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The effect of helminth co-infection on malaria in mice: A meta-analysis

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ABSTRACT

The question of how helminths may alter the course of concurrent malaria infection has attracted much interest in recent years. In particular, it has been suggested that by creating an anti-inflammatory immune environment, helminth co-infection may dampen both protective and immunopathological responses to malaria parasites, thus altering malaria infection dynamics and disease severity. Both synergistic and antagonistic interactions are reported in the literature, and the causes of variation among studies are not well understood. Here, meta-analysis of 42 mouse co-infection experiments was used to address how helminths influence malaria parasite replication and host mortality, and explore the factors explaining variation in findings. Most notably, this analysis revealed contrasting effects of helminth co-infection in lethal and resolving malaria models. Whilst co-infection exacerbated mortality and increased peak parasitaemia in ordinarily resolving malaria infections (*Plasmodium chabaudi* and *Plasmodium yoelii*), effects among lethal malaria infections (*Plasmodium berghei*) tended to be in the opposite direction with no change in parasitaemia. In the subset of experiments on cerebral malaria models (*P. berghei* ANKA strain in a susceptible host), helminth co-infection significantly delayed death. These findings are consistent with the hypothesis that depending on the existing balance of pro- and anti-inflammatory responses mounted against malaria parasites in a given host, immune responses elicited by helminth co-infection may either promote or inhibit malarial disease. However, despite such broad patterns, a prominent feature of this dataset was great heterogeneity in effects across studies. A key future challenge therefore lies in explaining the biological causes of this variation, including a more thorough exploration of non-immunological mechanisms of helminth-malaria interaction.

1. Introduction

Most natural host populations are exposed to a diverse community of parasites, and co-infection of hosts by multiple parasites is commonplace across a diverse range of systems (Petney and Andrews, 1998; Cox, 2001). Recent studies in vertebrates have indicated that interactions between co-infecting parasites can be pronounced and have important consequences for disease development, severity and transmission dynamics (Ezenwa et al., 2010; Telfer et al., 2010). Thus for any given parasite, understanding how it interacts with other parasites infecting the same host may be crucial for a deeper understanding of its biology and epidemiology, as well as effective disease control.

Two of the most prevalent types of human infection in the developing world, malaria and helminthiases, overlap extensively in their epidemiological distributions and frequently co-infect the same individuals (Cox, 2001; Mwangi et al., 2006; Brooker et al., 2007; Mazigo et al., 2010). The question of whether and how these two types of parasite might interact within co-infected hosts has attracted much interest and controversy (Druilhe et al., 2005; Nacher, 2006). Although interactions between helminths and malaria parasites could affect both parties, to date research has focused on the extent to which helminth co-infection influences malarial disease. This bias may reflect the greater human disease burden imposed by malaria compared with helminths (WHO, 2002), as well as the ongoing need to understand what causes variability in malaria infection outcomes; whilst some infections resolve quickly (so-called uncomplicated malaria), others give rise to symptoms falling within the clinical definition of 'severe malaria' including coma, severe anaemia and organ failure, coupled with *Plasmodium* parasitaemia (World Health Organization, 2000; Anstey and Price, 2007). Why only a fraction of individuals develop severe malaria is not well understood and variation in helminth co-infection has been investigated as a possible explanatory factor (Booth, 2006).

Co-infecting parasites may interact either positively (facilitation) or negatively (competition) via a range of mechanisms including resource competition, immune-mediated interactions and direct interference. To date, studies of helminth-malaria co-infection have focused largely on immune-mediated mechanisms, no doubt largely due to the known immunomodulatory effects of helminths (Maizels et al., 2004; van Riet et al., 2007; Hewitson et al., 2009; Grainger et al., 2010). Two major pathways have been

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proposed by which helminths might release malaria parasites from immune pressure and thereby facilitate their replication, both of which involve the dampening of pro-inflammatory immune responses (Hartgers and Yazdanbakhsh, 2006; Specht and Hoerauf, 2007). The first of these relies on a well-known trade-off within the immune system, namely that different T helper (Th) cell subsets tend to be induced by intracellular microparasites (Th1-dominated responses) and extracellular macroparasites (Th2-dominated responses) and these are mutually inhibitory (Jankovic et al., 2001). Thus it has been suggested that by polarizing immune responses towards Th2-type effector mechanisms, helminths will diminish the pro-inflammatory Th1-type mechanisms needed to kill malaria parasites. Second, helminths often elicit potent regulatory T cell (T_{reg}) responses, which suppress all types of cellular effector mechanisms including those against malaria parasites (Maizels and Yazdanbakhsh, 2003; Wammes et al., 2010). By these same pathways, it has also been hypothesised that helminth co-infection might protect against severe malarial disease (Hartgers and Yazdanbakhsh, 2006). Although the mechanisms underlying severe malaria pathogenesis are not fully understood and are likely to vary somewhat among manifestations, immunopathology has been strongly implicated; for example, cerebral malaria (CM) in both mouse models and humans is associated with a relative excess of pro- to anti-inflammatory cytokines (Artavanis-Tsakonas et al., 2003; Hunt and Grau, 2003). Despite diverse organ and system-specific manifestations of severe malaria (e.g. CM versus severe anaemia), it is thought they may stem from similar immunopathological pathways, involving runaway inflammatory cascades inadequately regulated by the host immune system (Schofield and Grau, 2005; Riley et al., 2006). Thus, if helminth-induced immunoregulation or Th2 polarisation limits such inflammatory cascades, it could potentially protect against severe malaria. Although less often considered, resource competition may also play an important role in helminth-malaria interactions, particularly in cases where helminths cause anaemia and might thus limit the availability of red blood cells for malaria parasites (Lwin et al., 1982; Antia et al., 2008; Mideo et al., 2008).

Several epidemiological studies have now sought evidence for effects of helminth co-infection on human malaria, with variable results (reviewed in Mwangi et al., 2006). Whilst some report an increased risk of clinical malaria in helminth co-infected individuals (Spiegel et al., 2003; Sokhna et al., 2004; Roussilhon et al., 2010), others find no effect (Shapiro et al., 2005; Bejon et al., 2008) or even suggest a protective effect (Lyke et al., 2005). Similarly, whilst studies in Thailand have reported negative associations between helminth co-infection and severe malaria (Nacher et al., 2001, 2002), African studies have failed to confirm these associations (Degarege et al., 2009) or suggested the opposite pattern (Le Hesran et al., 2004). Elucidating general patterns from the relatively small number of human studies is difficult, since they cover a range of helminth species (e.g. schistosomes and gut helminths) that are likely to interact differently with malaria parasites, involve diverse epidemiological settings and vary in study design, clinical definitions and other methodological factors. Perhaps more importantly, since most human studies are observational, correlated exposure to helminths and malaria parasites might generate spurious patterns of interaction (Booth, 2006). Thus, firm conclusions about helminth-malaria interactions in humans and the likely effect of antihelminthic treatment on the burden of malarial disease cannot yet be reached (Mwangi et al., 2006; Eziefula and Brown, 2008).

