Parasitic nematodes simultaneously suppress and benefit from coccidian coinfection in their natural mouse host

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Abstract

Within-host interactions among coinfecting parasites are common and have important consequences for host health and disease dynamics. However, these within-host interactions have traditionally been studied in laboratory mouse models, which often exclude important variation and use unnatural host-parasite combinations. Conversely, the few wild studies of within-host interactions often lack knowledge of parasite exposure and infection history. Here we exposed lab-reared wood mice (*Apodemus sylvaticus*) that were derived from wild-caught animals to two naturally-occurring parasites (nematode: *Heligmosomoides polygyrus*, coccidia: *Eimeria hungaryensis*) to investigate the impact of coinfection on parasite infection dynamics, and to determine if the host immune response mediates this interaction. Coinfection led to delayed worm expulsion and prolonged egg shedding in *H. polygyrus* infections and lower peak *E. hungaryensis* oocyst burdens. By comparing antibody levels between wild and colony-housed mice, we also found that wild mice had elevated *H. polygyrus*-IgG1 titres even if currently uninfected with *H. polygyrus*. Using this unique wild-laboratory system, we demonstrate, for the first time, clear evidence for a reciprocal interaction between these intestinal parasites, and that there is a great discrepancy between antibody levels measured in the wild versus those measured under controlled laboratory conditions in relation to parasite infection and coinfection.

Key words: *Heligmosomoides polygyrus*, *Eimeria hungaryensis*, coinfection, wild mice, laboratory systems.

Key findings

- Naturally occurring within-host parasite interactions can be investigated in controlled conditions
- *E. hungaryensis* coinfection prolongs *H. polygyrus* shedding and increases adult worm burdens
- Repeated *Eimeria* challenge leads to only partial immunity in wild-derived hosts and parasites
- Wild mice have elevated IgG1 levels, even in the absence of signs of *H. polygyrus* infection

Introduction

Individuals in their natural environment can be exposed to and infected with a multitude of parasites, either sequentially or simultaneously (Pedersen & Fenton, 2007; Telfer *et al.*, 2008), which can lead to within-host interactions between parasite species (Graham, 2008; Seabloom *et al.*, 2015). While interactions between microparasites (bacteria, protozoa, viruses) and macroparasites (e.g. helminths) can positively and negatively impact the outcome of disease (Salgame *et al.*, 2013), the direction of within-host interactions due to coinfection can be variable, resulting in both positive (facilitating) and negative (competitive) interactions. In addition, host demographic (e.g. sex, age, reproductive condition) and environmental variation can affect the strength and direction of parasite interactions (Gorsich *et al.*, 2014; Moreno *et al.*, 2013). Therefore, by developing an understanding of what determines the magnitude and direction of parasite interactions can help to alleviate host damage and guide new trajectories for improved treatment and control programs (Maizels *et al.*, 2012; Pedersen & Fenton, 2007).

Most studies which have investigated both the mechanisms and health impacts of within-host parasite interactions have used highly controlled laboratory environments, mainly utilising inbred laboratory mice raised under standard pathogen-free (SPF) conditions (Graham, 2008; Knowles, 2011). However, several recent studies have highlighted the importance of making the laboratory mouse model better reflect natural systems by introducing wild mouse microbiomes (Rosshart *et al.*, 2017), infection and coinfection (Maizels & Gause, 2014; Reese *et al.*, 2016) and by co-housing mice with pet-shop mice to allow exposure to pathogens and microbes (Beura *et al.*, 2016). For example, Beura et al. (2016) have shown that the immune cell composition of feral and pet-shop mice resembles that of human adults, whereas lab mice show very low levels of differentiated memory T-cells, similar to human neonates (Beura *et al.*, 2016). As a result of the often unrealistic conditions used in laboratory studies, and the accompanying difficulty in translating these results to the real world, researchers have begun to investigate the underlying mechanisms of parasite within-host interactions in natural environments. These studies have helped unravel the natural conditions under which parasites interact

and importantly what factors determine the impacts of these interactions on host health and disease (Ezenwa *et al.*, 2010; Ezenwa & Jolles, 2011; Friberg *et al.*, 2013; Knowles *et al.*, 2013; Pedersen & Antonovics, 2013; Turner *et al.*, 2011). However, these studies are also not without limitations, as both the exposure and infection history of wild animals is not usually known, which leads to uncertainty in understanding the causal relationships between parasite infection/exposure and host characteristics such as an animal´s immune phenotype (Abolins *et al.*, 2011). Further, wild animals often experience resource limitation at various times throughout their lives, which can potentially reduce their investment in specific aspects of their life-history, for example in the development of their immune system (Tate & Graham, 2015). To overcome the limitations of both laboratory and wild studies, and to better understand the causes and consequences of interactions between coinfecting parasites, field and laboratory approaches need to be better integrated.

Our previous field experiments revealed a negative interaction between two gastrointestinal parasites of wild wood mice (*Apodemus sylvaticus*), the nematode *Heligmosomoides polygyrus* and the coccidian microparasite *Eimeria hungaryensis*. We found that a single dose of the anthelmintic drug Ivermectin reduced *H. polygyrus* prevalence by 70%, but consequently led to a 15-fold increase in the burden of coinfecting *E. hungaryensis* 1 to 3 weeks post-treatment (Knowles *et al.*, 2013). However, it is currently unknown whether this interaction is mediated by the host's immune system (specifically the protective effect of *H. polygyrus* towards *E. hungaryensis*), or via competition through shared host resources. Furthermore, we were previously not able to test for a reciprocal interaction of *E. hungaryensis* on *H. polygyrus* in the field due to difficulties in treating coccidia in the wild.

Here, we developed a wild-like laboratory system using lab-reared wood mice kept under standard laboratory conditions. This colony of wood mice was derived from wild-caught animals collected from woodlands near Liverpool, UK, which were taken into captivity and purposefully outbred for many generations. In addition, we used parasite isolates of both *H. polygyrus* and *E. hungaryensis* that were collected for a Scottish wood mouse population. Together with the lab-kept wood mice, using these wild-derived parasite isolates means that here, we were able to overcome many of the

limitations of both laboratory and wild studies. First, we performed a controlled laboratory experiment to test i) the effect of infection and coinfection on helminth and coccidian shedding and burdens, ii) the effect of coinfection on protective immunity against *E. hungaryensis*, and iii) antibody responses during single and coinfection. Next, we made use of two independent field experiments using the same host and parasite species as in the lab experiment to directly compare antibody levels between lab-kept and wild wood mice and to assess how wild conditions, including frequent exposure to a diverse parasite community, impacts antibody levels. To measure the host immune response following parasite infection, we measured *H. polygyrus*-specific IgG1 and total faecal IgA levels. We chose to focus on these antibody types because they are both important during infection and can be measured in our non-model species using lab mouse reagents. IgG1 is the main antibody class responsible for worm clearance and establishment of protective immunity (Reynolds *et al.*, 2012), whereas IgA is the main antibody class found at mucosal sites (Macpherson *et al.*, 2012) and has been found to have anti-schizont and sporozoite activity during Eimeria infections in chickens (Davis *et al.*, 1978; Trees *et al.*, 1989).

To our knowledge, this study represents is a unique and novel experimental approach to investigate coinfection dynamics using a combination of a mammal host and both micro- and macroparasites, where both host and parasites were either directly wild-derived or the recent descendants of wild-derived animals. We believe that this novel approach may help bridge the current gap between wild and laboratory studies in understanding the causes and consequences of coinfection.

