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RESEARCH ARTICLE

Mechanisms and Consequences of Infection-induced Phenotypes

Helminth-associated changes in host immune phenotype connect top-down and bottom-up interactions during co-infection

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Abstract

1. Within-host parasite interactions can be mediated by the host and changes in host phenotypes often serve as indicators of the presence or intensity of parasite interactions.
2. Parasites like helminths induce a range of physiological, morphological and immunological changes in hosts that can drive bottom-up (resource-mediated) or top-down (immune-mediated) interactions with co-infecting parasites. Although top-down and bottom-up interactions are typically studied in isolation, the diverse phenotypic changes induced by parasite infection may serve as a useful tool for understanding if, and when, these processes act in concert.
3. Using an anthelmintic treatment study of African buffalo, *Syncerus caffer*, we tracked changes in host immunological and morphological phenotypes during helminth-coccidia co-infection to investigate their role in driving independent and combinatorial bottom-up and top-down parasite interactions. We also examined repercussions for host fitness.
4. Clearance of a blood-sucking helminth, *Haemonchus*, from the host gastrointestinal tract induced a systemic Th2 immune phenotype, while clearance of a tissue-feeding helminth, *Cooperia*, induced a systemic Th1 phenotype. Furthermore, the *Haemonchus*-associated systemic Th2 immune phenotype drove simultaneous top-down and bottom-up effects that increased coccidia shedding by changing the immunological and morphological landscapes of the intestine.
5. Higher coccidia shedding was associated with lower host body condition, a lower chance of pregnancy and older age at first pregnancy, suggesting that coccidia infection imposed significant condition and reproductive costs on the host.
6. Our findings suggest that top-down and bottom-up interactions may commonly co-occur and that tracking key host phenotypes that change in response to infection can help uncover complex pathways by which parasites interact.

KEYWORDS
bottom-up, coccidia, helminth, immunity, parasite, phenotype, top-down

1 | INTRODUCTION

Most animals harbour multiple parasite species simultaneously, and co-infection is the norm rather than the exception (Cox, 2001; Pedersen & Fenton, 2007; Petney & Andrews, 1998). Parasites co-occurring within a single host can interact with one another in multiple ways, with repercussions for both host and parasites. Because many within-host parasite interactions are mediated by the host (Griffiths et al., 2014), changes in host phenotype, particularly morphological, physiological and immunological changes, often serve as key indicators of the presence and/or intensity of certain parasite interactions. For example, different parasite species can deplete the same host resource such as red blood cells (e.g. hookworm and *Plasmodium* spp.; Melo et al., 2010) or modify habitat within the host (e.g. helminth-induced changes of the intestinal metabolome and facilitation of *Salmonella* colonization; Reynolds et al., 2017), resulting in resource mediated or 'bottom-up' ecological interactions. Likewise, down-regulation of host immune responses caused by certain parasites (e.g. helminths, retroviruses) can release other parasites from immune-associated control (Dietze et al., 2016; Morawski et al., 2017), resulting in classic 'top-down' ecological interactions. These different pathways (bottom-up vs. top-down) by which parasites interact are typically studied independently, with a few exceptions (Budischak, Sakamoto, et al., 2015; Graham, 2008), however, they are inextricably linked because the host resource pool and immune system are interconnected. Interestingly, the diverse phenotypic changes induced by interacting parasites during co-infection may serve as a useful tool not only for detecting isolated top-down and bottom-up interactions but also for identifying when these processes act in concert and understanding if such combinatorial interactions act to magnify the fitness costs of infection.

Helminths and protozoa are common parasite taxa that each affect a wide range of vertebrate and invertebrate hosts. Consequently, many hosts often harbour parasites from these two groups simultaneously (Clerc et al., 2018; Hürlimann et al., 2019), setting the stage for potential interactions. In some cases where a helminth and protozoan share the same host resource, local or systemic physiological changes in response to infection can facilitate bottom-up interactions. For example, in humans, blood-feeding nematodes (hookworms, *Necator americanus*) cause anaemia, a systemic physiological state that decreases the intensity of infection with the red blood cell dwelling protozoan that causes malaria *Plasmodium vivax* (Budischak, Wiria, et al., 2018). In rodents (*Peromyscus* sp. and *Apodemus* sp.), chemical clearance of nematodes that feed on intestinal cells increases the shedding of coccidia (*Eimeria* spp.) (Knowles et al., 2013; Pedersen & Antonovics, 2013), likely due to local changes in intestinal morphology or physiology that increase the availability of epithelial cells, a limiting resource for coccidia replication. Helminths and protozoa also induce distinct and antagonistic systemic immune responses in hosts, setting the stage for top-down interactions. For example, in poultry and cattle, coccidia infection biases the immune system towards IFN-γ-producing T-cells (T helper-1[Th1] cells) (Kim et al., 2019; Lucas, 2011; Yun et al., 2000), but helminths can suppress this Th1 dominant phenotype (Mabbott, 2018; Maizels, 2020). Similarly, helminth infection generally induces an immune phenotype dominated by IL-4-producing T-cells (T helper-2 [Th2] cells), but these Th2 responses can be down-regulated by IFN-γ-inducing parasites like coccidia, or as a result of general immunomodulation by other helminth species (Maizels, 2020). Consequently, perturbation of a helminth infection could change systemic host immunity with resulting top-down effects on coccidia. However, these effects likely occur in the context of simultaneous bottom-up interactions in the gastrointestinal (GI) tract, as is often the case for co-occurring GI helminths and protozoa. Identifying the presence of such concomitant interactions is challenging, but the distinct host phenotypic changes induced by different GI helminth species offers a unique opportunity to unravel the diverse mechanisms that operate during parasite co-infection.

Within the host GI tract, some helminth species feed on undigested intestinal contents (e.g. *Ascaris* spp.), whereas others feed on intestinal cells (e.g. *Cooperia* spp.) or blood (e.g. *Haemonchus* spp.) (Poulin, 2001). For GI protozoans such as coccidia, intestinal epithelial cells are a key resource and the availability of uninfected cells is critical for parasite establishment and replication (Bangoura & Bardsley, 2020; Kim et al., 2019). Therefore, helminth species, like *Cooperia*, that prey on and deplete intestinal cells may have strong bottom-up interactions with coccidia, mediated by resource competition. In contrast, helminths such as *Haemonchus* that feed on host blood are more likely to interact with coccidia through top-down mechanisms mediated by helminth-induced changes in the host immune phenotype. A third possibility is that GI helminths, regardless of resource exploitation strategy, may exert simultaneous top-down and bottom-up effects on coccidia by causing localized changes in immunity or morphology within the intestinal tract. Both blood feeding and intestinal grazing helminths can damage intestinal tissue, inducing wound-healing responses (Mishra et al., 2014; Nusse et al., 2018). However, the immune-mediated wound healing process can induce morphological changes in the intestine that may affect coccidia resource exploitation. For example, wound healing can lead to proliferation of epithelial cells (Barron & Wynn, 2011; Nusse et al., 2018), increasing the pool of suitable cells for coccidia replication. Alternatively, intestinal immune changes during and after helminth infection may antagonize the immune effector mechanisms that control coccidia infection (Barron & Wynn, 2011; Mishra et al., 2014). Thus, it is possible that a helminth-induced change in immune phenotype could increase the availability of resources (bottom-up effect) for coccidia or facilitate coccidia immune escape at the site of replication (top-down effect). This could boost the shedding of coccidia, with potential consequences for host fitness.