Alongside human field studies, a substantial literature on helminth-malaria co-infection has developed from controlled laboratory experiments in mouse models. Many studies have adopted a common experimental framework to specifically test the effect of helminth co-infection on the course of malaria, using a variety of *Plasmodium* species and inbred mouse lines to mimic different aspects of human malarial disease. Such experiments offer a tractable system in which to elucidate parasite interaction mechanisms. However, despite the controlled nature of these experiments, their findings are also variable, and a key challenge lies in explaining the biological or methodological reasons for this heterogeneity. Although a previous meta-analysis of mouse co-infection studies (Graham, 2008) has shed light on patterns and mechanisms of interaction between helminths and microparasites in general (bacteria, viruses and protozoa), it is unclear to what extent such broad patterns apply specifically to helminth-malaria co-infections. Moreover, although qualitative reviews have speculated about the causes of variation in findings among helminth-malaria co-infection studies (Hartgers and Yazdanbakhsh, 2006), no systematic analysis has been performed. The current study takes advantage of a recent increase in the number of published helminth-malaria co-infection experiments in mice to perform a meta-analysis with two major aims: first, to examine whether there are general trends across heterogeneous study systems indicating that helminth co-infection alters either (i) the ability of malaria parasites to replicate within the host or (ii) the severity of malarial disease. Second and perhaps more importantly, to explore the reasons for variation in these effects across studies and ask whether apparently contradictory findings from different experiments can be reconciled by an understanding of their biological underpinnings.

2. Materials and methods

2.1. Data collection and inclusion criteria

In 2010, the literature was searched for studies in laboratory mice (strains of *Mus musculus*) investigating the effect of helminth co-infection on the course of malaria, using keyword searches on Web of Science, PubMed and Google Scholar (http://wok.mimas.ac.uk/; http://www.ncbi.nlm.nih.gov/pubmed/; http://scholar.google.co.uk/) with the terms 'malaria', 'helminth', 'co-infection', '*Heligmosomoides*', '*Schistosoma*', 'schistosome', '*Plasmodium*' and 'cerebral malaria' in various combinations. No language limits were enforced during the literature search. Abstracts of all retrieved articles were scanned to determine whether the paper contained a helminth-*Plasmodium* co-infection experiment in laboratory mice and their references were checked for other potentially relevant studies. Once it was determined that a paper contained a helminth-*Plasmodium* co-infection experiment in mice, the full text was consulted to determine whether the study met the following specific inclusion criteria:

i. The study involved a controlled experiment in which *Plasmodium* parasitaemia and/or host mortality were monitored in two treatment groups of mice: those given malaria parasites alone (control group), and those given both helminths and malaria parasites (co-infected group).
ii. Helminth infections were initiated prior to or simultaneously with malaria infection but not afterwards.
iii. All mice given malaria parasites developed patent infections.
iv. Malaria parasites were administered by inoculation of infected red blood cells (iRBCs).
v. No chemotherapy, immunomodulatory agents or dead helminths were administered prior to measurement of the response variable in question.
vi. The mice used were not specific knockout strains.

One experiment was excluded under criteria 3 and 4, as malaria infections were initiated using sporozoite inoculation and not all mice developed patent malaria infections (Fernández Ruiz et al.,

2009); although the route of infection is likely to influence how co-infecting parasites interact, sufficient data to test this were not available as all other experiments challenged mice with blood-stage malaria infections. Two further results were excluded on the basis of criterion 5, as they involved administration of drugs (Legesse et al., 2004) or dead helminth larvae (Yan et al., 1997).

2.2. Response variables and effect size calculations

The effect of helminth co-infection on two response variables was examined, chosen to reflect two different processes within a malaria infection. These were: (i) peak *Plasmodium* parasitaemia, indicating the replication of malaria parasites within the host, and (ii) host mortality, to capture severe malarial disease. It was assumed that host mortality can be considered a surrogate of malaria pathology and that helminth infection per se plays a negligible role in host mortality. Supporting this assumption, 13 of 21 experiments reporting host mortality documented no mortality in a helminth-only treatment group over the same time period. Among the remaining eight experiments, three involved *Litomosoides sigmodontis* or *Heligmosomoides polygyrus* infections, neither of which cause host mortality in laboratory mice under normal circumstances, whilst five involved *Schistosoma mansoni*, which can lead to host mortality in prolonged infections. To test whether potential schistosome-induced mortality in these five experiments could have influenced conclusions from the mortality analysis, sensitivity analysis was performed in which they were excluded (see Section 3.4).

Mean peak parasitaemia and its S.D. for each treatment group was extracted using one of the following data forms (in order of preference), depending on result presentation: (i) peak parasitaemia measurements for individual mice; (ii) mean parasitaemia at its highest, in treatment groups where all mice survived; (iii) mean parasitaemia on the last day all mice were alive, in treatment groups where at least one mouse died (usually in cases where parasitaemia was increasing before death). This data was extracted either from statistics presented in the text, raw data received from authors or published figures using the free programme 'Datathief' (http://datathief.org/). For each experiment, these means and S.D.s were used to calculate Hedge's g and its associated variance using standard methods (Borenstein, 2009). Hedge's g is a standardised measure of the difference between two means from independent groups. Here, the magnitude of g indicates the difference in mean peak parasitaemia between co-infected and control mice, with positive values of g indicating that co-infected mice had higher peak parasitaemia and lower values indicating a lower peak parasitaemia. Wherever possible, data from identical replicates within an experiment were pooled before effect size calculation. For studies where data on means and S.D.s in each group were not available (7/37), g was calculated from F statistics or two-tailed P-values (Borenstein, 2009).

Host mortality effect sizes were only calculated for experiments in which at least one host died. In almost all cases, the day of death (or survival beyond the experimental period) could be determined for individual mice from figures and reported sample sizes or raw data supplied by authors. Standard calculations were then used to derive log hazard ratios (lnHR) and their associated variance, var(lnHR) (Parmar and Machin, 1995). Positive values of lnHR indicate higher mortality of co-infected mice compared with malaria-only controls. For one study (Helmby et al., 1998), data were presented only as the numbers of mice that died in each group. However, verbal description of times of death allowed a hazard ratio to be calculated and results were virtually identical when this approximate effect size was excluded (data not shown).

