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Antagonism between co-infecting gastrointestinal nematodes: A meta-analysis of experimental infections in Sheep☆

M.J. Evans [a,b,c,*], Y. Corripio-Miyar [b], A. Hayward [b], F. Kenyon [b], T.N. McNeilly [b,1], D.H. Nussey [c,1]

[a] Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin, UK
[b] Department for Disease Control, Moredun Research Institute, Penicuik, UK
[c] Institute for Ecology and Evolution, University of Edinburgh, Edinburgh, UK

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ABSTRACT

Gastrointestinal nematodes (GIN) have enormous global impacts in humans, wildlife and grazing livestock. Within grazing livestock, sheep are of particular global importance and the economics and sustainability of sheep production are greatly constrained by GIN infections. Natural infections are composed of co-infections with multiple species, and while some past work suggests species can interact negatively with one another within the same host, there is wide variation in reported patterns. Here, we undertook a systematic literature search and meta-analysis of experimental GIN co-infections of sheep to determine whether these experimental studies support the hypothesis of antagonistic interactions between different co-infecting GIN, and test whether aspects of parasite biology or experimental design influence the observed effects. A systematic search of the literature yielded 4848 studies, within which, we identified 19 experimental sheep studies comparing *post-mortem* worm counts across two co-infecting GIN species. Meta-analysis of 67 effects obtained from these studies provides strong evidence for interactions between GIN species. There was wide variation in the strength and direction of these interactions, but the global effect was significantly antagonistic. On average, there was a decrease in the number of worms of one species when a co-infecting species was also present, relative to a mono-infection with that species alone. This effect was dependent on the infectious dose and was rapidly lost after anthelmintic treatment, suggesting that live worms are required for the effect to occur. Individual parasite species varied in the extent to which they both exerted, and were subject to, these interspecies interactions, and these differences are more complex than simply co-localisation within the gastrointestinal tract. Antagonistic interactions between co-infecting GIN may feedback into their epidemiology as well as potentially affecting the clinical impacts of infection. Furthermore, the consequences of these interactions may be heightened when clinical interventions affect only one species within the co-infecting network. Whilst it was not possible to identify the causes of variation between GIN species in the impact of co-infection, these findings point to new avenues for epidemiological, clinical and mechanistic research on GIN co-infections.

1. Introduction

Gastrointestinal nematodes (GIN) infect over half the world's human population (Chan, 1997; Horton, 2003) and are near ubiquitous parasites in wildlife and grazing livestock. Within grazing livestock, sheep are the most numerous species globally (Gilbert et al., 2018) and are highly important to rural economies both in higher income countries and in lower income countries, where, alongside goats, they are particularly relied upon by people living in poverty, especially women (Sinn et al., 1999). However, the economics and sustainability of sheep production are greatly constrained by GIN infections (Charlier et al., 2020; Fitzpatrick, 2013; Mavrot et al., 2015; McLeod, 1995; Nieuwhof and Bishop, 2005) and are further threatened by the widespread development of anthelmintic resistance (Kaplan and Vidyashankar,

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* Corresponding author at: Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin, UK.
E-mail address: mike.evans@ed.ac.uk (M.J. Evans).

[1] Joint-Last Authors.

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2012; Rose et al., 2015a). These challenges have pushed the livestock industry to develop host genetics and farm management techniques that are less dependent on anthelmintics, and to invest in research that has generated enormous advances in GIN epidemiology, vaccines and immunology (Morgan et al., 2019). However, most research considers GIN species in isolation, whereas most natural infections are in fact complex co-infections of multiple species, affecting multiple sites within the GI tract. In temperate climates, the composition of these communities vary seasonally (Boag and Thomas, 1977) and geographically (Redman et al., 2019); however, variation in GIN community composition may also occur between age classes and between years even within a single farm (Evans et al., 2021).

Epidemiological models of livestock GIN continue to improve (McFarland et al., 2022; Rose et al., 2015b; Rose Vineer et al., 2020) but an understanding of interactions between co-infecting GIN species is vital for the construction of holistic multi-species models of GIN. Between-species differences in the ecologies of GIN parasites' free-living larval stages will undoubtedly contribute to observed variation in co-infection composition (O'Connor et al., 2006). However, interactions between species may also contribute to epidemiological patterns, as was postulated by Jackson et al. (1992) for *Teladorsagia circumcincta* and *Trichostrongylus vitrinus* - two species with similar free-living ecologies, but markedly different seasonal epidemiologies.

Interactions between co-infecting GIN could also have impacts on the effectiveness of clinical interventions, and the evolution of anthelmintic resistance. Lello et al. (2004) demonstrated in rabbits that the removal of a single species from a network of interacting parasites can have unexpected effects on the remaining species. Such situations may readily arise in veterinary practice, for example targeted treatment of *Haemonchus contortus* with salicylanilide drugs, use of monovalent anti-nematode vaccines, or use of broad spectrum anthelmintics where anthelmintic resistance is present in only some of the co-infecting species. If such interactions are antagonistic they could also have impacts on the evolution of anthelmintic resistance, via both competitive release (increased fecundity or survival of those worms surviving treatment) and subsequent competitive exclusion (reduced establishment of susceptible worms from *refugia*).

Interactions between GIN of sheep are therefore of clear importance, and there has been an increase in scientific interest in the composition of ovine GIN communities following the development of a metabarcoded ITS-2 sequence-based GIN speciation platform ('the Nemabiome') (Avramenko et al., 2015; Redman et al., 2019). However, this platform has almost exclusively been applied to cross-sectional studies utilising samples pooled from multiple individuals, which are ill-suited to identifying inter-specific interactions (Fenton et al., 2014). Experimental approaches are more powerful in that regard but the results can be hard to extrapolate beyond the specific experimental conditions. The review of co-infection experiments by Christensen et al. (1987) showed that interactions between ovine GIN were predominately antagonistic, but examples of synergistic interactions do exist (Kates and Turner, 1960; Lello et al., 2018; Turner et al., 1962; Turner and Colglazier, 1954). We therefore aimed to perform a meta-analysis of GIN co-infection experiments in sheep to test the hypothesis of antagonistic interactions between different co-infecting GIN, and determine whether the broad range of experimental results reported were affected by the parasite species, or by experimental design details.

