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Title
Competing for blood: the ecology of parasite resource competition in human malaria-helminth co-infections
Short title: Resource competition in malaria-worm co-infection

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AEW, FH, LJW, MMMK, LL, ES, TS & MY carried out the deworming trial. SAB & ALG analyzed the data in discussions with ES and MY. SAB and ALG wrote the manuscript. AEW, LJW, MMMK, LL, ES, & MY provided editorial feedback.

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Abstract

Ecological theory suggests that co-infecting parasite species can interact within hosts directly, via host immunity and/or via resource competition. In mice, competition for red blood cells (RBCs) between malaria and bloodsucking helminths can regulate malaria population dynamics, but the importance of RBC competition in human hosts was unknown. We analyzed infection density (i.e. the concentration of parasites in infected hosts), from a 2-year deworming study of over 4,000 human subjects. After accounting for resource-use differences among parasites, we find evidence of resource competition, priority effects, and a competitive hierarchy within co-infected individuals. For example, reducing competition via deworming increased *Plasmodium vivax* densities 2.8-fold, and this effect is limited to bloodsucking hookworms. Our ecological, resource-based perspective sheds new light into decades of conflicting outcomes of malaria-helminth co-infection studies with significant health and transmission consequences. Beyond blood, investigating within-human resource competition may bring new insights for improving human health.

Introduction

Ecology offers the tools and theory to study species interactions, but it has only recently been applied to parasites co-occurring within hosts. Two main ecological modes of parasite interactions are predicted, via the host's immune system or via resource competition (Graham 2008). Immune-mediated interactions have been demonstrated in numerous laboratory, wildlife, and human studies (Pedersen & Fenton 2007; Graham 2008). These interactions frequently occur in helminth-microparasite co-infections because the immune responses required to clear these disparate parasite types are mutually inhibitory (Abbas *et al.* 1996; Yazdanbakhsh *et al.* 2002) and often lead to an indirect, facultative interaction between helminths and microparasites. Yet, the frequency, strength, and consequences of bottom-up, resource competition for understanding infectious diseases has received far less attention. Within hosts, competition for a shared resource (e.g., red blood cells (RBCs)) could restrict growth of either or both parasite species. Indeed, when mice are co-infected with anemia-inducing helminths, interspecific competition for RBCs limits malaria population growth (Griffiths *et al.* 2015).

However, in human hosts, the impact of RBC resource competition even in the well-studied malaria-helminth co-infections is unclear. Malaria and soil transmitted helminth infections remain two of the most prevalent groups of human parasites (Brooker 2010; Murray *et al.* 2012). They co-occur in the same regions and often concurrently infect the same individuals (Salgame *et al.* 2013). Yet, despite dozens of studies over the past 40 years, the individual and public health consequences of malaria-helminth co-infection remain unresolved (Nacher 2011; Naing *et al.* 2013). Numerous studies have

found that helminths reduce malaria incidence, a possible consequence of resource competition. Yet comparable numbers of studies have documented that helminths increase or have no effect on malaria infections in people (Adegnika & Kremsner 2012). Here, we take a resource ecology approach in order to explain such varied outcomes.

Even in free-living systems, competition can be variable and difficult to detect. Competitive interactions are often complex, depending upon environmental conditions, order of establishment (i.e. priority effects), and the presence of multiple species (e.g. dominance hierarchies) (Clements 1938; Paine 1984). These same ecological principles can apply to parasite co-infections; for example, larval trematodes have competitive hierarchies within their snail intermediate hosts (Kuris 1990; Kuris & Lafferty 1994). Detecting such competition, however, relies on the proper scale of investigation. Most previous human co-infection studies have primarily focused on effects of helminths on the population-scale prevalence of malaria (i.e. proportion of hosts infected, reviewed in (Nacher 2011; Adegnika & Kremsner 2012; Naing *et al.* 2013), while far fewer have studied individual-level metrics like within-host density or infection intensity (i.e. number of parasites within each host (Bush *et al.* 1997), (Degarege *et al.* 2009; Kirwan *et al.* 2010; Kepha *et al.* 2016)), or infection severity (Nacher *et al.* 2002; Hesran *et al.* 2004; Degarege *et al.* 2009; Njua-Yafi *et al.* 2016). Although more frequently applied to macroparasites like helminths and ticks, the intensity or density of protozoan, bacterial, viral, and fungal parasites can also be quantified and may be important for individual health and disease transmission (Råberg 2012; Westerdahl *et al.* 2013; Cizauskas *et al.* 2015; Langwig *et al.* 2015; Carneiro Dutra *et al.* 2016). Like free-living species in the same habitat patch, interactions among parasites can only occur within co-infected

individual hosts. Thus, we postulate that density of each parasite species in infected hosts will be a more valuable measure of species interactions within a host than either prevalence or mean abundance, which include uninfected hosts (i.e. unoccupied habitat patches; (Bush *et al.* 1997)).

Crucially, our ecological approach to within-host competition also depends upon species' resource-use traits. Because resource needs vary according to life history and resource acquisition strategies of different parasites, taking a species specific approach is increasingly recognized as a key to understanding co-infection outcomes (Knowles 2011; Ramiro *et al.* 2016). Within hosts, it can be difficult to discern precisely what nutrient or tissue parasites are competing for, so the interactions among malaria, bloodsucking helminths, and host RBCs provide an opportune case study. Human malaria species utilize different resources (Simpson *et al.* 1999); for example, *Plasmodium vivax* (Grassi and Filetti) is a specialist on young RBCs (reticulocytes) and *P. falciparum* (Welch) is a generalist that can infect any age RBCs (Simpson *et al.* 1999). Helminths also differ in resource acquisition strategies that could have implications for co-infection interactions; for example, hookworms feed on blood while the giant roundworm *Ascaris lumbricoides* (L.) does not. Yet, in human co-infection studies, malaria species are frequently not identified or are pooled for analysis (Naing *et al.* 2013), and considering different types of helminths alone leads to conflicting outcomes across studies (Nacher 2011). Meanwhile, a meta-analysis of mouse malaria revealed that co-infection effects on both peak parasitemia and host mortality are malaria- and helminth-species specific (Knowles 2011). For instance, averaged across multiple studies, helminths increase mouse mortality risk due to *P. yoelii* by 7-fold, but cut mortality risk due to *P. berghei* by almost half.