Several papers reported multiple experiments where co-infected and control mice had been compared, for example using different mouse strains, helminth species, *Plasmodium* spp., inoculation doses or time intervals between helminth and *Plasmodium* co-infection. Wherever these involved independent groups of co-infected and control mice, separate effect sizes were calculated for each of these experiments. When multiple co-infected groups (for example differing only in inoculation dose or interval between infections) had been compared with a single malaria-only control group, a single effect size was calculated comparing pooled co-infected mice with control mice. In one study (Noland et al., 2008), two treatment groups infected with different helminth species were both compared with a single control group. Here, separate effect sizes were computed for each helminth species, despite the use of a common control group and therefore a small degree of pseudoreplication. Where reported statistics were insufficient to calculate an effect size and associated variance, or were reported for a subset of experimental replicates, further information was requested from authors, which proved fruitful in many cases. In the end, 11 experiments that would otherwise qualify for inclusion (10 experiments for the parasitaemia analysis, and one for the mortality analysis) could not be included due to insufficient quantitative data. The data source for calculation of each effect size is shown in Supplementary Tables S1 and S2. All effect size estimates were extracted by the author, and a random subset of 12 (six parasitaemia and six mortality effect sizes) were independently re-extracted under the same criteria by a colleague. Correlations between these paired estimates of effect size and variance were high, with the only differences occurring where data were inferred from figures (lnHR: $r = 1.00$, var(lnHR): $r = 1.00$, g: $r = 0.85$, var(g) $r = 0.78$).

2.3. Meta-analytic methods

Meta-analysis was performed using random effects meta-analytic models within the GLIMMIX platform of SAS (v9.2, SAS Institute, Cary, NC, USA). Effect sizes were weighted by their inverse variance to account for variation in sample sizes between experiments. First the overall mean effect size and 95% confidence intervals (CIs) in both the parasitaemia and mortality analyses were estimated, and heterogeneity in effect sizes was assessed by examination of forest plots. Subsequently, it was tested whether several moderator variables (methodological or biological features that vary among studies and could influence effect size) predicted effect size variation. As the datasets for both peak parasitaemia and mortality were relatively small (37 and 21 effects, respectively), only univariate analysis of moderator variables was performed. The following categorical variables were tested: *Plasmodium* sp., helminth sp., whether the helminth is thought to cause transient, chronic or no anaemia (as a measure of potential resource competition between helminths and malaria parasites), whether the *Plasmodium* strain is normally lethal within the particular mouse strain used, and whether the experiment involved a CM model or not (*Plasmodium berghei* ANKA strain in a CM-susceptible host strain). Variation in host genetic background was too great to test for an overall host strain effect; however, among studies that used the common C57/BL6 or BALB/c strains, the effect of mouse strain on effect size was tested. In addition two continuous moderator variables were considered: the dose of iRBCs administered and the interval in days between helminth infection and inoculation of malaria parasites (see Supplementary data S1 for further details of parasite classifications and variable coding).

Both the parasitaemia and mortality datasets contained instances where multiple experiments were included from a single paper (see Supplementary Tables S1 and S2). Such experiments may be considered non-independent. In the past, non-independence has been dealt with by taking a mean weighted effect size from studies before entry into meta-analysis. However, mixed model meta-analysis can deal with the non-independence of data

through incorporating random effects and thus avoid loss of information through data averaging (e.g. see Nakagawa et al., 2007; Cornwallis et al., 2009). In all analyses, to control for possible similarity among effect sizes calculated from the same publication, "Paper" was included as a random effect. Mean weighted effect sizes and their 95% CIs were estimated using restricted maximum likelihood (REML) and the Kenward Roger approximation for degrees of freedom. The Kenward and Roger (1997) method for estimating parameter estimate standard errors and denominator degrees of freedom is specifically designed for models with random effects and increases the accuracy of significance tests. The significance of moderator variables was assessed using Wald-type adjusted F statistics, where $P < 0.05$ was considered significant.

2.4. Assessing the potential for bias

An important problem in meta-analysis is the possibility that published studies form a biased subset of all studies on a subject, because non-significant results may be more likely to be filed away without submission or rejected for publication. This bias has the potential to increase the risk of a type I error (Sutton, 2009). The possibility of publication bias was explored via inspection of funnel plots as well as Spearman's rank correlations between effect size and sample size. Three post hoc sensitivity analyses were also performed to check the robustness of findings from the mortality analysis (see Section 3.4).

3. Results

Twenty-one papers were located containing data that could be used in this meta-analysis. Since these papers often contained results from multiple experiments, a total of 21 mortality effect size estimates and 37 parasitaemia effect size estimates were derived from this dataset, covering a diverse range of helminth-*Plasmodium* sp. pairings (Table 1; see Supplementary Tables S1 and S2 for full effect size details).

3.1. Helminth co-infection and peak Plasmodium parasitaemia

Visual inspection of the forest plot showed that effect sizes in the parasitaemia analysis were clearly heterogeneous (showed greater variance between experiments than within experiments; Fig. 1A). The mean weighted effect size across all experiments was positive with borderline significance ($g = 0.322$, 95% CI: −0.040 to 0.684, $t = 1.91$, $P = 0.077$). This suggests helminths generally tended to increase peak *Plasmodium* parasitaemia, although there is great variation in this effect. In this dataset, a unit increase in g corresponds to an increase of 11% in iRBCs (difference in % iRBC = 10.89g). Thus a value of $g = 0.322$ across the entire dataset indicates that on average helminth co-infection increased peak parasitaemia by 3.5%. None of the moderator variables examined significantly predicted effect size (Fig. 2, Supplementary Table S3). However, some subgroups of the data according to moderator variables showed significantly positive effect sizes (Fig. 2), most notably experiments involving the helminth *H. polygyrus* ($g = 0.959$, $t = 2.94$, $P = 0.011$), as well as those involving resolving malaria infections ($g = 0.527$, $t = 2.26$, $P = 0.038$), and in particular *Plasmodium yoelii* ($g = 0.879$, $t = 2.65$, $P = 0.015$). There was no evidence that the extent of helminth-induced anaemia predicted effect size ($F = 1.27$, $P = 0.321$), although the power to test this variable was limited by knowledge about the extent to which different helminths induced anaemia, in particular the commonly used nematode *H. polygyrus* (see Supplementary data S1). Among the subset of experiments involving *S. mansoni* ($n = 19$), the same moderator variables were tested, although again none significantly predicted effect size (Supplementary Table S3). The random effect 'Paper' explained little of the total variance in the dataset (5.63% in a model with no moderator variables), but was retained in all models to control for non-independence of experiments from the same paper.

3.2. Helminth co-infection and host mortality

Inspection of the forest plot revealed that effects of helminth co-infection on host mortality were again heterogeneous among

Table 1
Summary of papers examining the effect of helminth co-infection on *Plasmodium* parasitaemia and/or mortality in laboratory mice.