Pederson and Fenton (2007) described how co-infecting parasites may interact with each other negatively (via competition for space, consumption of resources, or stimulation of non-specific host responses) or positively (via mechanical facilitation, immunosuppression, or immune-polarisation). In general, interactions are predicted to be strongest for species occupying similar ecological niches, and Lello et al. (2004, 2018) and Lello and Hussell (2008) showed that by defining the ecological niches of GIN as a combination of their feeding habit and their predilection site, the strength and direction of co-infection interactions could be predicted. However, within the GIN species routinely studied in sheep, *H. contortus* is unique in feeding on blood, with the other species all considered 'mucosal browsers'. Due to the lack of replication across feeding habits, we therefore chose to also test whether the co-infection interactions were affected simply by the relationship between the parasites' anatomic predilection sites.

2. Methods

2.1. Systematic literature review

Our systematic literature review was conducted in accordance with PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) guidelines (Page et al., 2021b, 2021a) (see Fig. 1 for PRISMA diagram). The systematic literature search was performed in September 2022 (last search 2022–09–22) using CABabstracts, CABabstracts archive, MEDLINE, SCOPUS and Web of Science databases, searching in all fields. The search string was composed of four elements:

1. Synonyms for sheep:
   Sheep OR Lamb* OR Ovine OR "Ovis aries"
2. Synonyms for co-infections:
   "co-infect* " OR "co infect* " OR "coinfect* " OR "concomitant* " OR "concurrent* " OR synerg* OR antagonis* OR compet* OR interact* OR interspecific OR influenc* OR heterologous OR "cross-resistan* " OR "cross resistan* " OR "cross-immun* " OR "cross immun* "
3. Genera of species reported to infect sheep (including historic names) (Taylor et al., 2015), truncated in order to find references to both the genus and the associated clinical syndrome (e.g. *Haemonchus* and haemonchosis), or a truncation of the word 'nematode'. Following a scoping search specific exclusions were added to the truncation of '*Capillaria*' in order to avoid irrelevant references to 'capillary' and 'capillaries':
   Nematod* OR Bunostom* OR Camelostrongyl* OR (Capillar* NOT capillary NOT capillaries) OR Chabert* OR Cooper* OR Gaiger* OR Gongylone* OR Haemonch* OR Marshallag* OR Mecistocirr* OR Monodont* OR Nematodir* OR Oesophagostom* OR Ostertag* OR Oxyur* OR Parabrone* OR Skrjabine* OR Strongyloid* OR Teladorsag* OR Trichocephal* OR Trichostrongyl* OR Trichur*
4. Synonyms for nematode community diversity (adjacency operators dependent on database):

Nemabiome* or (Nematod* adj4\NEAR4\W/4 diversity) or (Nematod* adj4\NEAR4\W/4 community).

These four terms were linked to create the complete search string:

Sheep_synonyms AND ((Co-infection_synonyms AND Nematode_synonyms) OR Nematode_diversity_synonyms).

This search strategy yielded a library of 4157 studies. Backward and forward citation searching (using SCOPUS, Web of Science and Google Scholar) of all final eligible included papers yielded an additional 690 studies which were fed back into the library to give a total of 4847 studies. These were uploaded into the online Covidence application, which screened for duplicates automatically (Veritas Health Information, 2022). After final manual curation, this resulted in the removal of 2210 duplicate studies. The remaining 2637 studies were screened for relevance against their title and abstract (including any study that reported GIN species composition from co-infected sheep), resulting in the exclusion of 2496 studies. The full texts for the remaining 141 studies were then assessed for eligibility. 82 of these studies reported natural co-infections rather than experimental infection; 8 were secondary reports or conference abstracts without data, and 25 did not have the right study design. Those studies were excluded from the meta-analysis, but their meta-data were recorded, in order to assess the temporal and geographical representativeness of the included studies relative to the wider literature. This left 24 eligible studies that compared *post-mortem* worm counts of sheep infected with two GIN species (referred to hereafter as the 'principal species' and the 'co-infecting species') against