Methods

Mice

We used a colony of *Apodemus sylvaticus* that was derived from wild-caught animals collected from a woodland in the Wirral, United Kingdom around 5 years ago. Since then, the wood mice have been kept in standard laboratory conditions and purposely outbred to retain as much genetic variability

amongst animals as possible. The wood mouse colony is currently housed at the University of Edinburgh under standard laboratory conditions. For this experiment, we used 12 females and 12 males aged between 8-23 weeks (average 15 weeks ± 0.9 standard error). Prior to the start of the experiment, mice were housed in single-sex groups of 2-5 animals. After the start of the experiment, mice were housed individually in individual ventilated cages (Techniplast®, 1285L), with standard mouse chow and water ad libitum.

Parasites

Transmission stages of the two parasite species used in this experiment (*H. polygyrus* L3 larvae and sporulated *Eimeria* spp. oocysts) were derived from faeces collected from wild *A. sylvaticus*, trapped in September 2015 in a mixed woodland in Callendar park, Falkirk, UK (55.99 ° N, 3.77 ° W). To obtain *H. polygyrus* L3 larvae (the infective stage), we followed the protocol of Johnston et al. (2015) to hatch eggs from faecal material (Johnston *et al.*, 2015). In short, faecal samples were soaked in water to soften and then mixed with water-soaked charcoal (DARCO®, 20-40 mesh particle size, granular, 242268), which acted as a substitute for soil. A small amount of the faeces-charcoal mix was spread thinly on a damp filter paper (Whatman No. 40 Filter Paper circles, 1440055) in a plastic petri dish. These faecal cultures were placed in a plastic container layered with damp filter paper to create a humid environment, and stored in the dark at 17°C. After ~5 days, larvae migrated away from the charcoal mix to the edge of the filter paper and into the petri dish. Once larvae appeared in the petri dish, the filter paper was lifted onto a new petri dish and the larvae left behind were collected by flushing with tap water, and stored at 4°C. Faecal cultures were checked every 48h for the presence of new larvae.

To extract *Eimeria* spp. oocysts from faeces, we modified the method used by Ryley *et al.* (2009). In short, faecal samples were soaked in water to soften and spun down at 4,200 rpm for 10 min and the supernatant was removed. 20-30ml saturated salt solution was added to the faecal material, and samples were shaken vigorously to break up the pellets and spun down at 4,200 rpm for 10 min. the supernatant containing the oocysts was collected in a fresh tube and the salt concentration in the tube

was decreased by adding at least an equal amount of tap water to each tube. In order to pellet the oocysts, samples were spun again at 4,200 rpm for 10 min. The pelleted oocysts were then washed 3 times with water and kept in a 2% Potassium dichromate ($K_2Cr_2O_7$) solution to prevent bacterial and fungal growth and stored at 4°C.

After transmission stages of both parasites were isolated from wild wood mice, the isolates were screened using PCR diagnostics to ensure that no other known mouse parasites or pathogens contaminated the field isolates (IDEXX Bioresearch, Germany). To ensure infectivity and to accumulate enough transmission stages, both *H. polygyrus* larvae and *Eimeria* spp. oocysts were passaged three times through colony-housed *A. sylvaticus*. In the case of *Eimeria* spp., this allowed us to selectively passage oocysts that morphologically matched oocysts of *E. hungaryensis*. We henceforth refer to the *Eimeria* spp. isolate used in the experiment as *E. hungaryensis*, however molecular confirmation of its identity is pending.

Experimental design

On day 1 we randomly allocated mice to the following treatment groups: *H. polygyrus*-only, *E. hungaryensis*-only, coinfection and uninfected controls (Fig. 1). Animals in the *H. polygyrus*-only and coinfection groups received a dose of 80 *H. polygyrus* L3 larvae in 200ul water via oral gavage on days 1, 3 and 4 (total 240 L3 larvae), while *E. hungaryensis*-only and control groups received an equivalent dose of water on the same days. We used three infection doses because we were limited in the number of wild *H. polygyrus* larvae available; the larval concentration in our isolates was too low to administer the inoculation in a single dose, and we decided against trying to concentrate the inoculum further due to the risk of losing any larvae during this process.

On day 18, *E. hungaryensis*-only and coinfection groups received a single dose of 500 sporulated *E. hungaryensis* oocysts in 200ul water via oral gavage, while *H. polygyrus*-only and control mice received an equivalent dose of water. On day 32, all groups were challenged with a dose of 500 sporulated *E. hungaryensis* oocysts (from the same starting inoculum) in 200ul water via oral gavage.

On day 45, all animals were culled using an overdose of CO_2.

Over the course of the experiment, mice were sampled 3 times a week, starting on day 3. On each sampling occasion, we recorded body weight, took a small volume of host blood via tail bleed (2-10ul) and collected a fresh faecal sample. The blood was spun at 12,000 rpm for 10 min and serum was stored at -20˚C. We collected 3-6 faecal pellets per mouse per sampling time point and these were dry frozen at -80°C. The rest of the faecal pellets were weighed and stored in 10% buffered formalin at 4°C. to perform faecal egg counts by salt flotation (Dryden *et al.*, 2005) and microscopy. After animals were euthanized on day 45, the number and sex of all adult *H. polygyrus* worms in the small intestine was counted for each mouse. The experiment was conducted in two blocks (replicates), with 3 animals randomly assigned to each treatment group per block, giving a total of 6 mice per treatment group (3 males and 3 females).

One mouse failed to become infected with *E. hungaryensis* after the first *E. hungaryensis* challenge, but was successfully infected after the second *E. hungaryensis* challenge. We excluded this animal from our analysis on *E. hungaryensis* dynamics since we do not know the reason for the failed first infection. Additionally, animals in the second block were given their first *E. hungaryensis* challenge on day 16 instead of day 18 by mistake. This meant that oocyst shedding started on day 19 in in the first block and on day 17 in the second block. However, this had no influence on peak oocyst shedding or total oocyst shedding for the first challenge, as there was no significant effect of block in any of the statistical models (Table 1).

Immunological methods

To measure *H. polygyrus*-specific IgG1 from serum samples, we coated plates (Nunc™ MicroWell™ 96-Well Microplates) with *H. polygyrus* excretory-secretory antigen (HES, supplied by R. M. Maizels, 1.0µg/ml (Johnston *et al.*, 2015)) diluted in carbonate buffer overnight at 4˚C. Non-specific binding sites were blocked with Tris-buffered Saline (TBS) containing 4% Bovine Serum Albumin (BSA) at 37˚C for 2h. Twofold serial dilution of serum samples were prepared in cluster tubes

containing TBS-1% BSA, starting at 1:100. A serum-sample of laboratory *M. musculus* that were artificially infected with *H. polygyrus* was added to each plate as a positive control (supplied by R. M. Maizels). After plates were washed with TBS-0.1% Tween 20, sample dilutions were added to the plates (50µl per well) and incubated overnight at 4˚C. After washing, 50µl goat anti-mouse IgG1-HRP detection antibody (Southern Biotech, Lot J6908-MC69), diluted 1:2000 in TBS-1%BSA was added to each well and incubated at 37˚C for 1h in the dark. Plates were washed 4 times with TBS-Tween 20 and 2 times with $_{d}$H2O, before 50µl Tetramethylbenzidine (TMB) solution was added to each well. Plates were immediately covered to allow the enzymatic reaction to develop for seven minutes and the reaction was stopped with 50µl 0.18M Sulphuric acid. Absorbance was measured at 450nm. Cut-off values were calculated per plate as mean absorbance of blank wells plus 3 times the standard deviation of blank wells. The sample titre was determined as the denominator of the lowest sample dilution step that showed absorbance greater than the cut-off value.