Thus, we predict that considering resource-use characteristics of malaria and helminth species has the potential to provide new insight into the contradictory reports in human co-infection studies.

To test this prediction, we used a two-pronged study design that held promise for quantifying interactions among parasite species, despite the difficulty of detecting such interactions in nature (Fenton *et al.* 2014). Parasite interactions can be detected through controlled co-infection experiments. Indeed, such studies have made great contributions to our understanding of the strength and mechanisms of parasite interactions (Holmes 1961; Christensen *et al.* 1987; Behnke *et al.* 2001), but they are limited in their ability to capture a realistic environmental context. Field studies can provide this natural context, but then differentiating real interactions from processes like correlated exposure is challenging (Kuris & Lafferty 1994), and even when real interactions are occurring, they can be difficult to detect given the typically over-dispersed macroparasite distributions (Fenton *et al.* 2010, 2014). Here, we combine the strengths of a parasite removal experiment for differentiating true interactions from spurious correlations with the realism of a longitudinal study in a natural population. Though rarely applied, this combined approach has proved to be powerful for studying parasite interactions in natural populations (Jolles *et al.* 2008; Telfer *et al.* 2010; Johnson & Buller 2011).

Our "parasite removal experiment" was actually a placebo-controlled deworming trial in 3,491 human subjects on Flores Island, Indonesia. This work first established that deworming with albendazole had no lasting effect on malaria prevalence (Wiria *et al.* 2013), despite significantly altering immune regulatory molecules which resulted in enhanced anti-*P. falciparum* responses in dewormed individuals (Wammes *et al.* 2016).

114 The present study focuses on the individual-host scale, parasite density within infected
115 hosts, and key features of parasite life histories to reveal whether, when, and how these
116 parasites interacted within that group of human hosts. We estimate parasite density as the
117 concentration of malaria DNA in the blood and worm egg DNA the feces, which are
118 highly correlated with traditional blood smear and fecal egg counts (Verweij *et al.* 2007;
119 Wampfler *et al.* 2013), respectively. We then explore the hypothesis that RBC resource
120 competition affects co-infection outcomes by testing for effects of helminths on malaria
121 parasite density and vice versa. We highlight the importance of a species-specific
122 approach by comparing aggregated and helminth species-specific analyses.
123 If competition for RBCs drives malaria-helminth interactions, we predict that co-
124 infected individuals will have lower parasite infection densities and interactions will be
125 strongest for bloodsucking hookworms. This is because, like emigrating young animals
126 trying to find new territory when the number of appropriate habitat patches has been
127 reduced on the landscape, we hypothesize that RBC competition with co-infecting
128 hookworms will decrease the success rate of merozoites (i.e. the RBC seeking stage of
129 malaria) finding and infecting susceptible RBCs, thereby reducing malaria density. We
130 further hypothesize that malaria will decrease the food availability (i.e. RBCs) to co-
131 infecting hookworms, potentially reducing worm growth, survival, and reproduction,
132 with a net reduction in total egg output that will be captured by our measure of worm
133 infection density. Finally, we predict that experimentally removing helminths will relieve
134 RBC competition, allowing malaria to replicate more and achieve higher densities in
135 albendazole-treated individuals. Instead, if immune cross-regulation drives malaria-

helminth interactions, we predict higher parasite densities in co-infected individuals and reduced malaria density in dewormed individuals.

Materials and methods

As previously described for this clinical trial (Wiria *et al.* 2010, 2013), individuals ages two to adult were cluster-randomized by household to a deworming treatment (400 mg albendazole; n = 1729 individuals) or placebo (n = 1762 individuals) administered every three months over the study period (September 2008 to July 2010). To assess malaria and helminth infections, blood and fecal samples were collected at 0, 9, and 21 months after initial albendazole treatment.

Parasite assessment

To assess malaria infection status and estimate within-host density, blood samples were frozen until analysis by multiplex real-time Polymerase Chain Reaction (PCR) following established protocols (Wiria *et al.* 2010; Hamid *et al.* 2011; Kaisar *et al.* 2013). DNA was extracted from 200 µl of blood. Primers specific for *Plasmodium falciparum*, *P. vivax*, *P. ovale* (Stephens), and *P. malariae* (Laveran) were included in each PCR reaction (Wiria *et al.* 2010; Kaisar *et al.* 2013). Real time PCR assays of malaria infections are nearly 100% sensitive and specific (Roth *et al.* 2016), and are strongly correlated with traditional light microscopy blood smear counts (Wampfler *et al.* 2013). Samples were analyzed using the CFX real-time detection system (Bio-Rad Laboratories, USA) and CFX Manager™ software (Bio-Rad, version 1.0) (Kaisar *et al.* 2013). Lower cycle threshold (Ct) values indicate higher infection densities (Ct per µl blood or mg

feces) and each unit change indicates a 2-fold change in parasite density. Samples that failed to amplify by the 50 cycle count threshold were considered uninfected (Kaisar *et al.* 2013). As a second method of assessing malaria infection status, blood smears were examined for the presence of each malaria species using Giemsa staining and microscopy (Trape 1985; Petithory *et al.* 2005). Three species of malaria were detected: *P. vivax*, *P. falciparum*, and *P. malariae*. Prevalences of *P. falciparum* and *P. vivax* at the baseline (i.e. 2008) were 14.5 and 13.2%, respectively (Kaisar *et al.* 2013). For both malaria species, prevalences ranged between 3.6 and 15.2% for the remainder of the study with no significant differences between treated and control individuals (Wiria *et al.* 2013). *P. malariae* infections were too rare (1.9%) of samples (Kaisar *et al.* 2013)) to examine co-infection interactions.

