Within guild co-infections influence parasite community membership: a longitudinal study in African Buffalo

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Abstract

1. Experimental studies in laboratory settings have demonstrated a critical role of parasite interactions in shaping parasite communities. The sum of these interactions can produce diverse effects on individual hosts as well as influence disease emergence and persistence at the population level.
2. A predictive framework for the effects of parasite interactions in the wild remains elusive, largely because of limited longitudinal or experimental data on parasite communities of free-ranging hosts.
3. This four year study followed a community of haemoparasites in free-ranging African buffalo (*Syncerus caffer*). We detected infection by 11 haemoparasite species using PCR-based diagnostic techniques, and analyzed drivers of infection patterns using generalized linear mixed models to understand the role of host characteristics and season on infection

likelihood. We tested for (1) effects of co-infection by other haemoparasites (within guild) and (2) effects of parasites infecting different tissue types (across guild).

4. We found that within guild co-infections were the strongest predictors of haemoparasite infections in the buffalo; but that seasonal and host characteristics also had important effects. In contrast, the evidence for across-guild effects of parasites utilizing different tissue on haemoparasite infection was weak.
5. These results provide a nuanced view of the role of co-infections in determining haemoparasite infection patterns in free living mammalian hosts. Our findings suggest a role for interactions among parasites infecting a single tissue type in determining infection patterns.

Keywords: *Anaplasma*, haemoparasites, parasite ecology, *Theileria*, GLMM

Introduction

In natural populations, most hosts are infected with multiple parasites, most of the time (Fenton 2008, Cox 2001, Petney & Andrews 1998). Recent research in wild mammalian and avian host populations suggests that co-infections can play a central role in driving parasite dynamics (Fenton 2008, Tompkins et al. 2011, Ezenwa & Jolles 2015, Telfer et al. 2008) and infection risk (Telfer et al. 2011, Lello et al. 2004, Pedersen & Fenton 2007).

Formulating a general predictive framework for the direction and strength of parasite interactions is challenging, because of the taxonomic and functional diversity of parasites that infect natural host populations, and the complexity of their interactions with the host's immune system. Examining within-host parasite interactions through the lens of community ecology (Pedersen & Fenton 2007), where interactions among consumer species result from competition

for resources (“bottom-up interactions”) or "predation" by the immune system (“top-down”), has provided a useful starting point for understanding parasite interactions at a functional level.

Like free-living organisms, co-infecting parasites may modify each other’s dynamics through competition for shared resources, such as nutrients or physical space within the host. As such, one might expect competitive interactions between parasites that occur in the same organs and overlap in the resources they extract from the host. For example, studies of malarial parasites in human populations have shown that one of the limiting factors for co-infection with other closely related parasites is competition for red blood cells (Bruce & Day 2003). Similarly, helminthes that feed on blood can suppress population growth of haemoparasites that also rely on red blood cells as their primary food resource (Nacher 2002).

Top-down interactions among parasite species result from the parasites’ interactions with the host’s immune system (Graham 2008). Apparent competition can occur among parasites that are antigenically very similar, via cross-immunity (negative immune mediated interactions; (Cox 2001,Lello et al. 2004, Adams, Anderson &Windon 1989)). By contrast, dissimilar parasite species might facilitate one another if they elicit opposite, mutually incompatible immune responses (co-regulation; (Mosmann & Sad 1996)). This mechanism is particularly important in understanding interactions of macroparasites, such as helminthes, with microparasites like bacteria or viruses. In addition, some parasite species act to suppress host immunity, which may also contribute to facilitation of co-infecting parasite species. For example, immune suppression by the gastrointestinal nematode parasite *Trichostrongylus retortaeformis* increases the abundance of a second helminth species (*Graphidium strigosum*) in European rabbits; and both immune suppression and co-regulation may underlie facilitative effects of *T. retortaeformis* on myxoma virus infection (Cattadori, Boag & Hudson 2008, Boag et al. 2001).

Both resource-based and immune-mediated parasite interactions should generally be stronger among parasites occurring in physical proximity of one another, because of local resource depletion, and localized action of many immune signaling molecules (Murphy & Janeway 2008). Indeed, a recent meta-analysis of human co-infection studies showed that interactions were strongest between pairs of parasites that occupy the same organs within the host (Griffiths et al. 2014). However, few studies have yet compared the direction and strength of interactions among multiple parasite species in the same host population to evaluate the relative importance of resource-based and immune-mediated, localized and systemic interaction mechanisms.

Here, we examine the interactions within a diverse community of tick borne haemoparasites in free-living African buffalo (*Syncerus caffer*). Buffalo are host to a wide variety of infectious organisms, including gastrointestinal helminths and protozoa, ectoparasites such as ticks and mites, and viral and bacterial pathogens (Bengis, Grant & deVos 2006, Anderson & Rowe 1998, Beechler unpublished data, Gorsich et al. 2015). Many of these infections have been comparatively well described, because of concerns over potential spillover to livestock populations (Dion, VanSchalkwyk & Lambin 2011), making buffalo an ideal study system to investigate interactions among parasite and pathogen species in a wild mammalian host.

Our predictions for parasite interactions are summarized in Table 1, and key features of the parasites we included in the study can be found in Table S1. The most common haemoparasites in buffalo fall into two main genera; *Anaplasma* and *Theileria*. *Anaplasma* are obligate, intracellular bacterial pathogens inhabiting red blood cells that replicate by binary fission until they are transmitted to another host by their tick vector (Aubry & Geale 2011).

