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Intraspecific competition between co-infecting parasite strains enhances host survival in African trypanosomes

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Abstract. It is becoming increasingly clear that under natural conditions parasitic infections commonly consist of co-infections with multiple conspecific strains. Multiple-strain infections lead to intraspecific interactions and may have important ecological and evolutionary effects on both hosts and parasites. However, experimental evidence on intraspecific competition or facilitation in infections has been scarce because of the technical challenges of distinguishing and tracking individual co-infecting strains. To overcome this limitation, we engineered transgenic strains of the protozoan parasite *Trypanosoma brucei*, the causal agent of human African sleeping sickness. Different strains were transfected with fluorescence genes of different colors to make them visually distinguishable in order to investigate the effects of multiple-strain infections on parasite population dynamics and host fitness. We infected mice either with each strain alone or with mixes of two strains. Our results show a strong mutual competitive suppression of co-infecting *T. brucei* strains very early in infection. This mutual suppression changes within-host parasite dynamics and alleviates the effects of infection on the host. The strength of suppression depends on the density of the co-infecting strain, and differences in life-history traits between the strains determine the consequences of strain–strain competition for the host. Unexpectedly, co-infection with a less virulent strain significantly enhances host survival (+15%). Analysis of the strain dynamics reveals that this is due to the suppression of the density of the more virulent strain (−33%), whose degree of impact ultimately determines the physical condition of the host. The competitive suppression is likely caused by allelopathic interference or by apparent competition mediated by strain-specific immune responses. These findings highlight the importance of intraspecific variation for parasite–parasite and parasite–host interactions. To fully understand parasite and disease dynamics, the genetic diversity of infections must be taken into account. Through changes in parasite dynamics, intraspecific variation may further affect transmission dynamics and select for increased virulence of each strain. The precise mechanisms underlying mutual suppression are not yet understood but may be exploitable to fight this devastating parasite. Our results are therefore not only of basic ecological interest investigating an important form of intraspecific competition, but may also have applied relevance for public health.

Key words: Africa; host–parasite interaction; immune-mediated apparent competition; interference competition; intraspecific interaction; intraspecific variation; pathogen; population dynamics; Trypanosoma brucei.

Introduction

Ecologists have long studied interspecific variation and its implications for interactions between species (Gause 1934, Tilman et al. 1981, Wootton 1994, Maitland et al. 1997, Schmitz et al. 1997, Berlow 1999, Bruno et al. 2003). Species are often treated as homogeneous groups of individuals with average characteristics. However, in reality species do not consist of individuals with all the same mean properties. Rather, individuals and populations of one species can greatly vary in genotype and in important phenotypic traits. The distribution of this variability affects the behavior of populations and the interactions of populations with those of other species (Roughgarden and Diamond 1986, Thompson 1988). The role of intraspecific variability in modulating population dynamics and interspecific interactions is not understood in detail but may be of fundamental importance to accurately predict species interactions.

In host–parasite interactions, the effects of intraspecific variation are bound to greatly modulate the outcome of the interspecific interactions between the host and the parasite. Both the variability of the host

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and of the parasite can be significant. The success of a specific parasite genotype can depend on characteristics of the individual host encountered. Likewise, variability in the expression of parasite traits can determine which parasite strains are successful on a specific host genotype and which are not (Ebert et al. 1998, Ferguson and Read 2002, Lambrechts et al. 2005, Little et al. 2006, Salvaudon et al. 2007, Ben-Ami et al. 2008*b*).

Here we focus on effects that are likely to bear great significance in many if not all host–parasite systems: the effects of intraspecific parasite variability within an infected host, i.e., the effects of interactions between parasite strains on the parasite's population dynamics and the consequences these have for the host.