Source paper	Paper number	Host strain	Helminth	*Plasmodium* isolate	*n* Effect sizes (Parasitaemia)	*n* Effect sizes (Mortality)
Christensen et al. (1988)	1	Swiss Albino	*Echinostoma caproni*[a]; *Schistosoma mansoni*	*Plasmodium yoelii*	2	0
de Souza and Helmby (2008)	2	C57BL/6, BALB/c	*Heligmosomoides polygyrus*	*Plasmodium berghei* ANKA	2	2
Graham et al. (2005)	3	BALB/c	*Litomosoides sigmodontis*	*Plasmodium chabaudi* AS	1	0
Helmby (2009)	4	C57BL/6; BALB/c	*H. polygyrus*	*P. chabaudi* AS	1	2
Helmby et al. (1998)	5	C57BL/6	*S. mansoni*	*P. chabaudi* AS	1	1
Hoeve et al. (2009)	6	BALB/c	*Nippostrongylus brasiliensis*	*P. chabaudi* AS	1	0
Legesse et al. (2004)	7	Swiss Albino	*S. mansoni*	P. berghei ANKA	1	0
Lwin et al. (1982)	8	CBA/Ca	*S. mansoni*	*P. berghei*, *P. yoelii* 17X 1358, *P. yoelii* 17X 1368, *P. chabaudi*	8	3
Ngwenya (1982)	9	CD1; C57BL/6	*Trichinella spiralis*	*P. berghei* NYU-2	4	0
Noland et al. (2005)	10	BALB/c	*E. caproni*	*P. yoelii* 17X NL	1	1
Noland et al. (2008)	11	BALB/c	*E. caproni; H. polygyrus*	*P. yoelii* 17X NL	2	0
Sangweme et al. (2009)	12	BALB/c	*S. mansoni*	*P. yoelii* 17X NL	3	1
Segura et al. (2009)	13	C57BL/6	*H. polygyrus*	*P. chabaudi* AS	1	0
Specht et al. (2010)	14	C57BL/6	*L. sigmodontis*	*P. berghei* ANKA	1	1
Su et al. (2005)	15	C57BL/6	*H. polygyrus*	*P. chabaudi* AS	1	1
Tetsutani et al. (2008)	16	C57BL/6	*H. polygyrus*	*P. berghei* ANKA	1	1
Tetsutani et al. (2009)	17	C57BL/6	*H. polygyrus*	*P. yoelii* 17X NL	1	1
Waknine-Grinberg et al. (2010)	18	ICR HS	*S. mansoni*	*P. berghei* ANKA	2	2
Yoshida et al. (2000)	19	C57BL/6; A/J	*S. mansoni*	*P. chabaudi* AS	2	2
Laranjeiras et al. (2008)	20	BALB/c	*S. mansoni*	*P. berghei* NK65	1	1
Fernández Ruiz et al. (2009)	21	BALB/c	*L. sigmodontis*	*P. berghei* ANKA	0	2

[a] Synonymous with *Echinostoma revolutum* discussed in Christensen et al. (1988).

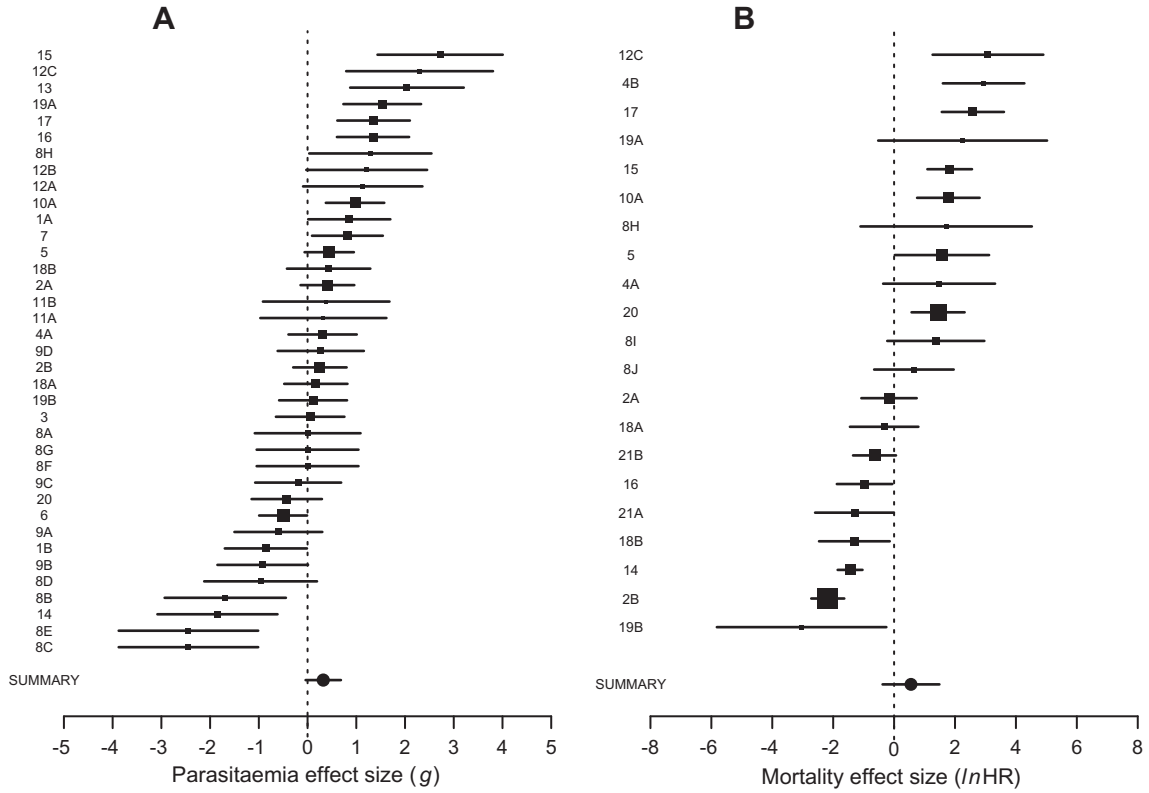

Fig. 1. Forest plots for meta-analysis investigating the effect of helminth co-infection on (A) peak *Plasmodium* parasitaemia and (B) host mortality, across experimental studies in mice. Positive effect sizes (values of Hedge's *g* or log hazard ratio *ln*HR) indicate a higher peak *Plasmodium* parasitaemia, or mortality, in co-infected hosts compared with hosts infected with *Plasmodium* alone. An effect size for each experiment (referenced by a letter and number, indicating the source paper and result, see Supplementary Tables S1 and S2) is shown with 95% confidence intervals (CIs), as well as the summary (mean weighted) effect size across all experiments. Point size for each experiment is proportional to the square root of the sample size.