Helminth infections were more common; over 80% of untreated individuals were infected and albendazole reduced prevalence to about 50% (Wiria *et al.* 2013). Using established protocols, helminth DNA was extracted from 100 mg of feces (stored at -20°C) suspended in 200 µl of PBS (Wiria *et al.* 2010) and analyzed using multiplex real-time PCR with species-specific primers (Verweij *et al.* 2007, 2009; Wiria *et al.* 2010; Hamid *et al.* 2011). Densities of two hookworms species: *Necator americanus* (Stiles) and *Ancyclostoma duodenale* (Dubini), and *A. lumbricoides* and *Strongyloides stercoralis* (Bavay) were detected. Parasite quantification by real-time PCR is highly correlated with fecal egg counts of intensity (Verweij *et al.* 2007), the traditional way of determining infection intensity of these parasites. PCR can even be more sensitive at detecting low-intensity helminth infections than such microscopy techniques (Meurs *et al.* 2017). Real-time PCR quantifications of these helminths have not been compared with adult worm

182 counts, but fecal egg counts were not correlated with adult worm counts in one post-
183 mortem study (Kannangara 1975). Nonetheless, helminth fecundity is correlated with
184 worm size (Poulin 2007; Walker *et al.* 2009) and hookworm egg counts are associated
185 with anemia across multiple studies (Smith & Brooker 2010), confirming that fecal egg
186 quantification techniques are a reasonable way to assess hookworm RBC resource use.
187 Low prevalences of *S. stercoralis* and *A. duodenale* precluded analysis of their individual
188 interactions with malaria species. Hookworms were pooled in the analyses because *N.*
189 *americanus* was found in over 98% of hookworm-positive samples, while *A. duodenale*
190 was found in only 7% positive samples, with 5% of individuals co-infected with both
191 hookworms. A fraction of each fecal sample was fixed in formalin (4%) for subsequent
192 microscopic examination using the formal ether concentration (FEC) method to detect the
193 helminth species noted above and *Trichuris trichura* (L.)(Allen & Ridley 1970; Wiria *et*
194 *al.* 2010). Real-time PCR data were used to quantify infection density and the
195 combination of real-time PCR-and microscopy were used to determine helminth infection
196 status.
197
198 *Red blood cell resources*
199 Red blood cell (RBC) counts were determined from blood samples collected at
200 each sampling time point in heparinized tubes. RBC counts were performed by a blood
201 analyzer (COULTER® Ac-T diff2™, Beckman Coulter, USA).
202
203 *Statistical analysis*

We tested if the density of each *Plasmodium* species within infected hosts, quantified using real-time PCR Ct value, differed by helminth co-infection status using the longitudinal data. With the experimental data, we tested if deworming affected the density of each *Plasmodium* species. We used separate linear mixed models for the density of *P. vivax* and *P. falciparum*. Each model also included age, sex, and sampling date, and random effects of individual nested within household to account for repeated measures and the blocked study design (Wiria *et al.* 2013). To test whether filtering by the type of co-infecting helminth clarified the interaction, we ran separate models adding helminth, hookworms (*N. americanus* and/or *A. duodenale*), or *A. lumbricoides* co-infection status to the models. Interactions between age and co-infection status were initially included in these models to account for potential effects of age-density relationships, but were dropped due to lack of significance. All statistical tables describe relationships with real-time PCR cycle thresholds, but for clarity given the inverse, exponential nature of real-time PCR data, we converted effect size estimates to fold-changes in density (fold change = $2^{estimate}$) for all figures (partial residual plots shown in supplemental materials).

Reciprocally, we investigated associations of each malaria species with the density of the two most prevalent helminths, *N. americanus* and *A. lumbricoides*. We used similar linear mixed models with real-time PCR Ct-values as the response. Models included age, sex, and sampling date, and a random effect of individual nested within household. To account for established effects of albendazole on helminth infections (Wiria *et al.* 2013), these models also included an interaction between deworming and

malaria co-infection. We also examined the evidence for interactions between the two worm species themselves (see Appendix S1 in Supporting Information).

To determine if there were any signatures of competition for red blood cells, we tested whether single and co-infections affected RBC counts. To investigate whether each parasite and experimental deworming (i.e. removing/reducing helminth competition) affected RBC counts, we used liner mixed models with a treatment-by-parasite status interaction, age, sex, and date. Individual nested within household was included as a random effect in this and all following hematological analyses. Next, the effects of parasite density on RBC count was examined with linear mixed models including treatment status, age, sex, and date as covariates. Original models included an interaction between treatment and parasite density, which was dropped due to lack of significance for all but the *P. vivax* model. Finally, for each *Plasmodium* species, we classified individuals as uninfected, infected with just malaria, infected with just hookworms, or infected with both malaria and hookworms. We then ran two linear mixed models testing if co-infection status affected RBC count. These models included an interaction with treatment, age, sex, and date as covariates.

Results

Resource competition explained density during worm-malaria co-infection

Interactions between helminths and *P. vivax* density were species-specific and aligned with our predictions under resource competition. Individuals co-infected with either of the bloodsucking hookworms had lower density *P. vivax* infections ($t = 2.18$, $df = 230$, $p = 0.030$; Fig. 1, see Table S1 in Supporting Information). This competitive

interaction was specific to hookworms; *P. vivax* density was not affected by overall helminth co-infection status or co-infection with *A. lumbricoides*, a non bloodsucking species (Fig. 1, see Fig. S1 and Table S1). Moreover, the direction of these associations is opposite to predictions of immune-mediated facilitation. Notably, this pattern suggesting malaria-hookworm competition was corroborated by experimental deworming data; compared to control individuals, removing helminths with albendazole generated higher *P. vivax* densities ($t = 2.19$, $df = 222$, $p = 0.029$; Fig. 2; see Table S2). Given the exponential nature of real-time PCR measures, the observed difference in mean density corresponds to a 2.75-fold increase in *P. vivax* density in dewormed individuals (Fig. 2; see Fig. S2).