In this study, we tracked changes in host immunological and morphological phenotypes during co-infection to investigate their role in mediating bottom-up and top-down parasite interactions and examined the repercussions for host fitness. To do this, we studied interactions between GI helminths and coccidia in a wild host (African buffalo, *Syncerus caffer*). In the study population, approximately 60% of individuals are co-infected with the intestinal tissue

feeding helminth, *Cooperia* (*C. fuelleborni*; hereafter *Cooperia*) and blood sucking *Haemonchus* species (*H. placei* and *H. bedfordi*; hereafter *Haemonchus*) (Budischak, Hoberg, et al., 2015). Coccidia infections are also common (Henrichs et al., 2016). By taking advantage of a long-term anthelmintic treatment experiment that disrupted the helminth community, we tested for the presence of interactions between the two helminths and coccidia and examined the mechanisms accounting for observed interactions. We predicted that if helminth-induced changes in intestinal physiology drive bottom-up interactions between helminths and coccidia, clearance of intestinal tissue-feeding *Cooperia* would have a stronger effect on coccidia. Alternatively, if changes in systemic immunity drive top-down effects, clearance of blood-feeding *Haemonchus* would have the stronger effect. We also predicted that changes in host systemic immunity could connect top-down and bottom-up effects through modification of tissue morphology or local immune responses in the GI tract. Finally, we evaluated the consequences of these interactions for host performance (body condition) and fitness (reproduction, survival).

2 | MATERIALS AND METHODS

2.1 | Animal sampling

South African National Parks (SANParks) granted permission to conduct this study in Kruger National Park (KNP), South Africa. Animal protocols were approved by the University of Georgia and Oregon State University Institutional Animal Care and Use Committees (UGA AUP no. A2010 10-190-Y3-A5; OSU AUP nos. 3822 and 4325). We captured and sampled 306 female African buffalo in KNP between June 2008 and August 2012. Individuals were captured on average every 180 days (range: 25–250 days) with approximately 6 (range: 3–9) captures per animal. At first capture, individuals were randomly assigned to a 'control' or 'anthelmintic' treatment group. At all captures, treated animals received an intra-ruminal, slow-release formulation of the drug fenbendazole (Panacur® bolus, Merck & Co), which has an estimated efficacy period of 140 days. The anthelmintic treatment effectively reduced worm burdens in buffalo during the drug efficacy period (Budischak, O'Neal, et al., 2018; Ezenwa & Jolles, 2015). After the drug efficacy period, reinfections were common and almost exclusively comprised of *Cooperia* (Budischak et al., 2016; Budischak, O'Neal, et al., 2018).

2.2 | Parasitological analyses

We quantified GI helminth and coccidia infections using faecal samples collected at capture (see Appendix S1 for detail on sample collection). A modified McMaster faecal egg counting (FEC) protocol was used to quantify strongyle nematode eggs and coccidian oocysts (Ezenwa, 2003a, 2003b). In addition, we cultured faecal samples to isolate third-stage nematode larvae, which were subjected to DNA barcoding for species-level identification using protocols described in (Budischak, Hoberg, et al., 2015). To estimate species-specific nematode abundance, we combined relative abundance information derived from barcoding with FEC data (Budischak, O'Neal, et al., 2018). Of 11 nematode species identified from barcoding, three were detected in >2.5% of captures: *Cooperia fuelleborni*, *Haemonchus placei* and *Haemonchus bedfordi* (Budischak, Hoberg, et al., 2015). Here, we focus on these three species, and given similarities in the effects of the two *Haemonchus* species on host physiology (Le Jambre, 1995), we combined them for analysis.

2.3 | Immunological and morphological analyses

We evaluated the host immune response to infection focusing on both systemic (blood) and local (intestinal) immunity (see Appendix S1 for detail on sample collection). To quantify systemic Th1 immune activity, we measured levels of IFN-γ in plasma following stimulation of peripheral blood leukocytes as described in (Ezenwa & Jolles, 2015). We quantified Th2 activity using a similar approach but focusing on the cytokine IL-4. To quantify local immunity and morphology in formalin-fixed sections of the GI tract, we counted the numbers of eosinophils, mast cells, T-lymphocytes, IgA positive leukocytes [immune traits] and assessed the degree of fibrosis (scarring, deposition of collagen in tissues [morphological trait]) in the mucosa of the abomasum and small intestine. We also examined the number of parietal cells, gastric cells which produce HCl [morphological trait], in the abomasum. For details on cell counting and fibrosis assessment see Appendix S1.

2.4 | Data analyses

We performed exploratory analyses to investigate co-dependencies between host, environmental and immunological traits in our dataset, and to check for the presence of outliers, homogeneity of variance and excess numbers of zeros. Final models were also checked for homoscedasticity, overdispersion, collinearity and temporal autocorrelation. For details on exploratory analyses and final model checks see Appendix S1.

To investigate whether anthelmintic treatment-associated clearance of *Haemonchus* and *Cooperia*, or only *Cooperia* affected coccidia, we examined the impact of treatment of animals with different *Haemonchus* and *Cooperia* burdens on future coccidia shedding. To do this, we fit binomial generalized linear mixed models (GLMMs) including total strongyle FEC, or *Cooperia* FEC and *Haemonchus* FEC, in the current capture as predictors of the presence of coccidia oocysts in the following capture. We also included animal ID as a random effect and controlled for herd membership, age and season, all factors that have been shown to influence coccidia shedding in African buffalo (Gorsich et al., 2014). To test if the relationship between helminth FEC and coccidia presence was impacted by treatment, we included treatment and two-way interaction terms between

treatment and strongyle FEC, or between *Cooperia* and treatment and *Haemonchus* and treatment, as predictors. We confirmed differences in the slope of the relationship between helminth FEC and coccidia shedding between treated and control animals using a post-hoc *Z* test of the model estimates with the 'glht' function in the 'MULTICOMP' R package (Hothorn et al., 2008).

