Complex interactions within the ectoparasite community of the eastern rock sengi (*Elephantulus myurus*)

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ABSTRACT

Concomitant infection with more than one parasite species is the rule in nature. Since co-infecting parasites are exploiting the same host, interspecific interactions at the infracommunity level are likely. The nature of such interactions can be expected to affect the distribution of parasites within host populations. Intraspecific interactions within the infracommunity are not easily discernible from cross-sectional studies and the focus of most of these studies lies on relationships between endoparasitic micro- and macroparasites. In the current study of the ectoparasite community of wild eastern rock sengis (*Elephantulus myurus*) we experimentally reduced tick and flea infestations and monitored ectoparasite burdens over the course of three years. We found a number of within-taxon facilitating interactions between tick species that might be the result of decreasing immune responses with increasing tick burden. In contrast, inter-taxon relationships appeared to be dominated by antagonistic relationships likely to be linked to competition over feeding sites. Only one of the observed interspecific interactions was reciprocal. Our experimental manipulation revealed additional antagonistic relationships that cross-sectional studies would not have captured. In addition, we found substantial long-term changes in the sengi ectoparasite community as a result of our experimental manipulation suggesting carry-over effects of our treatment. This study is the first that evaluates interspecific interactions within the entire ectoparasite community exploiting a mammalian host in Africa and highlights the complexity of interspecific interactions within an ectoparasite community.

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1. Introduction

The distribution of parasites across a host population tends to be highly skewed with a small proportion of the host population sustaining the majority of the parasite population (Woolhouse et al., 1997; Wilson et al., 2002; Poulin, 2007). Such asymmetries in parasite distribution are thought to be a result of individual differences between hosts in the exposure and susceptibility of hosts to parasites (Wilson et al., 2002). Abiotic factors such as seasonal variation in rainfall and temperature can affect developmental rates and survival of parasites in the environment but also the availability of resources for maintenance and reproduction of hosts and frequently results in seasonal variation in parasite burden (Altizer et al., 2006). Similarly, sex-specific strategies to maximize survival and reproductive output can result in differential resource allocation strategies into maintenance, reproduction and mate searches. Such

differences can be expressed as dimorphism in body size or sexual ornaments, ranging behaviour and immune function, all of which have been linked to sex biases in parasite burden (Moore and Wilson, 2002; Rolff, 2002; Wilson et al., 2002; Klein, 2004).

Much research has been dedicated to exploring the contributions of these abiotic and biotic factors on parasite burden and the vast majority of these focussed on a single parasite species. However, hosts are usually infested with more than one parasite species (Petney and Andrews, 1998; Behnke et al., 2001; Cox, 2001). Parasite species exploiting the same host can be expected to interact with each other like species of other ecological communities. Such interactions may be either through direct interference or indirect such as via competition for host resources (bottom-up regulation) or immune mediated (top-down regulation) and may be facilitating or antagonistic (Pedersen and Fenton, 2007). The nature and outcome of such interspecific interactions can be expected to contribute to the distribution of parasites within a host population and should be considered when exploring parasite distributions.

Although the number of studies providing evidence for interspecific interactions in parasite communities is increasing, they are largely biased towards those investigating interactions between helminths and microparasites (Lello et al., 2004; Cattadori et al., 2008;

Jolles et al., 2008; Ezenwa et al., 2010; Telfer et al., 2010; Moreno et al., 2013). This might be partially attributable to the comparatively well understood antagonism between host immune responses against these two groups of parasites (Graham, 2008; Tompkins et al., 2011). In contrast, possible interactions between members of the ectoparasite community living on a host have received much less attention and even fewer of these explore interactions between different ectoparasite taxa (Andrews and Petney, 1981; Andrews et al., 1982; Baer-Lehman et al., 2012; Pollock et al., 2012; Anderson et al., 2013).

Irrespective of the taxa studied, the vast majority of research into interspecific interactions between parasites relies on cross-sectional and observational data (Andrews and Petney, 1981; Lello et al., 2004; Ezenwa et al., 2010; Baer-Lehman et al., 2012; Moreno et al., 2013). Inferring interspecific interactions based on these data may result in erroneous conclusions regarding the prevalence and nature of such interactions due to confounding effects of similar temporal exposure or transmission routes (Fenton et al., 2010, 2014; Viney and Graham, 2013). In contrast, the selective experimental removal of certain parasite species from the community can reveal relationships not apparent in observational data (Hudson et al., 1998; Pedersen and Greives, 2008). Combining experimental manipulation with longitudinal sampling can help to elucidate the processes leading to the outcome documented in cross-sectional studies (Viney and Graham, 2013). Such approaches remain rare but were employed by two recent longitudinal studies of small mammal parasite communities (Knowles et al., 2013; Pedersen and Antonovics, 2013). By inducing perturbations through the application of an anti-helminthic drug these studies provided evidence for competitive interactions between species of the endoparasite community of two small mammal species of the northern hemisphere. In addition, one of these studies illustrated the transient nature of such perturbations and antagonistic relationships were only apparent for a short period (Knowles et al., 2013).

In the current study we employed experimental removal of target parasite taxa (i.e. ticks and fleas) to study interspecific relationships in the ectoparasite community of eastern rock sengis (*Elephantulus myurus*) in South Africa. Eastern rock sengis, also known as elephant shrews, belong to the order Macroscelidaea that comprises 19 species (Dumbacher et al., 2014) that are endemic to Africa. All members of this order lack a sexual dimorphism, are considered monogamous with overlapping home ranges within pairs and rely on an insectivorous diet (Rathbun, 1979; FitzGibbon, 1997; Skinner and Chimimba, 2005; Rathbun and Rathbun, 2006; Schubert et al., 2009). A large diversity of ectoparasitic arthropods has been recorded for sengis mostly comprising ticks, fleas and mites, but also a single louse species (*Neolinognathus elephantuli*) (Fourie et al., 1995, 2002, 2005; Segerman, 1995; Beaucournu et al., 2003; Harrison et al., 2011). Eastern rock sengis range in mass between 40 and 80 g. Their distribution extends from Mozambique north of the Zambezi River throughout the southern and eastern parts of Zimbabwe, eastern Botswana, wide parts of north-east South Africa as well as western Swaziland (Skinner and Chimimba, 2005). Rock sengis preferentially inhabit rocky outcrops and may be active during both day and night (Skinner and Chimimba, 2005). Their ectoparasite community is dominated by immatures of a large number of tick species (Fourie et al., 1995; Harrison et al., 2011; Horak et al., 2012). However, the dominant tick species is usually *Rhipicephalus warburtoni* which far outnumbers other tick species (Harrison et al., 2011; Lutermann et al., 2012a; Fagir et al., in press). In addition, several flea and mite species as well as *N. elephantuli* have been reported for *E. myurus* (Fourie et al., 1995; Beaucournu et al., 2003; Fagir et al., in press). After documenting the entire ectoparasite community of a wild sengi population for one year, we reduced the abundance of ticks and fleas experimentally over a period of two years and monitored the dynamics of the entire ectoparasite community. This constitutes the first study of this kind of small mammals in Africa and we aimed to determine the nature of interspecific relationships within the ectoparasite community of eastern rock sengis and to obtain first insights into the potential mechanism mediating these interactions. Although not the main focus of the current study given the previously reported seasonal patterns for ectoparasites in the study species and lack of sex-bias (Fagir et al., in press), we (1) expected marked temporal patterns while sex effects should be largely absent. In accordance with what was observed in similar studies for endoparasites we (2) hypothesized that the effects of the antiparasite treatment applied would be short-lived. As a result of such short-term effects we (3) predicted that interspecific interactions would be more apparent within the same trip compared to between trips. Furthermore, Based on the extraordinarily high prevalence (95–100%) and abundance (300.1 ± 23.8) of *R. warburtoni* we (4) hypothesized that this species would have many strong and antagonistic interactions with other members of the ectoparasite community.