Conversely, we detected no significant interactions between helminths and *P. falciparum*. Associations between *P. falciparum* density and helminth co-infection fit predictions of immune-mediated facilitation (i.e. higher density in co-infected individuals), yet none of these interactions were significant (all $p > 0.18$; Fig. 1, see Fig. S1 and Table S1). Again, the experimental treatment supported the longitudinal association data; deworming with albendazole did not affect *P. falciparum* density (Fig. 2, see Fig. S2 and Table S2). Thus, helminths had opposing associations with *P. vivax* and *P. falciparum* density. Only hookworms, and *N. americanus* in particular, were associated with reduced density of *P. vivax.*

Effects of malaria co-infection on helminth density also varied by parasite species. *P. vivax* co-infection was not associated with changes in the density of either helminth species (see Table S3), but *P. falciparum* co-infection was associated with lower *N. americanus* density (Fig. 3, see Table S3). Interestingly, in dewormed

individuals, the presence of *P. falciparum* was associated with a > 25-fold decrease in *N. americanus* density (see Fig. 3), but this interaction was marginally insignificant (p = 0.058, see Table S3).

Age-density relationships varied by parasite species

Age-density relationships confirm that these parasites also differ in their interactions with the host's immune system. As previously reported in this population (Kaisar *et al.* 2013), *P. falciparum* density declined with age, a signature of acquired immunity. Conversely, *P. vivax* density was independent of age (see Fig. S3 and Table S1). The helminths also showed opposing age associations; *N. americanus* density increased with age, but *A. lumbricoides* declined with age (see Fig. S3 and Table S3).

RBC resources were affected by malaria and by helminth removal

None of the four focal parasites affected RBC count (see Table S4), but a treatment by *P. falciparum* interaction was detected. Contrasts revealed that dewormed individuals had similar RBC counts regardless of *P. falciparum* infection status (p = 0.45), but control individuals infected with *P. falciparum* had lower RBC counts (p = 0.014; Fig. 4a). A similar pattern was observed for *N. americanus*, but the interaction was marginally insignificant (p = 0.060; Fig. 4c; see Table S4). If parasite density within infected hosts rather than presence/absence was examined, a negative relationship between *P. falciparum* and RBC count was detected (Fig. 5a; see Table S5). *P. vivax* density interacted with treatment such that among control individuals there was a non-significant negative slope between density and RBC count, but among dewormed

individuals this relationship was slightly positive (Fig. 5b; see Table S5). The densities of the two helminths were not associated with RBC count (Fig. 5c,d; see Table S8). Effects of co-infection on RBC count were difficult to detect because treatment status interacted with infection status (i.e. none, only hookworms, only malaria, both parasites), prompting the need for 8 separate contrasts for each malaria species, none of which proved significant (see Figure S4, Table S6).

Discussion

Utilizing data from a two-year randomized, placebo-controlled deworming trial, we found experimental and longitudinal evidence for resource competition among malaria and bloodsucking hookworms in human hosts. Our approach of filtering by both malaria and helminth species identity and examining estimates of parasite density within infected hosts, rather than prevalence, was critical to detecting co-infection interactions. Both the longitudinal and experimental evidence support our prediction of resource competition between *P. vivax* and helminths, and the correlational data further suggest that this effect is due to hookworm co-infection. These interactions were likely of magnitudes significant enough to impact human health (Tripathy *et al.* 2007; Barber *et al.* 2015); density of *P. vivax* infections was reduced 2.2-fold by hookworm infection, and, correspondingly, removing helminths with albendazole nearly tripled mean malaria density. Furthermore, RBC data suggest that *P. falciparu*m has the largest effect on resource availability, which was reduced in the presence of *P. falciparum* and in an density-dependent manner. Providing further evidence for resource competition between malaria and hookworms, both *P. falciparum* infection status and *P. vivax* density

318 interacted with deworming treatment to affect host RBC counts. Supporting the
319 hypothesis of resource competition, the lowest red blood cell counts were found in
320 control (i.e. non-dewormed) individuals with malaria. For *P. falciparum*, this interaction
321 was significant with infection status alone (yes/no), while for *P. vivax*, reduced RBC in
322 control individuals was seen only during high density infections. Reciprocally, since
323 deworming was associated with higher RBC counts during *P. falciparum* infection and
324 high density *P. vivax* infections, there may be net health benefits to deworming despite its
325 overall association with higher *P. vivax* density.

326 Further research would be required to identify the precise mechanism of *P. vivax*-
327 hookworm resource competition. While hookworms could share the preference of *P.*
328 *vivax* (Simpson *et al.* 1999) for reticulocytes, they are considered to be indiscriminant
329 bulk feeders that cut open blood vessels and eat all RBCs that flow out (Brooker *et al.*
330 2004), making this hypothesis unlikely. Hookworms may also ingest malaria-infected
331 RBCs, but this 'predatory' hypothesis could only explain the opposing associations with
332 the two *Plasmodium* species if hookworms prefer to prey upon *vivax*, which seems
333 unlikely if they are indeed unselective bulk feeders (Brooker *et al.* 2004). Alternatively,
334 hookworms could deplete RBCs at such a rate that hematopoiesis cannot keep up, or
335 qualitatively alter the processes of hematopoiesis and blood cell turnover. Indeed,
336 reticulocyte counts are reduced during severe *P. falciparum*-*N. americanus* co-infections
337 (Nacher *et al.* 2001). This reticulocyte reduction could be a product of the chronic anemia
338 and depleted iron stores typically caused by hookworms that can impair RBC production
339 (Smith & Brooker 2010). Thus, hookworm-induced anemia could explain the lower
340 density of the reticulocyte specialist, *P. vivax*, in hookworm co-infected hosts. Yet, we

found no evidence of RBC depletion in *N. americanus*-infected individuals in this population, but the marginal interaction with albendazole treatment suggests that longer-term hookworm infections may indeed reduce RBC counts. The *P. vivax* density-treatment interaction supports this hypothesis; RBC counts declined with *P. vivax* density in control hosts, but not dewormed hosts. A greater understanding of hookworm RBC usage and RBC production in chronically infected hosts will be key to explaining why they appear to be a superior competitor to *P. vivax*.