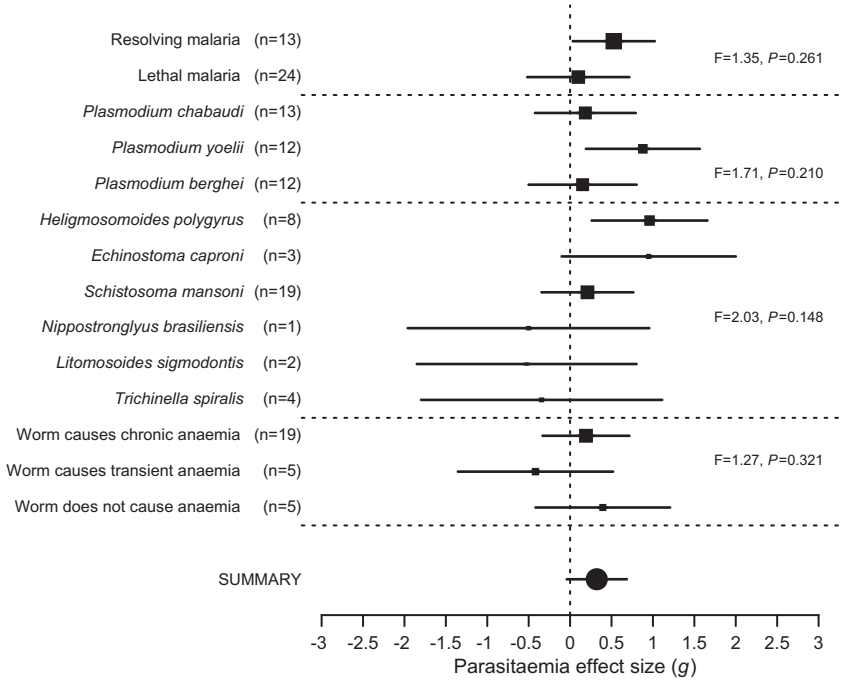

Fig. 2. The influence of moderator variables on the effect of helminth co-infection on peak *Plasmodium* parasitaemia among experiments in laboratory mice. Data points represent mean effect sizes (Hedge's *g*) and their 95% confidence intervals (CIs), as estimated from meta-analysis. Positive and negative values of *g* indicate that helminth co-infection increased or decreased peak *Plasmodium* parasitaemia, respectively. Where 95% CIs do not include zero, the effect can be considered statistically significant.

experiments (Fig. 1B). Across all studies there was no consistent effect of co-infection on host mortality, with overall mean weighted effect size positive but not significantly different from zero (*ln*HR = 0.554, 95% CI: −0.378 to 1.487, $t = 1.29$, $P = 0.221$).

However, variation in effect size was predicted by biological traits of the study systems examined. Three moderator variables strongly predicted effect size: whether the malaria infection was resolving or lethal (in the absence of helminths), *Plasmodium* sp., and whether a CM model was used. These three variables are to an extent collinear: whilst all CM models involve *P. berghei* and produce lethal infections, several experiments involving lethal malaria infections and/or *P. berghei* were not CM models (see Supplementary Table S2). Experiments using *Plasmodium*-mouse combinations that would ordinarily lead to resolving malaria infections had significantly higher mortality effect sizes than experiments using lethal *Plasmodium* models ($F = 26.90$, $P < 0.001$; Fig. 3). This finding was also reflected in effect size differences according to *Plasmodium* sp. ($F = 7.83$, $P = 0.005$, Fig. 3), as well as whether a CM model was being investigated ($F = 40.62$, $P < 0.001$, Fig. 3). In experiments using resolving malaria models, *Plasmodium chabaudi*, *P. yoelii* and non-CM models, mortality was significantly increased by helminth co-infection (Fig. 3). In lethal malaria models and/or *P. berghei* infections, helminth co-infection did not significantly alter mortality (Fig. 3, Supplementary Table S4). However among the smaller subset of experiments on CM models, mean mortality effect size was negative and significantly different from zero, indicating that helminth co-infection delayed death in these systems (*ln*HR = −1.099, 95% CI: −1.623 to −0.576, $t = -4.39$, $P = 0.0003$). No significant effects of mouse strain (C57BL/6 versus BALB/c), helminth species or helminth-induced anaemia were detected in the host mortality analysis (Supplementary Table S4, Fig. 3). The random effect 'Paper' explained between 0% and 67% total variance in mortality effect size, depending on which fixed moderator variable was fitted; when moderator variables pertaining to the type of malaria model were tested (i.e. those with substantial explanatory power) 'Paper' explained little variance, but explained more in models not containing these fixed effects. Regardless of the amount of variance explained, 'Paper' was retained in all models to control for non-independence of experiments from the same publication.

3.3. The link between mortality and parasitaemia effects

Since 15 of 21 experiments in the mortality analysis also provided parasitaemia data, it was possible to compare how co-infection affected both mortality and parasitaemia in this subset. Host mortality effect size increased with parasitaemia effect size ($F_{1,15} = 6.58$, $P = 0.022$, Supplementary Fig. S1), such that a co-infection-related increase in peak parasitaemia predicted higher mortality with co-infection. More strikingly, contrasting effects of co-infection on host mortality in resolving versus lethal malaria models were paralleled by contrasting effects of co-infection on peak parasitaemia ($F = 14.17$, $P = 0.0016$); whereas helminth co-infection significantly increased peak *Plasmodium* parasitaemia among normally resolving malaria infections ($g = 1.389$, 95% CI: 0.740, 2.038; $t = 4.6$, $P = 0.0004$), corresponding to a mean increase of 14.7% in iRBCs, co-infection had no significant effect on parasitaemia in lethal models ($g = 0.042$, 95% CI: −0.580, 0.663; $t = 0.15$, $P = 0.885$, Fig. 4).

3.4. Assessment of bias and sensitivity analyses

Although often difficult to detect with small datasets, publication bias was examined by inspection of funnel plots and Spearman's rank correlations between effect size (g or *ln*HR) and sample size. For both the parasitaemia and mortality meta-analyses, funnel plots showed no clear indication of bias (Fig. 5), and the rank correlations between sample size and effect size were non-significant (parasitaemia $r_s = 0.101$, $P = 0.551$; mortality $r_s = -0.260$, $P = 0.256$).