Multiple-strain infections are still poorly understood, both in terms of their prevalence in nature and of their effects on specific parasites or on host–parasite interactions in general. The occurrence of multiple-strain infections is well documented in malaria, where it has been shown that >85% of the infected population can carry multiple-strain infections (Babiker et al. 1999). There are increasing data on the prevalence of multiple-strain infections from other parasites. The emerging picture is that they are found in most parasite species for which the necessary genetic tools to detect them are available and researchers actively look for them (e.g., Sharp et al. 1997, Schmid-Hempel and Reber Funk 2004, Warren et al. 2004, Keeney et al. 2007, Balmer and Caccone 2008). They appear to be the norm rather than the exception.

Infections with more than one strain can be fundamentally different from infections with one strain and have important consequences both for the host and the parasite. For the host, they represent a broader challenge that complicates defense and immune response (Levin and Anderson 1999). For the parasite, they lead to direct or indirect interactions between strains that can alter within-host population dynamics and transmission between hosts and lead to novel selection pressures on and thus altered evolution of parasite life-history traits.

Direct interference competition by excretion of molecules that inhibit the growth of competing strains but not of the excreting strain (allelopathy) is well documented in bacteria (Kerr et al. 2002). This mechanism is also generally held to operate between different plant species, but it remains unclear how relevant it is in natural settings (Harper 1977). Indirect interactions between strains include competition for shared resources in the host, which is likely to be more intense than in competition between species due to the much stronger niche overlap as a consequence of the close relatedness of strains. Immune-mediated apparent competition is another form of indirect interaction. In immune-mediated apparent competition (Raberg et al. 2006), a strain suffers from an immune response that is elicited by another strain. The effector cells of the immune system are therefore analogous to a shared predator in predator–prey systems with two prey species (Holt 1977). Interactions between strains can also be commensalistic or mutualistic (Bruno et al. 2003). Many parasites have immunosuppressive properties (Hudson et al. 1976, Lello et al. 2004), so infection compromises an efficient immune response. Immunosuppression facilitates infection with further parasite species. Because strains are much more closely related and immunologically more similar than different species, facilitation is expected to be much more prominent in multiple-strain infections. Furthermore, specific parasite surface structures ("epitopes") can mutually interfere with the immune response against other epitopes (Gilbert et al. 1998, Plebanski et al. 1999), a phenomenon known as "altered peptide ligand" (Bertoletti et al. 1994, Klenerman et al. 1994), or they can restimulate an antibody response already mounted against a very similar epitope of another strain, leading to a suboptimal response and in effect benefiting that strain, a process known as "original antigenic sin" (Fazekas de St. Groth and Webster 1966, Klenerman and Zinkernagel 1998).

Any form of interaction between strains potentially changes selective pressures. Selection leads to the evolution of parasite life-history traits such as virulence (defined here as the level of harm caused by a parasite to its host). Virulence takes a central position either as an explanatory or response variable in most theoretical models of host–parasite and parasite–parasite interactions (Dieckmann et al. 2002). Classical theory predicts that competition between strains in most cases will select the parasites to evolve toward higher virulence (May and Nowak 1995, Van Baalen and Sabelis 1995, Frank 1996). On the other hand, virulence differences can be an important determinant of competition outcome between strains. For example, de Roode et al. (2005) and Ben-Ami et al. (2008*a*) have shown in rodent malaria and in the *Daphnia magna–Pasteuria ramosa* system, respectively, that the more virulent strain in mixed infections is competitively superior and has a higher probability of being transmitted.

Experimental evidence to distinguish between the different forms of interaction is very scarce in eukaryotic parasites, severely limiting our understanding of multiple-strain infections. Most empirical multiple infection studies investigate infections with (usually two) distinct species (Hudson et al. 2002, Poulin 2007), or they make indirect inferences by comparing single to mixed infections without assessing the contributions of the single strains in the mixed infection. One reason for the lack of experimental studies has been the technical challenge of distinguishing and independently observing the dynamics of different strains of one species in mixed infections.

