Epidemics 1 (2009) 162–167

Contents lists available at ScienceDirect

Epidemics

journal homepage: www.elsevier.com/locate/epidemics

The vicious circle and infection intensity: The case of *Trypanosoma microti* in field vole populations

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ARTICLE INFO

Article history:
Received 16 January 2009
Revised 23 May 2009
Accepted 26 May 2009

Keywords:
Infection dynamics
Trypanosomes
Disease ecology
Microtus
Wildlife epidemiology

ABSTRACT

Objective: In natural populations, infection and condition may act synergistically to trigger a vicious circle: poor condition predisposes to host infections, which further reduce condition, and so on. If this vicious circle originates from a reduced resistance to infection, it will not only result in greater proneness to becoming infected of those that are in poorer condition, but it may also cause infections of higher intensity. Here, we investigate the temporal relationship between host condition and intensity of infection by a specific pathogen using as a system the dynamics of the protozoan Trypanosoma microti in field vole (Microtus agrestis) populations.

Methods and results: With two years of longitudinal data from three monthly-sampled populations, we evaluated if individuals acquiring a high intensity of infection previously had lower haematological indicators of condition (red blood cells [RBCs] and lymphocyte counts) than those that acquired lower infection intensities. Also, the association of these indicators with past and present trypanosome blood levels was investigated. The individuals that developed high levels of parasitaemia were those that previously had low lymphocyte counts. Greater intensity of infection corresponded with lower RBCs only in low to moderate weight females, and no effect of intensity of infection on lymphocyte counts was observed. However, delayed effects of high trypanosome intensity were seen on both RBCs and lymphocytes.

Conclusions: The vicious circle may also result in high infection intensity: individuals in poor condition are not only more likely to become infected by one pathogen; they may also be the most important source of infection for that and for other pathogens, and thus key protagonists for parasite dynamics.

Introduction

It has recently been shown for cowpox virus, an endemic pathogen, that field voles (*Microtus agrestis* L. 1761) with a preceding poorer health condition were more likely to become infected in natural circumstances (Beldomenico et al., 2009). Another longitudinal study on voles has shown that there is a synergy between infection and condition: poor condition predisposes to host infections, which further reduce condition, and so on (Beldomenico et al., 2008a). Such synergy has also been established experimentally at the population level (Pedersen and Greives, 2008). If this vicious circle originates from a reduced resistance to infection, it will not only result in greater proneness to becoming infected of those that are in poorer condition; it may also cause infections of higher intensity, thus resulting in individuals that suffer a more severe disease and are a more significant source of infection. Here, we investigate the temporal relationship between host condition and intensity of infection by a specific pathogen under natural circumstances, using as a system the dynamics of the protozoan *Trypanosoma* (*Herpestoma*) *microti* Laveran and Pettit 1909 in field vole populations. As in the study of Beldomenico et al. (2008b), condition was characterised by haematological parameters. Red blood cells (RBCs) and lymphocytes were the proxies of condition. Low peripheral lymphocyte counts (lymphopenia), are generally an indication of immunosuppression or poor immunological investment, whereas low RBC levels (anaemia) are an indication of poor aerobic capacity and negative metabolic balance.

Trypanosoma microti is a stercorarian trypanosome specific to voles (Noyes et al., 2002). It is transmitted by fleas, which take up the protozoan from an infected individual during a blood meal. The trypanosomes then grow and divide in the hind gut of the insect, and may infect another host when they are shed in the flea faeces contaminating the skin, or alternatively when the hosts accidentally ingest the fleas or the flea faeces (Albright and Albright, 1991). An experimental study conducted in Kielder Forest (Northumberland,

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doi:10.1016/j.epidem.2009.05.002

UK), suggests that vector-independent transmission may also be taking place, probably involving aggressive behaviour that favours mechanical transmission (Smith et al., 2006). In field vole populations at Kielder, it was found that trypanosome prevalence is highly seasonal and is positively associated with past (1–3 months) flea infestation (Smith et al., 2005). The prevalences are highest in late summer–autumn and lowest in spring (Smith et al., 2005).