To examine the effect of helminths on the host systemic immune response, we assessed the impact of anthelmintic treatment on levels of the key Th2 cytokine, IL-4, in plasma. To do this, we fit a GLMM with treatment status as a predictor of IL-4 plasma concentration (Budischak, O'Neal, et al., 2018). Given the overdispersion in IL-4 values, we selected a negative binomial distribution for this response. The model included animal ID as a random effect to account for repeated sampling of the same individuals, and controlled for covariates known to have an impact on African buffalo cytokine levels, including body condition, season, herd membership and age (Budischak, O'Neal, et al., 2018). The model showed a significant positive effect of anthelmintic treatment on IL-4, similar to previous research on IFN-γ in buffalo (Ezenwa & Jolles, 2015), therefore, to explore whether anthelmintic effects on immunity were associated with elimination of a particular helminth species, we ran separate GLMMs in treated and control animals with both *Haemonchus* FEC and *Cooperia* FEC included as predictors of IL-4 or IFN-γ. These models also included animal ID as a random effect and previously described covariates. Since these models revealed a negative association between *Haemonchus* FEC and IL-4 (Table 1), we next tested if the effect of treatment on IL-4 was associated with possible relaxation of *Haemonchus*-mediated suppression of IL-4. To do this, we grouped individuals into those infected with both *Haemonchus* and *Cooperia* and those infected with *Cooperia* only and then compared IL-4 concentrations before and after anthelmintic treatment for treated individuals and for the first and second capture for control animals (reflecting the same time points used for treated animals). We did not include a '*Haemonchus* only' group because all *Haemonchus* infected individuals also had *Cooperia*. The model also included capture interval as a predictor to account for the potential effect of the time lag between captures on IL-4 differences between individuals. We used the same procedure for a dataset containing only *Cooperia* infected individuals. Both models included animal ID as a random effect and the same covariates described above for the cytokine GLMMs. We repeated this approach using IFN-γ as a response instead of IL-4 to test for suppressive effects of specific helminth taxa on IFN-γ. These models revealed a significant effect of *Haemonchus* on IL-4 and *Cooperia* on IFN-γ.

Next, we simultaneously assessed the impact of *Haemonchus* and IL-4 on coccidia shedding to examine whether the observed effects of *Haemonchus* on coccidia shedding were mediated by IL-4. We fit GLMMs that included *Haemonchus* FEC and IL-4 in one capture as predictors of coccidia shedding in the next capture. To account for the relationship between *Haemonchus* and IL-4 we included an interaction term between the two predictors. Like previous coccidia shedding models, we included season, herd membership, and age as covariates and animal ID as random effect. We fit separate models for treated and control animals because a *Haemonchus* effect on IL-4 was only observed among treated animals. The models revealed that IL-4 was positively correlated with coccidia shedding in treated animals, so to confirm these results with the entire dataset, we fit binomial GLMMs with IL-4 as predictor of coccidia shedding in next capture. These coccidia shedding models also included an interaction term between treatment and IL-4 to account for the observed

TABLE 1 Associations between *Haemonchus* and *Cooperia* burden and interleukin-4 (IL-4) and interferon gamma (IFN-γ) concentrations in blood of anthelmintic treated animals. Higher *Haemonchus* burdens were associated with lower IL-4 concentrations. *Cooperia* burden was not associated with IL-4 concentration. Neither *Haemonchus* nor *Cooperia* burden was associated with IFN-γ concentration. Generalized linear mixed models with animal ID included as a random effect. Models also controlled for the effects of body condition, herd, season, and age. Significant predictors are highlighted in bold. SE, standard error

Response	Predictor	*β*	SE	*Z*	*p*
IL-4 (Negative binomial)	(Intercept)	6.137	0.446	13.8	<0.0001
	Haemonchus FEC	−0.007	0.002	−3.1	0.0017
	Cooperia FEC	−0.001	0.0001	−1.6	0.1152
	Age	−0.0023	0.003	−0.7	0.4791
	Body condition	−0.149	0.143	−1.0	0.3005
	Herd (lower Sabie)	0.057	0.215	0.3	0.7898
	Season (wet)	−0.081	0.163	−0.5	0.6181
IFN-γ (Gaussian)	(Intercept)	0.962	0.544	1.769	0.077
	Haemonchus FEC	0.003	0.002	1.686	0.0918
	Cooperia FEC	0.000	0.001	0.004	0.9967
	Age	−0.005	0.004	−1.08	0.2802
	Body condition	−0.269	0.156	−1.722	0.085
	Herd (lower Sabie)	−0.105	0.267	−0.394	0.6937
	Season (wet)	−0.362	0.196	−1.849	0.0645

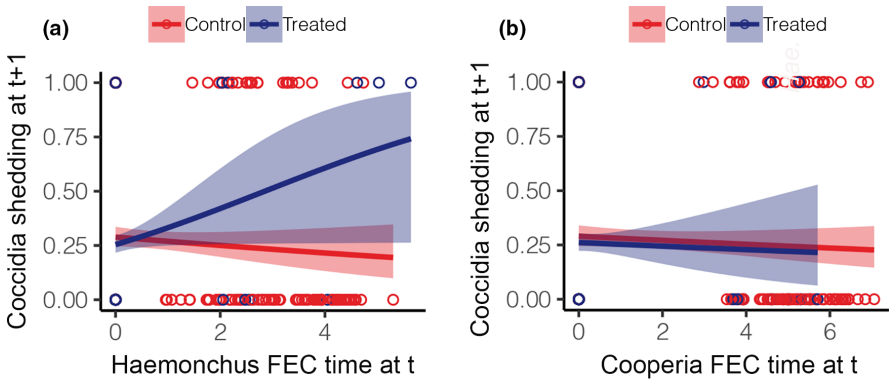

FIGURE 1 Anthelmintic treatment increases the likelihood of coccidia shedding in *Haemonchus* infected animals. (a) In treated animals, a higher *Haemonchus* faecal egg count (FEC) at one capture (*t*) was associated with a higher probability of coccidia shedding at the following capture ($t+1$), while there was no effect of *Haemonchus* FEC on future coccidia shedding probability in control animals (treated vs. control slope Z test = 4.29, $p<0.001$). (b) *Cooperia* FEC did not affect future coccidia shedding in treated or control animals.

effect of treatment on IL-4. Additionally, we controlled for potential effects of treatment on coccidia shedding through interactions with IFN-γ by adding an IFN-γ × treatment term as predictor. These models also included animal ID as a random effect and controlled for the effects of worm burden, herd membership, age and season on coccidia shedding. Finally, to confirm differences between treated and control animals in the slopes of cytokine levels on coccidia shedding, we performed a post-hoc Z test of the model estimates using the 'glht' function within the 'MULTICOMP' R package (Hothorn et al., 2008).