Given the commonness of multiple-strain infections and their likely important consequences, it is critical to develop experimental systems in which both the population dynamics of strains within hosts and the mechanisms mediating those dynamics can be investi-

gated experimentally. To that end we chose *Trypanosoma brucei* Plimmer and Bradford 1899, a unicellular protozoan blood parasite (Barrett et al. 2003), as our model system (see Plate 1). Trypanosomes are a major factor shaping the African continent. Human African trypanosomosis (sleeping sickness) is fatal if untreated and ranks second among parasitic diseases in sub-Saharan Africa only to malaria in terms of mortality (WHO 2002). Animal trypanosomosis (Nagana) prevents cattle farming and the use of work animals over huge areas of sub-Saharan Africa (Connor 1994) and thus profoundly influences the ecology and the socio-economic situation of the entire continent. The parasite reproduces clonally in the vertebrate host and facultatively recombines in the tsetse fly (*Glossina* spp.) vector (Jenni et al. 1986; see plate 1). To evade the immune response, parasites regularly undergo the process of antigenic variation. Almost weekly, the total exchange of cell surface structure generates a population of identical, but antigenically novel, parasites (Barry and Carrington 2004).

Here we investigate what effects multiple-strain infections have on within-host parasite dynamics and on host–parasite interactions. We engineered visually distinguishable transgenic *T. brucei* strains with different levels of virulence containing strain-specific fluorescent genes of different color (Balmer and Tostado 2006). We compared the population dynamics of two focal strains in single vs. mixed infections and their effects on mouse hosts to ask: (1) Do different strains of *T. brucei* interact in their vertebrate host? (2) Does strain virulence influence the outcome of interaction? (3) Does the population density of a strain influence the other strain? and, (d) What effect does the interaction between strains have on host fitness? To investigate the effect of virulence we generated two variants of different virulence for one of the strains and compared the effects of multiple infection when the co-infecting strains differed in virulence (variable-virulence experiment, VVE) to the effects when both had the same virulence (equal-virulence experiment, EVE). To investigate the effect of density we compared parasite dynamics of a focal strain co-infected with different concentrations of a second strain (density-dependence experiment, DDE).

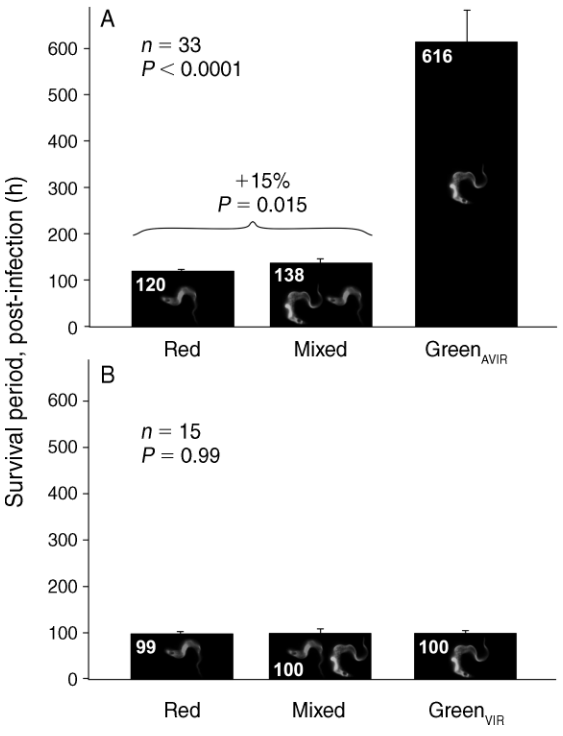

FIG. 1. Host response to mixed infections by strains of the protozoan parasite *Trypanosoma brucei*: mouse (*Mus musculus*) survival (mean + SE) post-infection in (A) the variable-virulence experiment (using the avirulent green strain, green$_{AVIR}$) and (B) the equal-virulence experiment (using the more virulent strain, green$_{VIR}$). All pairwise contrasts in (A) are significant: red vs. mixed, $t_{20} = 2.66$, $P = 0.015$; mixed vs. green$_{AVIR}$, $t_{20} = 8.37$, $P < 0.0001$; red vs. green$_{AVIR}$, $t_{20} = 8.73$, $P < 0.0001$.