For the faecal IgA ELISA, faecal extracts were prepared for each dry frozen faecal sample by soaking faecal pellets in a 3 to 1 volume of protease inhibitor solution (Complete Mini Protease Inhibitor Tablets, Roche, Cat No.: 11836153001). The extraction was then incubated for 1h at room temperature, after which samples were centrifuged at 12,000 rpm for 5min and the supernatant containing IgA removed. ELISA plates were coated with unlabelled goat anti-mouse IgA (Southern Biotech, Lot H7912-S233, 2 µg/ml) diluted in carbonate buffer overnight at 4˚C. Non-specific binding sites were blocked with TBS containing 4% BSA at 37˚C for 2h. Faecal extracts were diluted 1:100 in cluster tubes containing TBS-1% BSA and added to the plates as triplicates, 50µl per well. Two twofold serial dilutions of standard antibody (Purified mouse IgA, κ isotype control, BD Pharmingen, Lot 3039828) at 50µl per well were added to each plate. Plates were incubated overnight at 4˚C and then after washing, 50µl goat anti-mouse IgA-HRP (Southern Biotech, Lot G4512-V522D) diluted 1:4000 in TBS-1% BSA was added to each well and incubated at 37˚C for 1h in the dark. Plates were washed 4 times with TBS-Tween and 2 times with $_{d}$H2O, before 50µl TMB solution was added to each well and plates were immediately covered to allow the enzymatic reaction to develop for seven minutes. The reaction was stopped with 50µl 0.18M Sulphuric acid and absorbance at

450nm was measured. Sample concentrations of total faecal IgA were determined by fitting 4-parameter logistic regression to standard curves using online software (www.elisaanalysis.com, ©Copyright 2012 Elisakit.com Pty Ltd.).

Statistical analysis

All statistical analyses were performed using R software version 3.2.2 (R Development Core Team (2013), www.r-project.org).

Lab experiment

To analyse *H. polygyrus* dynamics, we tested for differences between the *H. polygyrus*-only and coinfection group in (i) the number of worms recovered at the end of the experiment (continuous, worm counts), (ii) the duration of egg shedding (continuous, number of days), (iii) the peak egg shedding (continuous, eggs/gram faeces rounded to the nearest integer) or (iv) total egg shedding (continuous, eggs/gram faeces rounded to the nearest integer).

To analyse *E. hungaryensis* dynamics, we split the analysis in two parts: first, we measured oocyst shedding dynamics after the first challenge of the *E. hungaryensis*-only and coinfection group. Second, we measured oocyst shedding dynamics after the second *E. hungaryensis* challenge on day 32 (first challenge for the *H. polygyrus*-only and control groups), hence all the treatment groups were analysed. For both analysis parts, we asked whether there were differences between (i) the peak oocyst shedding and (ii) the total oocyst shedding (both variables are continuous, oocysts/gram faeces rounded to the nearest integer).

To analyse *H. polygyrus*-specific IgG1 dynamics, we excluded the treatment groups that weren't challenged with *H. polygyrus* before day 8 as no *H. polygyrus*-specific IgG1 antibodies were detected before this point in any of the treatments. We tested whether there were differences between (i) *H. polygyrus*-specific IgG1 levels (continuous, log-transformed IgG1 titres) throughout the experiment

or (ii) *H. polygyrus*-specific IgG1 levels at the end of the experiment (day 45). We tested whether there were any differences between treatment groups in (i) total faecal IgA levels (continuous, ng/ml) throughout the experiment across all the treatment groups, as all mice were assumed to be producing IgA because this non-specific antibody is known to be involved in gut homeostasis (Macpherson *et al.*, 2012).

In all the models described above we included the following covariates: host sex (factor, male or female), age at the start of the experiment (continuous, number of days), mean body weight over the experimental period (continuous, grams) and experimental block (factor, A or B). Depending on the response variable, we ran either linear models if the response variable was normally distributed, or generalized linear models (GLM) if the response variable was not. For the models that tested dynamic responses over time, we also included the covariate day (continuous, number of day in the experiment) and an interaction between treatment and day. Those models were run as linear mixed effect models (LMM), including animal ID as a random term to control for repeated measures on the same individual.

Comparison lab vs field

In order to compare mean antibody levels between the lab and wild, we used data from three different sources. The first data set came from the coinfection experiment described in this paper (henceforth called "lab", n = 23), the second data set came from a cross-sectional field experiment conducted in 2013 in the Wirral Peninsula, UK (henceforth called "Liverpool", n= 54 (Clerc *et al.*, 2018)) and the third data set came from a longitudinal field experiment conducted in 2014 and 2015 in Falkirk, UK (henceforth called "Scotland", n = 89, Clerc *et al.* in prep). For the lab dataset, we used IgA data collected between days 17 and 33, which corresponds to the time-point of the first *E. hungaryensis* challenge. The IgA data thereby should reflect more closely the IgA levels to be expected in the wild, where the probability of *H. polygyrus* and *E. hungaryensis* infection is high. Further, we used *H. polygyrus*-specific IgG1 data collected at the end of the experiment (day 45), which should more closely reflect what is to be expected in the wild (chronic helminth infection). For the Scotland

dataset, we used data from first observations only, since the experiment subsequently involved anthelmintic treatment. Total faecal IgA and *H. polygyrus*-specific IgG1 levels were measured the same way for all datasets. In order to test the effect of *H. polygyrus* and *E. hungaryensis* infection on both antibody levels, we ran a linear model with either *H. polygyrus*-specific IgG1 or IgA as the response variable. The covariates included in each model were sex (factor, male or female), *H. polygyrus* infection (factor, yes or no), *E. hungaryensis* infection (factor, yes or no) and experiment (factor, lab, Liverpool or Scotland). To test whether the effect of *E. hungaryensis* on antibody levels depended on *H. polygyrus* infection, we also included an interaction between those two factors in each model.

Results

Lab experiment

Coinfected animals had on average over 2.5 times more worms than *H. polygyrus*-only infected animals by the end of the experiment (day 45; *H. polygyrus*-only: mean 13 worms ± 5 SE; coinfection: mean 35 worms ± 7 SE; negative binomial GLM: Effect of *H. polygyrus*-only treatment: Est = -1.18, $p < 0.0001$, Fig. 2, Table 1). We also found that age had a significant effect on the number of adult *H. polygyrus* worms harboured, with older mice harbouring fewer worms than younger mice. *H. polygyrus*-only and coinfected mice both started shedding *H. polygyrus* eggs on day 8, but the duration of egg shedding was significantly longer in coinfected animals than *H. polygyrus*-only (mean end of shedding on day 17 ± 1.5 SE vs 26 ± 4.5 SE respectively; Poisson GLM: Effect of *H. polygyrus*-only treatment: Est = -0.42, $p = 0.001$, Fig. 3A, Table 1). There was no significant difference in peak *H. polygyrus* shedding (*H. polygyrus*-only: mean 187 eggs per gram faeces ± 47 SE; coinfection: mean 180 eggs/gram faeces ± 44 SE, Table 1) nor in total *H. polygyrus* shedding over the experimental period between *H. polygyrus*-only and coinfected mice (*H. polygyrus*-only: mean 336 eggs/gram faeces ± 68 SE; coinfection: mean 260 eggs/gram faeces ± 62 SE, Table 1).