We explored the mechanisms underlying putative IL-4 facilitation of coccidia shedding by examining the impact of IL-4 on host intestinal immunity and morphology. Specifically, we calculated the sum of plasma IL-4 concentrations in an individual over its last four captures before tissue sampling and used this value as a predictor of the number of immune and non-immune cells and fibrosis in GI tract tissues. We chose a four capture cutoff because all sampled animals had at least four captures, making it simpler to standardize across individuals. Moreover, these four IL-4 measurements were taken over the 2-year period prior to tissue sampling, better representing the impact of a sustained Th2 immune phenotype on long term changes in intestinal immunity and morphology. We used GLMs with an interaction term between IL-4 and treatment to test for a possible modulatory effect of anthelmintic treatment on the impact of IL-4 on immune and morphological traits. Response variables included the number of eosinophils, mast cells, T-lymphocytes and IgA+ leukocytes in both the abomasum and small intestine (immune traits) and the number of parietal cells in the abomasum and level of fibrosis in the intestine (morphological traits). The cellular responses were modelled using a negative binomial error distribution, while fibrosis was modelled using a Gaussian error distribution. We also examined if these immune traits covaried with concurrent systemic IL-4 responses, as could be the case if the assessed immune cells were a source of IL-4. To do this, we used the same model structure, this time including IL-4 measured in the last sampling event only as a predictor of the immune traits. To avoid inflation of type I error due to multiple comparisons, we corrected all p-values using the Benjamini and Hochberg method implemented using the 'p.adjust' function of the 'MULTCOMP' R package (Hothorn et al., 2008). These analyses confirmed that IL-4 concentrations that preceded tissue sampling predicted some immune traits, while IL-4 concentrations at the time of tissue sampling did not covary with immune traits, allowing us to further test associations between these traits and coccidia shedding. We did this by using negative binomial GLMs with the relevant immune or morphological trait and an interaction with treatment as predictors of the total number of coccidia oocysts shed by an animal during its last five captures. Given the small sample size for this analysis ($n = 47$), we used several models instead of a single consolidated model to avoid model overfitting and convergence issues. To avoid type I errors and to weight predictors against each other, we compared models using the Akaike information criteria corrected for small sample size (AICc). We considered models with a delta AICc >7 to have minimal support (Burnham et al., 2011).

Finally, to examine whether coccidia predicted host fitness we explored the associations between coccidia shedding and host body condition, reproduction (pregnancy, lactation) and survival in a subsequent capture. Using separate GLMMs, body condition was modelled with a Gaussian error distribution, while pregnancy, lactation and survival were modelled as binomial responses (yes/no). In all models, coccidia shedding status (yes/no) was included as a binomial categorical predictor. These models controlled for effects of herd membership, age, anthelmintic treatment and season, all factors that impact body condition, reproductive stage or survival in African buffalo (Budischak, O'Neal, et al., 2018; Ezenwa & Jolles, 2015). The body condition model also controlled for capture interval to account for the time it takes an animal to improve or lose condition between subsequent captures (Budischak, O'Neal, et al., 2018). Models for lactation, pregnancy, and survival included body condition as a covariate to account for the effect of body condition on these parameters. All models included animal ID as a random effect. We also assessed the association between coccidia and age at first reproduction for a subset of 121 animals that were captured before their first pregnancy event. To do this, we fit a GLM with the total coccidia oocyst count preceding an animal's first reproduction event as a predictor of age at first pregnancy. We used total coccidia oocyst

shedding instead of coccidia shedding status (yes/no) as a predictor because age at first reproduction is a single time point metric impacted by cumulative events preceding the pregnancy event. This model also controlled for the impact of average body condition across all captures preceding first pregnancy, the number of captures preceding first pregnancy, anthelmintic treatment, herd membership and season.

3 | RESULTS

3.1 | Anthelmintic treatment-associated disruption of *Haemonchus*, but not *Cooperia*, increases coccidia shedding

We found no effect of treatment or the interaction between treatment and overall strongyle FEC on an individual's probability of shedding coccidia oocysts at its next capture (Treatment × Strongyle FEC; $\beta = 0.038 \pm 0.054$, $Z = 0.696$, $p = 0.486$, Table S1). However, when we decomposed strongyle FEC into species (*Cooperia* vs. *Haemonchus*), we found that treatment interacted with *Haemonchus* FEC, but not *Cooperia* FEC, to explain differences in coccidia shedding (Treatment × *Haemonchus* FEC; $\beta = 0.98 \pm 0.43$, $Z = 2.31$, $p = 0.0207$). Specifically, animals with high *Haemonchus* counts who received treatment at one capture were more likely to be shedding coccidia at the next, while the same pattern did not hold for control animals who did not receive treatment (Figure 1a). In contrast, there was no effect of the interaction between treatment and Cooperia FEC (Treatment × Cooperia FEC; $\beta = -0.40 \pm 0.28$, $Z = -1.43$, $p = 0.151$, Figure 1b, Table S2). These findings suggest that treatment of *Haemonchus* worms, in particular, facilitated coccidia oocyst shedding and the effect increased with intensity of prior *Haemonchus* infection.

3.2 | Increased systemic Th2 immunity in response to *Haemonchus* disruption predicts coccidia shedding

To understand whether the effect of *Haemonchus* disruption on coccidia shedding was mediated by the host immune response, we examined the effect of anthelmintic treatment on systemic immunity. Our prior work suggests that in buffalo, anthelmintic treatment increases concentrations of the Th1 cytokine, IFN-γ (Ezenwa & Jolles, 2015), and here we found similar effects on the Th2 cytokine, IL-4 (Treated: $\beta = 0.052 \pm 0.02$, $Z = 2.3$, $p = 0.027$, Table S3). We also found that for IL-4, but not IFN-γ, this effect was associated with *Haemonchus* as evidenced by a significant negative correlation between IL-4 and *Haemonchus* FEC among treated animals (Table 1). Moreover, animals infected with both *Haemonchus* and *Cooperia* prior to first treatment, but not those infected with *Cooperia* only, experienced a significant increase in IL-4 at their second (post-treatment) capture (Figure 2a,b, Table S4). Importantly, the effect was present among treated animals, but not controls (Figure 2a) indicating that treatment was responsible for the change in IL-4 among *Haemonchus* and *Cooperia* co-infected individuals. In contrast, after treatment, IFN-γ concentrations increased in animals with prior *Cooperia* infection, but not