MATERIALS AND METHODS

Parasite strains

The two strains of *Trypanosoma brucei brucei* used were isolated from a vertebrate host *Alcelaphus buselaphus cokii* (Coke's hartebeest, kongoni) in Serengeti National Park, Tanzania (STIB246BA-R1, "red"), and from the vector *Glossina fuscipes fuscipes* (tsetse fly) in Busoga, Uganda (STIB777AE-G1, "green$_{AVIR}$"), respectively. They differ substantially in mitochondrial and nuclear (Balmer et al. 2006) genotype. *Trypanosoma b. brucei* is an animal-infective form of *T. brucei* that is genetically identical to the human-infective host-range variant *T. b. rhodesiense* except for the lack of the serum resistance associated (*SRA*) gene conferring human infectivity to *T. b. rhodesiense* (Gibson 2005). Both strains were cloned (i.e., grown up from a single parasite individual) after isolation and then cultured in axenic medium prior to transfection and infection. They thus had time to accumulate mutations and are expected to contain some (low) level of genetic variability, as in natural infections. STIB246BA-R1 and STIB777AE-G1 were stably transfected with mRFP1 (red) and EGFP (green) fluorescent protein genes, respectively, as described previously (Balmer and Tostado 2006) to allow the tracking of both strains by color in mixed infections (see photographs in Figs. 1 and 3). The genes were inserted into the genome and are constitutively expressed in all parasite life stages. The two fluorescent strains are referred to as red and green$_{AVIR}$ for simplicity. Both strains exhibit different but highly reproducible virulence (Fig. 1A) and growth characteristics (Fig. 2A).

Mice

All experiments were conducted in accordance with institutional guidelines and federal regulations with outbred female white NMRI mice (*Mus musculus*; RCC, Itingen, Switzerland). Outbred mice were used

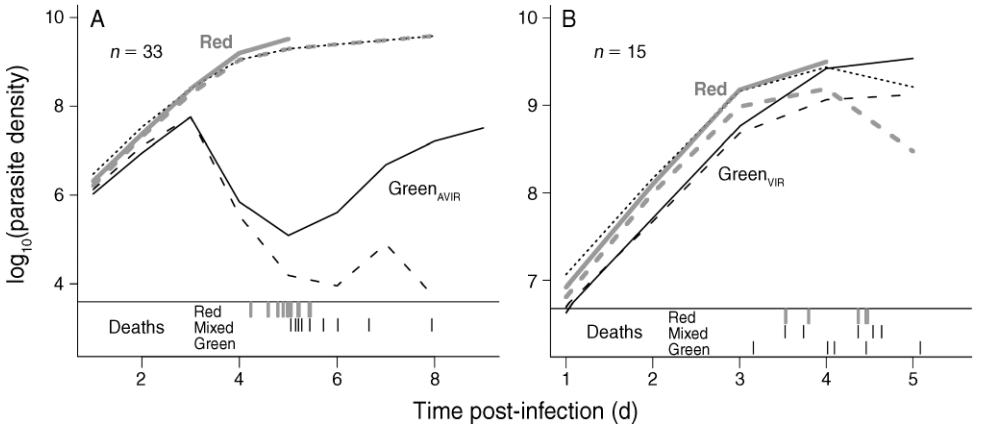

FIG. 2. Geometric mean parasite population growth curves per strain in (A) the variable-virulence experiment (using green$_{AVIR}$) and (B) the equal-virulence experiment (using the more virulent green$_{VIR}$). Curves end at the day of death of the last mouse in each treatment group except for the single infection of green$_{AVIR}$ in (A). The dotted black lines show the combined density of red and green$_{AVIR/VIR}$ in mixed infections. Each line is based on (A) 11 or (B) five repeatedly measured mice, respectively. The solid lines show single infection. The dashed lines show the density of red or green$_{AVIR/VIR}$ strains in mixed infections. The three mixed curves all derive from the same mice. Ticks above the *x*-axis indicate time of death of each mouse [outside the scale only for green$_{AVIR}$ in panel (A)]. Note the varying scales.

to be closer to the natural situation and because effects are more convincingly relevant if they can be shown even in genetically variable hosts. Mice were housed in cages with a maximum of five animals and only one mouse per treatment. They were mass-matched per cage (maximum ± 0.5 g) to ensure mouse masses did not differ by treatment. Mice were provided food and water ad libitum.