During the first *E. hungaryensis* challenge (Fig 3B day 18 onwards), there were no significant differences in peak oocyst shedding (Table 2, Fig. 4A) or total oocyst shedding (Table 2, Fig. 4B) between *E. hungaryensis*-only infected and coinfected mice. However, during the second challenge (Fig 3B day 32 onwards), all treatment groups had at least 50% lower peak oocyst shedding than control mice (*E. hungaryensis*-only: 17,279 oocysts ± 8,035 SE; coinfection: 12,110 oocysts ± 3,087 SE; *H. polygyrus*-only: 27,505 oocysts ± 12,271 SE; control: 52,910 oocysts ± 14,349 SE; Linear model: Effect *of H. polygyrus*-only: Est = -1.66, p = 0.006, effect of *E. hungaryensis*-only: Est = -2.38, p = 0.001, effect of coinfection: Est = -1.84, p = 0.003, Table 2, Fig. 4C). Further, total *E. hungaryensis* oocyst shedding was 63% lower during a second challenge compared to a first challenge (*E. hungaryensis*-only: 22,091 oocysts ± 8,919 SE; coinfection: 17,160 oocysts ± 3,342 SE; *H. polygyrus*-only: 58,733 oocysts ± 23,487 SE; control: 112,799 oocysts ± 34,366 SE; Linear model: Effect of *H. polygyrus*-only: Est = -6.64, p = 0.056, effect of *E. hungaryensis*-only: Est = -19.71, p < .0001, effect of coinfection: Est = -17.76, p < 0.0001, Table 2, Fig. 4D).

H. polygyrus-specific IgG1 antibodies were detectable from day 12 onwards for mice in the coinfection group, and from day 15 onwards in the *H. polygyrus*-only group (Fig 5A). After these time points, *H. polygyrus*-specific IgG1 titres increased steadily until around day 24, after which titres started to plateau (Fig. 5A). However, while the titre of *H. polygyrus*-specific IgG1 changed over time, there was no difference in the dynamics of *H. polygyrus*-specific IgG1, nor in the final amount of *H. polygyrus*-specific IgG1 at the end of the experiment between the *H. polygyrus*-only and the coinfected mice (Fig. 5A, table 3). Faecal IgA concentration varied substantially for all four treatment groups throughout the experimental period (Fig 5B) and was not significantly different between treatment groups over time (Table 3).

Comparison lab vs wild

We used data from this lab experiment and two previous field experiments to test for the difference in antibody levels between the wild and lab in both single and coinfected hosts (Fig. 6). We found no

difference in the prevalence of either parasite between the two field experiments for *H. polygyrus* (Scotland = 58.5%, Liverpool = 59.3%, p = 1.00) and *E. hungaryensis*/*Eimeria* spp. (Scotland= 32.0%, Liverpool = 18.5%, p = 0.101). However, Liverpool mice had higher IgA levels (Linear model: Effect of Liverpool origin: Est = 25.81, p < 0.0001, Table 4, Fig. 6C) and Liverpool IgA levels were also more variable compared to the lab and Scotland samples. In the Scotland samples, IgA concentrations were highest in the autumn 2014 season (Spring 2014: 18.8 ng/ml +/- 1.4 ng/ml, Autumn 2014: 24.1 ng/ml +/- 1.4 SE, Spring 2015: 19.8 ng/ml +/- 1.0 SE, ANOVA $F_{2,72} = 3.65$, p = 0.031). For *H. polygyrus*-specific IgG1, lab animals only had antibodies if they had been infected with *H. polygyrus* (Fig. 6D), whereas wild animals showed elevated *H. polygyrus*-specific IgG1 (Fig. 6E-F), even if they were not shedding *H. polygyrus* eggs at the time of sampling (Linear model: Effect of *H. polygyrus* infection: Est = 1.52, p = 0.013, Table 4). Interestingly, *E. hungaryensis* had a negative effect on *H. polygyrus*-specific IgG1 levels in the field, but this effect was stronger in mice that were not currently shedding *H. polygyrus* eggs at the time of sampling (linear model: *H. polygyrus* x *E. hungaryensis*: Est = 2.45, p = 0.045, Table 4).

Discussion

By using the offspring of originally wild-derived wood mice and their naturally co-evolved parasites *H. polygyrus* and *E. hungaryensis,* we were able to demonstrate the impact of coinfection on the infection dynamics of both interacting parasites, as well as total and parasite-specific antibodies in a controlled environment. This enabled us to demonstrate, for the first time, that the interaction between these parasites in this system is reciprocal. Our results confirmed our previous wild experiment findings (Knowles *et al.*, 2013), specifically that *H. polygyrus* has a negative effect on *E. hungaryensis* infection (but only on secondary challenge), but importantly were also able to demonstrate that *E. hungaryensis,* in turn, has a positive effect on *H. polygyrus* shedding and adult worm burdens. Further, thanks to our wild-like laboratory experiment, we directly compared antibody levels found in two independent field studies to those found under controlled laboratory conditions.

388 This highlighted that wild mice had much higher baseline parasite-specific antibody levels, potentially
389 suggesting much higher parasite exposure events, a series of trickle infections and/or a role for
390 immune-memory in mediating parasite within-host interactions in the wild. Our results also highlight
391 the crucial effect of coinfection for within-host parasite interactions, and enable us to gain a more
392 mechanistic insight into the underlying causes governing this previously identified interaction, which
393 was not possible from field-data alone due to unknown/uncontrolled levels of parasite exposure,
394 nutritional status and infection histories.
395
396 One key finding of our study, was that coinfection with *E. hungaryensis* increased the duration of *H.*
397 *polygyrus*-egg shedding and led to 2.5 times higher adult worm burdens compared to singly infected
398 mice. The higher worm burdens found in coinfected mice suggests that *E. hungaryensis* infection may
399 reduce resistance towards *H. polygyrus.* Both singly and coinfected mice started shedding eggs at the
400 same time and there was no difference in peak *H. polygyrus* egg shedding, suggesting that coinfection
401 did not impact susceptibility and worm establishment. Instead we propose that coinfected mice were
402 less able to expel adult worms from the gut, once coinfected, which resulted in much higher adult
403 worm burdens and a longer period of egg shedding. A previous coinfection study using *H. polygyrus*
404 and the bacterium *Bordetella bronchiseptica* conducted in laboratory mice found a similar effect; with
405 no difference in worm burdens 12 days post-infection, but coinfected animals had significantly more
406 worms at 24 and 48 days post-infection and a prolonged egg shedding period (Lass *et al.*, 2013). We
407 further found lower worm burdens in older compared to younger mice, which is consistent with recent
408 findings from a wild study we conducted, and likely represents a less mature immune system in
409 younger mice compared to older mice (Clerc et al., in review).
410
411 In our wild-like laboratory experiment, we also found that coinfection with *H. polygyrus* had no
412 significant effect on *E. hungaryensis* oocyst shedding during primary infection (Fig. 2B green and
413 grey lines). However, we then challenged all experimental groups, including the control and *H.*
414 *polygyrus*-only groups, with *E. hungaryensis*, which showed that there was a negative effect of *H.*
415 *polygyrus* infection during a secondary *E. hungaryensis* challenge leading to lower peak oocyst