Trypanosomes are generally considered to be of low pathogenicity for rodents, but there is evidence of anaemia caused by infection in microtines (Wiger, 1977). Other trypanosome species have a well documented pathogenicity. Anaemia associated with African trypanosomiases is a consistent and significant finding in humans (Woodruff et al., 1973) and other vertebrates (Hornby, 1921). Laboratory mice experimentally infected with *Trypanosoma brucei* experienced anaemia from 1 week after infection, which lasted for approximately 3 weeks (Amole et al., 1982). *Trypanosoma cruzi*, a stercorarian trypanosome that causes Chagas disease, has the ability to intensely suppress the immune system (Ouaissi et al., 2001). In mice and humans, the acute phase of Chagas disease is marked by a state of immunosuppression resulting from an inhibition of T lymphocyte proliferation (reviewed by Ouaissi et al., 2001).

Some existing evidence from trypanosome studies supports our hypothesis that individuals with impaired immunocompetence are more prone to suffer higher intensities of infection. In humans, HIV-positive patients show higher levels of *T. cruzi* parasitaemia (Sartori et al., 2002), and a recent experimental study using a rodent host (*Calomys callosus*) demonstrated that induced social stress gave rise to higher blood levels of this parasite (Santos et al., 2008).

Against this background, investigation on *T. microti*, based on detection of nucleic acids in blood using PCR, failed to find a significant demographic effect on wild (Kielder) populations of field voles (Smith, 2005). Nevertheless, effects of differential intensities of infection have not been explored. The goal of the present study, then, is to establish the relationship between haematological indicators of condition already explored in field vole populations (red blood cell and lymphocytes counts) and past, present and future levels of *T. microti* parasitaemia, to test two hypotheses:

(i) That the intensity of a trypanosome infection depends on the preceding condition of hosts.
(ii) That high infection intensities are associated with greater direct or delayed effects on indicators of condition than lower infection intensities.

Materials and methods

Sampling procedures

In Kielder Forest (Northumberland, UK), three sites with suitable habitat for field voles were sampled ('primary sessions') every 4 weeks over a two-year period (from April 2005 to March 2007) apart from 8-week gaps between November and February (Beldomenico et al., 2008b). At each site, a trapping grid measuring 50×50 m was established, with 100 Ugglan special live capture traps (Grahnab, Sweden), set at approximately 5 m intervals. In each primary session, the traps were checked for capture 5 times, at sunrise and before sunset (roughly 12-h intervals).

Individuals were uniquely and permanently marked on first capture with a small microchip transponder (Labtrac by AVID plc, UK). On first capture within a primary session, each vole was assessed for pelage (juvenile coat, first molt, adult coat), sex and body mass (to the nearest 0.5 g, using a spring balance). Body condition [coded BODYCOND in the analysis] was evaluated by estimating by palpation the degree of fat and muscle cover over the vertebral column and the pelvic bones, giving a score between 2 and 10 (Beldomenico et al., 2008b).

Haematological parameters

As described in detail by Beldomenico et al. (2008b), a haemogram was produced with blood collected from the tip of the tail of live individuals. Briefly, 2 µl of non-coagulated blood were diluted 1:20 in 4% acetic acid with 1% crystal violet and 1:5000 in PBS, to count white blood cells (WBCs) and RBCs (cells/µl), respectively, using Kova Glasstic® (Hycor Biomedical Ltd, Penicuik, UK) slides with grids and hence to determine their concentration. The rest of the blood sample was used to produce blood smears for differential WBC counts, which allowed the proportion of each WBC type and their concentration to be estimated, and the assessment of trypanosome infection intensity. Smears were air-dried, fixed with methanol and stained with Rapid Romanowsky Stain Pack - HS705 (HD Supplies, Aylesbury, UK).