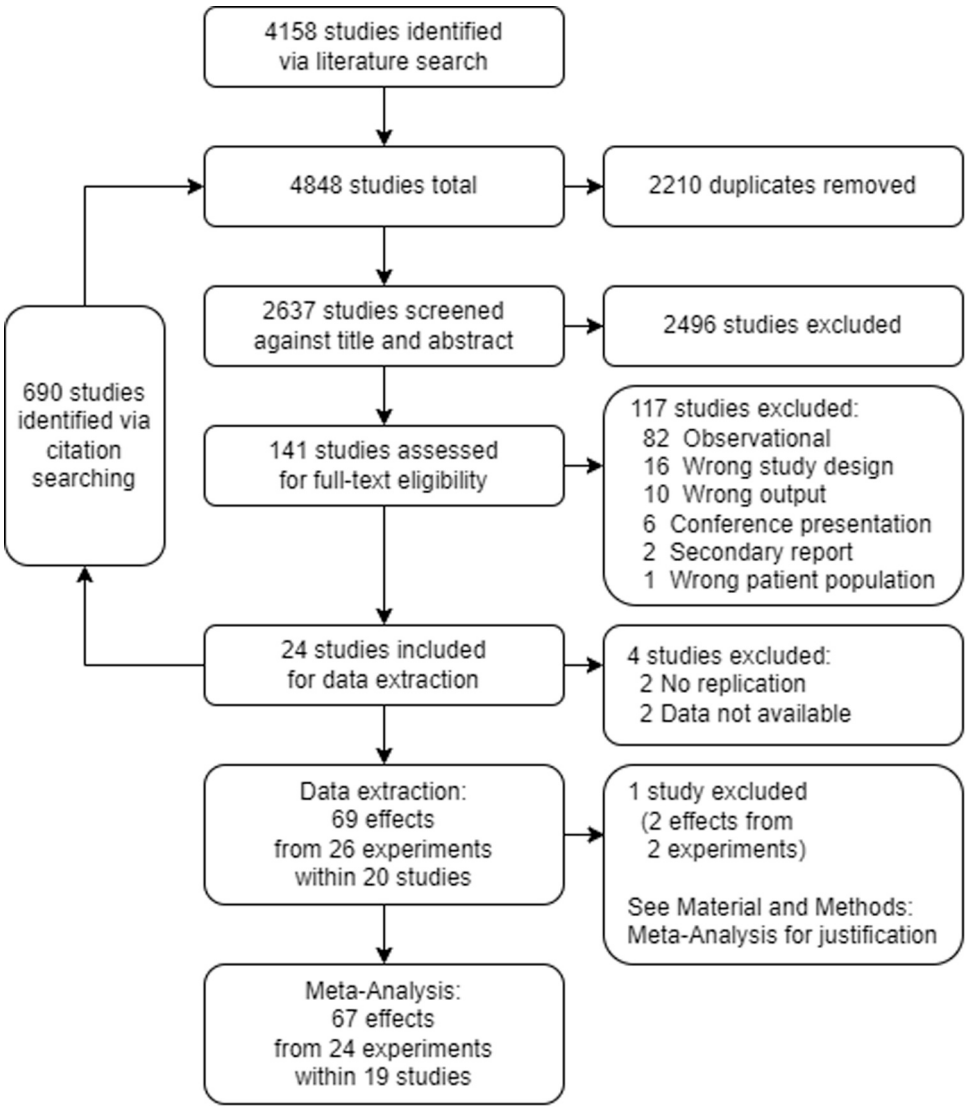

Fig. 1. PRISMA diagram illustrating study selection. All screening and data extraction was performed by the lead author (ME).

control sheep mono-infected with just a single species (i.e., the principal species) (Fig. 1).

2.2. Data extraction, effect size calculation

The standardized mean differences (SMD or Hedges' *g*) in *post-mortem* (principal species) total worm count (all worm life-stages) between the co-infected sheep and the mono-infected sheep were calculated from the mean, standard deviation (SD) and number of sheep (n) in each experimental group using the 'escalc(., measure = "SMD")' command in the 'metafor' package: (Hedges, 1981; Viechtbauer, 2010)

$$g = \frac{\overline{y}_1 - \overline{y}_2}{s_p}, where \quad s_p = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 - 1) + (n_2 - 1)}}$$

That command also generated the associated sampling variances using the large-sample approximation method (Hedges, 1982, equation 8). Where the SD was not available, it was back-calculated using the reported Standard Error (SE) and sample size (n). Where the mean and SD/SE were not available, raw data was used to calculate them (extracted from data tables or graphs using DataThief III software (Tummers, 2016)).

Of the 24 eligible studies, it was not possible to calculate effect sizes for four studies. In two this was due to a lack of replication (n = 1 in some groups) arising from either differing study objective (Kates and Turner, 1953) or the host death mid-experiment (Kates and Turner, 1960), and in the other two this was because neither the raw data, nor the SD/SE were reported, and no author contact details were available to trace the raw data (Blanchard and Wescott, 1985; Dash, 1981). Therefore, effect sizes were calculated from a total of 20 studies. As many studies contained multiple experimental groups (see below) a total of 69 effect sizes were obtained from 26 experiments within those 20 studies.

2.3. Experimental design metadata

During data extraction, the following methodological details were also recorded: the principal species; the co-infecting species; the breed of sheep (if stated); age at first infection (recorded in days, months assumed to contain 30 days, midpoint used if a range given); inter-infection period in days; and post-infection period (days from last infection until necropsy). In addition, for both the principal and the co-infecting species, the total infectious dose (total number of third stage larvae (L_3) administered - always identical in experimental and control groups) and the duration of the infection administration (number of days from first administration to final administration) were recorded. Experimental methods were then classified into four categories according to their design (Fig. 2). As only one experiment utilized the