Table 1. Comparing predicted associations between haemoparasites and the broader community utilizing the predictive framework proposed. '+' = positive association, '-' = negative association, 'Ø' = no association while () indicates a weak effect. A (-) in cross-immunity refers to negative effects due to activation of similar general immune pathways whereas '–' refers to actual cross-immunity due to antigenic similarity among closely related parasite species. White background indicates within the same body compartment while grey indicates across body compartment

	Anaplasma marginale* vs. *Anaplasma centrale	***Theileria* spp. vs. *Anaplasma* spp.**	***Anaplasma* sp. Omatjenne vs. *Anaplasma* spp.**	***Mycobacterium bovis* vs. Haemoparasites**	***Brucella abortus* vs. Haemoparasites**	**Worms vs. Haemoparasites**	**Coccidia vs. Haemoparasites**	**Ticks vs. Haemoparasites**
Resource overlap	–	(–)	Ø	Ø	Ø	(-)	Ø	(-)
Cross immunity	–	(–)	–	(–)	(-)	Ø	(-)	Ø
Co-regulation	Ø	Ø	Ø	Ø	Ø	+	Ø	(+)
Immune suppression	Ø	+	Ø	Ø	Ø	+	Ø	Ø
Vector	+	(+)	Ø	Ø	Ø	Ø	Ø	+
Predicted	-	+	-	(-)	(-)	+	Ø	+

Theileria are obligate, intracellular, protozoan parasites that inhabit two cell types within the vertebrate host; lymphocytes and red blood cells (McKeever 2009). *Theileria* parasites reproduce asexually within host lymphocytes until the lymphocyte bursts, at which point the parasite invades red blood cells for transmission by the tick vector (Bishop et al. 2004). Tick species implicated in vectoring *Anaplasma* and *Theileria* parasites in Southern Africa include several *Rhipicephalus* species (*R. decoloratus*, *R. microplus*, *R. evertsi evertsi*, *R. simus*) and *Hyalomma marginatum rufipes*. In our study population *Amblyomma hebraeum* and two *Rhipicephalus* species have been found to occur commonly (Anderson, Ezenwa & Jolles 2013).

Different haemoparasite species might interact through competition for the cells they use, via the host immune response, or through transmission by the same vector species. Since *Anaplasma centrale* and *marginale* inhabit (and destroy) red blood cells, one would expect

strong negative interactions through resource competition among these congeneric species. *A.* sp. Omatjenne is less well studied than the two other *Anaplasma* species, but appears to concentrate in certain white blood cells (monocytes, neutrophils) and endothelial cells, rather than red blood cells. Resource-overlap between *A.* sp. Omatjenne and the other Anaplasmas should thus be minimal. Theileria parasites primarily utilize white blood cells (lymphocytes), transferring to red blood cells only for uptake by the tick vector. Resource-based interactions between Theileria and *Anaplasma* species are thus expected to be absent or weakly negative. Immunological interactions between *Anaplasma* species should be also strongly negative, due to possible cross-immunity between these potentially antigenically similar congeneric parasites. On the other hand, *Theileria* are not closely related to *Anaplasma*, and cross-immunity in the sense of the host immune system recognizing similar surface markers on both taxa, is unlikely. Weak cross-immunity, if any, is thus expected only as resulting from non-specific activation of inflammatory responses required to curb infection by any intracellular parasite (Th1 responses), including both *Theilerias* and *Anaplasmas* (Aubry & Geale 2011, Mckeever 2009). However, because *Theilerias* utilize and destroy the host's immune effector cells (lymphocytes), they can have significant immunosuppressive effects (McKeever 2009), and might thus facilitate colonization and population growth of *Anaplasmas,* . Finally, *Anaplasma centrale* and *marginale* share a tick vector, which is distinct from the ticks that transmit *A.* sp. Omatjenne. *Theilerias* (Horak, Gallivan & Spickett 2011) utilize both tick species that the different *Anaplasmas* are found in. Based on shared vectors, one might thus expect to see positive associations between *Anaplasma central* and *marginale* , but not *A.* sp. Omatjenne. The *Theilerias* are expected to have weaker positive associations with all of the *Anaplasma* species, based on shared vectors. Observed association patterns are the net effect of these facilitative and competitive interactions, and

common exposure via tick vectors. Thus, overall, we expected to find strong negative associations between the three *Analasma* species, but positive effects of *Theilerias* on *Anaplasmas*.

Other infections tracked throughout the study include; bovine tuberculosis (*Mycobacterium bovis*), which localizes primarily in the respiratory tract, bovine brucellosis (*Brucella abortus*), which infects the host's reproductive and lymph organs; gastro-intestinal (GI) worms (nematodes of the genera *Cooperia* and *Haemonchus* and trematodes (Budischak, Jolles & Ezenwa 2012), *Schistosoma* spp., (Beechler, Ezenwa & Jolles 2015), coccidia, and ectoparasitic ticks (*Rhipicephalus evertsi evertsi, Rhipicephalus appendiculatus, Amblyomma hebraeum)*, (Anderson, Ezenwa & Jolles 2013). Overall, we expected effects of these other parasites on haemoparasites to be less pronounced than interactions among haemoparasites. GI helminths can bias host immunity to an anti-parasite response mediated by type 2 T-helper (Th2) cells (Mosmann & Sad 1996), which is antagonistic to and inhibits the Th1 responses required for effective defense against intracellular parasites such as *Theilerias* and *Anaplasmas*. As such, GI helminth infections might facilitate haemoparasite infections. Finally, we expected that infestation by ticks, which act as vectors for haemoparasites, would be positively correlated with haemoparasite infection likelihood.

Overall, we thus predicted (i) strong competitive interactions between closely related (congeneric) haemoparasites, (ii) facilitation of haemoparasite co-infections due to immunosuppressive effects of *Theileria* parasites, and (iii) stronger interactions within the haemoparasite guild than between haemoparasites and parasites infecting other host organs.