shedding in coinfected mice than in the control mice. Given that the negative effect of *H. polygyrus* on *E. hungaryensis* oocyst shedding was only found in the secondary challenge, this suggests that the interaction between the two parasites is not purely based on competition for shared host resources (Knowles et al. 2013), but likely also involves the host immune system. A study in laboratory mice found an effect of timing of worm coinfection on *Eimeria* infection (Rausch *et al.*, 2010); mice were infected with *E. falciformis* either 6 days or 28 days after *Heligmosomoides bakeri* infection and the results suggest that only early coinfection increased *E. falciformis* replication. This positive effect on *Eimeria* was accompanied by a reduced production of pro-inflammatory cytokines and an overall increase in the nematode-specific Th2 response, whereas chronic coinfection had no effect on *E. falciformis* replication (Rausch *et al.*, 2010). A key difference between the Rausch et al. (2010) and our current study was that we used two parasites that physically overlap in their infection site (duodenum), whereas *H. bakeri* (duodenum) and *E. falciformis* (caecum) do not physically overlap. This suggests that in the case of coinfection of parasites that share the same niche, there is potential for more direct interaction between diverging immune cell populations as well as for tissue structural effects due to disruption of epithelial tissue integrity by the helminths (Boyett & Hsieh, 2014; Bramhall & Zaph, 2017). Specifically, in the case of *H. polygyrus*, tissue damage can occur when larvae are emerging from the gut epithelium and/or when feeding in the lumen as adults, which can limit the pool of suitable epithelial cells available for *E. hungaryensis* to infect. Whilst, to our knowledge, no study has yet attempted to quantify the degree of available host cells for *Eimeria* infection in the case of helminth coinfection, a mathematical model of single-*Eimeria* infection found that host cell availability, specifically at high infection doses, could explain the so-called "crowding effect", where *Eimeria* fecundity decreases with increased infection dose due to a smaller pool and lifespan of available epithelial cells (Johnston *et al.*, 2001). It is possible that *H. polygyrus*-infection mimics the host cell availability of high-dose single *Eimeria* infections, due to tissue damage, but more work is needed to confirm this hypothesis. Ultimately, our results suggest that in order for *H. polygyrus* to exert a protective effect for the host against high *Eimeria* burden, the two parasites need to physically overlap during chronic *H. polygyrus* infection.

444 Overall, we show that the interaction between these two gut parasites is not uni-directional but
445 reciprocal, with different outcomes for the epidemiology of each parasite: whilst *H. polygyrus*
446 coinfection reduces the transmission potential of *E. hungaryensis* via a reduction in peak oocyst
447 shedding (see also Knowles *et al.*, 2013), *E. hungaryensis* coinfection can actually facilitate *H.*
448 *polygyrus* transmission via increased worm survival and prolonged egg shedding. This result
449 highlights that it is important to understand the direction in which parasites interact in order to make
450 predictions about the causes and consequences of within-host interactions. Combining experiments
451 conducted both in the lab and in the field represents a powerful tool to disentangle the underlying
452 causes of parasite within-host interactions and their directions.
453
454 In addition to disentangling the within-host parasite interactions, we aimed to test whether mice were
455 able to develop protective immunity to homologous *Eimeria* challenge, i.e. challenge with the same
456 strain of *Eimeria* spp. In contrast to laboratory mice and chickens, where protective immunity towards
457 homologous challenge is frequently observed (Pogonka *et al.*, 2010; Smith *et al.*, 2002; Steinfelder *et*
458 *al.*, 2005), wild mice are often found infected with one or multiple *Eimeria* species repeatedly over a
459 long period of time, with prevalence usually ranging around 30-50% (Higgs & Nowell, 2000;
460 Knowles *et al.*, 2013). In our controlled laboratory infections of wood mice with *Eimeria*, although
461 we found that peak and total *E. hungaryensis* oocyst shedding were lower in the secondary challenge,
462 protective immunity was incomplete, as both the *E. hungaryensis*-only and coinfection group still
463 shed significant *E. hungaryensis* oocysts after their second challenge. This lack of protective
464 immunity towards reinfection may result from partial immunity to all strains (imperfect homologous
465 immunity) or from complete immunity to only a subset of *E. hungaryensis* strains. Indeed, the *E.*
466 *hungaryensis* isolate used in this experiment likely consisted of multiple genetically different *E.*
467 *hungaryensis* strains and the original isolate was only passaged through colony housed wood mice
468 three times. Heterologous immunity has been demonstrated in chickens, where hosts infected with
469 one strain of *Eimeria maxima* were always protected against homologous challenge, whereas
470 protection from heterologous challenge varied from 0 to 100% and further depended on host genotype
471 (Smith *et al.*, 2002). This highlights that natural levels of genetic diversity within an infective dose,

472 which is highly likely to be the case in natural coccidian infection of wild wood mice (Higgs &
473 Nowell, 2000), may be an important reason why hosts are unable to mount substantial protective
474 immunity under natural conditions.
475
476 With regard to adaptive immunity to *H. polygyrus*, we found that *H. polygyrus*-specific IgG1 titres
477 increased following helminth infection, but that coinfection had no effect on the magnitude of *H.*
478 *polygyrus*-specific IgG1 in serum. This result resembles the findings of a laboratory mouse
479 experiment that showed that coinfection with *Toxoplasma gondii* did not impact the production of
480 *Fasciola hepatica*-specific IgG1 (Miller *et al.*, 2009). In contrast, Fairlie-Clarke *et al.* (2010) showed
481 reduced helminth-specific IgG1 levels in laboratory mice coinfected with either *Nippostrongylus*
482 *brasiliensis* and *Plasmodium chabaudi*, or *Litomosoides sigmodontis* and *P. chabaudi* (Fairlie-Clarke
483 et al., 2010). These conflicting results highlight the variable effect of microparasite coinfection on
484 helminth-specific antibody production. *H. polygyrus*-specific IgG1 is important for reducing adult
485 worm fecundity upon primary infection and plays a key role in worm expulsion after multiple *H.*
486 *polygyrus*-challenges (McCoy *et al.*, 2008). Because our study included only a single *H. polygyrus*
487 challenge, the delay in *H. polygyrus* expulsion following *E. hungaryensis* coinfection may have been
488 mediated by other immune factors. However, further work is needed to investigate the role of this
489 antibody in secondary *H. polygyrus* challenge in our co-evolved system. We were surprised by the
490 highly variable concentrations of total faecal IgA, which showed no discernible temporal pattern and
491 no difference between treatment groups. This meant that we found a significant block effect in the
492 IgA model, highlighting that the main portion of variation in IgA levels could not be explained by
493 either the experimental treatment groups or any other important covariates. In coccidian infections,
494 the precise role of IgA is still debated, but *E. maxima*-specific IgA levels have been shown to increase
495 markedly at 8 days post infection in infected chickens (Yun *et al.*, 2000). Additionally, parasite-
496 specific IgA levels have been shown to rise after secondary *H. polygyrus*-infection, while total
497 intestinal IgA levels stay constant after primary and secondary *H. polygyrus* infection (McCoy *et al.*,
498 2008). In the future, measuring both total and parasite-specific faecal IgA will give more insight into
499 how IgA may impact both coinfection and protective immunity to *Eimeria*.