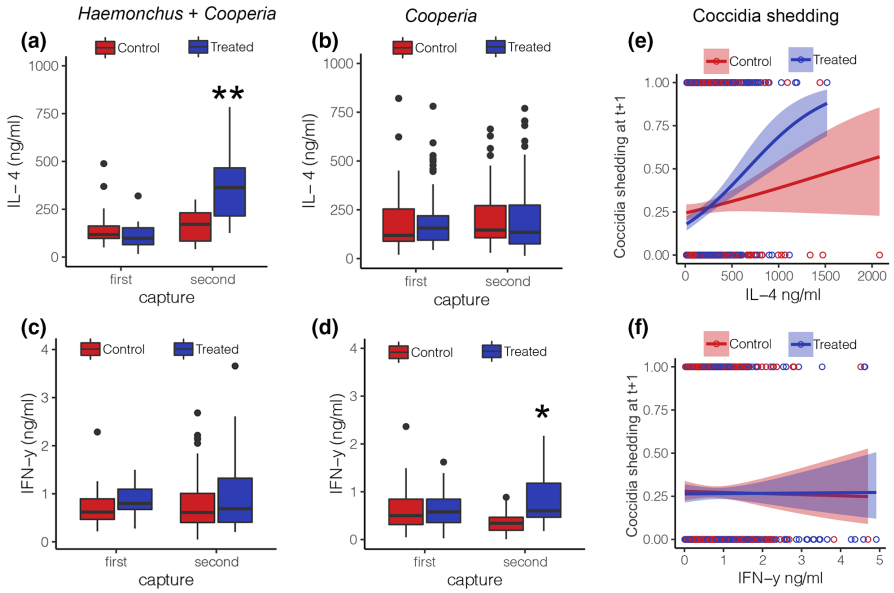

FIGURE 2 Anthelmintic treatment alleviates *Haemonchus* suppression of the Th2 immune response, favouring future coccidia shedding. Individuals infected with (a) *Haemonchus* and *Cooperia* but not those infected only with (b) *Cooperia* experienced a significant increase in systemic IL-4 levels after anthelmintic treatment. For the Th1 response, animals infected with (c) *Haemonchus* and *Cooperia* did not experience a significant increase in IFN-γ after anthelmintic treatment, but individuals infected only with (d) *Cooperia* did produce higher levels of IFN-γ after treatment. (e) In treated individuals, higher levels of IL-4 in the blood was associated with increased shedding of coccidia at the next capture. (f) However, in contrast to IL-4, IFN-γ levels in blood were not associated with future coccidia shedding (on boxplots asterisks denote statistical significance: $^{*}p = 0.01–0.05$, $^{**}p < 0.01$).

those with dual *Haemonchus* and *Cooperia* infection (Figure 2c,d, Table S5), suggesting that *Haemonchus* presence impaired the IL-4 response but not the IFN-γ response.

Given the observed association between *Haemonchus* and IL-4, we tested whether worm-associated immune modulation could explain patterns of coccidia shedding. In support, we found that in a model accounting for IL-4, the effect of *Haemonchus* on future coccidia shedding in treated animals disappeared (*Haemonchus* FEC; $\beta = 0.08 \pm 0.07$, $Z = 1.1$, $p = 0.293$, Table S6). Furthermore, among treated animals, higher concentrations of IL-4 predicted higher probabilities of coccidia shedding at the next capture (Treatment×IL-4; $\beta = 0.002 \pm 0.001$, $Z = 2.7$, $p = 0.006$; Figure 2e, Table S7). IFN-γ concentration did not have a significant effect on coccidia shedding (Treatment×IFN-γ; $\beta = 0.062 \pm 0.26$, $Z = 0.2$, $p = 0.81$, Figure 2f), suggesting that the effect of anthelmintic treatment on the Th2 (IL-4) and not Th1 (IFN-γ) immune response enhanced coccidia shedding. These results suggest that in treated animals, higher IL-4 concentrations, likely as a consequence of *Haemonchus* clearance, facilitated coccidia shedding.

3.3 | Systemic Th-2 immune phenotype modifies intestinal immunity and morphology favouring coccidia shedding

We found that historically higher exposure of intestinal tissue to IL-4, quantified as the sum of IL-4 concentrations in blood in the 2 years preceding tissue sampling, was associated with increased intestinal fibrosis among anthelmintic treated but not control animals (fibrosis: Treatment×IL-4; $\beta = 0.0006 \pm 0.0002$, $Z = 2.9$, $p = 0.045$; Figure 3a,b, Table S8), suggesting a higher sensitivity to IL-4 induced fibrosis among treated animals. In the small intestine, IL-4 also predicted higher numbers of IgA+ leukocytes (IL-4; $\beta = 0.022 \pm 0.0006$, $Z = 4.0$, $p = 0.001$) and lower numbers of T-lymphocytes (IL-4; $\beta = -0.002 \pm 0.0005$, $Z = -2.9$, $p = 0.045$) in both treated and control animals (Figure 3c–e, Table S8). In contrast, IL-4 did not predict the number of mast cells in the small intestine (IL-4; $\beta = 0.0025 \pm 0.0011$, $Z = 2.3$, $p = 0.102$). In the abomasum, historic IL-4 levels did not predict fibrosis, numbers of eosinophils, mast cells, or IgA, but treated animals had higher numbers of HCl producing parietal cells compared with controls (treated; $\beta = 0.45 \pm 0.16$, $Z = 2.7$, $p = 0.007$; Figure 3f, Table S8). Notably, there were no associations between immune (eosinophils, mast cells, IgA, T-lymphocytes) or morphological (fibrosis, parietal cells) tissue traits and IL-4 levels recorded at the time of tissue sampling (Table S9), suggesting that the traits evaluated were more likely a response to prior IL-4 concentrations and not a source of IL-4 at the time of sampling.

Furthermore, more fibrosis ($\beta = 5.048 \pm 2.0$, $Z = 2.5$, $p = 0.01$) and higher numbers of mast cells ($\beta = 0.16 \pm 0.07$, $Z = 2.1$, $p = 0.034$) and IgA+ leukocytes ($\beta = 0.004 \pm 0.002$, $Z = 2.0$, $p = 0.042$) in the intestine were associated with higher coccidia shedding (Figure 4a–d, Tables S10 and S11). In combination, these results suggest that anthelmintic treatment induced IL-4-dependent immunological and morphological changes in the intestine that facilitated coccidia shedding.