Methods

Sample collection and host data

African buffalo were captured in Kruger National Park (KNP), located in Mpumalanga Province, South Africa. Between June 23 and July 5 2008, 100 adult female buffalo (age 2-5) were captured in the Lower Sabie section in the south of the park, and fitted with radio collars to permit recapture. All buffalo were recaptured biannually from September of 2008 to August of 2012. Captures took place approximately six months apart with buffaloes being chemically immobilized with a mixture of M99 and azaparone. Sample collection took between five and fifteen minutes, after which the animal was reversed with M5050 and naltrexone. Samples collected during immobilization included blood, feces, and photographs to evaluate tick burdens. Parameters collected were age, body condition, horn size, pregnancy status and lactation status, using previously described methods (Jolles, Cooper & Levin 2005). Briefly, blood was collected by jugular venipuncture, collected into 10 mL EDTA coated vacutainer tubes, placed on ice and then transported to Veterinary Wildlife Services (VWS) in KNP. Feces were collected rectally and used for coccidia and intestinal parasite diagnostics. Lactation status was determined by manual milking of all four teats (Aly et al. 2011), and pregnancy status was determined by rectal palpation. This test has 100% sensitivity in Egyptian buffalo (*Bos bubalis)* after 51 days of gestation (Petney & Andrews 1998). Photos were taken of the axial, perianal, and inguinal areas for quantification of tick burdens (Anderson, Ezenwa & Jolles 2013). Age was determined by tooth eruption up to 5 years and then judged by tooth wear (Grimsdell 1973, Jolles 2007). Body condition was determined by palpation of fat reserves on ribs, spine, hips and tail on a scale of 1(emaciated)-5 (excellent) and then averaged. This measure has been used previously in buffalo and correlates well with kidney fat index (Ezenwa, Jolles & O'Brien 2009). Horn width was

measured at the widest portion of the horns (Jolles et al. 2008). Animal protocols for this study were approved by the University of Georgia (UGA), Oregon State University (OSU) and SANParks Institutional Animal Care and Use Committees (UGA AUP A2010 10-190-Y3-A5; OSU AUP 3822 and 4325).

Diagnostics

Methods for macro and microparasite diagnostics have been described previously. Briefly, fecal egg and oocyst counts were used to assess gastrointestinal (GI) nematode and coccidian. A modified McMaster method was used to process the collected feces and assess infection load after feces collection (Ezenwa 2003). Previous work has shown fecal egg count is positively correlated with adult worm burden (Budischak 2014). TB diagnostics were performed using a whole-blood gamma interferon assay (BOVIGAM, Prionics), optimized for African buffalo with a sensitivity of 86% and a specificity of 92% (Michel et al. 2011). Schistosome infection was diagnosed by strip ELISA that detects circulating anodic antigen. This test was performed according to (De Bont et al 1996) and (Agnew et al. 1995) using a UCP strip assay. Brucellosis infection status was diagnosed by ELISA (IDEXX Laboratories, Westbrook, Maine, USA) according to the manufactures protocol and optimized for use in African buffalo (Gorsich et al 2015). Tick burdens were quantified by counting the adult burden of *Rhipicephalus* spp. and *Ambylomma hebraeum* according to (Anderson, Ezenwa & Jolles 2013). Two *Rhipicephalus* species commonly occur in this buffalo population, *R. evertsi evertsi* and *R. appendiculatus* (Anderson, Ezenwa & Jolles 2013). The two *Rhipicephalus* species cannot be distinguished from one another on photographs taken at buffalo capture, so we refer to them collectively as

Rhipicephalus spp. However, *R. evertsi* was previously found to be far more abundant than *R. appendiculatus,* so most of the *Rhipicephalus* counted in this study are likely to be *R. evertsi*.

Molecular Diagnostics

Genomic DNA was extracted from 200 ul of whole blood using Quiagen QuiAmp DNA Mini Kit (QUIAGEN, Valencia, CA, USA) according to manufacturer's instructions. Extracted DNA was stored at -20^{o}C until haemoparasite diagnostics. Diagnostics for *Theileria sp., Ehrlichia sp., Anaplasma sp., Babesia sp.* and, *Hepatozoon sp.* were performed at Department of Veterinary Tropical Disease, University of Pretoria (Pretoria, South Africa) using Reverse Line Blot Hybridization (Gubbels et al. 1995, Chaisi et al. 2011) (RLB hybridization). Briefly a PCR was performed using genus specific primers for *Anaplasma, Theileria* and *Hepatozoon* (Bekker et al. 2002) and species-specific probes (Brothers et al. 2011) for five *Anaplasma* species, three *Ehrlichia* species, 13 *Babesia* species and sub-species, 15 *Theileria* species and sub-species and one *Hepatozoon* genus specific primer. The PCR protocol was performed according to (Nijhof et al. 2005) and probe sequences are presented in Table S2.

Statistical analysis

Effects of season, host characteristics and co-infecting parasites on likelihood (positive/negative) of haemoparasite infections were investigated using generalized linear mixed models (GLMM) with a binomial error structure and a logit link. We focused our analysis of factors contributing to infection patterns of haemoparasites on the three species that showed high though variable prevalence over the study period, *A. centrale, A. marginale, A. sp.* Omatjenne (*A. centrale* range*;* 14.3%-61.2%, *A. marginale* range*;* 20.9%-53.8%, *A. sp.* Omatjenne range;

7.7%-18.7%). The *Theileria* species were extremely common (total range: 61.5%-100%; Fig 1b) and the *Babesia, A. phagocytophilum,* and *E. ruminantium* were extremely rare (*Babesia spp*; 0-2%, *A. phagocytophilium;* 0-1%, *E. ruminantium*; 0-1%, Fig 1b). The *Theileria, Babesia, A. phagocytophilum,*and *E. ruminantium* species were not investigated due to lack of variance. Species were either always present or infrequently present.