2. Materials and methods

2.1. Study site and animal capture

Sengis were captured at Telperion/Ezemvelo Nature Reserve (25° 41′ S, 28° 56′ E). The reserve is approximately 11,000 ha in size and situated at the border between Gauteng and Mpumalanga Provinces, South Africa. Animals were captured between April 2010 and February 2013 four times a year (April/May, July/August, October/November and January February) to cover all seasons. Captures were conducted on eight rocky outcrops using 72 Sherman traps (H. B. Sherman Traps, Inc., Tallahassee, Florida) baited with a mixture of oats and peanut butter. The plots were comparable in size (0.5–0.7 ha) and over the study period the maximum number of individual known to be alive per plot ranged from 1 to 5 individuals. The distance between plots ranged from 3 to 5 km and surrounded by grassland making dispersal of individuals between plots unlikely. Furthermore, eastern rock sengis are known to be highly territorial and philopatric (Ribble and Perrin, 2005) and none of our marked animals was ever caught in more than one plot. Traps were brought out in parallel lines in an 18×3 grid with traps spaced approximately 10 paces apart. On each plot captures were conducted during four consecutive nights from late afternoon to early morning. We provided bedding during winter to prevent death due to exposure.

2.2. Ectoparasite collection and experimental treatment

All individuals captured were sexed and the entire body was thoroughly searched for ectoparasites by blowing into the fur and combing the hair back with tweezers. Ectoparasites encountered were removed with fine forceps and stored in 70% ethanol for later identification. Sengis were released at their site of capture in the afternoon after being marked individually with a combination of ear notches to ensure long-term identification. Parasite removal was repeated during each capture of an individual including recaptures within the same field trip. This approach allowed assessing the recruitment rates of ectoparasites when none of the other species are present on the host. During the second and third study year the animals were treated against ectoparasites with Frontline®, a topical tick and flea dip, before their release by applying it to the handlers' gloves and then rubbing it over the animals' body. This treatment was applied to all sengis from four of the study plots while animals on the remaining plots remained untreated. Frontline® was applied once during each field trip. This approach allowed us to contrast recruitment patterns of none-target ectoparasite species that did not experience either competitive or facilitating interactions with

the target species when invading the host with those that did (i.e. in untreated sengis).

For identification fleas, mites and lice were cleared and mounted following standard protocols while ticks remained unmounted. Details of the specimen processing and the keys used are provided elsewhere (Fagir et al., in press). For each individual sengi the number and developmental stages of every parasite species removed were noted. The larvae and nymphs of *R. warburtoni* and *R. arnoldi* (see section 3) are very similar and could thus not be distinguished reliably (Fagir et al., in press). Hence, we pooled data for these two species and refer to them as *R. warburtoni/arnoldi*. Similarly, the immature stages of *Haemaphysalis* spp. and *Ixodes* spp., respectively, have few distinguishing features and were thus pooled by genus.

2.3. Data analyses

We determined the prevalence (the number of hosts infested divided by the total number of hosts examined) and abundance (the total number of individuals of a parasite species recovered divided by the total number of hosts investigated; Bush et al., 1997) for each ectoparasite species collected. For the analyses developmental stages and sexes were pooled for each ectoparasite species.

The contributions of study year (i.e. first, second or third), season (autumn: April/May, winter: July/August, spring: October/November and summer: January February), treatment (untreated or treated) and sex as well as the presence of other ectoparasite species on the prevalence of a particular ectoparasite species were evaluated by using generalized linear mixed models (GLMMs) with a binomial error structure and a logit-link function. All two-way interactions between study year, season, treatment and sex were included in the model while parasite prevalences were included as main effects only. For parasite abundance we fitted GLMMs with a negative binomial error structure and a log-link function and included the abundance of other ectoparasite species as covariates. These analyses were carried out for ectoparasite species with a prevalence of more than 15% (see section 3). Due to the low prevalence and abundance of the majority of *Rhipicephalus* spp. we pooled these with counts for *Rhipicephalus distinctus* (see section 3) for analyses and refer to this group as 'Rhipicephalus spp.' throughout the remainder of the manuscript. *Rhipicephalus* spp. was the only ectoparasite that did not comply with a negative binomial distribution (k = 0.18, p = 0.0448) and we thus fitted a GLMM with a Poisson distribution and a log-link function when analysing the abundance of this tick species. In

addition, to the common five ectoparasite species we included lice (represented by a single species) and fleas as covariates in our models to ensure a large taxonomic coverage. The prevalence and abundance of flea species was low (see section 3). Consequently, we pooled all flea species for the analyses. This may have masked some potentially existing interactions or treatment effects but due to their low burdens no more detailed analyses were possible. In all models we included sengi ID nested in study plot as random effect to account for repeated sampling of the same study plots and individuals. The analyses for prevalence and abundance were carried out including only the first capture of an individual in a particular trip to identify the factors affecting longitudinal patterns in the ectoparasite community that would also be captured by cross-sectional sampling. In addition, we repeated these analyses including the data for recaptures of individuals within the same trip to investigate possible short-term effects of our treatment. For these analyses we added capture status (first capture or recapture) in the model and included all possible two-way interactions for this factor.

We identified the best fitting model based on the Akaike information criterion (AIC) by calculating the difference in AIC (ΔAIC) between all possible models (Burnham and Anderson, 2002). Independent factors were dropped sequentially from the full model starting with the interaction terms using a forward stepwise procedure. To further validate our final model we compared models with and without the variable of interest. Post-hoc analyses for significant terms were carried out using the least significant difference (LSD) for pairwise comparisons. We excluded one sengi from all our analyses. This individual carried an extraordinary high abundance of the tick *Rhipiciphalus appendiculatus* (n = 365) while this tick was otherwise rare (see section 3). In this individual *R. appendiculatus* entirely replaced the otherwise dominant tick species *R. warburtoni/ arnoldi* found in all other animals. All statistical analyses were conducted in IBM SPSS version 21 (IBM SPSS Statistics 21.Ink 2013). This study was approved by the animal ethics committee of the University of Pretoria (EC015-10) and permits were issued by the Gauteng Nature Conservation Board (permit no. CPF6-0041).

3. Results

Throughout the study we caught a total of 98 individuals (54 males, 44 females) between one and 14 times. From these a total of 87,276 ectoparasites from at least 18 species were recovered (Table 1). The immatures of no less than eleven species of ticks were

Table 1

Ectoparasite species collected and their infestation parameters on *Elephantulus myurus* in the Ezemvelo/Telperion Nature Reserve. Highlighted in bold are totals for the five main parasite taxa.

Taxon	Species	Total	Prevalence (%)	Abundance (±SE)
Ticks	*Rhipiciphalus appendiculatus*	665	0.8	2.49 ± 2.483 (0–653)
	Rhipiciphalus warburtoni/arnoldi tot	51,122	80.9[a]	194.94 ± 13.327 (0–998)
	Rhipiciphalus distinctus	140	20.2[a]	0.53 ± 0.145 (0–30)
	Rhipiciphalus decoloratus	1	0.6	0.01 ± 0.074 (0–1)
	Rhipiciphalus evertsi evertsi	10	4.4	0.05 ± 0.273 (0–2)
	Rhipiciphalus lanulatus	1	0.5	0.01 ± 0.074 (0–1)
	Rhipiciphalus exothalmus	2	0.5	0.01 ± 0.148 (0–2)
	Rhipicentor nuttalli	144	17.2[a]	0.55 ± 0.110 (0–14)
	Haemaphysalis spp.	28	6.1	0.11 ± 0.033 (0–6)
	Ixodes spp.	910	34.0[a]	3.46 ± 0.780 (0–99)
	Ticks total	**53,023**	**99.5**	**287.35 ± 214.107 (0–998)**
Mites	Trombiculidae larvae (chiggers)	**34,054**	**74.8**[a]	**129.48 ± 18.810 (0–3571)**
Lice	*Neolinognathus elephantuli*	**133**	**5.7**	**0.51 ± 0.271 (0–68)**
Fleas	*Demeillonia granti*	47	4.4	0.26 ± 1.364 (0–10)
	Xenopsylla brasiliensis	6	3.3	0.3 ± 0.179 (0–1)
	Chiastopsylla godfreyi	1	0.5	0.1 ± 0.074 (0–1)
	Dinopsyllus ellobius	5	1.0	0.03 ± 0.305 (1–4)
	Cthenophalides felis damarensis	7	2.7	0.04 ± 0.243 (0–2)
	Fleas total	**66**	**8.0**	**0.25 ± 0.080 (0–11)**