Conversely, density of *P. falciparum*, the RBC generalist, was not affected by resource competition with hookworms. However, density of the most common hookworm species was significantly reduced in *P. falciparum* co-infected hosts, suggesting that *P. falciparum* may outcompete *N. americanus* for RBCs. Indeed, *P. falciparum* infection was associated with reduced RBC counts, suggesting that species of malaria could limit resources available to *N. americanus* in co-infected hosts. This effect on *N. americanus* egg shedding could result from decreased larval establishment, increased mortality rate, and/or reduced fecundity in co-infected hosts. Further supporting that RBC-competition is mediating these interactions, a non-bloodsucking helminth, *A. lumbricoides,* was not associated with malaria density during co-infection or RBC counts. While nutrient limitation has long been suggested as a possible mechanism for human malaria-helminth interactions (Murray *et al.* 1978), this study provides the strongest evidence to-date that resource-mediated interactions, likely via host RBCs, can occur within co-infected people.

The opposing age-density patterns for *N. americanus* and *A. lumbricoides* match intensity and prevalence patterns from other endemic regions (Dunn *et al.* 2016), and

suggest that individuals can acquire at least partial immunity to *A. lumbricoides* or that exposure to this fecal-oral transmitted parasite declines with age. Reciprocally, the positive age-density relationship observed for *N. americanus* could suggest increased susceptibility or exposure to this parasite, acquired by contact with contaminated soil, with age. Age-intensity relationships for the two malaria species have been reported previously (Kaisar *et al.* 2013), but suggest that individuals develop some degree of protection that reduces *P. falciparum* intensity. Thus, as expected, the malaria and helminth species affecting this population represent diverse life histories, in terms of age-dependence of transmission and resource use, and tendency to achieve chronic, repeated, or relapsing infections. By accounting for host age statistically in our models, we were able to show that associations of within-host density, independent of host age, underlie the co-infection and deworming effects discussed above.

Despite the decades of malaria-helminth studies (reviewed in (Nacher 2011; Adegnika & Kremsner 2012), only a small number are of similar study design and thus directly comparable to ours. First, there have been few deworming trials in human populations to study malaria-helminth interactions (Murray *et al.* 1978; Kirwan *et al.* 2010; Kepha *et al.* 2016). Only one of these trials examined intensity and, like us, found no effect of helminths on *P. falciparum* (Kepha *et al.* 2016). Second, although cross-sectional studies are more numerous than deworming trials, most focus on prevalence rather than the quantity of parasites in infected hosts. We detected negative effects of hookworms on *P. vivax* density, while the only previous hookworm-malaria intensity study reported a positive interaction (Degarege *et al.* 2009). However, that study did not differentiate among malaria species (Degarege *et al.* 2009) and the reticulocyte bias of *P.*

387 *vivax* varies by strain (Lim *et al.* 2016), which could affect the strength of resource
388 competition with hookworms. Not differentiating among malaria species may also
389 explain why the three studies examining associations of *A. lumbricoides* and malaria
390 intensity are in complete disagreement, reporting positive (Hesran *et al.* 2004), negative
391 (Nacher *et al.* 2002), or no evidence of (Degarege *et al.* 2009) interactions. Overall, we
392 suggest that more studies focusing on intensity or density within infected hosts,
393 separately examining various malaria and helminth species, and considering their diverse
394 resource and immunomodulatory traits, are necessary for testing the generalizability of
395 the resource-mediated interactions we detected and further elucidating the helminth
396 malaria co-infection patterns found over the past 4 decades.
397 Some of the more complex interaction patterns we observed make sense in light
398 of community ecology competition theories. First, differential interactions among
399 hookworms and the two malaria species suggest a competitive hierarchy. Such
400 hierarchies are common in ecological communities, such as herbaceous plants (Keddy &
401 Shipley 1989) and rocky intertidal invertebrates (Paine 1984), where, dependent upon
402 environmental conditions, there is a predictable pecking order of competitive outcomes.
403 The reductions in density in co-infected hosts suggest that *P. falciparum* is a dominant
404 RBC competitor to hookworms, and that hookworms outcompete *P. vivax*, the
405 reticulocyte specialist. The RBC data support the idea that *P. falciparum* is a dominant
406 competitor in this system. Specifically, *P. falciparum* reduced RBC counts in a density-
407 dependent manner, suggesting that hosts could not fully compensate for blood loss due to
408 this parasite. Outside of larval trematodes in mollusks (Kuris 1990; Kuris & Lafferty
409 1994), competitive hierarchies among parasites have rarely been investigated. Second,

410 the community ecology concept of priority effects can explain why *P. falciparum* effects
411 on hookworm density were marginally stronger in dewormed hosts. Arriving first within
412 a host (i.e., habitat patch) may confer a general competitive advantage. For example, toad
413 tadpoles survive and grow larger when they arrive in a pond before the larger leopard
414 frog tadpoles (Alford & Wilbur 1985). Similarly, *P. falciparum* may outcompete young
415 hookworms, but have weaker effects on adult helminths. Priority effects during co-
416 infection have been documented for strains of the same parasite (Devevey *et al.* 2015)
417 and among parasite species (Hoverman *et al.* 2013). Thus, community ecology theories
418 appear useful for understanding interactions among malaria and helminths, and may also
419 provide new insight into other co-infecting taxa (Graham 2008).
420 Although parasite quantification in infected hosts (i.e. intensity or density) can
421 provide new insights into co-infection interactions that prevalence information cannot,
422 measuring infection quantity does pose challenges. For example, adult helminth density
423 can only be approximated via reproductive metrics (such as fecal egg excretion) in live
424 hosts. Furthermore, malaria genome copy number varies over the schizogonic cycle.
425 Nonetheless, our real-time PCR methods of quantifying infection density have been
426 technically validated and strong positive correlations between Ct scores and true infection
427 intensities are demonstrable (e.g., (Verweij *et al.* 2007); for further discussion, see
428 Appendix S1). Like all measurement errors, these challenges in quantifying helminth and
429 malaria density make detecting interactions more difficult. Yet, despite these sources of
430 variation, we were able to detect negative associations between hookworms and *P. vivax*
431 density and *P. falciparum* and hookworm density, suggesting they are robust interactions.