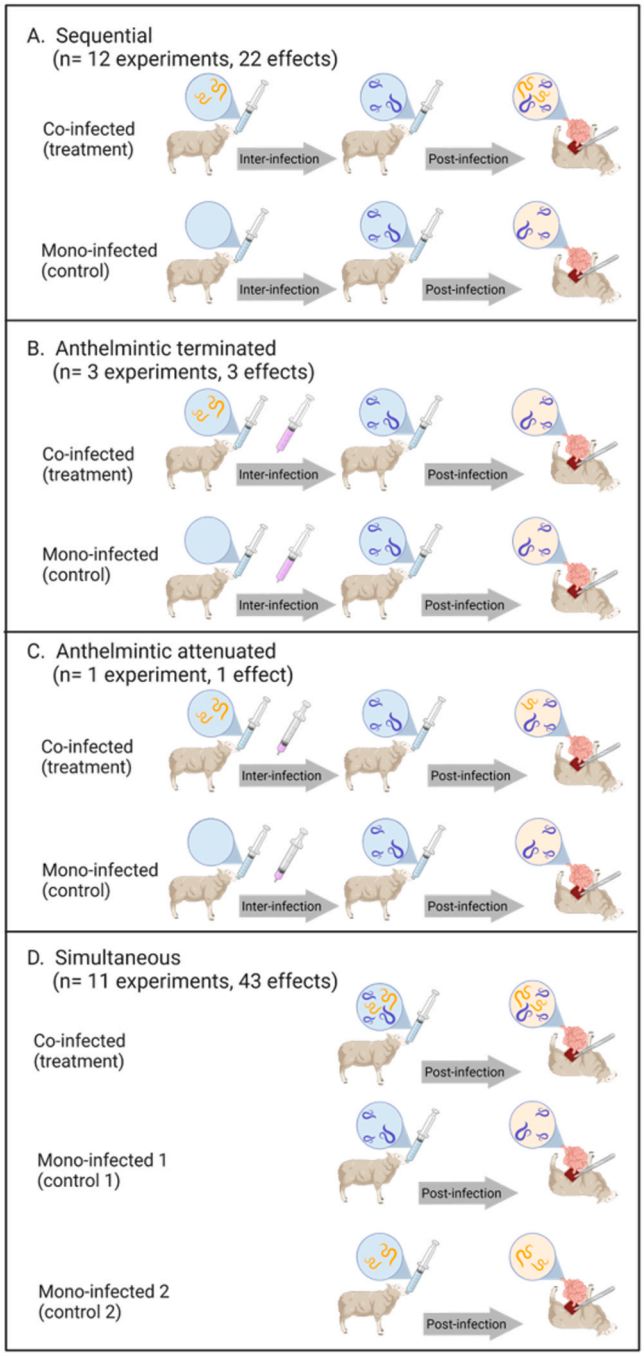

Fig. 2. Experimental design classifications (created with BioRender.com). A: Sequential. Co-infected sheep were infected with the co-infecting species (yellow) and subsequently infected with the principal species (blue); control sheep were mono-infected with the principal species (blue) only. *Post-mortem* worm counts of the principal species (blue) in the co-infected group were compared against those in the mono-infected group. B: Anthelmintic terminated. Design as per A, except with a therapeutic dose of anthelmintic administered between the co-infecting species (yellow) and the principal species (blue) in order to eliminate the co-infecting species. C: Anthelmintic attenuated. As per B, except using a subtherapeutic dose of anthelmintic intended to reduce the intensity of the co-infecting species without eliminating it. Note B & C were combined for analysis into a single 'Anthelmintic' group. D: Simultaneous. Co-infected sheep were infected with the principal species and the co-infecting species simultaneously; control sheep were mono-infected with the principal species only. In some cases, mono-infections with both species were performed, therefore two effects could be obtained (co-infected vs mono-infected 1 - blue worms are the principal species; or co-infected vs mono-infected 2 - yellow worms are the principal species).

'anthelmintic attenuated' design, this category was collapsed with the 'anthelmintic terminated' design, giving a total of three experimental design categories: sequential, simultaneous and anthelmintic.

GIN species were then classified according to their predilection for either the abomasum (stomach) (*Haemonchus contortus, Teladorsgia circumcincta, Trichostrongylus axei)* or intestines (*Nematodirus battus, Nematodirus spathiger, Trichostrongylus colubriformis, Trichostrongylus vitrinus*). An additional variable 'Anatomic Direction' with three levels was then assigned: 'Within Organ' (both species infect the same site); 'Downstream' (co-infecting species infects the abomasum and the principal species infects the intestines); and 'Upstream' (co-infecting species infects the intestines and the principal species infects the abomasum).

In some experiments, sheep were euthanized for necropsy at various time points after the last infection. These animals provided separate effect sizes, although they are clearly not entirely independent of each other. Similarly, some studies compared multiple experimental groups against a common control group (e.g. Species A co-infected with Species B vs Species A mono-infection; and Species A co-infected with Species C vs Species A mono-infection). Also, some studies compared a single experimental group against multiple control groups (Fig. 2D). To account for this interdependence, individual studies were divided into separate experiments, with effect sizes considered to be derived from the same experiment if they fitted any of the examples given above. (More in-depth descriptions of the original authors' methodologies are included in the complete dataframe, accessible in the supplementary materials.) Further interdependence may exist between studies due to the use of similar sheep breeds, parasite strains, necropsy protocols etc. by authors operating within wider research groups. Studies were therefore classified into 'research groups' if they shared any author with any other study. The following hierarchy of independence was therefore produced: Research Group (10) > Study (20) > Experiment (26) > Effect (69) (Table S1).

2.4. Meta-analysis

All analysis was conducted in RStudio v2022.12.0 using R v4.2.2 (R Core Team, 2022). Meta-analytic modelling was performed using the 'metafor' package (Viechtbauer, 2010). Plots were created using 'orchaRd' (Nakagawa et al., 2021), 'tidyverse' (Wickham, 2017), 'cowplot' (Wilke, 2018) and 'tmap' (Tennekes, 2018) packages. The dataset and R scripts can be accessed at DOI: 10.17632/275d8w3x3j.1.

We conducted our meta-analysis using multi-level mixed-effects maximum likelihood models. A random-effects model was first constructed by fitting research group, study and experiment as a three-tier nested random effect, in order to account for interdependence between multiple effects. This random effects model was used to estimate the global effect and the 95% confidence interval (CI) and 95% prediction interval around the global effect. In addition, the proportion of heterogeneity explained by the random effects structure was estimated by calculating I^2 for the random-effects model using the 'i2_ml' command from the 'orchaRd' package (Nakagawa et al., 2021) and comparing it against I^2 for a null model with no random effects.

The effects of 'Experimental Design', 'Co-Infecting Parasite Dose', 'Co-infecting Species', 'Principal Species' and 'Anatomic Direction' were then tested by adding each to the random effects model as a single moderator. Each moderator was judged to be significant where the Wald-type χ^2 test for moderators (Q_M) provided a P-value < 0.05. A more normal distribution for 'Co-Infecting Parasite Dose' was achieved using a natural logarithm (ln) transformation prior to fitting.

To assess the significance of potential confounding methodological variables on SMD, the following experimental design data were also tested as single moderators: 'Co-infection Duration' (as a continuous moderator in days); 'Co-Infection Duration' (discretised into 'single bolus' or 'multiple bolus/trickle infection'); and 'Inter-Infection Interval' (as a continuous moderator in days). The following methodological

details were not tested as for each effect they were controlled for by identical conditions in the within-experiment control group: the principal parasite dose; the principal parasite infection duration; the age at experiment onset; and the post-infection period.

All significant single moderators were then fitted together into a multiple moderator model. Moderators were considered significant after controlling for the other moderators if the Wald-type χ^2 test (Q_M) provided a P-value < 0.05. To control for potential publication bias 'Publication Year' and $\sqrt{1/\tilde{n}_i}$ *where* $\tilde{n}_i = \frac{n_{1i}n_{2i}}{n_{1i}+n_{2i}}$ were also fitted to the multiple moderator model (Nakagawa et al., 2022). As the $\sqrt{1/\tilde{n}_i}$ term was statistically significant it was replaced in the model by $1/\tilde{n}_i$ as recommended by Nakagawa et al. (2022). That replacement had no effect on the significance of any of the other moderators, therefore only the latter is reported in the results (both versions are present in the Supplementary Material).