Assessment of trypanosome infection intensity

Trypanosomes were monitored by examining blood smears microscopically. Each smear was thoroughly examined for trypomastigotes. On the basis of the number of 400× fields in which trypanosomes were detected, the intensity of infection was categorised into three levels: '*Not detected*', no trypomastigote found in the thorough examination; '*Low intensity*', up to 90% of the fields with trypomastigotes; and '*High intensity*', more than 90% of the fields with trypomastigotes. Albeit arbitrary, the cut-off point for infection intensity was intended to reflect the observation that infected individuals tended either to have few fields with trypomastigotes, or most fields had them. It should be acknowledged that within the 'not detected' category (omitted from the analysis) there are both non-infected individuals and those with very low intensity infections.

Statistical analysis

The analysis was restricted to data collected from late June to October 2005 and 2006, as during the rest of the year prevalences were lower (<20%) and high intensities of infection rare (Fig. 1). Because juveniles have very different and rapidly varying haematological parameters (Beldomenico et al., 2008b), only post-juvenile voles were considered. For the purposes of our analyses, age was approximated using categories on the basis of pelage and weight. Individuals with a juvenile coat and ≤17 g in weight were classed as 'juvenile'. Post-juveniles were categorised as 'adult' or 'young' on the basis of capture histories as being older and younger than 90 days, respectively. In the absence of enough trapping history, post-juveniles were classified as adult if they weighed 22 g or more, and otherwise young. It is worth remarking that in our age classification, 'adult' does not necessarily imply that the individual is breeding.

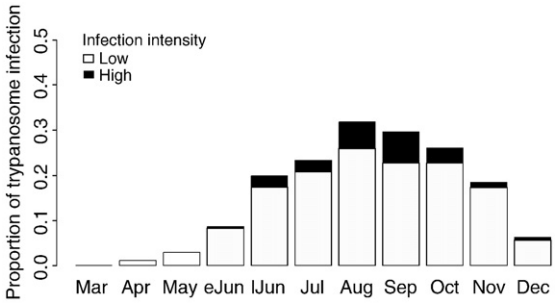

Fig. 1. Seasonality of infection with *Trypanosoma microti* in post-juvenile field voles. Proportions for different infection intensities: low = white; high = black. (eJun = early June; lJun = late June).

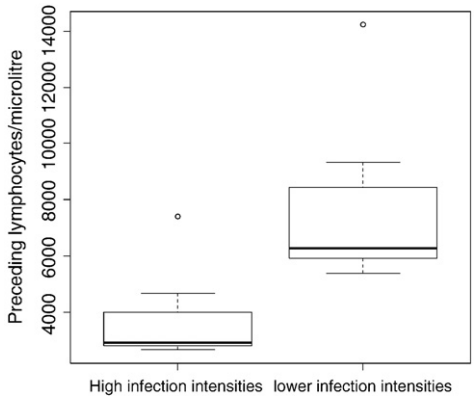

Fig. 2. Lymphocyte levels before (4 weeks previously) natural infection with *Trypanosoma microti* for field voles that acquired high infection intensities and others that developed lower levels of parasitaemia.

Two different approaches were used to address the hypotheses:

(i) The intensity of a trypanosome infection depends on the preceding condition of hosts.

The subset of data used for this analysis consisted of post-juvenile individuals with no trypanosomes detected at a given trapping session (T_0) but with trypanosomes detected 4 weeks later (T_{+1}). The prediction is that there will be significantly lower counts of lymphocytes and RBCs at T_0 in those that at T_{+1} develop high intensities of infection compared to those that develop low intensities.

The subset of post-juvenile individuals with recorded lymphocyte and RBC counts at T_0 that have high intensities at T_{+1} were only eight. Given this low number conforming to the selection criteria, the only way to address confounding phenomena, always present in observational studies (Dohoo et al., 2003), was to carry out a matched design. Therefore, for each individual that developed a high intensity of infection, an individual that developed a low intensity one (a "control") was assigned, matched for trapping session, trapping site, sex and body weight (the individual with the closest body weight was chosen). For one individual with a high infection intensity, there was no suitable control to assign (but its lymphocyte and RBC counts were within the range of the counts of those with higher infection), and the reduced number of suitable controls in general precluded repeating the analysis with randomised combinations of controls. Consequently, the analysis was done using a paired *t*-test, after converting the variables to approximate normality (Lymphocytes$^{0.1}$ and RBCs$^{0.5}$).