Virulence variants of strain STIB777AE-G1 (green)

To investigate the effects of parasite virulence, two variants of strain green, the less virulent of the two strains, were created. The original strain, called green$_{AVIR}$ was passaged through a mouse for 25 days (~75 parasite generations), which resulted in a higher-virulence variant called green$_{VIR}$ with a virulence very similar to red (Fig. 1B).

Experimental designs

In the variable-virulence experiment (VVE), 33 female 26-day-old mice (11 per treatment) were infected intraperitoneally with 1.5×10^6 parasites of red (red treatment), 4.5×10^6 parasites of green$_{AVIR}$ (green treatment), or 1.5×10^6 parasites of red and 4.5×10^6 parasites of green$_{AVIR}$ ("mixed" treatment). Relatively high inocula were used to stress parasite strain over host individual effects and because previous experiments had shown that this decreased random variation in the response. The green$_{AVIR}$ was infected at a higher dose because of its slower growth to maximize chances of detecting even weak interaction effects between the strains. As the VVE showed strong treatment effects, the sample size was reduced to five mice per treatment in subsequent experiments in accordance with national ethics guidelines.

The design of the second equal-virulence experiment (EVE) primarily differed from the VVE in that the more virulent variant green$_{VIR}$ was used instead of green$_{AVIR}$. Thirty-three-day-old mice were infected with 1×10^7 parasites of red, 1×10^7 parasites of green$_{VIR}$, or 1×10^7 parasites of red and 1×10^7 parasites of green$_{VIR}$ (mixed treatment) and kept in cages with one mouse per treatment.

Additionally, a fourth "intermediate" treatment was added to the VVE, which received 1×10^7 parasites of red but only 1×10^5 parasites of green$_{VIR}$ to assess effects of the density (inoculum size) of the competing strain on the focal strain red whose inoculum size was kept constant. This analysis is referred to as the "density-dependence experiment" (DDE).

Uninfected control mice were included in all experiments to provide baseline information on host parameters. They were not included in any analyses presented here because comparisons are always between multiple and single infections, not between infected and non-infected hosts.

Measurements and statistical analysis

Repeated measurements were taken from each mouse before infection and then again every 24 hours. We measured parasite density per strain (no./mL), mouse mass (g), erythrocyte (red blood cell) concentration (no./mL), thrombocyte (platelet) concentration (no./mL), and blood glucose level (mmol/L). Parasite and blood cell counts were measured with a Becton Dickinson FACScan flow cytometer (Becton, Dickinson, Franklin Lakes, New Jersey, USA) from the first drop of tail blood as described previously (Balmer and Tostado 2006). Glucose was measured immediately from the second blood drop using a MediSense Precision Q.I.D. blood glucose meter (Abbott Laboratories, Abbott Park, Illinois, USA).