Understanding the mechanistic underpinnings of malaria-helminth interactions is critical for improving human health and disease management outcomes. In our study population, deworming had minimal effects on malaria prevalence (Wiria *et al.* 2013), yet had strong effects on *P. vivax* density. Both immune- and resource-mediated interactions can affect density, arguing for wider inclusion of resource competition and species identities in co-infection studies, regardless of the interaction mechanism of interest to the researchers leading a given study. The greater than two-fold higher malaria densities observed in albendazole-treated hosts may affect symptom severity and mortality risk (Tripathy *et al.* 2007; Barber *et al.* 2015). However, treated individuals had higher RBC counts during *P. falciparum* infection, and did not experience the same degree of *P. vivax*-density related declines in RBC count as untreated controls. Thus, deworming may still provide net health benefits to this population. At the population scale, higher parasitemia can increase the likelihood of successful transmission to mosquito vectors (Ross *et al.* 2006). Reciprocally, low density infections, as observed with *P. vivax*-hookworm co-infection, can also contribute substantially to transmission by leading to longer infection duration because cases often go undetected and untreated (Roberts 2016). Hence, interactions between malaria and helminths not only have consequences for individual health, but also population-level disease transmission and the success of treatment and eradication programs. Although rapid, species-specific diagnosis of co-infections to inform treatment decisions may pose logistical challenges, the potential public health gains from being prepared to mitigate effects of deworming on non-target infections may ultimately justify this type of approach.

Supporting Information

Additional Supporting Information will be available in the online version of the article.

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663

Figure Legends

Figure 1. Hookworm co-infection is associated with lower *P. vivax* density. Fold-change in density (± 1se) of *P. falciparum* and *P. vivax* in co-infected individuals compared to individuals without worms. Due to the inverse, doubling nature of real-time PCR cycle threshold (Ct) values, fold changes were quantified by exponentiating two to inverse of each general linear model estimate. Asterisks indicate significant differences from zero in *Plasmodium* density for each comparison of single versus co-infected individuals. To view partial residual plots see Figure S1 and for statistical details see Table S1.

Figure 2. Deworming increases *P. vivax* density. Effect of albendazole treatment on *Plasmodium* density shown by the fold-change in density (± 1se) compared to untreated individuals. Due to the inverse, doubling nature of real-time PCR cycle threshold values, fold changes were quantified by exponentiating two to inverse of each general linear model estimate. Asterisks indicate significant differences from zero in malaria density. To view partial residual plots see Figure S2 and for statistical details see Table S2.

Figure 3. *P. falciparum* co-infection is associated with lower hookworm density. Density (± 1se) of *N. americanus* (Na), quantified as the inverse of real-time PCR cycle threshold (ct) value, grouped by whether individuals were co-infected with *P. falciparum* (+ Pf) for control and albendazole-treated individuals. Data are partial residuals from a

general linear model including co-infection status, age, date, sex, and random effects of individual within household. For statistical details see Table S3.

Figure 4. RBC was affected by infection status and treatment. a) *P. falciparum* infection status interacted with albendazole treatment to affect red blood cell count (RBC; x 10^6 cells/μL). The analogous interaction was marginal for c) *N. americanus* ($p = 0.06$). The interaction was not significant for b) *P. vivax* or d) *A. lumbricoides*. No main effects of parasite presence or treatment were detected for any species. For statistical details see Table S4.

Figure 5. RBC was affected by infection density and treatment. Red blood cell count (RBC; x 10^6 cells/μL) was negatively associated with a) *P. falciparum* density (cycle threshold (Ct)), and not associated with the density of c) *N. americanus* or d) *A. lumbriocoides* (dashed lines = non-significant). The relationship between RBC and b) *P. vivax* density differed between control and albendazole treated individuals. Due to the inverse, doubling nature of real-time PCR cycle threshold (Ct) values, lower Ct values represent exponentially higher densities. Plots a, c, and d show partial residuals from general linear models including parasite density*treatment, age, date, sex, and random effects of individual within household. Since partial residuals can not be calculated for interactions, plot b shows raw data. For statistical details see Table S5.

Figure 1.