Pairwise post hoc testing for all levels of categorical moderators that were significant in the multiple moderator model was performed with Benjamini-Hochberg correction using the 'multcomp' package (Hothorn et al., 2008).

One study (Herlich, 1965) had very large SEs and was therefore considered a potential outlier. It also had a much longer co-infection duration and utilised a pair of parasites (*Cooperia oncophora* and *Cooperia pectinata*) not represented in any of the other studies, therefore its inclusion would have prevented fitting the multiple moderator meta-regression. Consequently, this study (two effects) was excluded from all the meta-analyses reported below. For completeness the single moderator meta-regressions were repeated without excluding that study and no change to the effects or conclusions were seen (Supplementary Materials).

3. Results

Multi-level meta-analysis indicates that sheep co-infected with two GIN species usually have significantly fewer worms of the principal species than sheep mono-infected with just the principal species (Fig. 3). The global estimate for the standardized mean difference (SMD) in *post-mortem* worm counts of co-infected sheep compared to mono-infected control sheep (β_{global}) was -0.732 (95% confidence interval (CI) $= -1.03$ - -0.431, $Z = -4.78$, $P < 0.001$). Total heterogeneity was substantial ($I^2 = 62.60\%$), whilst heterogeneity in the random-effects model was moderate ($I^2 = 52.31\%$), indicating that the nested random effects structure accounted for 20.3% of the inter-effect heterogeneity.

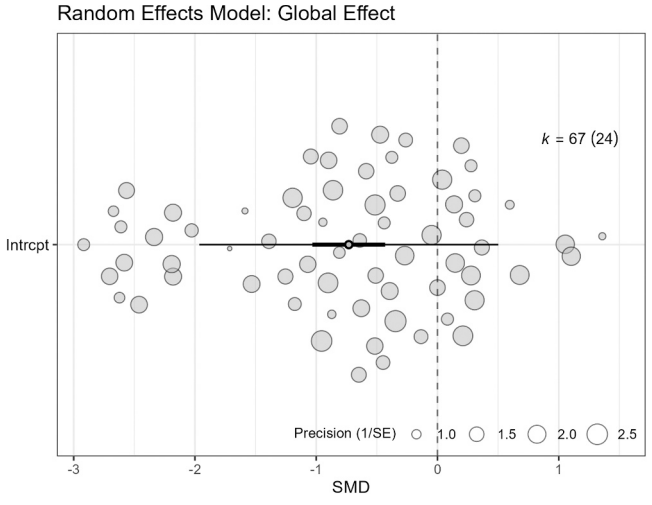

Fig. 3. Orchard plot showing significant effect of GIN co-infection on the Standardised Mean Difference (SMD) in *post-mortem* worm count of the principal GIN. Circles show individual effects, with their diameter inversely proportional to the standard error (SE) of the SMD; central circle shows the global estimate from the random effects model, with the thick lines showing the 95% confidences intervals associated with that estimate; the thin lines show the 95% prediction interval for the model; k shows the number of effects, with the number of experiments in brackets.

Single moderator meta-regression analysis showed the anatomic direction of the co-infection had no significant effect on *post-mortem* worm counts ($Q_M = 1.30$, df $= 2$, $P = 0.523$) (Fig. 4). Co-infection duration had no significant effect either as a continuous moderator ($Q_M = 2.18$, df $= 1$, $P = 0.140$) or as a two-level discrete moderator (bolus vs trickle) ($Q_M = 1.22$, df $= 2$, $P = 0.269$). Similarly, the inter-infection interval in days had no significant effect ($Q_M = 1.64$, df $= 1$, $P = 0.201$). Significant single moderator effects were identified for: experimental design ($Q_M = 8.68$, df $= 2$, $P = 0.013$); ln(co-infecting parasite dose) ($Q_M = 5.18$, df $= 2$, $P = 0.023$); co-infecting species ($Q_M = 13.46$, df $= 5$, $P = 0.019$); and principal species ($Q_M = 13.27$, df $= 6$, $P = 0.039$). Therefore these variables were carried forward into the multiple moderator meta-regression, where they all remained significant (Table 1).

Sequential co-infection experiments without the use of anthelmintics had significantly more negative effects on worm count than sequential co-infection experiments where an anthelmintic was administered between infections (post hoc $t = -3.28$, $P_{(BH\ adjusted)} = 0.005$). Simultaneously administered co-infections occupied an intermediate position relative to those other experimental designs, although the 95% CI did not cross the zero, suggesting simultaneous GIN co-infections also interact negatively (Fig. 5A). There was also a significant negative effect of ln(Co-Infecting Dose), indicating that higher doses of co-infecting worms exerted greater antagonism on the principal infection (Fig. 5B).

GIN species differed significantly in both the degree to which they inhibited a co-infecting GIN (Fig. 5C) and the degree to which they were inhibited by a co-infecting GIN (Fig. 5D). No significant pairwise comparisons between GIN species were found on Benjamini-Hochberg adjusted post hoc testing; however visual examination of the 95% CIs suggests *T. vitrinus*, *T. axei* and *H. contortus* may have more negative effects on co-infecting GIN (Fig. 5C), and at the same time *T. vitrinus* and *H. contortus* may be more greatly affected by a co-infecting GIN compared to the potentially more resilient *T. axei* (Fig. 5D).

There is no evidence that these results were significantly affected by publication bias, given publication year and $1/\tilde{n}_i$were non-significant in the multiple moderator meta-regression (Table 1 and Fig. S1).

The majority of inter-effect heterogeneity was explained together by experimental design, co-infecting species, principal species and co-infecting parasite dose, whilst controlling for publication year, effective sample size, research group and study. I^2 for the multiple moderator model was 13.18%, indicating a 74.81% reduction in the heterogeneity present in the random-effects model.