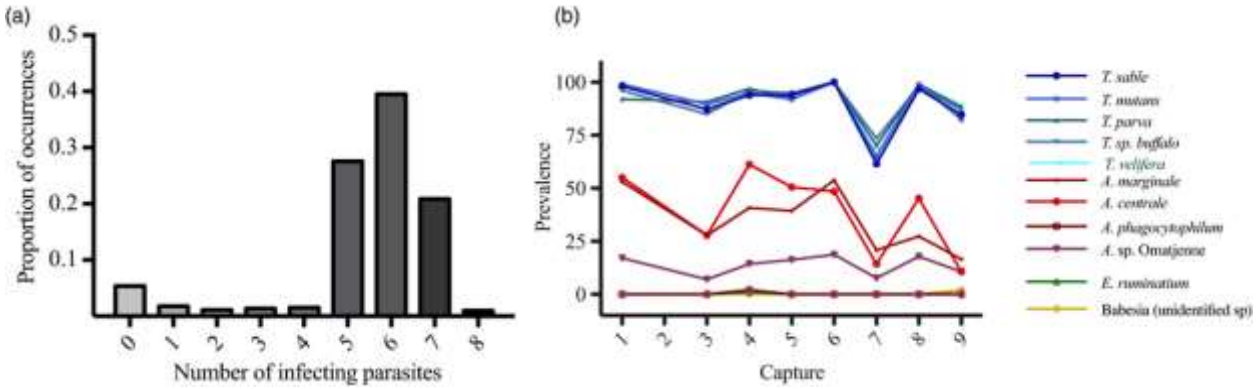

Figure 1. (a) Proportions of co-infections in African buffalo. There were 729 occurrences from 120 unique animal IDs. There were 11 co-infections possible with the following species; *Anaplasma centrale*, *A. marginale*, *A. phagocytophilium*, *A.* sp. Omatjenne. *Ehrlichia ruminantium*, *Theileria* sp. sable, *T.* sp. buffalo, *T. mutans*, *T. velifera*, *T. parva*, *Babesia* spp. (b) Prevalence of blood parasite species in buffalo by capture period. Capture 1 took place in June–July 2008 and the second capture took place over the next 3 months. No blood samples were collected during capture two and blood collection resumed during capture 3. Each re-capture took place over the subsequent 3 months. Each capture period alternates the season the animal was captured in. Capture 1 (June–July 2008) was during the dry season, Capture 2 was during the wet season and so on.

Infection status for three focal haemoparasite species was examined as a dependent variable, and animal ID was included as a random effect, reflecting the longitudinal nature of our dataset, where each animal was re-captured every six months for four years. In addition to the three parasites used as dependent variables, all haemoparasites were included as independent variables with the following exceptions: Only one *Theileria* species was included as an independent variable in our models, because occurrence of all five *Theileria* species detected in the buffalo were highly correlated within individual hosts, and could not be considered

independent variables (Table S3). We picked *T. parva* as our representative *Theileria* species, because it is the best-studied of the *Theileria* species present, due its role as the main *Theileria* pathogen of concern in livestock in southern Africa. Three parasites (unidentified *Babesia, A. phagocytophilium, E. ruminantium)* were dropped because they de-stabilized the models, due to their extreme rarity in the dataset. In addition, samples collected during capture 2 were not available for analysis due to lack of available sample.

Independent variables were added into the model as main effects only: season, animal traits (age, horn width, body condition, reproductive status), and co-infection by other haemoparasites (*Anaplasma centrale, A. marginale, A. sp. Omatjenne, Theileria parva),* ectoparasites (ticks: *Amblyomma hebraeum, Rhipicephalus* spp.), gastro-intestinal parasites (strongyle worms, Coccidia, Schistosomes), and systemic bacterial pathogens *Mycobacterium bovis* and *Brucella abortus*. Season was coded as a two level binary variable (wet= October – March; dry= April - September). Horn width is a proxy for individual quality in buffalo, which has been shown to be associated with parasite burden (Ezenwa & Jolles 2008). Because buffalo horns grow throughout the lifetime of the animal, horn width was regressed against age and the residuals used in our analyses. Infection statuses for bTB, all blood parasites, and brucellosis were coded as binary variables (positive/negative). Schistosome antigen titer, coccidian oocysts/gram and strongyle eggs/gram were log-transformed to account for aggregated parasite distributions, and coded as continuous variables. Making use of our longitudinal sampling design, we considered potential effects of both simultaneous co-infections and infections during the prior time step by including an independent, binary variable to represent previous haemoparasite infection (i.e. haemoparasite infection at time *t* might be associated with concurrent co-infections at time *t*, or prior infection at time *t-1*.) Tick data were only collected on

a subset of the study population. As such, the effects of ticks on *A. centrale, A. marginale* and *A. sp.* Omatjenne infection were investigated by performing a separate set of models using the same modeling approach with the appropriate subset of observations.

Model selection was performed to minimize AIC but if models were within 2 points of each other, the most parsimonious model was selected. Initial demographic model selection was done by forward selection with Animal ID as a forced random term. Independent predictors considered were season, age, condition, horn width residuals, and reproductive status. Using the final demographic model from each parasite, independent parasite predictors (GI helminths, bTB, brucellosis, and other haemoparasites) were added using forward selection to select the most parsimonious model with the lowest AIC. A Bonferroni correction for multiple tests was used to correct for type one error present when using multiple tests (statistical significance adjusted from $p<0.05$ to $p<0.0125$). All statistical analyses were performed using R, v.3.0.1 (R core Team 2013) with the *lme4* package (Bates et al. 2013).

Results

1. Parasite epidemiology

Overall, buffalo were re-captured between two and seven times at six month intervals for a total of 729 samples from 120 buffalo (capture 1 n= 87; capture 3 n= 86 ; capture 4 n= 99; capture 5 n=93 ; capture 6 n= 84; capture 7 n=91; capture 8 n= 91; capture 9 n= 98). We detected the following eleven haemoparasites: *Anaplasma centrale, A. marginale, A.* sp Omatjenne, *Ehrlichia ruminantium*, *Theileria parva, T. mutans, T.* sp (buffalo), *T.* sp (sable), *T. velifera*, and a *Babesia* species. *A. phagocytophilum* and *Babesia sp.* had not been detected in wild buffalo previously. No *Hepatozoon sp.* was detected at any time period. The majority of infections

detected were co-infections, with few buffalo having no parasites and the majority harboring a diverse community at every time point (Fig 1a). The median number of co-infecting haemoparasites was 6 (range: 0-8).