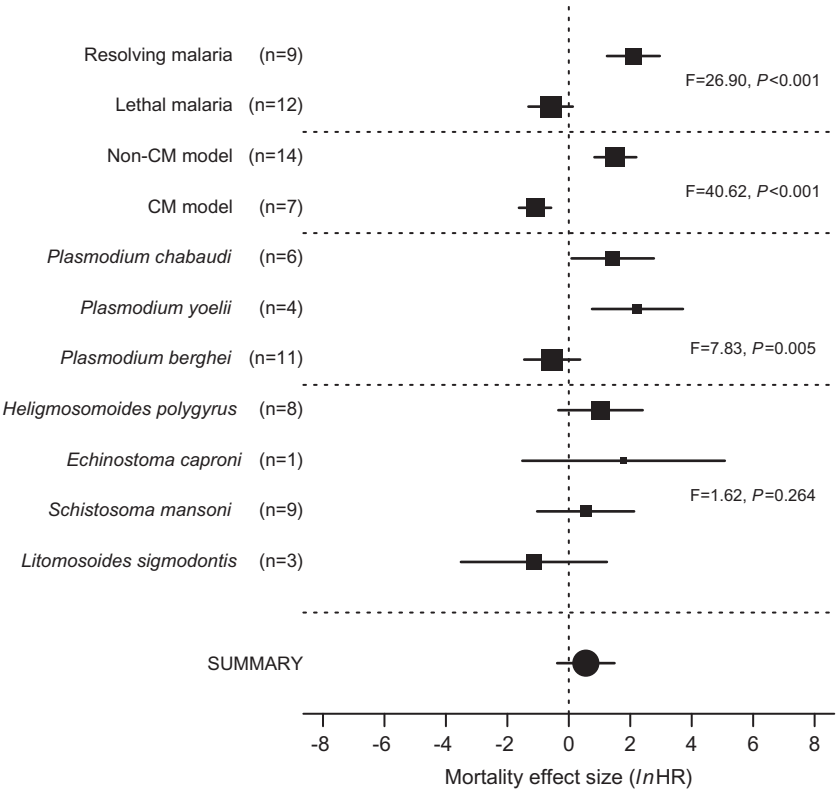

Fig. 3. The influence of moderator variables on the effect of helminth co-infection on host mortality among experiments in laboratory mice. Data points represent mean effect sizes (log hazard ratios, *ln*HR) and their 95% confidence intervals (CIs), as estimated from meta-analysis. Positive and negative values of *ln*HR indicate that helminth co-infection increased or decreased peak *Plasmodium* parasitaemia, respectively. Where 95% CIs do not include zero, the effect can be considered statistically significant.

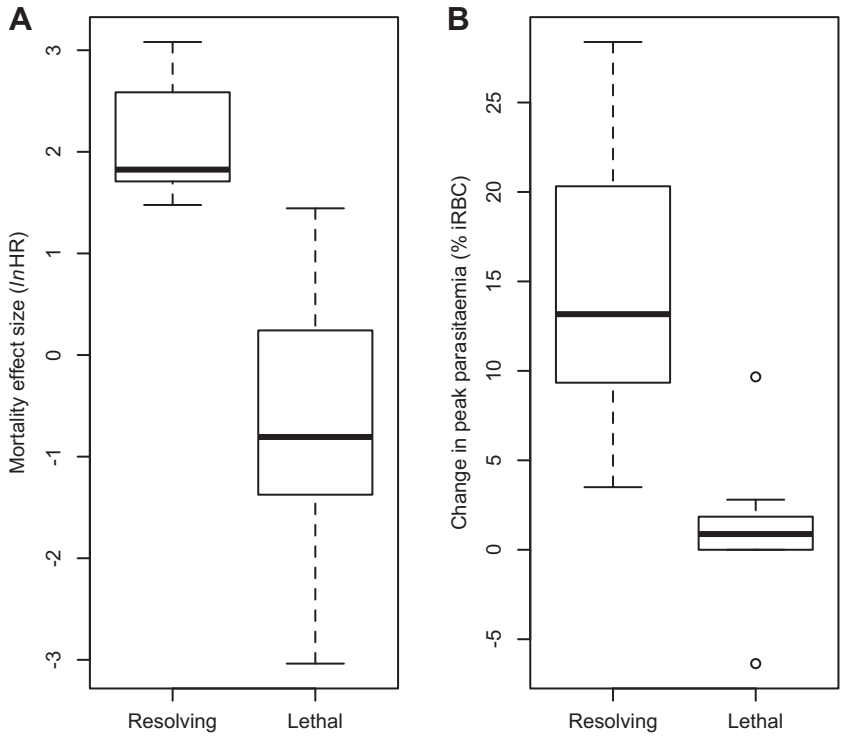

Fig. 4. The effect of helminth co-infection on (A) host mortality and (B) peak *Plasmodium* parasitaemia, according to whether the *Plasmodium*-mouse combination used ordinarily leads to a resolving or lethal malaria infection. Only experiments where at least one host died are included. Positive log hazard ratios (*ln*HR) indicate higher host mortality in co-infected hosts compared with hosts infected with *Plasmodium* alone. Changes in peak parasitaemia indicate the absolute difference in the proportion of infected red blood cells (% iRBC) between hosts with a helminth co-infection and a *Plasmodium* infection alone. Bold horizontal lines show the median effect size, boxes indicate the hinge (25–75% quartile) and error bars are 1.5 times the hinge, with points outside this range shown as open circles.

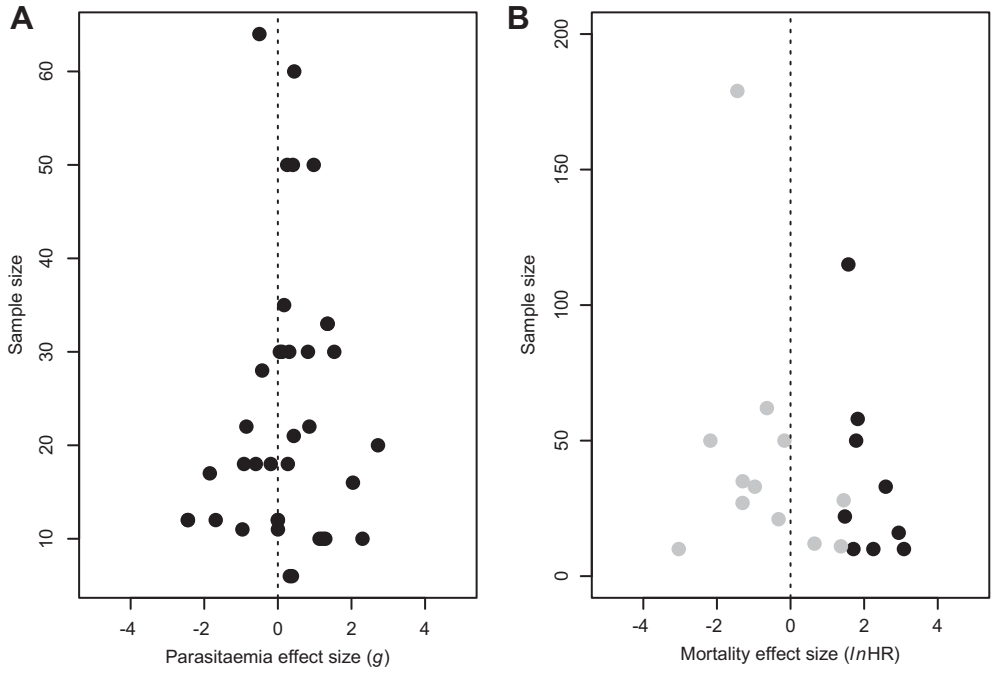

Fig. 5. Funnel plots showing the relationship between effect size and experiment sample size for meta-analyses examining the effect of helminth co-infection on (A) peak *Plasmodium* parasitaemia and (B) host mortality, in laboratory mice. In (B), black dots represent experiments using resolving malaria models (*Plasmodium chabaudi* and *Plasmodium yoelii* infections), whilst grey dots indicate those using lethal malaria models (all except one involving *Plasmodium berghei*), to illustrate the difference in effect sizes between these two types of experiment.