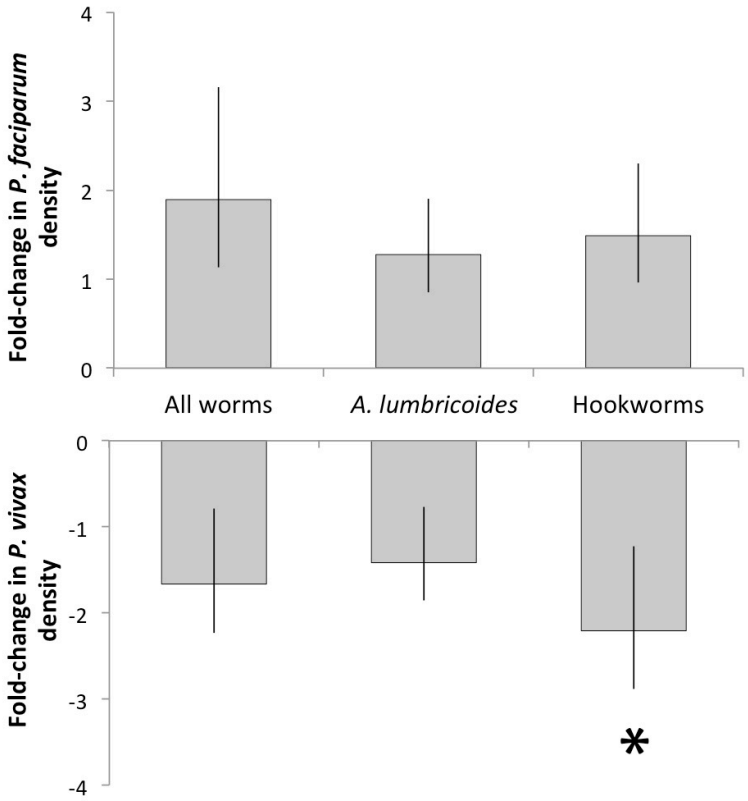

Fold-change in P. faciparum density
4
3
2
1
0
All worms
A. lumbricoides
Hookworms
Fold-change in P. vivax density
0
-1
-2
-3
-4
*

Figure 2.

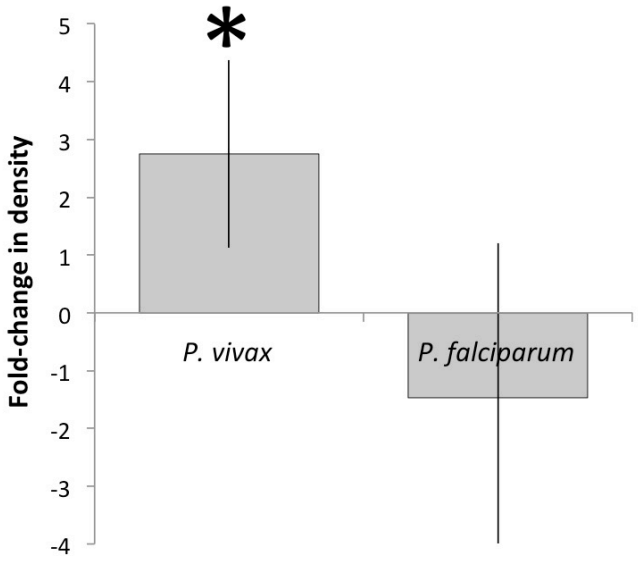


Figure 3.

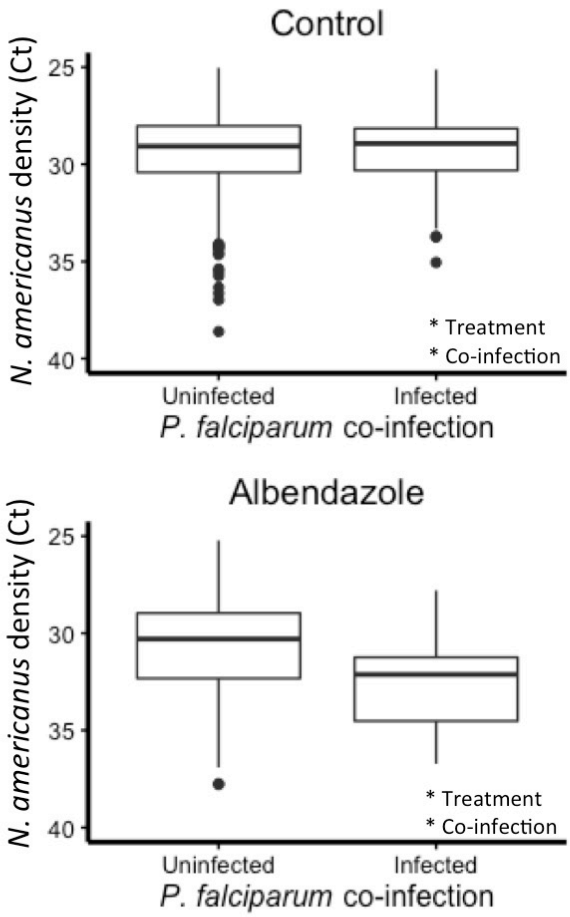

Control
N. americanus density (Ct)
25
30
35
40
* Treatment
* Co-infection
Uninfected
Infected
P. falciparum co-infection
Albendazole
N. americanus density (Ct)
25
30
35
40
* Treatment
* Co-infection
Uninfected
Infected
P. falciparum co-infection

Figure 4.