Anaplasma centrale, *Anaplasma marginale*, *Anaplasma sp.* Omatjenne were detected at intermediate and variable prevalences over time (*A. centrale;* 14.3%-61.2%, *A. marginale;* 20.9%-53.8%, *A. sp.* Omatjenne; 7.7%-18.7%; Fig 1b). The prevalence of *A. centrale* was far more variable over time than the prevalence of *A. marginale*, even though the mean prevalence of the two parasites was quite similar. *A. sp.* Omatjenne maintained a consistent low prevalence in the study group of buffalo (Fig 1b).

Three haemoparasite species occurred only once across all capture periods. In May-August 2012, two buffalo were found to be positive for the *Babesia spp.* catchall probe and in November 2009 – February 2010, two buffalo were positive for *Anaplasma phagocytophilum* and one buffalo was positive for *Ehrlichia ruminantium* (Fig 1b).

2. Factors structuring parasite community

2.1 Within Guild Associations

Haemoparasite infection patterns were found to be strongly influenced by other co-infecting haemoparasites after controlling for host characteristics and season (Table S4). The odds of *A. centrale* infection were decreased when co-infected with *A. sp.* Omatjenne (OR= 0.34 p value= 0.0015, Table S4), but increased almost seven-fold when co-infected with *T. parva* (OR=6.62 p value= 0.0063, Table S4). Similarly, odds of *A. marginale* infection were decreased in hosts co-infected with *A. sp.* Omatjenne (OR=0.41 p value= 0.0071, Table S4) and greatly increased when co-infected with *T. parva* (OR=3.71 p value= 0.010, Table S4). Odds of *A. sp.* Omatjenne

infection were decreased when currently co-infected with *A. centrale* and *A. marginale* (OR= 0.34 p value= 0.00062, OR= 0.45 p value= 0.018, Table S4). These associations are summarized in Fig 2.

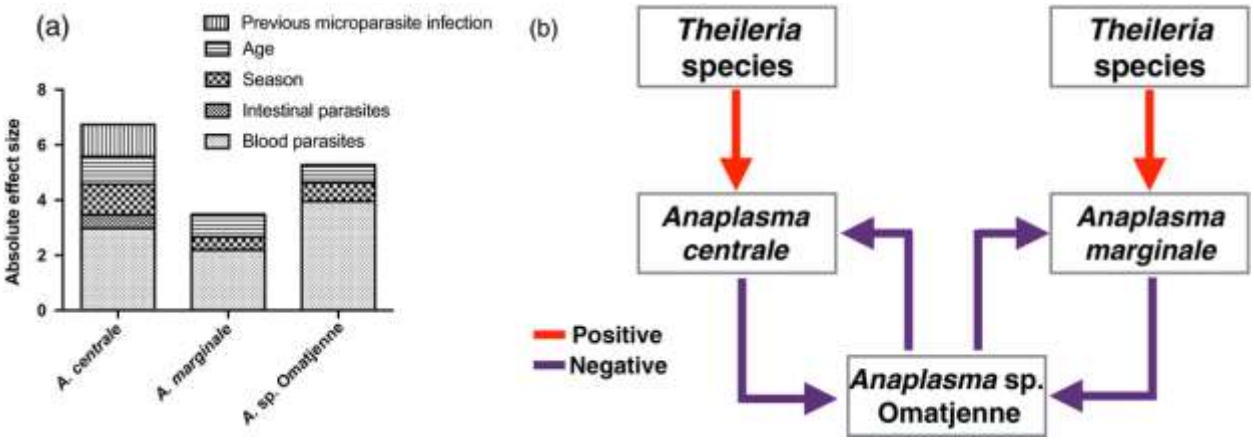

Figure 2. (a) Comparing the absolute standardized effect sizes for each significant predictor on outcomes of haemoparasite infection taken from Table S4. Standardized effect size calculated by dividing continuous predictors (age and coccidia burdens) by two standard deviations while not transforming the binomial predictors. Only significant effects are included. Marginally significant estimates are not included. Each group of parasites is the sum of the absolute value of the estimates from the GLMM. (b) With in guild haemoparasite interactions between *Anaplasma centrale*, *Anaplasma marginale*, *Anaplasma* (*Ehrlichia*) spp. Omatjenne, and *Theileria parva*. Red arrows indicate a positive interaction, purple arrows indicate a negative interaction.

There were no statistically significant influences of haemoparasites at the previous time step on the likelihood of infection at the current time step. Previous infection with *A. marginale* did provide explanatory power to the model for *A. centrale* and was retained during model selection, but was not significant after accounting for multiple tests (Table S4).

2.2 Across Guild Interactions

Ectoparasites

Abundance of the ticks *Rhipicephalus* spp. and *Ambylomma hebraeum* at the current time step always added explanatory power to the models but never had statistically significant effects on haemoparasite infection status (Table S4).

Gastrointestinal parasites

As the number of coccidian oocysts per gram increased, the odds of concurrent infection with *A. centrale* increased significantly (OR=1.14 p value= 0.0028, Table S4). The amount of circulating schistosome antigen at the current time step added explanatory power to the models but was never significant (Table S4)

Bacterial infection of the respiratory and reproductive tracts

Current infection with bTB was eliminated from all three models during the selection process. Current infection with brucellosis added explanatory power to the models for all three focal haemoparasite species but was never statistically significant (Table S4).

Previous bTB infection was never a significant predictor for focal haemoparasite infections but added explanatory power to the models for *A. marginale* and *A. sp.* Omatjenne. Previous brucellosis infection added explanatory power to the models for *A. centrale* and *A. marginale* but was never significant (Table S4).

