team at Armidale, NSW without whom this work could not have been undertaken, and Andrea Graham (Princeton University) for insightful discussions.

References

[1] Ferrari, N, Cattadori, IM, Rizzoli, A & Hudson, PJ. 2009 *Heligmosomoides polygyrus* reduces infestation of *Ixodes ricinus* in free-living yellow-necked mice, *Apodemus flavicollis*. *Parasitol* **136**, 305-316. (doi:10.1017/s0031182008005404).

[2] Lass, S, Hudson, PJ, Thakar, J, Saric, J, Harvill, E, Albert, R & Perkins, SE. 2013 Generating super-shedders: co-infection increases bacterial load and egg production of a gastrointestinal helminth. *J R Soc Interface* **10**, 20120588. (doi:10.1098/rsif.2012.0588).

[3] Lello, J, Boag, B, Fenton, A, Stevenson, IR & Hudson, PJ. 2004 Competition and mutualism among the gut helminths of a mammalian host. *Nature* **428**, 840-844. (doi:10.1038/nature02490).

[4] Jolles, AE, Ezenwa, VO, Etienne, RS, Turner, WC & Olff, H. 2008 Interactions between macroparasites and microparasites drive infection patterns in free-ranging African buffalo. *Ecology* **89**, 2239-2250. (doi:10.1890/07-0995.1).

[5] Randall, J, Cable, J, Guschina, IA, Harwood, JL & Lello, J. 2013 Endemic infection reduces transmission potential of an epidemic parasite during co-infection. *Proc R Soc B Biol Sci* **280**. (doi:10.1098/rspb.2013.1500).

[6] Telfer, S & Bown, K. 2012 The effects of invasion on parasite dynamics and communities. *Funct Ecol* **26**, 1288-1299. (doi:10.1111/j.1365-2435.2012.02049.x).

[7] Lello, J, Norman, RA, Boag, B, Hudson, PJ & Fenton, A. 2008 Pathogen interactions, population cycles, and phase shifts. *Am Nat* **171**, 176-182. (doi:10.1086/525257).

[8] Brockmeier, SL, Loving, CL, Nicholson, TL & Palmer, MV. 2008 Coinfection of pigs with porcine respiratory coronavirus and *Bordetella bronchiseptica*. *Vet Microbiol* **128**, 36-47. (doi:10.1016/j.vetmic.2007.09.025).

[9] Furze, RC, Hussell, T & Selkirk, ME. 2006 Amelioration of influenza-induced pathology in mice by coinfection with *Trichinella spiralis. Infect and Immun* **74**, 1924-1932. (doi:10.1128/iai.74.3.1924-1932.2006).

[10] Graham, AL, Lamb, TJ, Read, AF & Allen, JE. 2005 Malaria-filaria coinfection in mice makes malarial disease more severe unless filarial infection achieves patency. *J Infect Dis* **191**, 410-421. (doi:10.1086/426871).

[11] Fenton, A. 2008 Worms and germs: the population dynamic consequences of microparasite-macroparasite co-infection. *Parasitol* **135**, 1545-1560. (doi:10.1017/s003118200700025x).

[12] Steenhard, NR, Jungersen, G, Kokotovic, B, Beshah, E, Dawson, HD, Urban, JF, Jr., Roepstorff, A & Thamsborg, SM. 2009 *Ascaris suum* infection negatively affects the response to a *Mycoplasma hyopneumoniae* vaccination and subsequent challenge infection in pigs. *Vaccine* **27**, 5161-5169. (doi:10.1016/j.vaccine.2009.05.075).

[13] Graham, AL. 2008 Ecological rules governing helminth-microparasite coinfection. *PNAS* **105**, 566-570. (doi:10.1073/pnas.0707221105).

[14] Lello, J & Hussell, T. 2008 Functional group/guild modelling of inter-specific pathogen interactions: A potential tool for predicting the consequences of co-infection. *Parasitol* **135**, 825-839. (doi:10.1017/s0031182008000383).