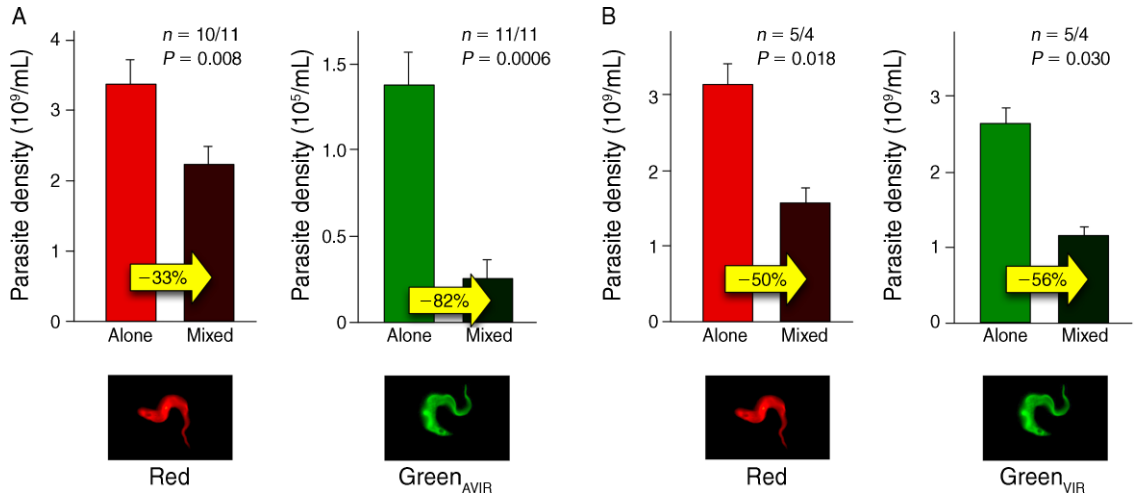

FIG. 3. Comparison of parasite densities (mean + SE) of both strains alone or in mixed infections on (A) day 5 or (B) day 4 post-infection in (A) the variable-virulence experiment (using $green_{AVIR}$) and (B) the equal-virulence experiment (using the more virulent $green_{VIR}$). The yellow arrows indicate the relative competitive suppression of each strain in mixed infections. Sample size *n* is given as sample size of singly/doubly infected mice. Note the varying scales.

Proportions of slender, intermediate, and stumpy parasite forms were not quantified because hardly any (<1%) non-slender forms were detected by eye at any time during repeated checks throughout the experiments.

Survival post-infection (hours) was recorded and differences between treatments assessed for overall significance and significance of pairwise contrasts between treatments by linear mixed-effects models in R version 2.4 (R Development Core Team 2005). Because mice were mass-matched by cage, "cage" was included as random effect where it significantly improved the model as determined by the likelihood ratio test. Parasite densities, anemia (erythrocyte loss), thrombocytopenia (thrombocyte loss), and hypoglycemia (glucose deficiency) were compared for both strains separately between single and mixed infection treatments by linear mixed-effects models on day 5 post-infection in VVE. In EVE, comparisons were made on day 4 because infections were more virulent due to increased inoculum sizes. To assess if the suppression of parasite density in mixed infections of VVE and EVE was significantly different between strains, $\log_{10}$-parasite densities ($\log_{10}$-transformed to transform the data to a proportional scale) were compared between treatments by linear models with the appropriate contrasts (Venables and Ripley 2002). Anemia, thrombocytopenia, and hypoglycemia were calculated by subtracting erythrocyte (or thrombocyte and glucose, respectively) counts on day 5 (VVE) or 4 (EVE, DDE) from the average erythrocyte (or thrombocyte and glucose, respectively) counts of day 0 (before infection) and day 1 (post-infection) and dividing by the average of days 0 and 1. For variables where this analysis did not reveal significant differences between treatments, all data points were included in a single linear mixed-effects model, i.e., the time course of those variables was modeled as a quadratic function with "treatment," "time post-infection," and the interaction between treatment and time post-infection as factors, to increase statistical power. The data were adjusted for unequal variances in treatment groups where it significantly improved the model as determined by the likelihood ratio test. In the DDE, there was a clear theoretical expectation that increasing amounts of co-inoculated $green_{VIR}$ would lead to a unidirectional change in red population density. We therefore treated the number of $green_{VIR}$ as an ordered factor and used a contrast estimating a linear trend to test for a significant treatment effect (Pinheiro and Bates 2000).

RESULTS

Parasite population growth and host survival

Virulence (measured as host survival post-infection) and population growth patterns differed drastically between the two *T. brucei* strains red and $green_{AVIR}$ but were very constant within each strain. The green $_{AVIR}$ was approximately five times less virulent than red (Fig. 1A) and was initially controlled presumably by the host immune system, leading to a sharp population decline after an initial peak (Fig. 2A). The $green_{VIR}$, the higher virulence variant of $green_{AVIR}$, exhibited the same virulence as red (Fig. 1B) and a similar growth pattern (Fig. 2B).