By comparing the antibody levels observed in our controlled environment to those measured in two field experiments, we showed that *H polygyrus* infected lab mice produced more specific IgG1 compared to wild mice. Further, wild mice had elevated *H. polygyrus*-specific IgG1 levels, even when they were not currently shedding *H. polygyrus* eggs, whereas lab-kept mice produced no *H. polygyrus*-specific IgG1 in the absence of the parasite. This likely reflects the repeated infectious challenges that wild animals face in natural environments (Tinsley *et al.*, 2012), and might suggest protective immunity in mice that were not shedding any *H. polygyrus* eggs at the time of sampling. We further observed that *Eimeria* infection had a significant negative effect on antibody levels in the wild, irrespective of the presence of *H. polygyrus* eggs (although the effect was stronger for *H. polygyrus*-specific IgG1 if *H. polygyrus* eggs were present). Interestingly, the negative effect of *Eimeria* infection on IgG1 titres was not consistent across field sites, as it was much stronger in the Liverpool population, where we also found overall higher IgA levels. This could, at least in part, be linked to seasonal effects; the Liverpool data were only collected in autumn, whereas the Scotland data were mainly collected in spring (2 of the 3 trapping sessions), and IgA levels were significantly higher in the autumn session compared to the spring sessions in Scotland. In the spring, mice reach their peak reproductive period and a study in field voles has shown that this is the time point of lowest expression of pro- and anti-inflammatory immune markers, suggesting a trade-off between resource allocation to immunity and reproduction (Jackson *et al.*, 2011). Further, strong seasonal changes in wood mice gut microbiome have been shown previously, with an overall more diverse bacterial community in late summer/autumn, and specifically higher abundances of *Alistipes* and *Heliobacter* (Maurice *et al.*, 2015). Since commensal bacterial species are triggers of intestinal IgA expression (Macpherson *et al.*, 2012), our finding of higher IgA levels in the autumn could suggest a higher capacity of the autumn-microbiome in triggering IgA expression, leading to higher baseline IgA levels irrespectively of parasite burdens. Alternatively, higher autumn IgA levels could also be caused by an accumulation of parasite exposure events during the spring and summer months, compared to mice sampled in the spring before a peak in parasite exposure. Both these hypotheses need further testing

528

529 By testing this natural host-parasite-parasite combination under controlled conditions, we offer a
530 novel perspective on the within-host parasite interactions. Due to our unique experimental set-up
531 (wild-derived hosts and co-evolved parasites that naturally interact), we were also able to compare our
532 findings from the lab to previous findings from wild mouse populations, which confirmed our
533 previous evidence of a negative interaction, but importantly identified a new positive interaction,
534 demonstrating a previously unknown reciprocity in this interaction. Our results call for a more
535 profound understanding of the effects of frequent exposure and force of infection on parasite and
536 immune dynamics, which will enable the design of more realistic lab experiments and increase
537 awareness of the importance of wild study systems.

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539

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545

546 *Ethics*

547

548 All procedures on animals conducted in accordance with the Animals (Scientific Procedures) Act
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Figure legends

Figure 1: Schematic representation of infection schedules for each treatment group. Parasite dose per single inoculum were 80 L3 larvae in 200ul water for *H. polygyrus*, 500 sporulated oocysts in 200ul water for *E. hungaryensis*, or 200ul water without parasites.

Figure 2: Number of adult *H. polygyrus* worms recovered from mice at day 45 of the experiment. Points represent means ± standard errors.

Figure 3: Parasite dynamics during the experimental period for A) *H. polygyrus*, B) *E. hungaryensis*. Points represent means ± standard errors. Black = Control, grey = *E. hungaryensis*-only, red = *H. polygyrus*-only, green = Coinfection. Solid black arrows denote *H. polygyrus* challenge events, dashed black arrows denote *E. hungaryensis* challenge events.

Figure 4: Peak and total *E. hungaryensis* shedding at different time points in the experiment. A) Peak *E. hungaryensis* shedding during the first *E. hungaryensis* challenge, B) total *E. hungaryensis* shedding during the first *E. hungaryensis* challenge, C) peak *E. hungaryensis* shedding during the second *E. hungaryensis* challenge, and D) total *E. hungaryensis* shedding during the second *E. hungaryensis* challenge. Points represent means ± standard error.

Figure 5: Antibody dynamics during the experimental period for A) *H. polygyrus*-specific IgG1 and B) total faecal IgA. Points represent means ± standard errors. Black = Control, grey = *E. hungaryensis*-only, red = *H. polygyrus*-only, green = Coinfection. Solid black arrows denote *H. polygyrus* challenge events, dashed black arrows denote *E. hungaryensis* challenge events.

Figure 6: Comparison of antibody levels between lab and field studies. Top row = Total faecal IgA, bottom row = *H. polygyrus*-specific IgG1. A and D represent the lab experiment, B and E represent the Scotland field study, and D and F represent the Liverpool field study. Data is split by *H. polygyrus*

734 infection (x-axis) and *E. hungaryensis* (or *Eimeria* spp.) infection (green dots) or *E. hungaryensis* (or

735 *Eimeria* spp.) uninfected (black dots). Points represent means ± standard errors.

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Table legends

Table 1: Modelling results for *H. polygyrus* worm burdens at day 45, last day of egg shedding, peak egg shedding, and total egg shedding (n = 12). Each column represents a single model, each row represents a model covariate. Each cell contains the covariate estimate and p value. Comparison levels for factors are given in brackets. *Hp* stands for *H. polygyrus.* **** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ˙ $p < 0.1$.

Table 2: Modelling results for *E. hungaryensis* peak oocyst shedding for challenge 1 (n = 11), total oocyst shedding at challenge 1 (n = 11), peak oocyst shedding for challenge 2 (n = 23) and total oocyst shedding at challenge 2 (n = 23). Each column represents a single model, each row represents a model covariate. Each cell contains the covariate estimate and p value. Cells containing NA represent covariates that were not included in the model. Comparison levels for factors are given in brackets. *Hp* stands for *H. polygyrus, Eh* stands for *E. hungaryensis.* **** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ˙ $p < 0.1$.

Table 3: Analysis results for *H. polygyrus*-specific IgG1 dynamics, *H. polygyrus*-specific IgG1 levels at day 45 and total faecal IgA dynamics. Each column represents a single model, each row represents a model covariate. Each cell contains the covariate estimate and p value. Cells containing NA represent covariates that were not included in the model. Comparison levels for factors are given in brackets. *Hp* stands for *H. polygyrus, Eh* stands for *E. hungaryensis.* **** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ˙ $p < 0.1$.

Table 4: Analysis results for *H. polygyrus*-specific IgG1 and total faecal IgA comparison between laboratory, Liverpool and Scotland sites. Each column represents a single model, each row represents a model covariate. Each cell contains the covariate estimate and p value. Comparison levels for factors are given in brackets. *Hp* stands for *H. polygyrus, Eh* stands for *E. hungaryensis.* **** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ˙ $p < 0.1$.