[15] Maizels, RM, Hewitson, JP & Smith, KA. 2012 Susceptibility and immunity to helminth parasites. *Curr Opin Immunol* **24**, 459-466. (doi:10.1016/j.coi.2012.06.003).

[16] Wolfson, W. 2013 Parasites R Us: Coronado Biosciences recruits parasitic worms to treat autoimmune disease. *Chem Biol* **20**, 135-136. (doi:10.1016/j.chembiol.2013.02.008).

[17] Amarante, AFT, Rocha, RA & Bricarello, PA. 2007 Relationship of intestinal histology with the resistance to *Trichostrongylus colubriformis* infection in three breeds of sheep. *Pesqui Vet Bras* **27**, 43-48.

[18] Anthony, RM, Rutitzky, LI, Urban, JF, Jr., Stadecker, MJ & Gause, WC. 2007 Protective immune mechanisms in helminth infection. *Nat Rev Immunol* **7**, 975-987. (doi:10.1038/nri2199).

[19] Reynecke, DP, van Wyk, JA, Gummow, B, Dorny, P & Boomker, J. 2011 Validation of the FAMACHA (c) eye colour chart using sensitivity/specificity analysis on two South African sheep farms. *Vet Parasitol* **177**, 203-211. (doi:10.1016/j.vetpar.2009.08.023).

[20] Bethony, J, Chen, JZ, Lin, SX, Xiao, SH, Zhan, B, Li, SW, Xue, HC, Xing, FY, Humphries, D, Yan, W, et al. 2002 Emerging patterns of hookworm infection: Influence of aging on the intensity of *Necator* infection in Hainan Province, People's Republic of China. *Clin Infect Dis* **35**, 1336-1344. (doi:10.1086/344268).

[21] Bundy, DAP, Kan, SP & Rose, R. 1988 Age-related prevalence, intensity and frequency-distribution of gastrointestinal helminth infection in urban slum children from Kuala-Lumpur, Malaysia. *Trans R Soc Trop Med Hyg* **82**, 289-294. (doi:10.1016/0035-9203(88)90450-6).

[22] Onyemelukwe, GC & Musa, BO. 2001 T-lymphocyte subsets in patients with hookworm infection in Zaria, Nigeria. *Afr J Med Med Sci* **30**, 255-259.

[23] Quinnell, RJ, Bethony, J & Pritchard, DI. 2004 The immunoepidemiology of human hookworm infection. *Parasite Immunol* **26**, 443-454. (doi:10.1111/j.0141-9838.2004.00727.x).

[24] Morassutti, AL & Graeff-Teixeira, C. 2012 Interface molecules of *Angiostrongylus cantonensis*: Their role in parasite survival and modulation of host defenses. *Int J Inflam* **2012**, 512097-512097. (doi:10.1155/2012/512097).

[25] Hotez, PJ, Zhan, B, Bethony, JM, Loukas, A, Williamson, A, Goud, GN, Hawdon, JM, Dobardzic, A, Dobardzic, R, Ghosh, K, et al. 2003 Progress in the development of a recombinant vaccine for human hookworm disease: The human hookworm vaccine initiative. *Int J Parasitol* **33**, 1245-1258. (doi:10.1016/s0020-7519(03)00158-9).

[26] Ogechi, NRI & Maduka, AB. 2015 Effect of experimental single *Ancylostoma caninum* and mixed infections of *Trypanosoma brucei* and *Trypanosoma congolense* on the humoural immune response to anti-rabies vaccination in dogs. *J Coast Life Med* **3**, 491-494.

[27] Quinnell, RJ, Woolhouse, MEJ, Walsh, EA & Pritchard, DI. 1995 Immunoepidemiology of human necatoriasis - correlations between antibody-responses and parasite burdens. *Parasite Immunol* **17**, 313-318. (doi:10.1111/j.1365-3024.1995.tb00897.x).

[28] Pearson, MS, Tribolet, L, Cantacessi, C, Periago, MV, Adela Valerio, M, Jariwala, AR, Hotez, P, Diemert, D, Loukas, A & Bethony, J. 2012 Molecular mechanisms of hookworm disease: Stealth, virulence, and vaccines. *J Allergy Clin Immunol* **130**, 13-21. (doi:10.1016/j.jaci.2012.05.029).