2.3 External Factors

Season was always a significant predictor with odds of infection always increasing greatly during the wet season for *A. centrale, A. marginale* and *A. sp.* Omatjenne (*A. centrale:* OR= 3.35 p value= <0.0001, *A. marginale:* OR=1.63 p value= 0.012, *A. sp.* Omatjenne: OR= 2.05 p value=0.0089, Table S4). With each year increase in age, the odds of infection with *A. centrale* and *A. marginale* decreased slightly but significantly (*A. centrale:*OR= 1.02 p value= 0.0002 *A. marginale:* 1.02 p value= 0.00064, Table S4) Condition added explanatory power to the model for *A. centrale* but was never significant (Table S4).

Discussion

This longitudinal study followed haemoparasite infections in 120 buffalo over a four year time period, detecting eleven species of haemoparasite, two of which had not been described in buffalo before (*Anaplasma phagocytophilum* and an unknown species of *Babesia*). Five blood parasite species (*Theilerias*) were almost universally present in most buffalo while three species showed intermediate but variable prevalence in the study population over time. Three species (*A. phagocytophilum, Babesia spp., E. ruminantium*) only occurred during a single time period. Focusing on the three species with variable prevalence (*A. centrale, A. marginale,* and *A. sp.* Omatjenne) we discovered that co-infections by other haemoparasites were the most consistent and significant predictors for infections with the all of the focal *Anaplasma* species (Fig 2a). Consistent with our predictions, pairwise interactions between *A. sp.* Omatjenne and each of the other two Anaplasma species were strongly negative, and immunosuppressive *Theileria* showed facilitative effects on each *of* the focal *Anaplasmas*. However, counter to our expectations, we did not detect a negative association between *A. centrale* and *A. marginale*. Season, age and body condition also affected likelihoods of infection for our focal haemoparasites but there was little evidence of associations with macro- and microparasites infecting other organ systems than the blood.

Within Guild Co-infections

We observed strong interactions among haemoparasites, and overall, the direction of these effects was in accordance with predictions based on resource overlap and immune-mediated interactions. However, some of the interactions, or lack of interactions were not as

expected. Specifically, we did not detect strong reciprocal negative effects of *A. centrale* and *A. marginale*.

Two of our focal parasites, *A. centrale* and *A. marginale,* have been comparatively well studied, whereas *A.* spp. Omatjenne is a newly described strain, the phylogenetic and host relationships of which have not been studied in detail. *A. centrale* is very closely related to *A. marginale* (Kocan et al. 2010) and has been used as a live attenuated vaccine against the more virulent *A. marginale* (de la Fuente et al. 2005). Both parasites inhabit erythrocytes and replicate by binary fission (Aubry & Geale 2011). Based on these similarities, we expected strong negative interactions between *A. centrale* and *A. marginale* due to resource competition and cross immunity (Aubry & Geale 2011)(Table S4). Our findings show that this is not the case, with no effects of *A. centrale* infection on the probability of *A. marginale* infection and vice versa. It is possible that resources might not be limiting in the host because the resupply rate of erythrocytes outstrips use by the parasites when the host is able to limit parasite replication and maintain resource supply. The expectation of strong interactions between closely related species may be misleading for congeners that coexist within the same host compartment. Closely related species occupying the same habitat should either exclude one another, or evolve to occupy different ecological niches within their shared environment, effectively circumventing competition (Rohde 1979, Ravigne, Dieckmann, & Olivieri 2009). Thus, if *A. centrale* and *A. marginale* share an evolutionary history of occupying buffalo red blood cells, and still co-occur commonly in the same host individuals, perhaps we should not expect them to interact strongly. Strong negative interactions between similar parasite species might more reasonably be expected where endemic parasites are confronted with closely related emerging parasites that are novel in the context of the particular host-parasite system under study.

In line with expectations, we did observe consistently negative associations between the *A. centrale* / *A. marginale* and *A. sp.* Omatjenne (Table S4, Fig 2). While little is known about the life cycle and host-parasite interactions of *A. sp.* Omatjenne, it is considered to be an apathogenic variant of *E. ruminantium* (Allsopp et al. 2007). Parasites in the genus *Ehrlichia* use monocytes, neutrophils and endothelial cells in blood vessels to evade the immune system and replicate within intracellular vacuoles (Allsopp 2010). The observed negative associations may thus not result from competition for host resources. Instead, they may be due to activation of immune pathways that limit the replication of both pathogens. The *Anaplasma spp* and *A. sp.* Omatjenne are placed within the family Anaplasmataceae and are controlled by similar inflammatory responses (Aubry & Geale 2011). Primary infection by *A. marginale* and *A. centrale* may thus increase the inflammatory response, which could accelerate the removal of invading *A. sp.* Omatjenne (Liebenberg et al. 2012). In addition, the most common tick species seen on our study buffalo, *A. hebraeum*, is the dominant vector for another haemoparasite, the pathogenic *E. ruminantium* (Norval & Horak 2004). As such, we expected to see a positive association between *A. hebraeum/E. ruminantium* (Table 1). Despite high *A. hebraeum* burdens, we only found a single instance of *E. ruminantium* infection. Our study thus suggests that unlike cattle, buffalo populations may not maintain *E. ruminantium* infections, and that *E. ruminantium* infections in buffalo may most often be due to spillover from cattle. The herd examined was largely confined to the interior of the park with animals not traveling to the edges of the park, where they are more likely to break out or mix with domestic animals that enter the park. (Spaan 2015). Sampling herds that mix with cattle more frequently could address the hypothesis that *E. ruminantium* in buffalo results primarily from contact with ticks infected by cattle.

Theileria parva had striking positive effects on two focal haemoparasites, suggesting facilitation of *A. centrale* and *A. marginale* by *T. parva* or associated *Theilerias*. *T. parva* inhabits two cell types in the vertebrate host, lymphocytes and erythrocytes. The parasite invades lymphocytes and reproduces asexually until the lymphocyte bursts, releasing merozoites to invade erythrocytes for uptake and transmission by the tick vector (Bishop et al. 2004). The destruction of lymphocytes during *T. parva* replication could result in facilitation of other haemoparasites (McKeever 2009). In addition, a recent observational study suggests that *T. parva* actively suppresses the inflammatory response needed to combat other haemoparasite infections (Okagawa et al. 2012) Lymphocyte depletion and suppression of inflammatory responses may both contribute to the observed positive associations between *Theileria* and *A. centrale* and *A. marginale* (Table S4, Fig 2).