Table 1

Model covariate	***Hp* worms at day 45**	**Last *Hp* shedding**	**Peak *Hp* shedding**	**Total *Hp* shedding**
Sex (male)	-0.14, p = 0.797	-0.05, p = 0.832	-0.07, p = 0.901	-1.35, p = 0.765
Age	0.02, **p = 0.005** **	0.002, p = 0.532	0.01, p = 0.926	0.09, p = 0.118
Mean body weight	0.02, p = 0.813	-0.01, p = 0.688	-0.11, p = 0.179	0.11, p = 0.843
Treatment (Hp)	-1.18, **p < .0001** ***	-0.42, **p = 0.001** ***	-0.10, p = 0.745	0.85, p = 0.728
Block (B)	0.45, p = 0.173	-0.12, p = 0.410	0.55, p = 0.173	5.24, p = 0.100

Table 2

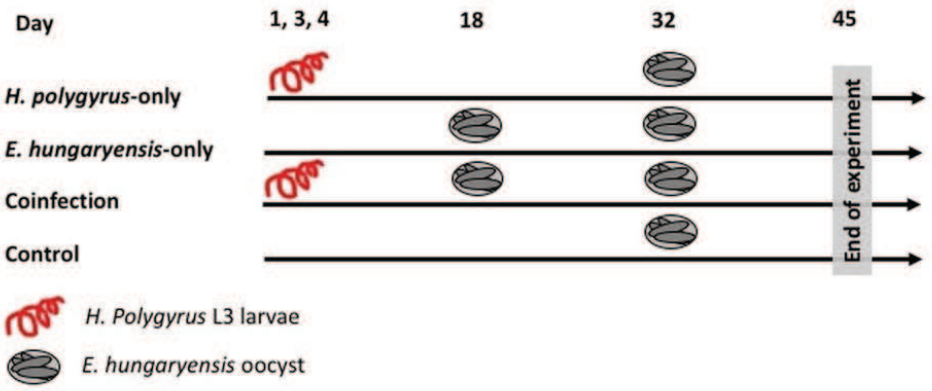
Model covariates	**1st peak *Eh* shedding**	**1st total *Eh* shedding**	**2nd peak *Eh* shedding**	**2nd total *Eh* shedding**
Sex (male)	0.30, p = 0.491	-5.85, p = 0.609	-0.36, p = 0.590	4.97, p = 0.241
Age	-0.01, p = 0.683	0.27, p = 0.644	-0.02, **p = 0.009** **	-0.10, p = 0.069 ·
Mean body weight	-0.10, p = 0.150	0.39, p = 0.802	0.09, p = 0.244	-0.16, p = 0.741
Treatment (Eh)	0.31, p = 0.265	-2.53, p = 0.718	-2.38, **p = 0.001** **	-19.71, **p < .0001** ***
Treatment (Hp)	NA	NA	-1.66, **p = 0.006** **	-6.64, p = 0.056 ·
Treatment (Coinfection)	NA	NA	-1.84, **p = 0.003** **	-17.76, **p < .0001** ***
Block (B)	-0.24, p = 0.762	10.99, p = 0.608	0.71, p = 0.133	-4.15, p = 0.154

Table 3: Analysis results for *H. polygyrus*-specific IgG1 dynamics, *H. polygyrus*-specific IgG1 levels at day 45 and total faecal IgA dynamics. Each column represents a single model, each row represents a model covariate. Each cell contains the covariate estimate and p value. Cells containing NA represent covariates that were not included in the model. Comparison levels for factors are given in brackets. *Hp* stands for *H. polygyrus, Eh* stands for *E. hungaryensis.* **** p < 0.001, ** p <0.01, * p < 0.05, ˙ p < 0.1.

Model covariates	***Hp* IgG1**	***Hp* IgG1 day 45**	**Total IgA**
Sex (male)	-0.69, p = 0.433	-0.31, p = 0.890	-0.68, p = 0.416
Age	-0.01, p = 0.733	0.01, p = 0.673	-0.004, p = 0.883
Mean body weight	0.06, p = 0.627	-0.16, p = 0.621	0.14, p = 0.141
Treatment (Hp)	-1.51, p = 0.161	-0.03, p = 0.982	-0.39, p = 0.693
Treatment (Eh)	NA	NA	-0.45, p = 0.681
Treatment (Coinfection)	NA	NA	-0.62, p = 0.535
Day	0.26, **p < .0001** ***	NA	-0.03, p = 0.244
Block (B)	0.58, p = 0.176	-1.67, p = 0.917	3.15, **p < .0001** ***
Treatment (Hp) x Day	0.04, p = 0.117	NA	0.007, p = 0.981
Treatment (Eh) x Day	NA	NA	0.03, p = 0.426
Treatment (Coinf) x Day	NA	NA	0.05, p = 0.121

Table 4: Analysis results for *H. polygyrus*-specific IgG1 and total faecal IgA comparison between laboratory, Liverpool and Scotland sites. Each column represents a single model, each row represents a model covariate. Each cell contains the covariate estimate and p value. Comparison levels for factors are given in brackets. *Hp* stands for *H. polygyrus, Eh* stands for *E. hungaryensis.* **** p < 0.001, ** p <0.01, * p < 0.05, ˙ p < 0.1.

Model covariates	***Hp* IgG1**	**Total IgA**
Sex (male)	0.28, p = 0.585	-2.08, p = 0.384
Hp infection (yes)	1.52, **p = 0.013** *	-1.74, p = 0.544
Eh infection (yes)	-1.53, p = 0.064 ˙	-7.42, p = 0.059 ˙
Experiment (Scotland)	3.30, **p < .0001** ***	5.21, p = 0.140
Experiment (Liverpool)	3.24, **p < .0001** ***	25.81, **p < .0001** ***
Hp infection x Eh infection	2.45, **p = 0.045** *	3.08, p = 0.547



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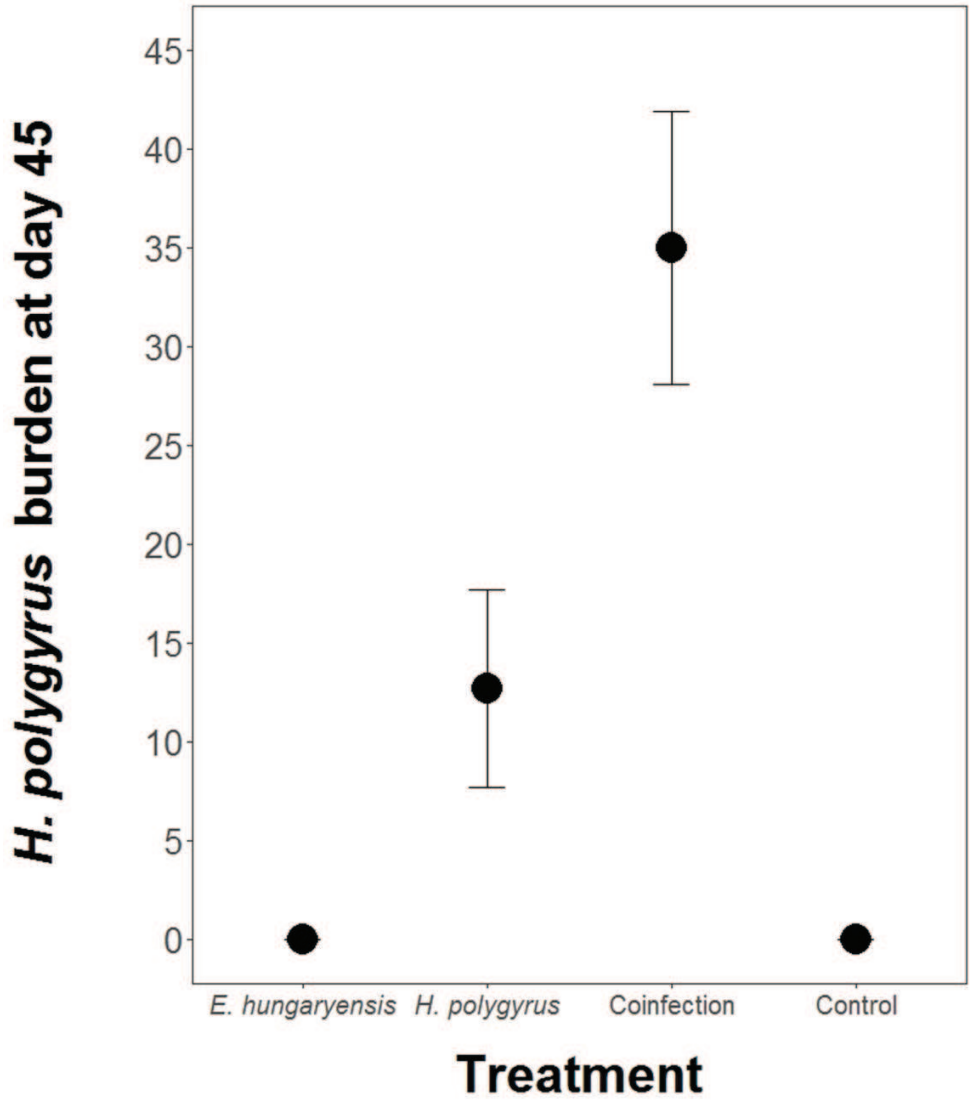