There were marked differences in the temporal and the geographic distributions of experimental studies identified during the literature search, compared to the excluded observational reports. The observational reports were globally distributed across 41 countries (Fig. 6A), whereas the experimental infections were performed in only 5 countries (Fig. 6B). The cumulative number of experimental studies increased approximately linearly through the 1960 s to the 1990 s but plateaued after the millennium; in contrast, the cumulative number of observational studies has expanded more exponentially with the greatest number of annual publications (7) in 2021 (Fig. 6C).

4. Discussion

There is strong evidence from this meta-analysis that GIN species co-infecting sheep interact with each other, and although there is wide variation the general effect is predominately antagonistic, leading to a decrease in the number of worms of one species, relative to a mono-infection. This effect is also dose dependent, with greater antagonism seen at greater infectious doses. Further, GIN species vary in both their effect on co-infecting species and their susceptibility to such effects, but these interactions appear more complex than simply the relationship

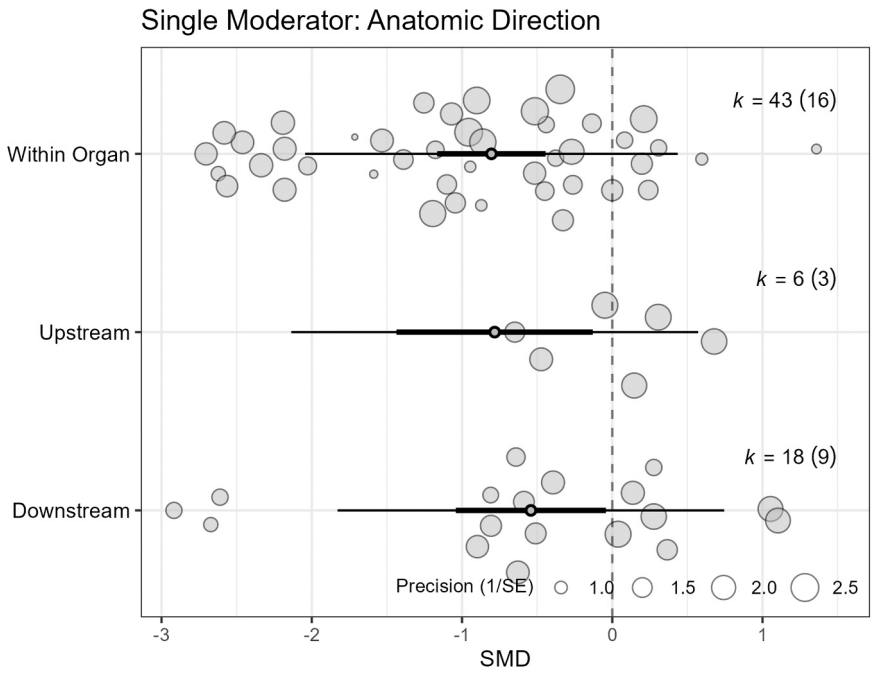

Fig. 4. Orchard plot showing the lack of effect of anatomical direction on the Standardised Mean Difference (SMD) in *post-mortem* worm count of the principal GIN.

Table 1
Results from the multiple-moderator meta-regression. Q_M, DF and P refer to the test for significant differences in effect size due to the moderator. Within moderators the estimates, 95% CIs, Z and P are relative to the reference level. Significantly different groups of moderator levels according to post hoc pairwise testing are indicated by different letters.

Variable	Level	Q_M	DF	*P*	Estimate	95% CI	*Z*	*P*	*Post hoc*
Intercept	Intercept				21.17	-87.33–129.67	0.382	0.702	
Experimental design	Anthelmintic	10.91	2	0.004					a
	Sequential				-1.39	-2.27 - – 0.565	-3.30	0.001	b
	Simultaneous				-0.700	-2.00–0.607	-1.05	0.295	ab
Co-infecting species	*H. contortus*	13.35	5	0.020					a
	N. spathiger				0.396	-0.857–1.65	0.619	0.536	a
	T. circumcincta				0.498	-0.487–1.48	0.991	0.322	a
	T. axei				-0.355	-1.46–0.754	-0.623	0.530	a
	T. colubriformis				0.879	-0.367–2.13	1.38	0.167	a
	T. vitrinus				-0.934	-1.95–0.085	-1.80	0.072	a
Principal species	*H. contortus*	15.19	6	0.019					a
	N. battus				0.926	-1.06–2.92	0.912	0.362	a
	N. spathiger				1.07	-0.082–2.23	1.82	0.069	a
	T. circumcincta				1.08	0.285–1.88	2.66	0.008	a
	T. axei				2.66	0.775–4.54	2.77	0.006	a
	T. colubriformis				0.709	-0.613–2.03	1.05	0.293	a
	T. vitrinus				-0.155	-0.904–0.594	-0.405	0.686	a
ln(Co-infecting dose)		4.62	1	0.032	-0.343	-0.655–0.030	-2.15	0.032	
Year		0.095	1	0.759	-0.009	-0.063–0.046	-0.307	0.759	
$1/\tilde{n}_i$		3.29	1	0.070	-2.39	-4.98–0.192	-1.81	0.070	

between their anatomic locations, given that factor was not significant in the single moderator analysis. This finding may simply reflect a lack of power given the small number of experiments examining upstream effects. However, it could also suggest that the mechanisms responsible for interactions among GIN (e.g. immunity, resource competition) are not local, but operate across the GI tract. This could have important implications for the development of immunity against natural mixed infections across multiple sites. Combinations of other life history factors may also be driving the observed variation between species, and specific parasite pair combinations may be important; however, unfortunately there were insufficient studies to fit an interaction between the co-infecting and principal species in the multiple moderator model.