In cross-sectional parasite community datasets, positive and negative correlations between parasites species can occur due to correlated exposure and/or variation in host susceptibility that affects multiple parasites, rather than interactions among the parasites themselves. In our longitudinal dataset, observed associations between haemoparasites are based on temporal concurrence or asynchrony of infections, and are thus less likely to result from fixed (including genetic) variation in host susceptibility. We attempted to control for effects of correlated exposure in our analyses by including tick burdens as explanatory variables. Moreover, many of the observed associations between haemoparasites were negative, making correlated exposure implausible as a mechanism underlying these patterns. In addition, we controlled for host body condition, reproductive status and season to account for some of the mechanisms that might underlie temporal variation in host susceptibility. Following other longitudinal wildlife infection studies (Telfer et al. 2010); we thus interpret associations between

haemoparasites, to occur, at least in part, due to interspecific interactions among parasites. To conclusively infer interactions between haemoparasite species, experimental infections or parasite removals would be needed. Experimental parasite removal studies in wildlife have been conducted by treating hosts with anthelminthic drugs, effectively reducing GI parasite burdens (Fenton, Viney & Lello 2010, Knowles et al. 2013, Pedersen & Antonovics 2013). Some bacterial haemoparasite infections can be treated with antibiotics (Bowman 1999), but protozoan haemoparasites are considerably more difficult to remove (Bowman 1999). In addition, tick burdens can be controlled with acaricides, limiting transmission of haemoparasites for brief periods of time (Norval & Horak 2004). Bacterial haemoparasite and tick removal studies may thus present a way forward for studying the complex interactions among this group of parasites.

Between Guild Interactions

We detected no significant associations between bTB infection, gastrointestinal helminthes, schitosomes or ticks with haemoparasites. We did observe a single cross compartment association, where the number of coccidia increased the likelihood of infection with one of our focal haemoparasites, *A. centrale*. Coccidia are microparasites inhabiting the intestinal wall of the vertebrate host (Bowman 1999); as such, we did not expect detectable interactions between coccidia and *Anaplasmas,* based on resources or immunologic mechanisms. The observed association between coccidian and *A.* central might be mediated indirectly, via behavioral or physiological host traits. For example, low-ranking buffalo may be forced to graze less desirable areas including those contaminated by high tick burdens and fecal matter. However, in this case one would expect to see additional positive interactions between other GI parasites and tick-borne haemoparasites. Additionally, coccidia have been shown to degrade host

condition in many species, including cattle (Bowman 1999). Perhaps coccidian infection results in a loss of body condition leading to an energetic deficit and the inability of the host to control a secondary *A. centrale* infection. However, in this case one would expect to see associations between poor condition and *A. centrale*. Very little is yet known about effects of coccidian infection on red blood cell parameters and immune responses in African buffalo. Experimental manipulation of *A. centrale* and coccidian infection status would be needed to trace the mechanisms underlying this enigmatic interaction.

Overall, associations between haemoparasites and parasites that use host lung, gastrointestinal, skin or reproductive tissue were only weakly supported in this study. These results concur with findings presented in a recent meta-analysis of human co-infection studies, which suggested that interactions are strongest within guilds of parasites that occupy the same organs within the host (Griffiths et al. 2014). Our study is significant in this context, because to our knowledge, it is the first study to assess the relative importance of interactions within and between different parasite guilds , within a single host population through time.

Based on this study of microparasitic and macroparasitic infections in African buffalo, a framework for predicting parasite interactions according to (i) resource use, (ii) interactions with the host's immune system, and (iii) location of infection within the host, performs well in explaining broad patterns of parasite co-occurrence. However, this simple framework was unable to predict some pairwise parasite interactions accurately. We conclude that, for a finer-scale predictive understanding of parasite interactions, refinements to the existing framework will be needed. For example, we might need to take account of shared evolutionary history and niche partitioning among sympatric parasites, as well as a more nuanced view of parasite effects on host immune function. More generally, our study illustrates how parasite communities within

hosts can present tractable study systems to test ideas in community ecology: Hosts are clearly distinct, replicated habitat patches for observing species interactions, animal physiology provides methods for quantifying habitat traits relevant to the interacting species, and behavioural studies can yield information about connectivity of habitat patches. The added layer of immune-mediated parasite interactions differs from the ecology of free-living species – offering opportunities to extend our frameworks for understanding species interactions to accommodate the nestedness of life within life.

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Supplementary Information

Table S1: Summary information for each parasite included in our study. Due to the diversity and complexity of various immune effectors, immunity is only considered to the cross regulatory Th1/Th2 level. Information is based on the best information available for cattle and African buffalo.

Parasite	Parasite Type	Cell type utilized	Vertebrate Immunity	Transmission	Citation
Hemoparasites					
Anaplasma centrale	Bacterium	Red blood cell	Th1	*R. evertsi evertsi*	Kocan *et al* 2010, Mckeever 2006
Anaplasma marginale	Bacterium	Red blood cell	Th1	*R. evertsi evertsi*	Kocan *et al* 2010, Mckeever 2006
Anaplasma (sp) Omatjenne	Bacterium	Endothelial cell	Largely unknown, likely Th1	*A. hebraeum*	Mckeever 2006, Allsopp *et al* 1997
. *Theileria spp*	Protozoan	Lymphocytes and red blood cells	Th1	*A. hebraeum* *R. evertsi evertsi*	Bishop *et al* 2004, Mckeever 2006
GI parasites					
Strongyle species	Strongyle worm	Gut lumen	Th2	Fecal contaminated food	Pedersen and Antonovics 2013, Cox 2001
Coccidia species	Protozoan	Gut epithelium	Th1	Fecal contaminated food	Pedersen and Antonovics 2013
Schistosome species	Trematode worm	Mesenteric arteries	Acute: Th1 Chronic: Th2	Contact with infected water	Pearce and MacDonald 2002
Other Microparasites					
Brucella abortus	Bacterium	Mammary tissue	Th1	Aborted fetus and associated discharge	Gorsich *et al* 2015
Mycobacterium bovis	Bacterium	Alveolar tissue	Th1	Infected sputum	Ezenwa *et al* 2010
Ectoparasites					
Amblyomma hebraeum	Ticks	Skin feeding on blood	Innate Immunity	Questing ticks	Norval and Horak 2004
Rhiphecephalus evertsi evertsi	Ticks	Skin feeding on blood	Innate Immunity	Questing ticks	Norval and Horak 2004