(ii) Higher infection intensities are associated with greater direct or delayed effects on indicators of condition than lower infection intensities. These analyses were conducted using linear mixed effects models with random intercepts (LMMs), using the statistical software package R (The R Foundation for Statistical Software; http://www.r-project.org). The approach consisted of adding the variable related to trypanosome infection intensity (together with all two-way interaction terms between infection and the existing explanatory variables) to the previously constructed optimal models that described the variation in RBC and lymphocytes for the same dataset (Beldomenico et al., 2008b), to evaluate the contribution of parasitism intensity. The intention of this multivariable analysis approach was to control for potential distortion of the associations investigated due to the confounding and effect modification phenomena that may occur when ignoring variables previously identified to be associated with the dependent variables (Dohoo et al., 2003). These optimal models (variables explained below) are:

$RBC^{0.5} \sim weight + weight^2 + season + sex + year + density\ lag\ 6 + weight * year + weight^2 * year +$ random effects (vole id# and site*trap session)

$Lymphocytes^{0.1} \sim age + season + sex + body\ \ condition + year + recaptured/new + density\ \ lag\ \ 6 + sex * body\ \ condition + sex * density\ lag\ 6 + age * year + season * recaptured/new + age * recaptured/new +$ random effects (vole id# and site*trap session)

Because an association of low levels of haematological indicators of condition and high intensity of parasitaemia may arise if, in general, those with poor condition are the ones manifesting high infection intensities (i.e. high infection intensity is a consequence, not a cause, of poor condition), the RBC and lymphocyte counts 4 weeks in the past (T_{-1}) were respectively included as explanatory

Table 1
LMMs showing intensity of *T. microti* infection (low/high) and other variables associated with RBC counts in post-juvenile field voles.

Current RBC counts and present intensity of infection				
Model =	$RBC^{0.5} \sim WGT + SEX + RBC.T_{-1} + Tryps.T_0 + Tryps*WGT + Tryps*SEX$ Random effects: SITE*SESSION; VOLE_ID.			
Term	Coefficients	Std. error	*p*-value	ΔAIC[a]
Intercept	2346	280	<0.0001	–
WGT	−3.45	9.35	0.7137	–
SEX (male)	−17	109	0.8752	–
$RBC.T_{-1}$	0.00003	0.000017	0.0612	+2
$Tryps.T_0$ (high intensity)	−2352	671	0.0007	–
WGT*$Tryps.T_0$ (high intensity)	77	26	0.0041	+7
SEX (male)*$Tryps.T_0$ (high intensity)	1127	337	0.0011	+9
Current RBC counts and intensity of infection 4 weeks previously				
Model =	$RBC^{0.5} \sim SEASON[sin] + SEASON[cos] + WGT + YEAR + Tryps.T_{-1} + RBC.T_{-1} + WGT*YEAR$ Random effects: SITE*SESSION; VOLE_ID.			
Term	Coefficients	Std. error	*p*-value	ΔAIC[a]
Intercept	1419	1057	0.1820	–
SEASON[sin]	−340	176	0.0559	+2
SEASON[cos]	−999	343	0.7857	0
WGT	41.7	28	0.1442	–
YEAR (2nd)	362	540	0.5031	–
$RBC.T_{-1}$	0.00004	0.000016	0.0246	+5
$Tryps.T_{-1}$ (high intensity)	−341	147	0.0223	+4
WGT*YEAR (2nd)	−35	20	0.0827	+2

The models include infection intensity at different times (current and past).
[a] AIC value changes if the single term is dropped from the model.