[29] Audebert, F, Vuong, PN & Durette-Desset, MC. 2003 Intestinal migrations of *Trichostrongylus retortaeformis* (Trichostrongylina, Trichostrongylidae) in the rabbit. *Vet Parasitol* **112**, 131-146. (doi:10.1016/s0304-4017(02)00386-2).

[30] McClure, SJ, Emery, DL, Bendixsen, T & Davey, RJ. 1998 Attempts to generate immunity against *Trichostrongylus colubriformis* and *Haemonchus contortus* in young lambs by vaccination with viable parasites. *Int J for Parasitol* **28**, 739-746. (doi:10.1016/s0020-7519(98)00040-x).

[31] Murphy, L, Nalpas, N, Stear, M & Cattadori, IM. 2011 Explaining patterns of infection in free-living populations using laboratory immune experiments. *Parasite Immunol* **33**, 287-302. (doi:10.1111/j.1365-3024.2011.01281.x).

[32] Stankiewicz, M & Hadas, E. 2000 Immunomodulation of lambs following treatment with a proteasome preparation from infective larvae of *Trichostrongylus colubriformis*. *Parasitol Res* **86**, 422-426. (doi:10.1007/s004360050688).

[33] Murphy, L, Pathak, AK & Cattadori, IM. 2013 A co-infection with two gastrointestinal nematodes alters host immune responses and only partially parasite dynamics. *Parasite Immunol* **35**, 421-432. (doi:10.1111/pim.12045).

[34] Lyndal-Murphy, M & Macarthur, FA. 1993 Anthelmintic resistance in sheep. In *Australian Standard Diagnostic Techniques for Animal Diseases.* (eds. L.A. Corner & T.J. Bagust). Melbourne, CSIRO for the standing Committee on Agriculture and Resource Management, Melbourne.

[35] Abbot, KA, Taylor, M & Stubbings, LA. 2012 *Sustainable Worm Control Strategies for sheep. A Technical Manual for Veterinary Surgeons and Advisers.* 4th ed. Santa Rosa, CA, Context Publications.

[36] Beasley, AM, Kahn, LP & Windon, RG. 2010 The periparturient relaxation of immunity in Merino ewes infected with *Trichostrongylus colubriformis*: Endocrine and body compositional responses. *Vet Parasitol* **168**, 51-59. (doi:10.1016/j.vetpar.2009.12.012).

[37] Macarthur, FA, Kahn, LP & Windon, RG. 2013 Immune response of twin-bearing Merino ewes when infected with *Haemonchus contortus*: Effects of fat score and prepartum supplementation. *Livest Sci* **157**, 568-576. (doi:10.1016/j.livsci.2013.08.017).

[38] Team, RC. 2014 R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

[39] Thomas, RJ, Vaughan I. R. & Lello J. . 2013 *Data Analysis with R Statistical Software. A Guidebook for Scientists.* Cardiff, Eco Explore.

[40] Courtney, CH, Parker, CF, McClure, KE & Herd, RP. 1983 Population dynamics of *Haemonchus contortus* and *Trichostrongylus* spp in sheep. *Int J Parasitol* **13**, 557-560. (doi:10.1016/s0020-7519(83)80027-7).

[41] McClure, SJ, Emery, DL, Wagland, BM & Jones, WO. 1992 A serial study of rejection of *Trichostrongylus colubriformis* by immune sheep. *Int J for Parasitol* **22**, 227-234. (doi:10.1016/0020-7519(92)90106-u).

[42] Dobson, RJ, Waller, PJ & Donald, AD. 1990 Population-dynamics of *Trichostrongylus colubriformis* in sheep - the effect of infection-rate on the establishment of infective larvae and parasite fecundity. *Int J Parasitol* **20**, 347-352. (doi:10.1016/0020-7519(90)90150-l).

[43] Diez-Tascon, C, Keane, OM, Wilson, T, Zadissa, A, Hyndman, DL, Baird, DB, McEwan, JC & Crawford, AM. 2005 Microarray analysis of selection lines from outbred populations to identify genes involved with nematode parasite resistance in sheep. *Physiol Genomics* **21**, 59-69. (doi:10.1152/physiolgenomics.00257.2004).