Three sensitivity analyses were performed to confirm that findings from the mortality analysis were robust. These tested whether conclusions were altered by exclusion of (i) a very large experiment that carried approximately six times the average weight in the dataset (experiment 14; Specht et al., 2010; see Supplementary Table S2); (ii) experiments using *Schistosoma* that had not confirmed a lack of mortality in a helminth-only treatment group (since schistosome infection alone can cause mortality, see Section 2.2); and (iii) all experiments under criteria i or ii above. Full details of these analyses are reported in Supplementary Tables S5–S7. Briefly, the major finding that the effect of helminth co-infection on mortality varied strongly between resolving and lethal malaria models, different *Plasmodium* spp. and CM versus non-CM systems remained robust in all sensitivity analyses. The finding that helminth co-infection significantly delayed death in CM models was also upheld (range of mean *ln*HR among CM models in sensitivity analyses = −1.099 to −0.769, $0.002 < P < 0.07$; Supplementary Tables S5–S7).

4. Discussion

Despite variable reports in the literature, here meta-analysis reveals predictable effects of helminth co-infection on malaria parasite replication and disease in mice, which critically depend on biological characteristics of the model system involved. Although effects were not strongly predicted by helminth attributes, there was clear variation in the effect of co-infection according to the type of malaria model: whereas co-infection increased mortality and peak parasitaemia in ordinarily resolving *Plasmodium* infections, it had far less effect on lethal *Plasmodium* infections and even tended to delay death in CM models. These results suggest that depending on the existing immune interaction between a given host and malaria parasites, addition of a helminth co-infection may have contrasting effects on malarial disease.

In both human and rodent malaria, successful resolution of an infection involves striking the right balance between inflammatory immune responses necessary for parasite clearance and anti-inflammatory responses that regulate this process and prevent immunopathology, with the timing of both being crucial (Artavanis-Tsakonas et al., 2003; Schofield and Grau, 2005; Riley et al., 2006; Walther et al., 2009). In resolving rodent malaria infections, blood parasitaemia is effectively controlled by an early and robust pro-inflammatory response (Jacobs et al., 1996; Su and Stevenson, 2000, 2002), which is kept under control by the anti-inflammatory cytokines IL-10 and TGF-β (Linke et al., 1996; Omer et al., 2000; Li et al., 2003). Excessive inflammatory cytokines and/or low levels of anti-inflammatory cytokines have been implicated in severe malarial disease, including experimental CM (Hunt and Grau, 2003; Sanni et al., 2004; Mitchell et al., 2005). Indeed, a key difference between resolving and lethal rodent *Plasmodium* infections seems to be the relative levels of pro- and anti-inflammatory cytokines they elicit; for example, levels of the regulatory cytokine TGF-β are found at much high levels during the acute stage of resolving *P. chabaudi* and *P. yoelii* infections compared with lethal *P. berghei* infections, and are at least partially responsible for modulating disease in the latter (Omer and Riley, 1998). Similarly, variation in virulence among conspecific clones of *P. chabaudi* is associated with the balance of pro- and anti-inflammatory cytokines they elicit, with more virulent clones tending to induce more pro- than anti- inflammatory cytokines (Long et al., 2006, 2008).

Helminth infections tend to induce strong Th2 as well as regulatory T cell responses, both of which are capable of dampening inflammatory responses (Maizels and Yazdanbakhsh, 2003; Maizels, 2009). Thus helminth co-infection, similar to the regulatory arm of the host immune system, may function as a double-edged sword during malarial infection. On the one hand, helminth-induced anti-inflammatory responses could inhibit control of *Plasmodium* replication, but on the other may prevent immunopathology. The results of this meta-analysis broadly support this idea: whereas helminth co-infection exacerbated mortality and increased peak parasitaemia within ordinarily resolving malaria infections (*P. chabaudi* and *P. yoelii*), in lethal models and particularly CM models, effects on mortality were in the opposite direction. Interestingly, despite the limitations of existing studies in human populations, data from Thailand are reminiscent of this pattern. A series of studies from this region indicated that individuals infected with soil-transmitted helminths (particularly *Ascaris lumbricoides*) had an increased risk of mild malaria episodes, but were also at reduced risk of developing various forms of severe malarial disease (see Nacher, 2008 for a review). Although it seems likely that contrasting effects of helminth co-infection in resolving and lethal malaria models arise from immunological differences in how parasites and hosts interact (e.g. the balance of pro- and anti-inflammatory responses), it cannot be ruled out that some other unidentified difference between *Plasmodium* spp. underlies these patterns. Further co-infection experiments with lethal and non-lethal clones of the same *Plasmodium* sp. could shed light on this possibility.

Although effects of helminth co-infection on *Plasmodium* replication were heterogeneous, the general trend was for helminths to increase peak *Plasmodium* parasitaemia (Fig. 2), an effect that was stronger in experiments involving resolving malaria infections, especially *P. yoelii*, and/or the helminth *H. polygyrus*. Moreover, increased mortality due to co-infection in resolving malaria models was accompanied by an increase in peak parasitaemia (Fig. 4). Importantly, several studies demonstrated the reversibility of such effects upon administration of anti-helminthic drugs (Noland et al., 2005; Sangweme et al., 2009), indicating the necessity of an ongoing helminth infection for these interactions to occur. Immunological data from some of these experiments suggest helminth-induced reductions in pro-inflammatory immune responses are an important mechanism underlying these effects: either malaria-specific IFN-γ or TNF-α responses (Helmby et al., 1998; Su et al., 2005; Noland et al., 2008; Tetsutani et al., 2009) and/or serum levels of IFN-γ (Helmby et al., 1998; Su et al., 2005; Segura et al., 2009) were lower in co-infected mice compared with those infected by malaria parasites only. These findings support those of a recent meta-analysis which showed that the extent to which helminths reduced microparasite-specific production of IFN-γ predicted the direction and magnitude of their effect on microparasite density (Graham, 2008). Exactly how the presence of a co-infecting helminth modulates anti-malarial immune responses remains to be elucidated. Su et al. (2005) suggested that Th2 cytokines were unlikely to be crucially involved in the interaction between *H. polygyrus* and *P. chabaudi*, since co-infection affected parasitaemia and mortality similarly in wild type and STAT6$^{-/-}$ mice, which have disrupted signalling pathways for the Th2 cytokines IL-4 and IL-13 (Kaplan et al., 1996). Given the evidence here for consistent effects of helminth co-infection on parasitaemia in resolving malaria infections, future work to disentangle the contribution of alternative immune pathways to this process are now warranted.