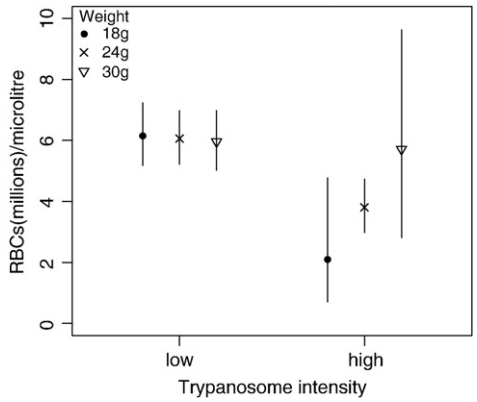

Fig. 3. RBC counts as predicted by the LMM evaluating the association with current intensity of *T. microti* infection. Simulation for August 2005, for females of three different weights. Error lines represent 95% confidence intervals.

variables. In this way, we can evaluate the association between intensity of infection and condition beyond the baseline haematological status of each individual.

For each response (RBC and lymphocytes), two models were constructed. The first evaluated the association between blood cell levels and current intensity of infection (Tryps.T_0; low/high). The second evaluated the same relationship, but using the intensity of infection 4 weeks in the past (Tryps.T_{-1}; low/high), in an attempt to reflect possible chronic/long-term effects of high intensities. Looking at blood cell levels at different times is valid because intensities of infection usually do not remain constant (see Results).

In these analyses, the explanatory variable of interest is the intensity of infection by trypanosomes (a dichotomous variable: low/high). The other variables were as follows. SEX was male or female and YEAR either 2005 or 2006. Seasonality was assessed by using two sinusoidal components (SEASON[sin] + SEASON[cos]) (Beldomenico et al., 2008b). A previous study determined that body weight was a better descriptor than age for RBC, while age as categorised here was a better descriptor than weight for lymphocytes. Therefore, for lymphocytes, AGE (among post-juveniles) was approximated categorising individuals into 'adult' or 'young', as described above. For RBC, body weight was used (WGT) (Weight + Weight^2 when appropriate). DENSITY refers to number of animals per grid (0.3 has) at a given trapping session and was estimated using Huggins's closed capture models within a robust design (Huggins, 1989; Kendall and Nichols, 1997). The time lag used was 6 months, as indicated by the previously developed optimal models (Beldomenico et al., 2008b). Finally, the variable R/N established if individuals were 'recaptured' or 'newly captured'. As voles sampled at the same site in the same month shared the same population level covariates, analyses also included the interaction between site and month as a random effect (SITE*SESSION) (Telfer et al., 2005). The unique identification number given to each vole (VOLE_ID) was added as a random effect to account for non-independence of samples from the same animal.

Subsequently, the model was restricted by the Akaike Information Criterion (AIC) (Akaike, 1974). Variables were removed unless they reduced the AIC by more than 2 units when included. Many of the variables included in the previously-derived optimal models were dropped, as we were working with a reduced subset of observations. Therefore, the final models reported only include variables that were significantly important to explain the variation in haematological indicators of condition in the subset of data employed. The number of observations that allowed the analyses was 129 when examining current *T. microti* infection and 154 when infection in the past was examined.

Results

The seasonality of *T. microti* infection was very marked (Fig. 1), with highest prevalences and most of the medium and high intensities concentrated from June to October. Within the subset of observations included in the study (post-juveniles captured from June to October), 1377 (73.7%) had no infection detected, 410 (22.0%) had low infection intensities, and 81 (4.3%) had high levels of parasitaemia. Of those voles that had any level of infection at a given time, 80% showed a reduction of intensity one month later. Only one (2.6%) of the high infection intensities that could be followed longitudinally remained high 4 weeks later. Except for this case (the second in that series), all high infection intensities recorded were preceded by no infection detected 4 weeks earlier.

The intensity of the infection depends on the preceding levels of lymphocytes

In every case, the matched individual that developed a lower infection intensity had a higher preceding lymphocyte level than the

Table 2
LMMs showing intensity of *T. microti* infection intensity and other variables associated with lymphocyte counts.