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Table S2:Genus and species specific probes used in this study a Symbols used to indicate degenerate positions: R = A/G; W = A/T;Y = C/T

Genus/species	Oligonucleotide Probe sequence (5`-3`)
Ehrlichia/Anaplasma genus-specific	GGG GGA AAG ATT TAT CGC TA
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A
Anaplasma centrale	TCG AAC GGA CCA TAC GC
Anaplasma marginale	GAC CGT ATA CGC AGC TTG
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG
Anaplasma sp. (Omatjenne)	CGG ATT TTT ATC ATA GCT TGC
Theileria/Babesia genus-specific	TAA TGG TTA ATA GGA RCR GTT G
Theileria genus-specific	ATT AGA GTG CTC AAA GCA GGC
Babesia genus-specific 1	ATT AGA GTG TTT CAA GCA GAC
Babesia genus-specific 2	ACT AGA GTG TTT CAA ACA GGC

Babesia bicornis	TTG GTA AAT CGC CTT GGT C
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT
Babesia canis canis	TGC GTT GAC GGT TTG AC
Babesia canis rossi	CGG TTT GTT GCC TTT GTG
Babesia canis vogeli	AGC GTG TTC GAG TTT GCC
Babesia divergens	ACT RAT GTC GAG ATT GCA C
Babesia felis	TTA TGC GTT TTC CGA CTG GC
Babesia gibsoni	CAT CCC TCT GGT TAA TTT G
Babesia major	TCC GAC TTT GGT TGG TGT
Babesia microti	GRC TTG GCA TCW TCT GGA

Table A2: Continued

Babesia sp. (sable)	GCG TTG ACT TTG TGT CTT TAG C
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG
Theileria annulata	CCT CTG GGG TCT GTG CA
Theileria bicornis	GCG TTG TGG CTT TTT TCT G
Theileria buffeli	GGC TTATTT CGG WTT GAT TTT
Theileria equi	TTC GTT GAC TGC GYT TGG
Theileria lestoquardi	CTT GTG TCC CTC CGG G
Theileria mutans	CTT GCG TCT CCG AAT GTT
Theileria ovis	TTG CTT TTG CTC CTT TAC GAG
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria separate	GGT CGT GGT TTT CCT CGT
Theileria taurotragi	TCT TGG CAC GTG GCT TTT
Theileria velifera	CCT ATT CTC CTT TACGAG T
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
Theileria sp. (kudu)	CTC CAT TGT TTCTTT CCT TTG
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C

Hepatozoon genus-specific	GCT TTG TAA TTG GAA TGA TAG A

Table S3: Chi square tests for independence between *Theileria* species

Combinations	Chi Squared Statistic	Degrees of freedom	p value
T.mutans/T.parva	39.86	728	1
T. mutans/T. sp. buffalo	38.33	728	1
T. mutans/T. sp. sable	43.99	728	1
T. mutans/T. velifera	40.39	728	1

Table S4: Associations between *A. centrale* (n=562 observations), *A marginale* (n=566 observations), *A.* sp. Omatjenne (n=561observations), and the broader parasite community with 120 unique animal ID's. Tick data was available on only a small subset of the study group so separate analysis were performed with the predictors from each final model (n=254 observations from 114 animals) Generalized Linear Mixed Models (GLMM) fit by Maximum Likelihood estimate were used. $^{*}<0.05$, $^{**}<0.01$, $^{***}<0.0001$

	A. centrale			*A. marginale*			*A sp. Omatjenne*		
Blood parasites	Est.	Z value	Effect	Est.	Z value	Effect	Est.	Z value	Effect
A. centrale							-1.09	-3.47**	↓
A. marginale	0.30	1.34					-0.79	-2.41**	↓
A. marginale $_{t-1}$							-0.56	-1.85	
A. sp. Omatjenne	-1.08	-3.23**	↓	-0.88	-2.69**	↓			
T. parva	1.85	3.13**	↑	1.31	2.57**	↑	1.49	1.98	
GI parasites									
Coccida species (oocysts/gram)	0.13	2.99**	↑						
Strongyle species (eggs/gram)	-0.093	-1.98					-0.076	-1.30	
Schistosomes (circulating antigen)	-0.002	-1.32		-0.00003	-0.017		0.0018	1.23	
Other Microparasites									
TB_{t-1}				-0.078	-0.37		0.087	0.32	
Brucellosis	0.68	1.28		-1.061	-1.78		0.11	0.42	
Brucellosis$_{t-1}$	-1.18	-2.18*		1.15	1.91				
Ectoparasites									
A. herbraeum (burdens)	-0.0025	-1.47		0.00055	0.63		-0.0004	-0.71	
R. evertsi evertsi (burdens)	0.014	1.27		0.0029	0.49		-0.001	-0.24	
Host and environmental factors									
Season (wet)	1.15	5.2***	↑	0.49	2.51*	↑	0.70	2.54*	↑
Age	-0.018	-3.78***	↓	-0.015	-3.41***	↓	-0.012	-2.12*	↓
Condition	0.30	1.69							