Our results are unable to definitively state how individual species vary from each other in their co-infection interactions but the observed differences provide interesting points for discussion. Lello et al. (2018) proposed that *H. contortus* has a facilitative effect on co-infecting *T. colubriformis* (mediated via immunosuppression); however, the multiple moderator model suggests *H. contortus* has antagonistic effects on co-infecting GIN in general, raising questions about how the host response against *T. colubriformis* may differ from other species. Lello et al. (2018) also suggested that blood-feeding by *H. contortus* may make it particularly vulnerable to serum antibodies raised against a co-infecting species. That idea is supported by previous vaccine trials against *H. contortus* using antigens from *Ostertagia ostertagi* (Smith et al., 2000) and *T. circumcincta* (Smith et al., 2001), and receives further support from the strongly negative estimate for *H. contortus* as the principal parasite in our meta-analysis (Fig. 5D). *T. vitrinus* was negatively affected by co-infection with *T. circumcincta* (an abomasal species), and the authors of those experiments proposed that this was due to a vulnerability of this species to pH changes induced by co-infection

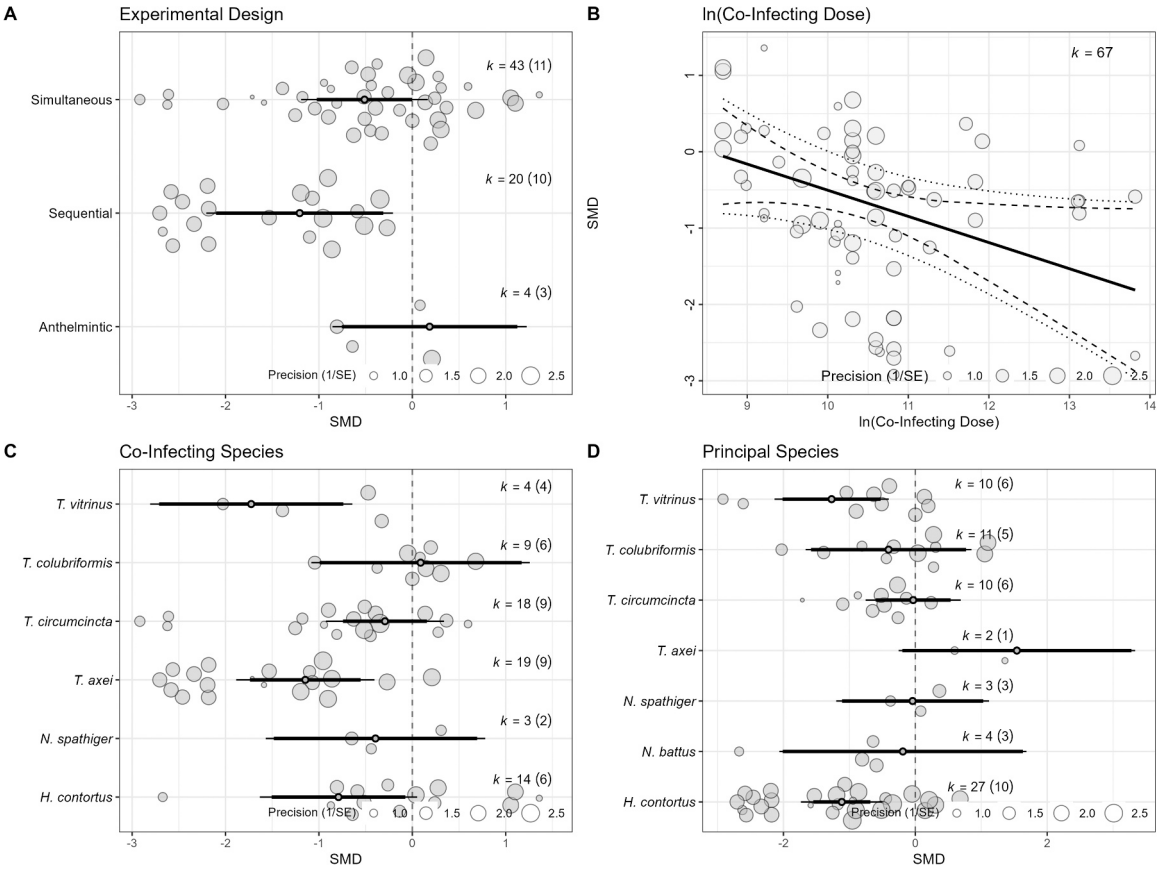

Fig. 5. Outputs from the multiple moderator meta-regression (Table 1) describing effects of experimental design and GIN species on *post-mortem* worm count of the principal GIN. Orchard plots (A, C & D) as described for Fig. 3. B: Bubble plot as per Orchard plots but with the solid line representing the estimated effect, the dashed lines representing the 95%CI around the effect, and the dotted lines representing the 95% prediction interval. SMD = Standardised Mean Difference in *post-mortem* worm count of the principal GIN.

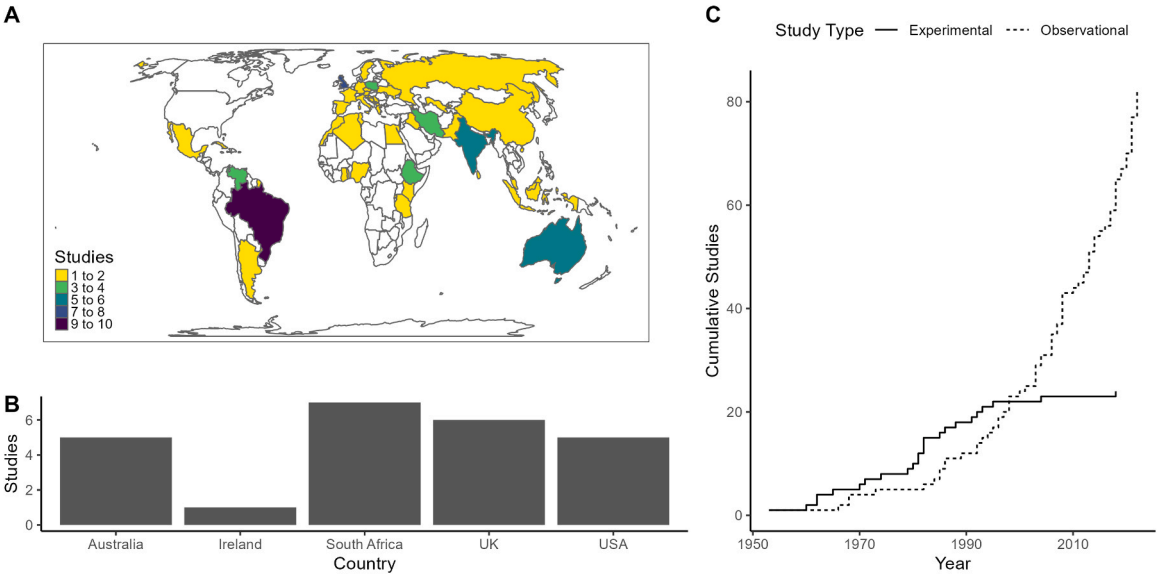

Fig. 6. Choropleth map (A) and bar chart (B) showing total number of observational (A) and experimental (B) studies from each country identified during the systematic literature review. C: Cumulate frequencies of the studies in A and B against time.