[a] Indicates the most prevalent and abundant species.

collected making ticks the most speciose, but also the most prevalent and abundant taxa found on sengis (Table 1). Of these, four species (*R. warburtoni/arnoldi*, *R. distinctus*, *Rhipicentor* (*Rc.*) *nuttalli* and *Ixodes* spp.) occurred at prevalences exceeding 15%, one (*Haemaphysalis* spp.) was found at a prevalence of 6.1%, while the remaining tick species were rare (Table 1). With a total of more than 30,000 larvae, Trombiculidae (chiggers) were the second most prevalent and abundant ectoparasite species sustained by sengis (Table 1). Although with five species the species richness of fleas was high, their prevalence and abundance was comparatively low. Similarly, the single louse species recovered (*N. elephantuli*) occurred at a lower prevalence and abundance when compared with the common tick species (Table 1).

3.1. Temporal and sex effects

Based on 181 first captures, the prevalence of chiggers but none of the other species differed significantly between study years (Tables S1 and S2). Similarly, the prevalence differed significantly between study years for chiggers and *Ixodes* spp. but none of the other parasite species when recaptures within trips (n = 262) were included in the analyses (Tables S1 and S2). For both species it was significantly lower during the first (chiggers: 69.7%, Ixodes spp.: 5.0%) compared to the last year (98.2% and 22.6%, respectively. p ≤ 0.029). For chiggers (97.7%, LSD: p < 0.0001) but not *Ixodes* spp. (19.3%, LSD: p = 0.070) this was also the case between the first and the second years.

The GLMMs indicated that with the exception of *Rc. nuttalli* the abundance of all ectoparasite species differed significantly between study years (Table 2). However, models did not converge for *Rhipicephalus* spp. and *Ixodes* spp. and posthoc analyses did not confirm significant differences between years for these species (p ≥ 0.104). In contrast, the abundance of *R. warburtoni/arnoldi* was significantly lower during the third (198.1 ± 20.8) compared to the first (303.8 ± 35.6, LSD: p = 0.001) and second study year (252.6 ± 28.6, LSD: p = 0.028) but did not differ significantly between the first two years (LSD: p = 0.117). Conversely, the abundance of chiggers was significantly lower during the first year (17.9 ± 4.4) compared to the second (155.5 ± 35.6) and third study years (109.7 ± 23.2; p < 0.0001 for both) but did not differ significantly between the second and third years (LSD: p = 0.146). When considering all capture data the GLMMs confirmed a significant fluctuation in the abundance of *R. warburtoni/arnoldi* and chiggers between study years but not any of the other species (Table 3). The *R. warburtoni/arnoldi* abun-

dance was significantly greater during the first year (58.0 ± 11.1) compared to the second (10.0 ± 2.5) and third years (21.5 ± 3.4, p < 0.0001 for both) and was significantly lower during the second than the third year (LSD: p = 0.001). In contrast, chigger abundance was significantly lower during the first year (0.2 ± 0.1) compared to the second (42.6 ± 12.8) and third year (28.0 ± 6.3, p ≤ 0.001) but did not differ significantly between the second and third year (LSD: p = 0.187).

Based on first captures only, the prevalence of *Rc. nuttalli* and *Ixodes* spp. differed significantly between seasons (Tables S1 and S2, Fig. S1a). Similarly, the abundance of the three most prevalent ectoparasite species (*R. warburtoni/arnoldi*, *Ixodes* spp. and chiggers) varied significantly between seasons (Table 2, Fig. S2). Since seasonal fluctuations in ectoparasite burdens of the study population have been reported elsewhere (Fagir et al., in press) we will not further report on this here. These patterns were largely confirmed when recaptures were included in the analyses (Tables 3, S3–S8). However, instead of *Rc. nuttalli* the prevalence of chiggers differed significantly between seasons (Table S3, Fig. S1b). Overall, the patterns found were in accordance with those previously reported for the study population (Fagir et al., in press).

The interaction between year and season was significant for the prevalence of *Rhipicephalus* spp. irrespective of whether all data or first captures only were considered (Tables S1–S5). In first captures the prevalence was significantly lower in autumn compared to summer during the first study year (LSD: p = 0.005, Fig. 1a) and in autumn compared to spring during the second year (LSD: p = 0.027, Fig. 1a). In contrast, it differed significantly between all seasons except winter and summer (LSD: p = 0.226) during the third study year (p ≤ 0.044, Fig. 1a). Furthermore, the prevalence of *Rhipicephalus* spp. was significantly greater during the last compared to the first study year in autumn (LSD: p = 0.003) while the opposite was true in summer (LSD: p = 0.022, Fig. 1a). None of the remaining comparisons was significant (p ≥ 0.079). In contrast, the abundance of other *Rhipicephalus* spp. was significantly greater for first captures during autumn and summer (p ≤ 0.011, Fig. 1b) and was not significantly different between any of the seasons for recaptures (p ≥ 0.071). None of the remaining posthoc comparisons were significant. When all captures were considered but not for first captures only, the interaction between year and season was significant for the prevalence of *Ixodes* spp. (Tables S1–S5). It was significantly lower in autumn of the first compared to the second and third year (p ≤ 0.029) but did not differ significantly between years for any other season

Table 2
Final GLMMs for the abundance of the five most common ectoparasite species of *E. myurus* in Ezemvelo/Telperion (first captures only).

Variable	*R. warburtoni/arnoldi*		*Rhipicephalus* spp.		*Rc. nuttalli*		*Ixodes* spp.		Chigger	
	F	p	F	p	F	p	F	p	F	p
Year	7.537	**0.001**	3.219	**0.043**	0.164	0.849	3.199	**0.044**	43.048	**<0.0001**
Season	27.394	**<0.0001**	1.390	0.248	1.809	0.148	10.530	**<0.0001**	63.983	**<0.0001**
Treatment	0.133	0.716	0.000	0.988	1.102	0.295	0.728	0.395	0.449	0.504
Sex	–	–	0.0001	0.974	6.481	**0.012**	0.286	0.594	4.144	**0.043**
Year × season	–	–	–	–	2.292	**0.010**	2.454	**0.027**	8.008	**<0.0001**
Year × treatment	–	–	4.774	**0.010**	3.158	**0.045**	0.665	0.516	–	–
Year × sex	–	–	2.141	0.121	1.371	0.257	0.364	0.696	1.189	0.307
Season × treatment	3.200	**0.025**	2.980	**0.033**	0.036	0.991	1.378	0.252	1.808	0.148
Season × sex	–	–	1.019	0.386	0.782	0.506	0.119	0.949	0.727	0.538
Treatment × sex	–	–	–	–	0.086	0.770	3.029	0.084	–	–
R. warburtoni/arnoldi	–	–	–	–	–	–	0.120	0.729	–	–
Other *Rhipicephalus* spp.	–	–	–	–	–	–	–	–	1.656	0.200
Haemaphysalis spp.	–	–	1.401	0.238	–	–	0.860	0.355	0.672	0.414
Rc. nuttalli	–	–	4.451	**0.036**	–	–	2.194	0.141	4.246	**0.041**
Ixodes spp.	2.816	0.095	6.030	**0.015**	–	–	–	–	–	–
Fleas	–	–	–	–	–	–	–	–	0.002	0.965
Chigger	–	–	2.649	0.106	6.621	**0.011**	0.001	0.981	–	–
N. elephantuli	–	–	–	–	–	–	–	–	–	–

–: factor dropped from the final model.
P-values highlighted in bold indicate significant effects.

Table 3
Final GLMMs for the abundance of the five most common ectoparasite species of *E. myurus* in Ezemvelo/Telperion (including recaptures).