This meta-analysis indicated that helminth co-infection significantly delayed death in the subset of experiments using CM models (Fig. 3). All seven CM studies included in this study, indeed all experiments involving *P. berghei* ANKA strain, showed negative mortality effect sizes (indicating delayed death with co-infection), although of varying magnitude and significance. These studies involved in approximately equal proportions the helminths *L. sigmodontis*, *H. polygyrus* and *S. mansoni*. Another study, excluded from meta-analysis on the basis that irradiated larvae were used as well as live larvae (Yan et al., 1997; see Section 2.1), similarly

showed protection against CM with *Brugia malayi* co-infection. Furthermore, these studies reported that co-infected mice that did not develop CM died later with high parasitaemia and suspected severe anaemia (Yan et al., 1997; Specht et al., 2010; Waknine-Grinberg et al., 2010). Thus, there appear to be general patterns in these protective effects across helminth genera. Whilst the underlying mechanisms require further investigation, several findings from the studies included here are noteworthy. Specht et al. (2010) show that concurrent *L. sigmodontis* infection delays death in C57BL/6 mice with *P. berghei* ANKA, an effect that is abrogated in IL-10 knockout mice, supporting the critical role for this filiaria-induced anti-inflammatory cytokine in CM pathogenesis (Kossodo et al., 1997). Interestingly, this protective effect was not associated with a change in IFN-γ responses but did involve reduced infiltration of CD8+ T cells to the brain, a process previously implicated in CM pathogenesis (Amante et al., 2007). Members of the same laboratory demonstrated a striking protective effect of filarial infection on the probability of becoming infected with malaria parasites following exposure to *P. berghei* sporozoites, which was also IL-10-dependent (Fernández Ruiz et al., 2009). These findings are consistent with the demonstrated importance of regulatory cytokines in balancing pro-inflammatory responses in CM pathogenesis (de Souza and Riley, 2002; Hunt and Grau, 2003; Wu et al., 2010).

Despite the usefulness of meta-analysis for revealing general patterns of helminth-*Plasmodium* interaction, emergent conclusions are limited to the context in which these interactions were examined. All experiments reviewed here involved inoculation of blood-stage *Plasmodium* parasites and most investigated the effects of pre-existing chronic helminth infections. The process of becoming co-infected will inevitably be far more variable in natural populations, and the timing and sequence of co-infection are likely critical to the form and outcome of parasite interaction. Indeed, the importance of timing for co-infection outcome is visible in the current dataset. For example, Waknine-Grinberg et al. (2010) reported that whereas co-infection with *S. mansoni* apparently protected against CM, these effects were only apparent when the patent egg-laying stage was present. Similarly, although *H. polygyrus* increased host mortality in *P. chabaudi* infections, the mechanisms underlying this interaction clearly differed according to the developmental stage administered; whilst larval *H. polygyrus* exacerbated liver pathology through induction of a potent inflammatory response (Helmby, 2009), chronic *H. polygyrus* led to systemic down-regulation of anti-malarial inflammatory responses (Su et al., 2005). Thus, the helminth dose and stage of infection (acute versus chronic) may be key factors influencing the size and direction of effect on malaria parasites. There is also limited evidence from humans that dose effects may be important in determining helminth-*Plasmodium* interactions (Nacher et al., 2002). Experiments that test the importance of co-infection order, the timing, dose and method of administration will be critical to more fully understand the generality of these effects across a more natural range of co-infection scenarios, including those experienced by humans. They might also offer important clues as to the underlying mechanisms of interaction across different systems.

Although no clear signal of resource competition influencing helminth-*Plasmodium* interactions was detected in this meta-analysis, the power to detect such effects was limited by insufficient data on whether certain helminths caused anaemia (in particular, the commonly used nematode *H. polygyrus*) and a limited number of helminth species investigated. Thus whilst immune-mediated mechanisms seem to play a central role in the effects here, resource-based interactions could explain residual heterogeneity in effects. For example, Hoeve et al. (2009) demonstrate a negative effect of *Nippostrongylus brasiliensis* co-infection on *P. chabaudi* peak parasitaemia and subsequent experiments on this system suggest this may be attributable to the transient anaemia induced during larval migration through the lungs (K. Fairlie-Clarke, personal communication). A role for resource competition was also suggested by the meta-analytical study of Graham (2008), in which the effect of macroparasite co-infection on microparasite growth was lower in systems with potential RBC competition. Resource-based facilitation of *Plasmodium* replication is also possible, for example in cases where helminth-induced anaemia stimulates production of reticulocytes, a preferred cell type for invasion by some *Plasmodium* spp. including *P. berghei*. Indeed, a study published after the search date of this meta-analysis provides preliminary evidence for such facilitation (Bucher et al., 2011). Other (non-erythrocytic) forms of resource-based interaction are also possible during helminth-*Plasmodium* co-infection, for example if liver damage induced by helminths, such as schistosome granuloma formation, interferes with the development of *Plasmodium* liver stage parasites. An important future challenge for this field will be to design and conduct experiments capable of teasing apart key resource- and immune-mediated mechanisms of interaction, and assess the relative importance of each in helminth-malaria interactions.

From heterogeneous results on helminth-*Plasmodium* interactions in the experimental literature, this meta-analysis reveals consistent patterns which suggest a dual role of helminth co-infection in malarial disease: whilst in resolving malaria infections helminths can increase host mortality as hosts fail to control *Plasmodium* replication, this is not the case in lethal malaria models, where if anything helminths tend to reduce malaria-related mortality. Thus these findings suggest there is no single answer to the question of how co-infecting helminths affect malaria, but that it depends critically on how a host and malaria parasites interact in the absence of helminths. Given such evidence for interactions at the phenotypic level, the stage is now set to elucidate the cellular and molecular pathways by which they arise and more fully understand why they vary across systems. Future experiments that dissect the importance of co-infection dynamics (order and timing effects), as well as the relative influence of immune- and resource-mediated interactions on the outcome of co-infection across a broad range of systems, will provide a greater mechanistic understanding of these effects and help assess their relevance within natural co-infected populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2011.05.009.

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