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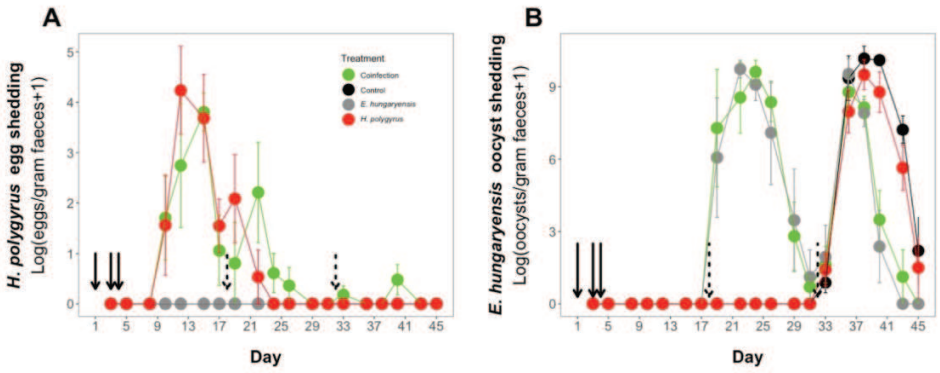

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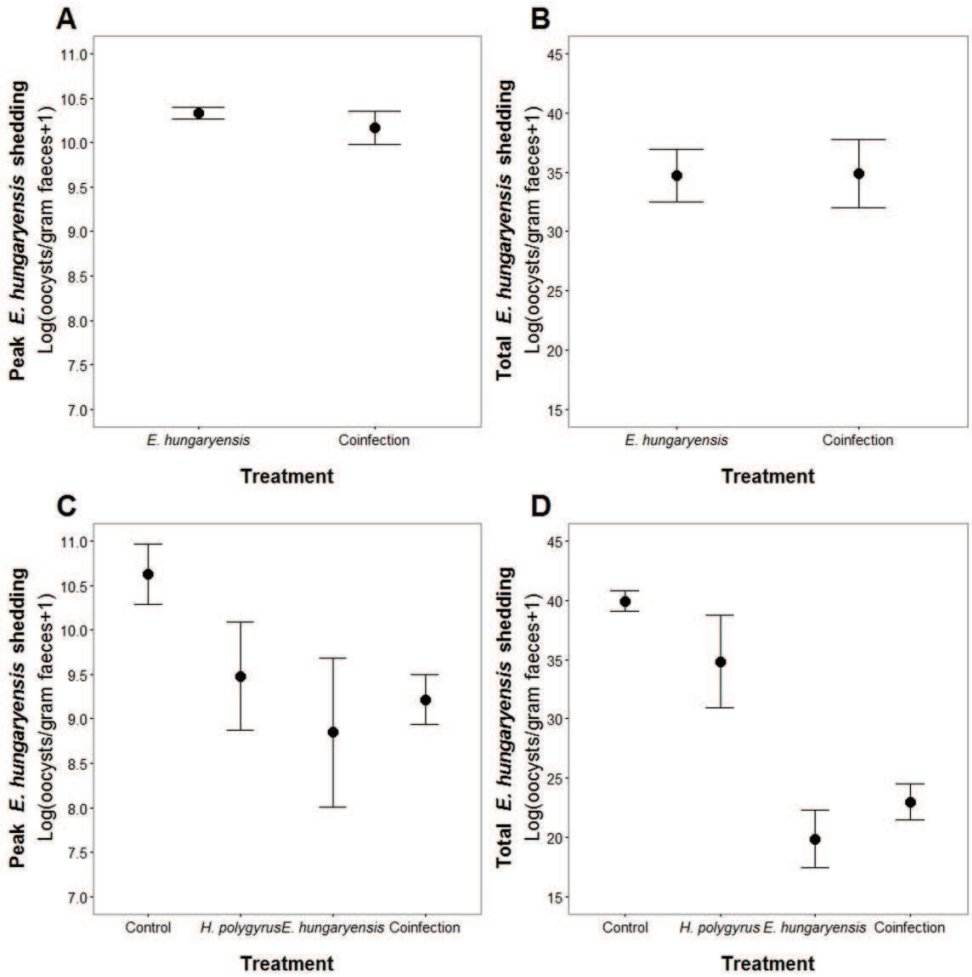

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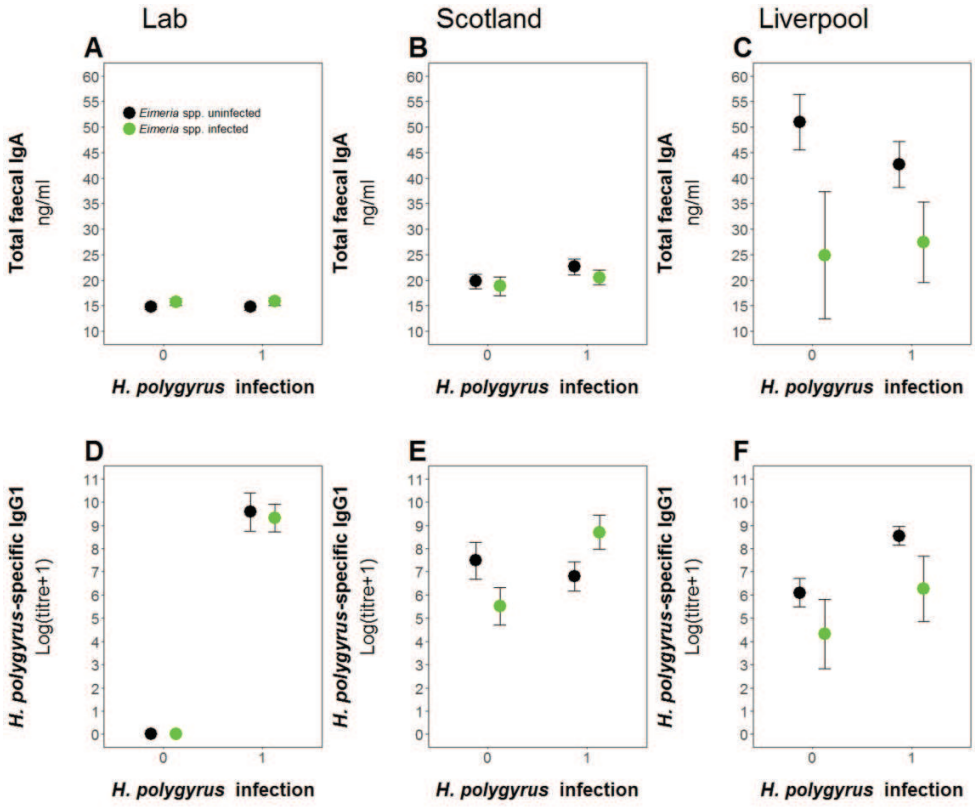

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