Variable	*R. warburtoni/arnoldi*		*Rhipicephalus* spp.		*Rc. nuttalli*		*Ixodes* spp.		Chigger	
	F	p	F	p	F	p	F	p	F	p
Year	23.359	**<0.0001**	0.000	1.000	0.040	0.961	1.305	0.273	54.524	**<0.0001**
Season	35.970	**<0.0001**	0.117	0.950	2.469	0.063	14.077	**<0.0001**	64.812	**<0.0001**
Treatment	1.578	0.210	0.000	0.994	2.707	0.101	0.619	0.432	0.242	0.623
Sex	14.326	**<0.0001**	0.264	0.608	7.828	**0.006**	1.078	0.300	6.928	**0.009**
capture	465.000	**<0.0001**	0.000	1.000	3.852	**0.051**	18.433	**<0.0001**	141.534	**<0.0001**
Year × capture	29.408	**<0.0001**	8.929	**<0.0001**	0.374	0.688	0.335	0.716	18.001	**<0.0001**
Season × capture	25.462	**<0.0001**	4.988	**0.002**	0.214	0.886	–	–	3.930	**0.009**
Treatment × capture	2.999	0.085	9.146	**0.003**	0.014	0.906	–	–	0.471	0.493
Sex × capture	15.083	**<0.0001**	–	–	0.002	0.964	1.238	0.267	–	–
Year × season	1.897	0.082	2.468	**0.025**	3.850	**0.001**	1.935	0.076	9.070	**<0.0001**
Year × treatment	1.032	0.358	–	–	4.874	**0.008**	–	–	3.767	**0.025**
Year × sex	–	–	1.499	0.226	1.681	0.188	0.892	0.411	2.358	0.097
Season × treatment	–	–	3.900	**0.010**	0.077	0.973	–	–	0.736	0.532
Season × sex	1.600	0.190	1.473	0.223	1.090	0.354	–	–	–	–
Treatment × sex	–	–	–	–	0.021	0.885	2.705	0.101	3.384	0.067
R. warburtoni/arnoldi	–	–	7.961	**0.005**	–	–	0.405	0.525	–	–
Rhipicephalus spp.	–	–	–	–	–	–	0.086	0.770	4.079	**0.045**
Haemaphysalis spp.	0.316	0.575	2.923	0.089	–	–	1.237	0.267	–	–
Rc. nuttalli	0.253	0.615	5.092	**0.025**	–	–	5.267	**0.023**	5.520	**0.020**
Ixodes spp.	–	–	6.114	**0.014**	–	–	–	–	1.444	0.231
Fleas	4.104	**0.044**	2.019	0.157	–	–	0.000	0.992	0.030	0.862
chigger	–	–	–	–	8.000	**0.005**	0.580	0.447	–	–
N. elephantuli	–	–	–	–	1.213	0.272	0.331	0.566	0.412	0.522

–: factor dropped from the final model.
P-values highlighted in bold indicate significant effects.

(p ≥ 0.102, Fig. S3). As a result *Ixodes* spp. prevalence was significantly greater during summer than any other season during the first year (p ≤ 0.042, Fig. S3). In contrast, it was significantly higher in autumn than in winter and summer during the second and compared to spring during the third year (p ≤ 0.020, Fig. S3).

The interaction between year and season was significant for the abundance of *Rc. nuttalli* and chiggers irrespective of whether only first captures or all captures were considered (Tables 2, 3, S6–S8). In contrast, this was only the case when first captures were considered for *Ixodes* spp. (Tables 2, 3, S6). The abundance of *Rc. nuttalli* was
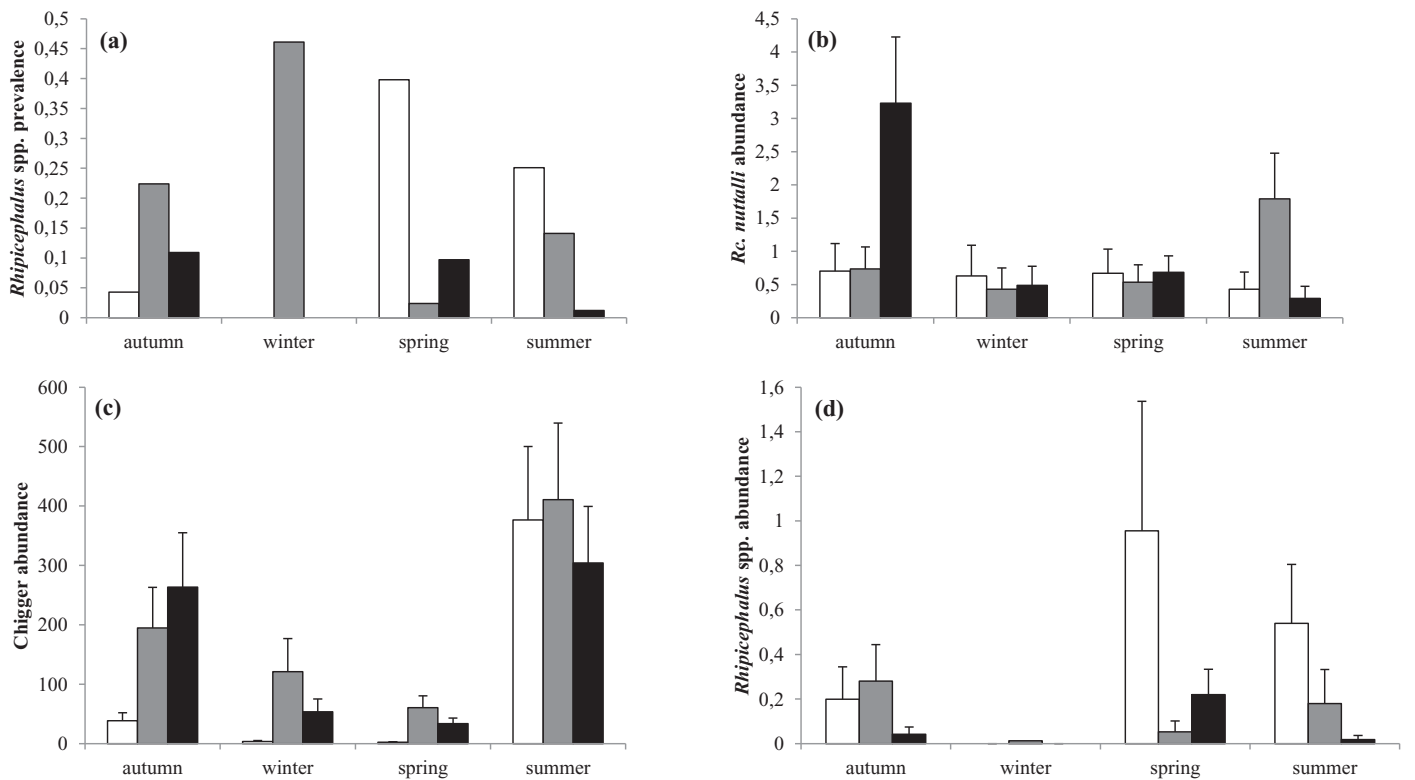

Fig. 1. Seasonal variation in the (a) prevalence of *Rhipicephalus* spp. as well as the abundance of (b) *R. warburtoni/arnoldi*, (c) chiggers and (d) other *Rhipicephalus* spp. on sengis in Telperion/Ezemvelo Nature Reserve between study years. Displayed are means ± SE. Open bars indicate the first, grey bars the second and black bars the third study year.

significantly greater in autumn compared to all other seasons (p ≤ 0.014, Fig. 1b). In addition, it was significantly higher in the autumn of the third year compared to the autumn of the previous two years (p ≤ 0.050) and significantly lower in the summer of the second compared to summer of the first year (p = 0.046, Fig. 1b). None of the remaining pairwise comparisons were significant (p ≥ 0.060). Posthoc tests did not confirm the significant interaction between year and season for *Ixodes* spp. (p ≥ 0.207). The chigger abundance was significantly lower for all seasons except summer (p ≥ 0.414) in the first compared to the other two years (p ≤ 0.039 for all, Fig. 1c). In addition, it was greater in summer compared to winter and spring in all study years and in autumn compared to spring (p ≤ 0.048, Fig. 1c). Furthermore, with the exception of the second year (LSD: p = 0.362) the abundance of chiggers was significantly lower in winter compared to autumn (p ≤ 0.023, Fig. 1c). In autumn, chigger abundance was significantly lower compared to summer during the first (LSD: p = 0.006), but not the other study years (p ≥ 0.113). It did not differ significantly between winter and spring in any of the study years (p ≥ 0.279). Only when all data were considered was the abundance of *Rhipicephalus* spp. significantly lower in winter compared to summer (LSD: p = 0.033) during the first year and compared to spring during the third year (LSD: p = 0.043, Fig. 1d). In addition, it was significantly greater in the summer of the first year compared to the last year (LSD; p = 0.036, Fig. 1d). None of the remaining seasons differed significantly (p ≥ 0.063).