Current lymphocyte counts and present intensity of infection				
Model =	Lymphs$^{0.1}$ ~ YNG/ADLT + Lymphs.T_{-1} + Tryps.T_0 Random effects: SITE*SESSION; VOLE_ID.			
Term	Coefficients	Std. error	*p*-value	ΔAIC[a]
Intercept	2.626	0.102	<0.0001	–
YNG/ADLT (adult)	−0.183	0.054	0.0009	+8
Lymphs.T_{-1}	8×10^{-6}	4×10^{-6}	0.0333	+2
Tryps.T_0 (high intensity)	0.068	0.043	0.1103	−0.6
Current lymphocyte counts and intensity of infection 4 weeks previously — post-juveniles				
Model =	Lymphs$^{0.1}$ ~ YNG/ADLT + DENSITY-6 + Tryps.T_{-1} + DENSITY-6*Tryps Random effects: SITE*SESSION; VOLE_ID.			
Term	Coefficients	Std. error	*p*-value	ΔAIC[a]
Intercept	2.752	0.102	<0.0001	–
YNG/ADLT (adult)	−0.182	0.051	0.0005	+11
DENSITY-6	−0.0012	0.0006	0.0575	–
Tryps.T_{-1} (high intensity)	−0.272	0.056	<0.0001	–
DENSITY-6*Tryps.T_{-1} (high intensity)	0.005	0.001	0.0001	+16

The models include infection intensity at different times (current and past).
[a] AIC value changes if the single term is dropped from the model.

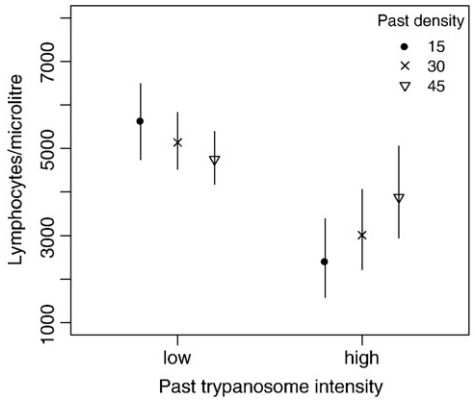

Fig. 4. Lymphocyte counts as predicted by the LMM evaluating the association with past *T. microti* infection. Simulation for adult individuals. Past vole densities (6 months previously): 15 and 45 represent the number of animals per grid at the 1st and 3rd quartiles, respectively. Error lines represent 95% confidence intervals.

one that developed a high infection intensity. The mean difference was 4005 lymphocytes/μl (ranging from 115 to 11328 lymphocytes/μl) ($t = 3.665$; $df = 6$; $p = 0.010$), meaning that those that developed high infection intensities had on average 52% lower lymphocyte counts before the infection than those that developed lower infection intensities (Fig. 2). Preceding RBC counts were not significantly different between individuals that developed high intensities of infection and those that developed lower intensities ($t = -1.55$; $df = 6$; $p = 0.086$).

The effect on haematological indicators of condition was greater for high infection intensities

RBC counts were negatively correlated with current trypanosome infection intensity (Table 1), but this association was significant and of substantial magnitude only in females, especially in those of relatively low body mass (Fig. 3). Males did not show a significant negative association. If the interactions (sex*Tryps.T_0 and weight*Tryps.T_0) are removed, AIC increases by 86 units, and the association with trypanosome intensity becomes non-significant ($p = 0.616$).

A significant negative correlation was found with RBCs when the effects of prior infection intensities (4 weeks previously) were examined. Individuals with a previously high intensity of infection had on average ~116,000 RBCs/μl less than those with a previously low infection intensity (Table 1).

No association was observed between lymphocyte counts and present infection intensity (Table 2). An association of lymphocyte counts with past intensities of infection (4 weeks previously) was found, but it was only observed after low or moderate densities (6 months previously) (Fig. 4). When past densities were high (4th quartile and above), the difference was not significant. When the interaction with past density is removed, the effect of past trypanosome intensity is not quite significant ($p = 0.054$), but retaining trypanosome intensity in the model significantly improves the goodness of fit ($\Delta AIC = 2$).