3.4 | Coccidia shedding affects body condition and reproduction

To evaluate whether the increase in coccidia shedding associated with anthelmintic treatment had implications for host fitness, we examined the potential effects of coccidia on host body condition, reproduction, and survival while controlling for the effect of anthelmintic treatment. Active coccidia shedding at one capture was associated with lower body condition (coccidia status [yes]: $\beta = -0.195 \pm 0.06$, $Z = -3.09$, $p = 0.0019$, Table S12) and a lower likelihood of pregnancy (coccidia status [yes]: $\beta = -0.35 \pm 0.17$, $Z = -2.02$, $p = 0.043$, Table S13) at a subsequent capture, but not lactation status (coccidia status [yes]: $\beta = 0.0631 \pm 0.232$, $Z = 0.27$, $p = 0.785$, Table S14) or survival (coccidia status [yes]: $\beta = 0.329 \pm 0.56$, $Z = 0.59$, $p = 0.557$, Table S15). There was also a strong association between cumulative coccidia shedding and the age of first reproduction. The total number of oocytes shed prior to first reproduction was positively associated with age at first reproduction (cumulative coccidia shedding [OPG]: $\beta = 0.00002 \pm 0.00001$, $Z = 2.44$, $p = 0.015$, Table S1), such that an increase in coccidia shedding of 100 OPG correlated with a delay in the age of first pregnancy of 1 month. The associations between coccidia and body condition and reproduction did not depend on anthelmintic treatment status (Tables S12–S16), suggesting that coccidia infection imposed energetic and reproductive costs on the host irrespective of the presence of helminths.

4 | DISCUSSION

Helminths are common members of animal parasite communities (Cox, 2001; Pedersen & Fenton, 2007; Petney & Andrews, 1998), and an increasing number of studies show that helminth-associated changes in host immune phenotypes can drive top-down interactions between helminths and coinfecting parasites (Desai et al., 2021; Osborne et al., 2014; Reese et al., 2014). Likewise, there is also evidence that physiological and morphological modification of the host environment by helminths can drive bottom-up interactions (Ramanan et al., 2016; Reynolds et al., 2017). However, most studies do not account for the potential that top-down and bottom-up effects may occur simultaneously. In this study, we quantified diverse ways in which helminths modify the host phenotype as a tool to examine the multiple pathways by which helminths interact with coccidia. We found that anthelmintic treatment-associated loss of a blood-feeding helminth (*Haemonchus*) changed the host systemic Th2 immune phenotype. This systemic change was associated with simultaneous top-down and bottom-up effects on coccidia in the GI tract as suggested by changes in the immune (number of leukocytes, IgA) and morphological (fibrosis) landscapes of the intestine, respectively. Furthermore, higher coccidia shedding was associated with a reproductive cost on hosts, suggesting an indirect effect of this parasite-associated phenotypic change on host fitness. Specifically, we found that every 100 egg per gram increase in *Haemonchus* egg shedding in host faeces decreased plasma IL-4 concentrations by

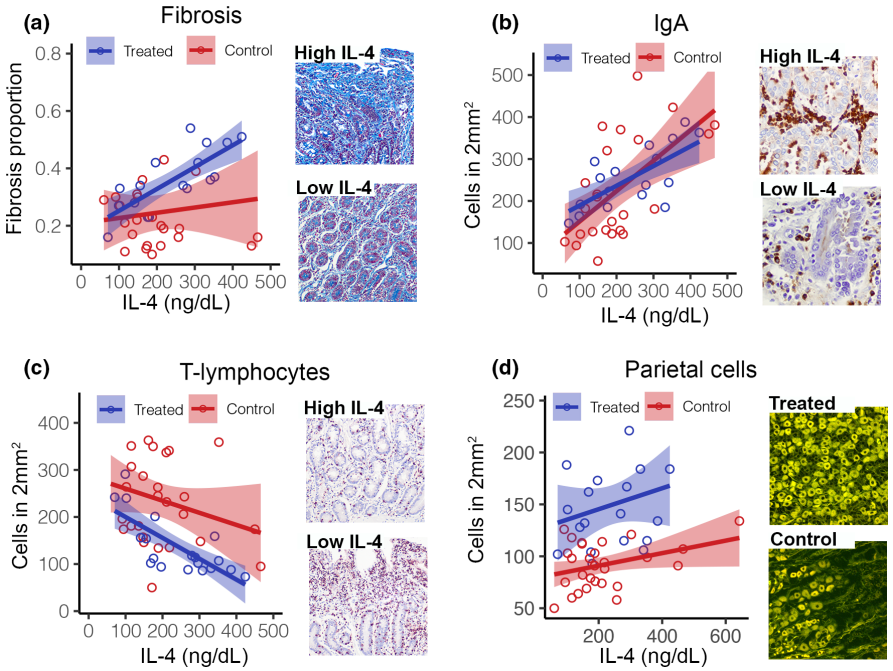

FIGURE 3 Anthelmintic treatment and historic IL-4 levels are associated with intestinal morphology and immunity. (a) Anthelmintic treated individuals with higher levels of IL-4 throughout the study had more fibrosis in the intestinal lamina propria. Inset figures show intestinal fibrosis (dark-blue colour) in individuals exposed to high (~400 ng/dl) or low (~100 ng/dl) IL-4 concentrations throughout the study (Masson's trichome stain). (b) Anthelmintic treated and control individuals with higher levels of IL-4 had more IgA+ leukocytes in the intestinal lamina propria. Inset figures show intestinal IgA+ leukocytes (dark-brown cells) in animals exposed to high (~400 ng/dL) or low (~100 ng/dL) IL-4 levels throughout the study (IgA immunohistochemistry). (c) Anthelmintic treated and control individuals with higher levels of IL-4 had fewer T-lymphocytes in the intestinal mucosa. Inset figures show intestinal T-lymphocytes (dark-brown dots) in animals exposed to high (~400 ng/dL) or low (~100 ng/dL) IL-4 throughout the study (CD3 immunohistochemistry). (d) Anthelmintic treated individuals had higher numbers of parietal cells in the abomasum independent of IL-4 levels. Inset figures show parietal cells (round, apple-green fluorescent cells) in treated and control individuals (autofluorescence at 550 nm wavelength).