For none of the parasite species did the prevalence differ significantly between the sexes irrespective of whether all data or only first captures were considered (Tables S1–S5). In contrast, the abundance of *Rc. nuttalli* and chiggers was significantly greater for male (*Rc. nuttalli*: 1.0 ± 0.2; chigger: 82.6 ± 16.8) than female sengis (*Rc. nuttalli*: 0.5 ± 0.1; chigger: 54.8 ± 11.8) both for first captures and when all captures were considered (Tables 2, 3, S6–S8). In contrast, male-biased abundances of *R. warburtoni/arnoldi* were only apparent when recaptures were included in the analysis (female: 16.5 ± 3.0, male: 32.3 ± 5.2, Tables 2, 3, S6–S8). Neither the interaction between year and sex, nor the interaction between season and sex was significant (Tables 2 and 3).

3.2. Treatment effects

Our treatment did neither affect the prevalence of abundance of any of the parasites considered irrespective of whether only first captures were considered or recaptures were included (Tables 2, 3, S1, S4). In contrast, the interaction between year and treatment was significant for the prevalence and abundance of *Rhipicephalus* spp. and the abundance of *Rc. nuttalli* (Tables 2, 3, S1, S2). However, the GLMM for *Rhipicephalus* spp. prevalence did not converge and posthoc comparisons did not confirm the significance for *Rhipicephalus* spp. In contrast, the abundance of other *Rhipicephalus* spp. was significantly lower in the second year than the two other study years (p ≤ 0.039, Fig. 2a). None of the remaining pairwise comparisons was significant (p ≥ 0.627). The *Rc. nuttalli* abundance of untreated sengis was significantly lower during the first compared to the last year (LSD: p = 0.046) and it was also significantly lower than that of treated individuals during the first year (LSD: p = 0.007, Fig. 2b). None of the remaining pairwise comparisons was significant (p ≥ 0.079). These patterns were confirmed when recaptures were included in the analysis (Tables 3, S7, S8). In contrast, the interaction between year and treatment became significant for the abundance of chiggers. It was significantly lower during the first study year compared to the following two years for both untreated and treated animals (p ≤ 0.026, Fig. 2c). None of the remaining posthoc comparisons was significant.

The interaction between season and treatment was significant for the abundance of *R. warburtoni/arnoldi* and the other *Rhipicephalus* spp. when only first captures were considered (Tables 2,
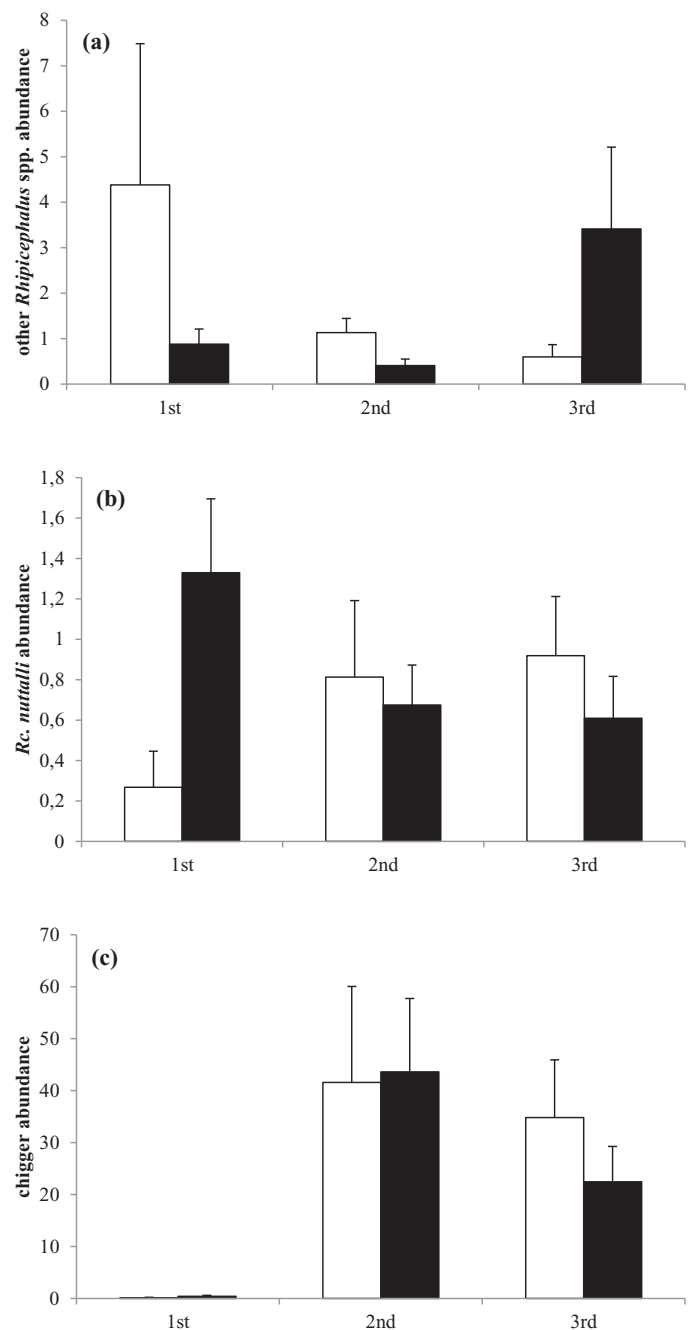

Fig. 2. Effects of antiparasite treatment in the three study years on the abundance of (a) *Rhipicephalus* spp., (b) *Rc. nuttalli* and (c) chiggers. Displayed are means ± SE. Open bars indicate untreated and filled bars treated animals.

S6). The treatment did not significantly affect the abundance of *R. warburtoni/arnoldi* within a season (≥0.063) or between autumn and spring (LSD: p = 0.585) and summer and winter (LSD: p = 0.955) for untreated or between autumn and winter for treated animals (LSD: p = 0.140). In contrast, all other pairwise comparisons between seasons differed significantly for untreated and treated individuals (p ≤ 0.025, Fig. S4a). The abundance of *Rhipicephalus* spp. was significantly lower in winter compared to summer (LSD: p = 0.018, Fig. S4b). In contrast, for treated individuals it was significantly lower during winter compared to autumn and spring (p ≤ 0.024) while it was significantly greater during summer compared to autumn and spring (p < 0.025 for both, Fig. S4b). None of the remaining pairwise comparisons was significant (p ≥ 0.062). When all capture data were

considered, the interaction between season and treatment was significant for other *Rhipicephalus* spp. only (Tables 3, S7). Posthoc comparisons confirmed that the patterns reported for first captures only.

The interaction between treatment and sex was significant for the prevalence of *Ixodes* spp. when all captures were included in the analysis (Tables S3–S5). The *Ixodes* spp. prevalence of treated males (29.2%) was significantly greater than that of females (9.2%, LSD: p = 0.042) while it did not differ between the sexes for untreated individuals (males: 7.1%, females: 14.9%, LSD: p = 0.337). As a result the abundance of *Ixodes* spp. differed significantly between treatments for males (LSD: p = 0.027) but not females (LSD: p = 0.492).