Discussion

Our results provide evidence that, under natural conditions, the intensity of *T. microti* infection is dependent on the preceding health status of the host, and that the effect of the pathogen on host condition is dependent on infection intensity.

Lymphopenic voles were more likely to show high intensities of infection 4 weeks later, suggesting that immunosuppressed voles are more prone to suffer intense infections. Depletion of T-cell subpopulations results in exacerbated parasitism in experimental Chagas disease (Tarleton et al., 1994), and rodents subject to social stress experience higher intensities of *T. cruzi* infection (Santos et al., 2008). Our results indicate that the findings of these experimental studies may occur in natural settings and be of substantial magnitude.

In these natural field vole populations, the higher the intensity of infection with *T. microti*, the lower were the RBC counts, either measured simultaneously (in lighter females) or 4 weeks later. It might be argued that an individual with a compromised condition is expected to show both low RBC levels and high intensities of infection, with the infection not necessarily being the cause of the anaemia. However, because the analysis included an adjustment for previous RBC levels, a delayed effect was observed (i.e. first high intensities, then lower RBC counts), and the effect of potential confounders was controlled, a causal explanation is favoured.

Microcytosis (Fiennes, 1970), hypoferremia (Woo and Kobayashi, 1975), and low plasma-iron turnover rates (Dargle et al., 1979) have all been observed during chronic African trypanosomiasis, suggesting impaired erythropoiesis. During the acute phase of the disease, anaemia has been attributed to antibody-dependent haemolysis (Amole et al., 1982). An antibody-induced haemolysis might explain the failure to find evidence for a direct effect of high infection intensities in males, as during the breeding season testosterone inhibits antibody production while female hormones stimulate it (Grossman, 1985). A later effect might be the result of the chronic phase of the infection, when the capacity for producing RBCs is jeopardised.

A simultaneous association between *T. microti* and lymphocyte levels was not observed. A month later, nevertheless, greater infection intensities were followed by lower lymphocyte counts. This pattern was clearer and significant when past densities were low or moderate, perhaps because the association with high trypanosome intensities becomes apparent only after the negative influence of high past densities on lymphocyte counts (Beldomenico et al., 2008b) is removed. This interaction is probably a result of the ambiguous meanings lymphocyte counts may have (Beldomenico et al., 2008b). Apart from being a measure of immunocompetence, lymphocyte counts also tend to rise in some chronic infectious diseases. Thus, because greater prevalences of infection are expected after high past densities (Burthe et al., 2006; Smith et al., 2006), variations in lymphocyte counts in this case might be reflecting both a variation in immunocompetence and differential antigenic stimulation.

The presence of interactions (with sex, weight and past density) indicates that the patterns described are not necessarily to be found in all sub-categories of individuals. However, the present results put forward an important element that should be considered in order to better understand the synergy between poor condition and infection (Pedersen and Greives, 2008; Beldomenico et al., 2008a): the intensity of infection. So, individuals in poor condition are more likely to become infected, and those infections are more likely to be intense: thus the impact on condition would be greater. Moreover, animals in nature live in pathogen-rich environments, and this synergy is likely to extend to other pathogens, hence triggering a vicious circle. Therefore, individuals in poor condition are not only more likely to become infected by one pathogen: they may also be the most important source of infection for that and for other pathogens, and thus key protagonists for parasite dynamics.

Acknowledgments

Pablo Beldomenico is a fellow of the Argentine Council for Science and Technology (CONICET). We thank Gill Telford, Roslyn Anderson, Jenny Rogers and Gemma Chaloner for their assistance in the field, and Sue Jopson for her work in the animal house. This work was funded by

Wellcome Trust grant 075202/Z/04/Z to M. Begon and a NERC Dorothy Hodgkin Award to PB.

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