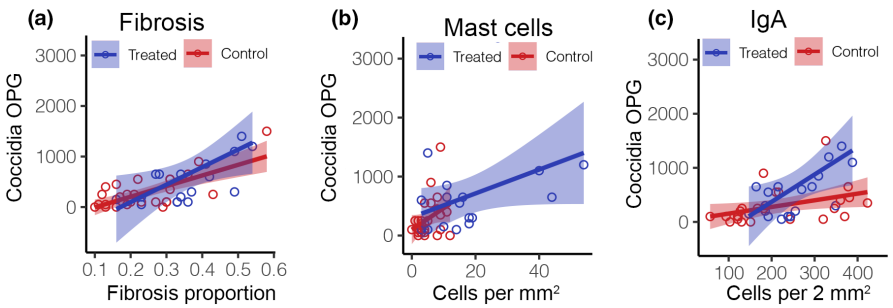

FIGURE 4 Gastrointestinal morphological and immune traits are associated with coccidia shedding. (a) Anthelmintic treated and control animals with more intestinal fibrosis shed more coccidia oocysts per gram of faeces (OPG) throughout the study. (b) Anthelmintic treated and control individuals with more mast cells in the intestine shed more coccidia throughout the study. (c) Anthelmintic treated and control animals with more IgA+ leukocytes in the intestine also shed more coccidia throughout the study

~100 ng/dl, leading to a decline in coccidia shedding of 18%. This level of decline in coccidia shedding was equivalent to a 0.4-point increase in body condition (measured on a scale from 1 to 5), a 6% increased probability of pregnancy, and an age of first reproduction that was 1 week earlier. These findings support the idea that identifying and tracking 'informative' phenotypic changes induced by key parasites can be used as a tool to understand the nature and consequences of parasite interactions for both co-infecting parasites and hosts.

In wild mammals and humans, several studies have reported increased shedding of intestinal protozoa after the elimination of GI helminths (Blackwell et al., 2013; Knowles et al., 2013; Pedersen & Antonovics, 2013). In all these examples, the helminths likely compete directly for intestinal epithelial cells or space with the protozoa (bottom-up effects). Based on this evidence, we predicted that in our study system, *Cooperia*, a helminth that feeds on intestinal cells, would have stronger impacts on coccidia shedding than *Haemonchus*, a blood-feeding helminth that lives in the stomach, given a greater

likelihood of direct competition with coccidia. Contrary to our initial prediction, clearance of *Haemonchus* and not *Cooperia* increased coccidia shedding, suggesting a role for top-down or immune-mediated interactions. *Haemonchus* are highly pathogenic helminths that cause anaemia and significant damage to the stomach (Besier et al., 2016; Le Jambre, 1995). Therefore, hosts usually develop a strong Th2 immune response to this parasite characterized by recruitment of eosinophils and mast cells, and the production of mucus and IgA at the site of infection, effector mechanisms that usually control parasite numbers and sometimes clear infection (Bowdridge et al., 2015; Escribano et al., 2019). However, this helminth has evolved immunoregulatory mechanisms to avoid clearance. For instance, some *Haemonchus* gut enzymes and structural proteins are potent inhibitors of the Th2 immune response in livestock (Lu et al., 2020; Wen et al., 2021). Similarly, we found that clearance of *Haemonchus* induced a systemic Th2 immune phenotype characterized by increased levels of IL-4, suggesting that this parasite also suppresses Th2 immunity in buffalo. Interestingly, among *Haemonchus* infected individuals, there was no significant difference in average IL-4 levels between treated and control animals, only changes in an individual's own value before and after treatment. This suggests that *Haemonchus* modulates IL-4 levels within normal ranges and not below some baseline threshold. In the case of *Cooperia*, although there were no effects on the host Th2 response, this helminth impaired Th1 responses (IFN-γ). This difference could be due to differing host defence strategies against these parasites since *Haemonchus* is 'resisted' by buffalo via a Th2 response and *Cooperia* is 'tolerated' (Budischak, O'Neal, et al., 2018). However, how *Haemonchus* and *Cooperia* suppress Th2 and Th1 immune responses, respectively, is not well-understood. Studies in cattle and goats suggest that a key mammalian regulatory cytokine, IL-10, could be one of the mediators of immunosuppression for both parasites (Ehsan et al., 2020; Matamoros-Mercado et al., 2021). In our system, IL-10 could play a similar role, but additional mediators are likely involved since for *Haemonchus*, the regulatory signal affected only the Th2 response, while for *Cooperia* only the Th1 response was affected.

The Th2 immune response evolved to prevent and repair tissue damage inflicted by large, extracellular parasites (Cortés et al., 2017; Gause et al., 2013). Therefore, upon exposure to extracellular threats, Th2 effector mechanisms are activated by IL-4. Some of these mechanisms include production of cytokines such as IL-13 and TGF-β, immunoglobulins such as IgA, and the attraction and proliferation of eosinophils, mast cells and alternatively activated macrophages (Gause et al., 2013; Maizels, 2020). These effector mechanisms can clear helminth infection through stimulation of mucus production or direct attacks on helminths. Many of these components also take on the role of repairing the tissue damage inflicted by helminths through regeneration or scarring (Gieseck et al., 2018). Regeneration is the process of restoring lost tissue components, while scarring is the replacement of lost tissue by collagen and fibroblasts (fibrosis) (Gause et al., 2013). It is unclear why under some circumstances Th2 effector mechanisms induce regeneration and other times scarring, but in our study, we found evidence of both in different host compartments. In the abomasum, the site of *Haemonchus* infection, there was regeneration of parietal cells following helminth clearance although this was not associated with systemic IL-4 levels. It is possible that the presence of *Haemonchus* inhibited regeneration as an important survival strategy for the parasite since parietal cells decrease the stomach pH, making the environment less suitable for gastric helminths (Mihi et al., 2013). In the intestine, a site not directly affected by *Haemonchus* infection, the Th2 systemic immune phenotype induced fibrosis. Interestingly, this was the only trait that increased in response to IL-4 exclusively in treated animals, suggesting that helminths suppress fibrosis in the intestine in response to IL-4. In laboratory rodents, in the context of systemically up-regulated IL-4, helminth-induced immunoregulatory pathways such as IL-10 and TGF-β tend to decrease tissue regeneration and promote fibrosis (Gause et al., 2013; Gieseck et al., 2018). This suggests that helminth species with immunoregulatory properties may ameliorate the fibrotic effect of the Th2 response. Interestingly, intestinal fibrosis was an important predictor of total coccidia shedding. This could be related to changes in the intestinal landscape that enhance suitability of intestinal epithelial cells for coccidia or impair the immune response against coccidia. For instance, intestinal fibrosis can be associated with the epithelial to mesenchymal transition, a process where intestinal epithelial cells acquire 'fibroblast like features' and migrate to the intestinal lamina propria, increasing the turnover of epithelial cells (Lenti & Di Sabatino, 2019). This could increase the pool of 'new' or uninfected cells for coccidia. Additionally, fibroblasts, the cells responsible for fibrosis, decrease proliferation and migration of T-lymphocytes in the intestine (Pinchuk et al., 2008; Roulis & Flavell, 2016), the most important effector cells for control of coccidia (Kim et al., 2019; Lucas, 2011). Although these potential links between fibrosis and coccidia shedding have not been confirmed, the evolutionarily conserved host response to helminth infection across mammals and its facilitatory effect on coccidia shedding we describe here suggest that these mechanisms could represent important pathways by which a systemic Th2 immune phenotype can favour coccidia replication across a range of host–parasite systems.