3.3. Effects of capture status

Capture status affected the ectoparasite prevalence and abundance for all species but *Rhipicephalus* spp. (Tables 3, S3, S4, S7, S8). For all species the prevalence was significantly lower for recaptures compared to first captures. In contrast, while parasite abundance was significantly lower in recaptures compared to first captures for *R. warburtoni/arnoldi* (2.06 ± 0.49 vs. 257.67 ± 28.94), *Ixodes* spp. (0.03 ± 0.02 vs. 0.44 ± 0.22) and chiggers (0.66 ± 0.27 vs. 65.64 ± 12.86), the opposite was true for *Rc. nuttalli* (0.79 ± 0.13 vs. 1.52 ± 0.49) although the latter just failed to be significant (p = 0.051, Tables 3, S7, S8).

The interaction between year and capture status was significant for the abundance of *R. warburtoni/arnoldi*, *Rhipicephalus* spp. and chiggers (Tables 3, S7, S8). For all of these species, abundance was significantly greater in first captures compared to recaptures (p ≤ 0.048, Fig. S5). In addition, first captures sustained significantly lower abundances of *R. warburtoni/arnoldi* during the third study year compared to the previous two years (p ≤ 0.048, Fig. S5a). In contrast, *R. warburtoni/arnoldi* abundance differed significantly between all years for recaptures (p ≤ 0.001, Fig. S5a). For chiggers, for both first captures and recaptures the abundance was significantly lower during the first compared to the second and third years (p ≤ 0.034, Fig. S5b). None of the remaining comparisons was significant (p ≥ 0.292).

The interaction between season and capture status was significant for *R. warburtoni/arnoldi*, *Rhipicephalus* spp. and chigger abundance (Tables 3, S7, S8, Fig. S7). Seasonal patterns of first captures for all three species were the same as those of the main effect and for *R. warburtoni/arnoldi* and chiggers sengis had significantly greater ectoparasite abundances when first captured compared to recaptures (p < 0.0001 for all, Fig. S6a and c).

The interaction between treatment and capture was significant for *Rhipicephalus* spp. abundance (Table 3). However, the model did not converge and none of the posthoc comparisons was significant (p ≥ 0.993). Furthermore, the interaction between sex and capture status was significant for *R. warburtoni/arnoldi* (Tables 3, S7, S8). The abundance of *R. warburtoni/arnoldi* was significantly greater for first captures compared to recaptures for both sexes (p ≤ 0.0001 for both). However, while it did not differ significantly between the sexes during first capture (females: 244.5 ± 33.2, males: 271.5 ± 34.9, LSD: p = 0.452) it was significantly greater for males (3.8 ± 1.0) compared to females (1.1 ± 0.3) for recaptures (p = 0.003).

3.4. Evidence for direct interactions between parasites

When only first captures were considered, a limited number of direct interactions were found. The prevalence of *Rc. nuttalli* was significantly higher in sengis infested with *N. elephantuli* (70.3%) compared to those not infested with lice (32.1%, Tables S1, S2). This result was confirmed when all data were considered (Tables S3–S5). In addition, the abundance of *Rhipicephalus* spp. increased significantly

with the abundance of *Rc. nuttalli* and *Ixodes* spp. (Fig. 3a and b). In contrast, *Rc. nuttalli* and chiggers had a significant negative effect on each other (Tables 2, S6). However, the abundance of chiggers decreased more steeply with increasing *Rc. nuttalli* abundance (Fig. 3c) than vice versa (Table S6).

When all capture data were considered, additional direct interactions became apparent. The prevalence of *Ixodes* spp. was significantly lower when chiggers were present (6.4%) than when they were not (25.7%, Tables S3–S5). The abundance of *Rhipicephalus* spp. increased significantly with increases in abundance of *R. warburtoni/arnoldi* (Fig. 4a), *Rc. nuttalli* and *Ixodes* spp. (Table S7). At the same time, chigger abundance increased significantly with *Rhipicephalus* spp. abundance (Table S8, Fig. 4b). Similarly, the abundance of *Ixodes* spp. increased significantly with an increasing abundance of *Rc. nuttalli* (Table S8, Fig. 4c). In contrast, the
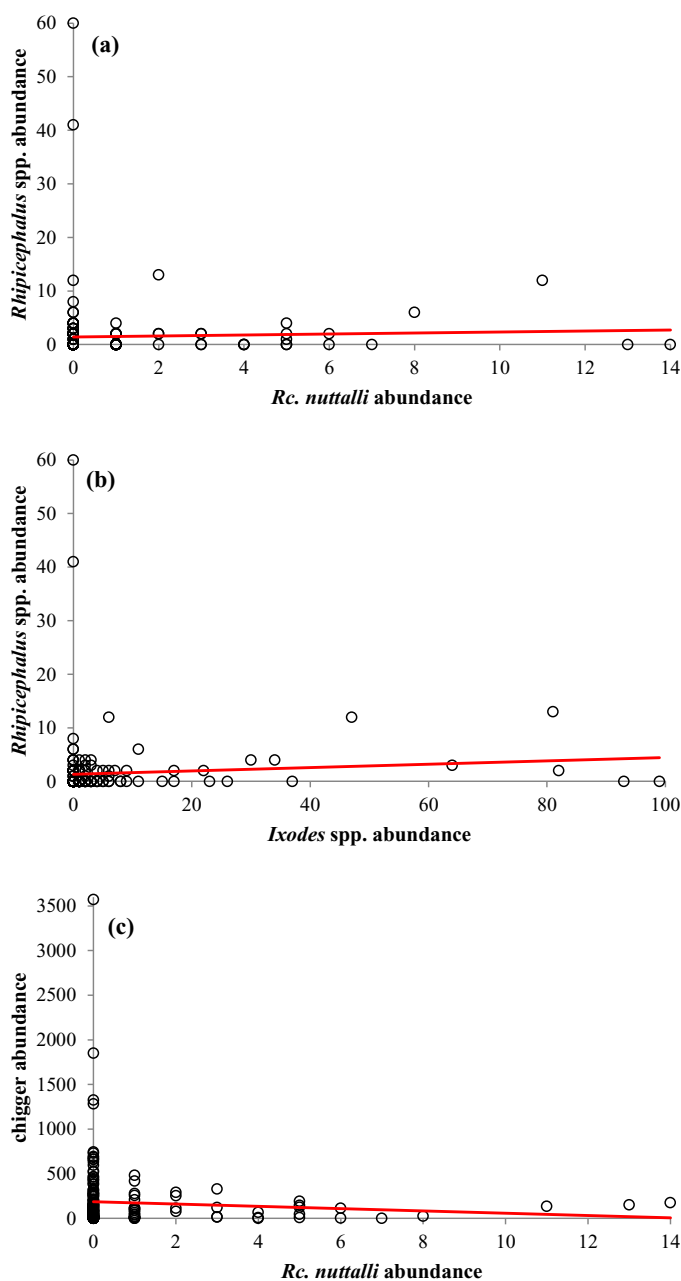