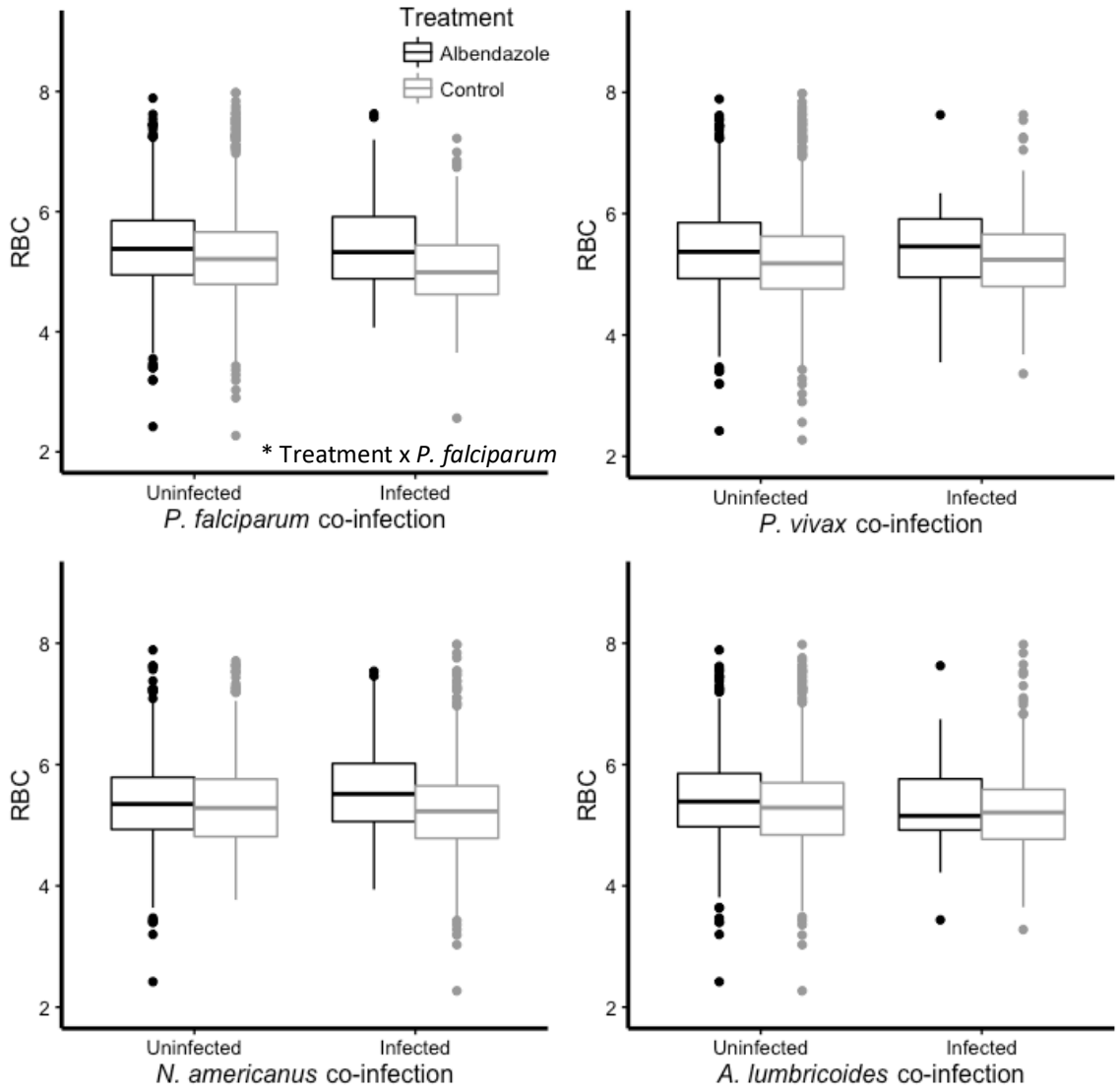
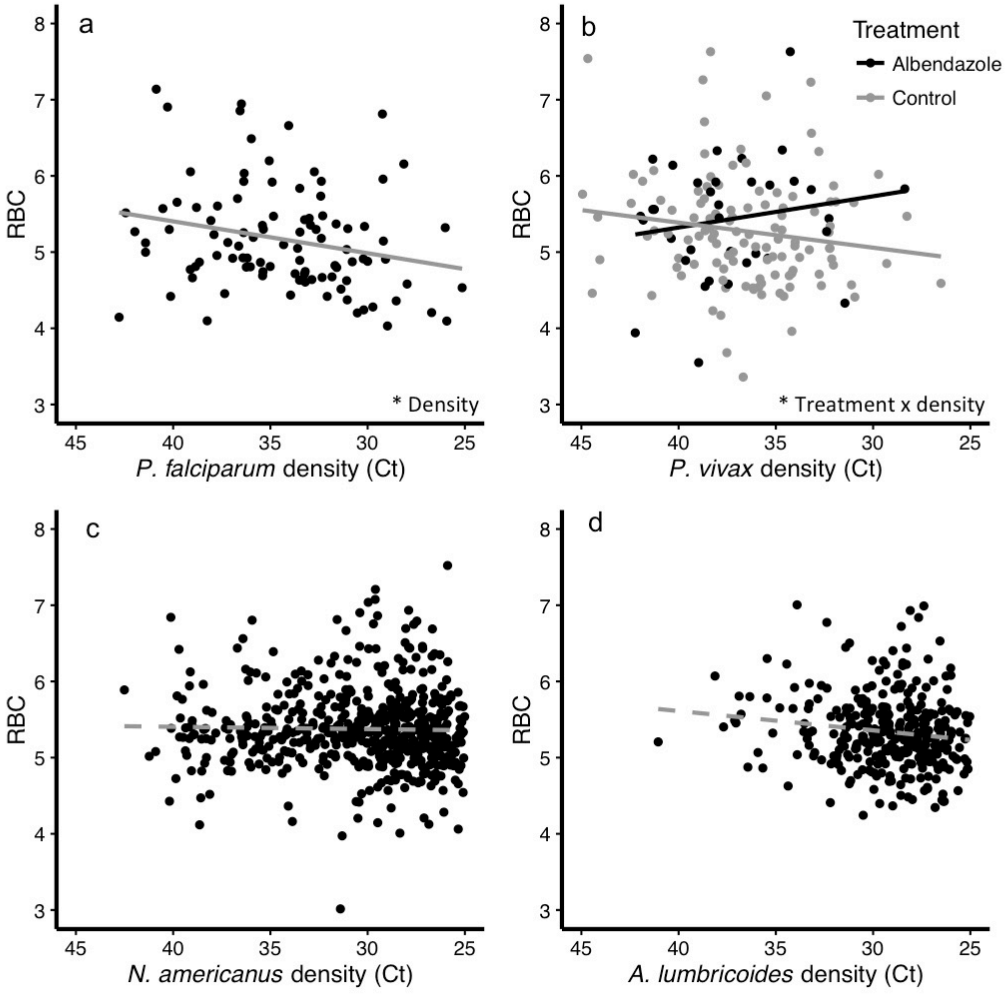

Treatment
Albendazole
Control
RBC
8
6
4
2
* Treatment x P. falciparum
Uninfected
Infected
P. falciparum co-infection
P. vivax co-infection
N. americanus co-infection
A. lumbricoides co-infection

Figure 5.


a
b
c
d
RBC
Treatment
Albendazole
Control
* Density
* Treatment x density
P. falciparum density (Ct)
P. vivax density (Ct)
N. americanus density (Ct)
A. lumbricoides density (Ct)