In addition to the induction of fibrosis, a systemic Th2 phenotype was associated with lower numbers of T-lymphocytes and more IgA. IL-4 can induce proliferation of some T-cell subpopulations in the intestine such as T-regs and Type II CD4+ T-cells although other subpopulations such as type I (IFNγ secreting) T-cells can be diminished in the presence of IL-4 (Varyani et al., 2017). The decrease in T-cells in response to IL-4 could also indicate a decrease in Type I proinflammatory T-lymphocytes, although this is difficult to confirm since we did not differentiate T-lymphocyte subpopulations. Alternatively, the decrease in T-lymphocytes could reflect a switch in host investment towards B-lymphocytes. IL-4 is a well-established driver of proliferation of B-lymphocytes and IgA secreting plasma cells in the intestinal mucosa (Xiong & Hu, 2015). In our study, we saw a positive association between IL-4 and IgA+ leukocytes, suggesting there could have been a significant increase in B-lymphocytes (plasma cell precursors) and IgA producing plasma cells in the intestine, which could have occurred at the expense of T-lymphocytes. Interestingly,

this increase in IgA production in parallel with IL-4 could have had a positive effect on coccidia shedding. Most studies in domestic animal models have found that although IgA increases in response to coccidia challenge, the number of coccidia oocysts in faeces does not decrease (Matos et al., 2017; Yun et al., 2000), suggesting that IgA is an inefficient response to coccidia infection. Similarly, intestinal mast cells, which did not respond to IL-4 in our study, but were positively associated with coccidia shedding, also represent an inefficient response to coccidia in animal models (Matos et al., 2017; Yun et al., 2000). Interestingly, both IgA and mast cells have been associated with stimulation of regulatory T-cells, which suppress adaptive and innate immunity against intracellular pathogens (Li et al., 2020; Mazzoni et al., 2006). Therefore, changes in the intestinal immune landscape, partially induced by the Th2 systemic immune phenotype, could favour the shedding of coccidia.

Coccidia parasites are widespread in free-ranging animal populations and while some species cause little damage to the host, others are highly pathogenic and cause severe disease and mortality (de Gouvea Pedroso et al., 2020; Hakkarainen et al., 2007). In bovines, *Emeria bovis*, *E. zuernii* and *E. bareillyi* are some of the most pathogenic species and have been associated with weight loss, enteritis, diarrhoea and death (Bangoura & Bardsley, 2020; Dubey, 2018). In our study, we did not differentiate coccidia to species level although pathogenic species such as *E. bovis* and *E. zuernii* are common in South African cattle (Matjila & Penzhorn, 2002). Additionally, cross sectional studies in African buffalo have found negative effects of coccidia on body condition, especially in animals co-infected with GI helminths (Gorsich et al., 2014). Similarly, we observed that individuals shedding coccidia had reduced body condition and likelihood of pregnancy. Increased coccidia shedding was also associated with an older age of first pregnancy. These findings suggest that coccidia infection likely imposes fitness costs on buffalo, and any factor that increases coccidia shedding, like clearance of *Haemonchus*, has the potential to result in fitness declines. However, *Haemonchus* itself imposes fitness costs on hosts, for example, via condition-mediated effects on reproduction and survival (Budischak, O'Neal, et al., 2018) and possibly by inducing anaemia (Budischak et al., 2012). Given the dose-dependent association between *Haemonchus* burden at clearance and coccidia shedding (Figure 1a), the coccidia-related costs of worm clearance likely apply disproportionately to the most highly *Haemonchus*-infected individuals, but it is not clear if the fitness costs of worm clearance, in terms of increased coccidia shedding, exceed the direct costs of intense *Haemonchus* infection. Our past work has shown that in buffalo, haemoglobin levels, a key marker of anaemia, decline with increasing *Haemonchus* burden in a dose-dependent manner (Budischak et al., 2012). In this study's dataset, there was a weak association between haemoglobin level and *Haemonchus* burden, but haemoglobin was not associated with either age at first reproduction or body condition (see Appendix S2). Thus, our data do not support a cost of *Haemonchus* presence that exceeds the coccidia-related cost of *Haemonchus* clearance. However, future work is needed to unpack the likely multifactorial ways in which parasite interactions translate into changes in host fitness.

While our study suggests that *Haemonchus* infection dampens coccidia shedding through changes in the host immune phenotype, other complementary mechanisms not explored here are possible. For instance, helminths can produce molecules that have direct effects on host tissues or microparasite (e.g. bacteria) replication (Midha et al., 2018). These types of effects are challenging to address without experimental infection studies, which are complicated to perform in natural systems. However, as tools from proteomics and metabolomics become more available for field studies (Brunetti et al., 2018), approaches that look simultaneously at hundreds of molecules present in small concentrations in animal tissues could identify new pathways by which parasites interact.

Co-infection is nearly universal in wildlife systems, however, there is still limited understanding of the mechanisms that drive parasite interactions within hosts. Here, we used helminth-induced changes in host phenotypes to understand the combinatorial effects of top-down and bottom-up interactions on host and parasites. We showed that experimental clearance of helminths induced a systemic Th2 phenotype that facilitated coccidia shedding via linked top-down and bottom-up interactions. Importantly, these effects were linked to fitness costs on the host. Our findings suggest that tracking of key host phenotypes that change in response to infection can help unmask complex pathways by which parasites interact.

AUTHOR CONTRIBUTIONS

Mauricio Seguel and Vanessa O. Ezenwa conceived the study; Mauricio Seguel, Sarah A. Budischak, Anna E. Jolles and Vanessa O. Ezenwa designed the methodology; all authors contributed to data collection; Mauricio Seguel analysed the data; Mauricio Seguel and Vanessa O. Ezenwa wrote the paper with feedback from all authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data associated with this study are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.00000006j (Seguel et al., 2022).

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SUPPORTING INFORMATION

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