Fig. 3. Relationships between the abundance of (a) *Rc. nuttalli* and (b) *Ixodes* spp. and the abundance of *Rhipicephalus* spp. and the (c) abundance of *Rc. nuttalli* and the chigger abundance when only first captures are considered.
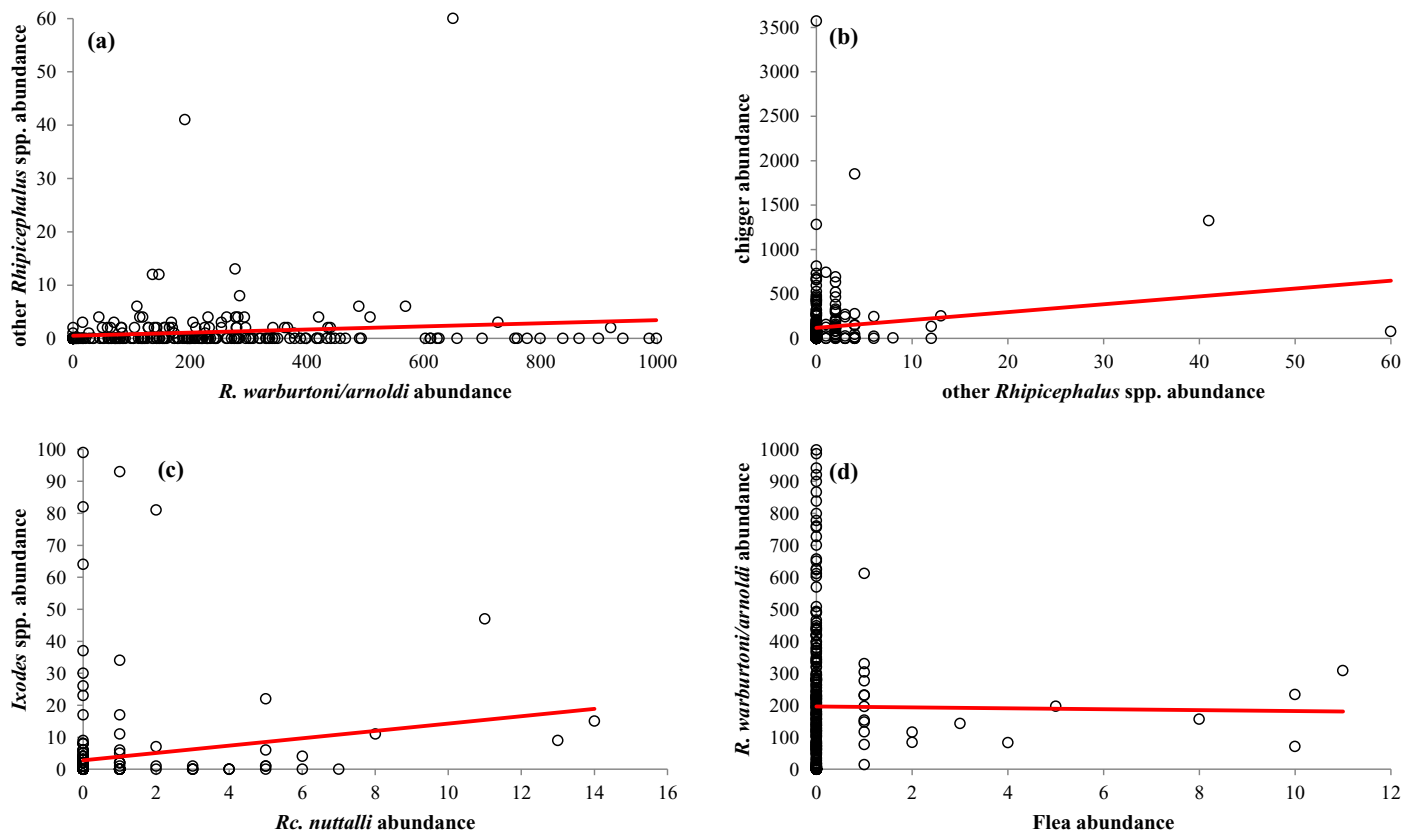
Fig. 4. Relationships between the abundance of (a) *R. warburtoni/arnoldi* on the other *Rhipicephalus* spp. abundance, (b) other *Rhipicephalus* spp. on the abundance of chiggers, (c) *Rc. nuttalli* on the abundance of *Ixodes* spp. and (d) fleas on the abundance of *R. warburtoni/arnoldi* when all capture data are considered.

abundance of *R. warburtoni/arnoldi* decreased significantly with increasing flea abundance (Tables 3, S7, Fig. 4d). In addition, *Rc. nuttalli* abundance decreased significantly with increasing chigger abundance (Tables 3, S7) while it in turn had a significant negative effect on the abundance of chiggers (Tables 3, S8).

4. Discussion

4.1. Ectoparasite distribution patterns

Seasonal fluctuations were pronounced for the abundant ectoparasite species, *R. warburtoni/arnoldi* and chiggers, but much less evident for the less abundant ones. These patterns are largely in accordance with those previously reported for the study species (Lutermann et al., 2012a; Fagir et al., in press) and thus will not be further discussed. However, the differences in seasonal peaks in burden for the main parasites suggest that interspecific interactions between these species may be dynamic, particularly when considering those between *R. warburtoni/arnoldi* that are present all year round. Their constant presence in large numbers might furthermore have masked some competitive interactions accounting for the low number of direct interactions found when only considering cross-sectional data.

The abundance of *Rc. nuttalli* and chiggers was male-biased in the cross-sectional data. In addition, a male bias in *Ixodes* spp. prevalence and the abundance of *R. warburtoni/arnoldi* was observed when recaptures were considered. Since the study species does not exhibit a sexual dimorphism and ectoparasite burden has previously been shown to be independent of both body mass and length (Lutermann et al., 2012b; Fagir et al., in press), physiological mechanisms such as the immunosuppressive properties of testosterone

may account for this observation (Hughes and Randolph, 2001). However, male testosterone levels in the study species are generally low (Medger et al., 2012) and hence behavioural mechanisms are likely to be more important for this sex bias. In support of this hypothesis male home ranges of eastern rock sengis are reported to be almost twice as large as those of females (Ribble and Perrin, 2005). Greater male ranges could also explain the higher recruitment rates observed for *Ixodes* spp. and *R. warburtoni/arnoldi* when recaptures were considered. However, these did not result in an overall sex bias for either of these species, possibly because the extraordinary large abundance of *R. warburtoni/arnoldi* results in spatial constraints with regard to attachment sites for both species.

4.2. Ectoparasite recruitment rates and treatment effects

The capture status of an individual affected its ectoparasite burden. Not surprisingly these were significantly lower for all but one species. The recruitment rates of ectoparasites observed in sengis recaptured within the same trip were low compared to the abundance observed during first captures. This suggests that the daily exposure of sengis in the study population is rather low even for the most abundant species of parasite. With the exception of the chiggers, all of the ectoparasite species are specific for sengis or they are the preferred host (Fourie et al., 1992, 2002; Harrison et al., 2011, 2012). Consequently, the comparatively low sengi density in the study area may account for this observation. Nevertheless, for *R. warburtoni/arnoldi* and chiggers, recruitment rates mirrored fluctuations in annual and seasonal abundance suggesting that observed abundances on the sengis during their first capture reflect variations in host exposure to these parasites with year and season.

We did not find any direct effects of our treatment in the current study. This is not unexpected for the cross-sectional data since the time elapsed between consecutive field trips exceeded the period indicated by the manufacturer for the effectiveness of Frontline® of four to six weeks for ticks that has been confirmed in several laboratory studies (Widermann, 2000; Dryden et al., 2008; Kužner et al., 2012). However, this cannot account for the lack of treatment effects when recaptures were included. The abundance of *R. warburtoni/arnoldi* was significantly reduced in the years where the treatment was applied suggesting that our treatment was effective. We suggest that the low number of treated recaptures as well as overall recruitment rates may account for the lack of treatment effects within trips observed in the current study.