with abomasal parasites (Coop et al., 1988; Jackson et al., 1992). This hypothesis is potentially supported by the fact that Roy et al. (2004) experiment co-infecting *T. vitrinus* with *T. colubriformis* (another intestinal species) provided three effect sizes close to zero (Fig. 5D). Our meta-analysis suggests that *T. axei* may be more resilient to the effects of a co-infecting GIN, albeit based on data from a single experiment. This species is arguably the most generalist of GIN species, capable of infecting many host species and potentially infecting the duodenum in

addition to its abomasal predilection site. This generalist role may give it a wide ecological niche and the flexibility to potentially modify its site of infection and thereby mitigate the antagonism of a co-infecting species, as suggested by Pustovoi (1972).

A further striking finding of our meta-analysis was that the effects of a co-infection were lost following anthelmintic treatment. This suggests that either live parasites are necessary to mediate their effects, or that any indirect mechanisms are quickly lost after the clearance of the first species. Experimental design was significant both as a single and as a multiple moderator, and the pairwise comparison between sequential and anthelmintic-treated designs was significant on post hoc testing. Simultaneous co-infections also had smaller effect sizes than sequential co-infections. Whilst post hoc testing of this pairwise comparison was not significant, it is intuitive that effects would be greater if one species has first either modified its environment to suit its own niche, or has pre-stimulated non-specific host immune or physiological responses. The indication that live parasites are necessary for antagonistic effects to occur raises important questions about the underlying mechanisms through which parasites may mediate antagonistic effects (e.g. via parasite excretory-secretory products) and why mucosal immune responses may be so short lived as to be lost quicky after anthelmintic treatment.

Although this meta-analysis has provided strong support for several important findings, the ability to investigate species-specific factors was limited due to the low number of reports relative to the number of potential pairwise combinations of parasites. The potential range of species interactions is even greater when the limited geographical distribution of the included studies is considered against the globally distributed observational reports. Although it would be interesting to expand the range of species studied, the number of pairwise comparisons would expand exponentially, and it would be unfeasible to test so many pairwise co-infections in vivo. Further, two-species co-infections are only a single step towards the biological reality of the multi-species co-infections that occur in the field. There is therefore value in studies utilising complex natural infections. The scope of this meta-analysis was also limited, in that it was only possible to assess the effect of co-infection on worm number, rather than fecundity or pathology. There is evidence that GIN co-infections may reduce worm egg production (Dobson and Barnes, 1995; Jackson et al., 1992; Mapes and Coop, 1971) and that their pathologic impact on the host may be positive or negative (Coop et al., 1986; Steel et al., 1982; Sykes et al., 1988). There is hence also a need for further work on the impacts of co-infection interactions.

Two clear avenues for future research therefore open from this meta-analysis: firstly, studies of natural GIN co-infections able to identify multi-species interactions and quantify their impacts; and secondly, further controlled co-infection experiments aimed at identifying the mechanisms underlying them. The development of high throughput techniques for quantifying GIN species offers the potential for observational studies of natural co-infection dynamics (across the global breadth of host biomes). In contrast to the findings of this meta-analysis, observational studies of natural infections have generally reported positive correlations between co-infecting species (Barger, 1984; Cabaret and Hoste, 1998; Diez-Baños et al., 1992; Hoste and Cabaret, 1992; Morales et al., 2006; Rehbein et al., 1997; Stear et al., 1998; Sweeny et al., 2012). However, those studies were all cross-sectional, a study design which Fenton et al. (2014) showed to have limited power to identify interspecies interactions in co-infections. To resolve this issue, future studies should consider longitudinally sampling a large number of individual hosts with high temporal granularity. Within such studies, the collection of good quality long-term measures of host fitness/production (e.g. lamb growth and survival, and ewe rearing success and longevity) would provide the most meaningful measure of the impact of co-infection dynamics, and the contextualisation alongside species-specific pasture larval counts would enable the greatest epidemiological inference. Analysing the effects of anthelmintic treatment or monovalent vaccines on GIN community composition would provide further insight and could be designed as deliberate perturbation experiments or could utilise clinical samples from faecal egg count reduction tests or vaccine trials. Once these studies have identified significant interaction pathways, it would be valuable to return to controlled co-infection experiments targeting the causative mechanisms. Such studies could be in vivo, for example focussing on the influence of co-infections on mucosal immune responses along sites in the GI tract. Alternatively, they could be in vitro, perhaps looking for evidence of direct communication between nematode species, or using organoid models to examine whether changes in epithelial phenotypes (e.g. gastric remodelling (Faber et al., 2022)) are evidence of niche alterations that may affect the invasion success of a subsequent heterologous infection.

CRediT authorship contribution statement

M.J. Evans, Y. Corripio-Miyar, A. Hayward, F. Kenyon, T.N. McNeilly, D.H. Nussey: Conceptualization, Methodology, Writing – review & editing. **M.J. Evans, A. Hayward, D.H. Nussey**: Formal analysis. **M.J. Evans**: Investigation, Data curation, Writing – original draft. **M.J. Evans, A. Hayward**: Visualization. **Y. Corripio-Miyar, A. Hayward, F. Kenyon, T.N. McNeilly, D.H. Nussey**: Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.110053.

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