[44] Sutherland, I & Scott, I. 2010 The immune response to parasites. In *Gastrointestinal Nematodes of Sheep and Cattle. Biology and Control.* (pp. 211-233. Oxford, Wiley-Blackwel.

[45] Zhao, AP, Urban, JF, Anthony, RM, Sun, R, Stiltz, J, Van Rooijen, N, Wynn, TA, Gause, WC & Shea-Donohue, T. 2008 Th2 cytokine-induced alterations in Intestinal

smooth muscle function depend on alternatively activated macrophages. *Gastroenterology* **135**, 217-225. (doi:10.1053/j.gastro.2008.03.077).

[46] Coyne, MJ & Smith, G. 1992 The mortality and fecundity of *Haemonchus contortus* in parasite-naïve and parasite-exposed sheep following single experimental infections. *Int J Parasitol* **22**, 315-325. (doi:10.1016/s0020-7519(05)80009-8).

[47] Doyle, EK, Kahn, LP, McClure, SJ & Lea, JM. 2011 Voluntary feed intake and diet selection of Merino sheep divergently selected for genetic difference in resistance to *Haemonchus contortus. Vet Parasitol* **177**, 316-323. (doi:10.1016/j.vetpar.2011.01.043).

[48] Charlier, J, van der Voort, M, Kenyon, F, Skuce, P & Vercruysse, J. 2014 Chasing helminths and their economic impact on farmed ruminants. *Trends Parasitol* **30**, 361-367. (doi:10.1016/j.pt.2014.04.009).

[49] Gruner, L, Cortet, J, Sauve, C & Hoste, H. 2004 Regulation of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* worm populations by grazing sheep with differing resistance status. *Vet Res* **35**, 91-101. (doi:10.1051/vetres:2003043).

Figure Titles and Legends

Figure 1. A schematic description of the experimental protocol. Coinfection and mono-infection groups of animals were infected twice weekly for 10 weeks (shaded box) and the animals were then sampled (10 / infection group, and 3 for the control group) after 6, 10, 14 and 18 weeks post initial infection.

Figure 2. Effect of coinfection on within-host parasite dynamics. The predicted number of (a) *T. colubriformis* adult worms by time post initial infection and infection group and (b) *H. contortus* hypobiosed larvae by infection group. Error bars are the 95% confidence intervals. In (a) the *T. colubriformis* mono-infection group is denoted by the

closed grey squares, and the coinfection group by the crossed diamonds, the black arrow represents the last day of larval dosing and the grey arrow represents the first day by which the last larval dose may potentially have reached adulthood. Groups have been offset by one day to aid visualisation.

Figure 3. Immune responses during coinfection. (a) The predicted PC1 scores of jejunal immune response, with time post initial infection and *T. colubriformis* infection group (i.e. mono- and coinfection). The *T. colubriformis* mono-infection group is denoted by the closed grey squares and the coinfection group is denoted by the crossed diamonds. (b) Predicted anti-*H. contortus* IgG1 titre concentration through time post initial infection for the control (open black circles), *H. contortus* mono-infection (solid black circles) and coinfected (crossed diamonds) groups. In (a) and (b) groups have been offset by one day to aid visualisation. Error bars are the 95% confidence intervals. The black arrow represents the last day of larval dosing and the grey arrow represents the first day by which the larvae from the last dose may have reached adulthood.

Figure 4. Jejunal immune responses shown as the bootstrapped number (*per* villus-crypt unit) of (a) eosinophils, (b) goblet cells, (c) globule leukocytes and (d) score of goblet cells with granules. Treatment groups have been offset by one day to aid visualisation. Error bars are the 95% confidence intervals. The solid black line and dashed lines represents the bootstrapped mean for the control treatment group and its 95% confidence intervals, respectively. Grey squares are *T. colubriformis* mono-infection, solid black circles are *H. contortus* mono-infection and crossed diamonds are coinfection.