4.3. Evidence for antagonistic community interactions

Contrary to our prediction we did not find a large number of antagonistic interactions between the dominant ectoparasite species, *R. warburtoni/arnoldi*, and other members of the ectoparasite community of eastern rock sengis. However, the prevalences of *Ixodes* spp. and chiggers as well as the abundances of *R. warburtoni/arnoldi* and chiggers varied substantially between years. While the abundance of the former was significantly reduced during the years during which the sengis were treated against ticks, chigger prevalence and abundance as well as the prevalence of *Ixodes* spp. increased significantly. Although there was no direct correlation between the abundances of these species our results suggest an antagonistic interaction between *R. warburtoni/arnoldi* and *Ixodes* spp. as well as chiggers. Such antagonism could also account for the observed drop in the abundance of *R. warburtoni/arnoldi* in summer that coincides with the peak in chigger abundance, while the abundance of this tick is substantially greater prior to and after the peak in chigger burden. In addition, an antagonistic interaction between *R. warburtoni/arnoldi* and chiggers would account for the observed increases in *Ixodes* spp. prevalence in autumn and chigger abundance in autumn, winter and summer during the second and third study years when our treatment would have reduced the number of *R. warburtoni/arnoldi*. The antagonistic relationship between both *R. warburtoni/arnoldi* and the two other species may also explain why a direct antagonistic relationship between *Ixodes* spp. and chiggers was only apparent when recaptures within trips were considered in the analyses. The absence of similar annual fluctuation in the remaining tick species may be linked to their generally low abundances. Ectoparasite communities that largely consist of parasite species that spend the majority of their life-cycle off-host, such as in the case of ticks, mites and fleas, can be expected to be characterized by a high turn-over rate. Consequently, our manipulation of recruitment rates should allow higher invasion rates for species that are prevented from invading a particular host by the presence or recruitment of another parasite species (e.g. *R. warburtoni/arnoldi*). Hence, although all ectoparasites were removed initially the increased recruitment rates of other species provide corroborating evidence for competitive relationships between species.

The observed carry-over effects of our treatment do not support the hypothesis that our experimental manipulation will only result in short-term perturbations of the ectoparasite community. It also contrasts markedly with the high resilience observed in endoparasite communities of small mammals (Knowles et al., 2013). These differences may be linked to the markedly different life-cycles of the target species concerned. While the nematodes targeted by Knowles et al. (2013) have a direct life cycle, ticks spend the majority of their life off-host and moult to the next stage after each completed meal (Sonenshine, 1991). The experimental reduction of the proportion of the immature *R. warburtoni/arnoldi* population that feeds successfully and subsequently moults into the next

stage could substantially reduce the adult tick population and in turn the next generation of immatures that infest sengis. Interestingly, the abundance of *R. warburtoni/arnoldi* was significantly greater during the first study year compared to the following two years when our experimental manipulation was applied. This suggests that the manual removal of this tick once per season does affect the *R. warburtoni/arnoldi* population to a much lesser degree than the action of Frontline® which extends over several weeks.

Since chiggers are not haematophagous it is unlikely that the antagonistic interaction between chiggers and the two tick species is mediated by direct competition for host resources (Pedersen and Fenton, 2007). In contrast, it appears likely that both species compete for attachment sites on the host as has been suggested for *Ixodes pacificus* and chigger mites parasitizing western fence lizards (*Sceloporus occidentalis*) in North America (Pollock et al., 2012). Similar spatial competition has been proposed between endoparasites of rodents (Knowles et al., 2013; Pedersen and Antonovics, 2013). Since larval and nymphal ticks are substantially larger than chiggers (D.M. Fagir and H. Lutermann, personal observation) the size difference alone may give the former a competitive advantage over the latter. This hypothesis is supported by the observation that in sengis the vast majority of *R. warburtoni/arnoldi* larvae attach on the ridges of the ear pinnae and nymphs usually attach on the lower back while chiggers are found on the rear and around the base of the tail. In contrast, in sympatric rodent species *R. warburtoni/arnoldi* is largely absent (Fagir et al., 2014) and chiggers are generally found attached to the ear ridges (D. M. Fagir and H. Lutermann, personal observation). Future studies should include a quantification of attachment sites to further corroborate this hypothesis. A similar antagonism may also explain the negative correlation between the prevalence of *Ixodes* spp. and the abundance of *Rc. nuttalli* and chiggers. Interestingly, the latter was the only species pair where such effects were mutual with the slope being much steeper for the negative effect of *Rc. nuttalli* on chiggers than vice versa, probably a consequence of the substantially greater abundance of chiggers. The antagonistic relationship between *Rc. nuttalli* and chiggers could also account for the observed increases in abundance of *Rc. nuttalli* in recaptured sengis compared to first captures contrary to what we observed in chiggers.

Despite their low prevalence and abundance, we found evidence for inter-taxon antagonistic effects of fleas on *R. warburtoni/arnoldi* when recaptures where considered. This may partially be explained by the different life-cycles of these two taxa. Unlike ticks, the most prevalent and abundant fleas retrieved in the current study complete their entire life-cycle on the host living in its fur (Segerman, 1995). Frontline® is not effective against flea eggs and we did not remove these when collecting parasites. Hence fleas may have hatched and established new populations faster than *R. warburtoni/arnoldi* was recruited once the protective effects of Frontline® had worn off. At the same time, with accumulating number of *R. warburtoni/arnoldi* interference competition between *R. warburtoni/arnoldi* and fleas may have compensated for this effect resulting in no significant effect in our long-term data supporting our hypothesis that treatment effects on the main ectoparasite species would be short-lived.

4.4. Facilitating community interactions

In contrast to the antagonistic relationships between the ectoparasite taxa harboured by sengis we found evidence for facilitating effects within a taxon. The abundance of *Rc. nuttalli* and *Ixodes* spp. had a significant positive effect on other *Rhipicephalus* spp. abundance. Similarly, we found a positive effect of the abundance of *R. warburtoni/arnoldi* on other *Rhipicephalus* spp. when recaptures were considered. This observation suggests that competition for attachment sites or direct resource competition (i.e. blood) does not

govern the relationships between tick species. However, since attachment site was not recorded in the current study, we cannot entirely exclude the possibility that the different tick species adjusted their attachment sites depending on the presence of the other species. Such a mechanism has repeatedly been suggested for tick species exploiting the same host in reptiles (Andrews and Petney, 1981; Andrews et al., 1982). This possibility has received surprisingly limited attention in mammals, particularly small mammals, and the available studies are mostly focussed on inter-stadial rather than inter-specific interactions (Baer-Lehman et al., 2012; Anderson et al., 2013). However, the availability of alternative attachment sites is likely to decrease with an increasing abundance of other tick species and hence our results are more likely to be linked to indirect effects mediated via the host immune system (Pedersen and Fenton, 2007). The feeding of ticks triggers an intricate cascade of innate and adaptive immune responses by the host (Brossard and Wikel, 2004). With an increasing number of attacking ticks such a response might be weakened due to resource depletion and hence facilitate the attachment and feeding of additional ticks. There are currently no immunological data available for the study species and hence the nature of such a mechanism remains unknown for the study species. However, previous studies suggest that eastern rock sengis do not develop a resistance against ticks (Du Toit et al., 1994) suggesting that each new attack may result in a progressively weaker immune response. Experimental studies are needed to address this possibility in the future.

Contrary to the antagonistic inter-taxon relationship observed, *N. elephantuli* and *Rhipicephalus* spp. appeared to facilitate the establishment of *Rc. nuttalli* and chiggers, respectively. These are the only positive inter-taxon interactions found in the current study. If *Rhipicephalus* spp. avoids spatial competition with the other tick species by using different attachment sites as has been suggested for other tick species (Andrews and Petney, 1981; Andrews et al., 1982; Baer-Lehman et al., 2012), they may at the same time reduce spatial competition with chiggers that appear to share attachment site preferences with *R. warburtoni/arnoldi*. At the same time there is no reason to assume that *Rhipicephalus* spp. would trigger host immune responses that differ from those of the other tick species and consequently immune-mediated facilitation is unlikely to account for the observed effect of *Rhipicephalus* spp. on chiggers.

In conclusion, our study is the first evaluating interspecific interactions of the entire ectoparasite community exploiting a mammalian host in the wild. We found evidence for a number of within-taxon facilitating interactions between ectoparasite species probably mediated by the host immune system. At the same time, inter-taxon relationships appeared to be dominated by antagonistic relationships likely to be linked to competition over feeding sites. Only one of the observed interspecific interactions was reciprocal. Combining longitudinal and short-term observations in the current study revealed several interspecific relationships that cross-sectional studies would not have captured. The experimental perturbation employed in the current study resulted in substantial changes in ectoparasite community composition suggesting long-term effects of our treatment. Our results highlight the complexity of interspecific interactions within an ectoparasite community and stress the need for longitudinal studies on small mammal ectoparasites.

Conflict of interest

The authors declared that there is no conflict of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.ijppaw.2015.02.001.

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