Unexpectedly, the mixed infection (with the highest total inoculum size) was not the most virulent treatment in the variable-virulence experiment (VVE). Rather, mixed infection significantly reduced the virulence of *T. brucei* infection compared to the single infection with red alone (15% longer host survival; Fig. 1A). Both parasite strains significantly suppressed each other in the mixed infection of the VVE (Fig. 3A). The combined population size of red and $green_{AVIR}$ in the mixed infection

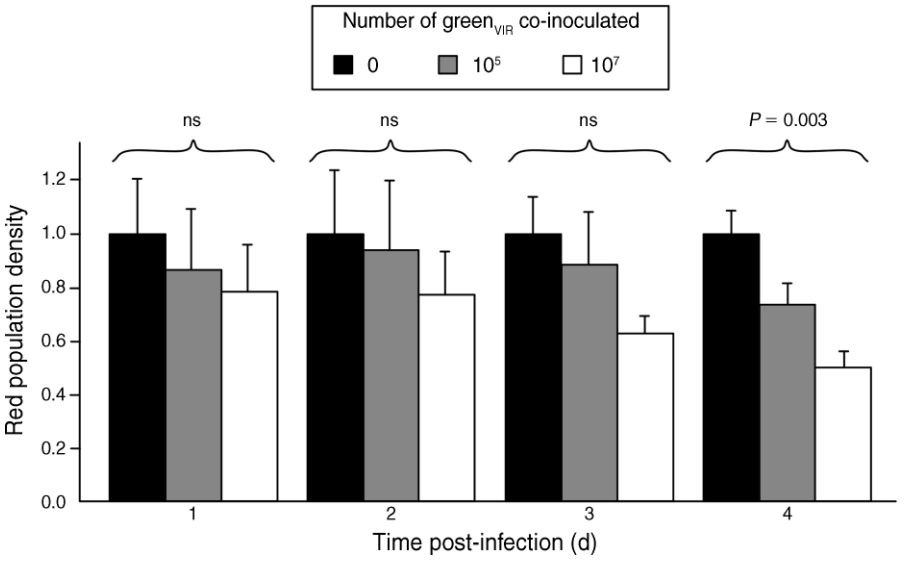

FIG. 4. Density dependence of strength of suppression. Dependence of competitive suppression of strain red on the density (inoculum size) of green$_{VIR}$ in the density-dependence experiment (DDE). Daily population densities (mean + SE) of strain red competed against three densities of green$_{VIR}$ are shown. Population densities are scaled to 1 for each day to visualize the relative differences between treatments. Significance of the treatment effect is indicated for each day separately.

reached levels significantly below that of the single red infection in the VVE (Fig. 2A; density on day 5 post-infection: −33%, $F_{1,9} = 11.63$, $P = 0.008$). The observed suppression of parasite population growth was thus due to an active inhibition, and not simply a numerical consequence of a limitation of the maximal attainable population size. The green$_{AVIR}$ was significantly more suppressed by its competitor than red ($t = 3.14$, $P = 0.003$) in the VVE. In the equal-virulence experiment (EVE), significant mutual competitive suppression occurred as well (Fig. 3B) but this did not translate into a difference in host survival between treatments (Fig. 1B) and the strains did not differ significantly in how much they were suppressed ($t = 0.58$, $P = 0.57$). Also, in contrast to the VVE, the combined parasite density reached in the mixed infection of the EVE was as high as in any of the single infections ($P > 0.3$ for both strains). Thus, taken alone the EVE might have been interpreted as indicating that the growth of the strains in mixed infections is limited due to a maximal attainable total population size, while the VVE reveals that this is not the case.

There was a significant effect of the inoculum size of green$_{VIR}$ on the strength of suppression of strain red in the density-dependence experiment (DDE) (Fig. 4). From day 1 on (although statistically significant only on day 4 post-infection owing to the sample size), increasing densities of green$_{VIR}$ led to an increasingly stronger suppression of the red population growth.

Host condition

Three measures of host condition (anemia, thrombocytopenia, and hypoglycemia) showed strong responses to infection in most treatments of the VVE and the EVE

TABLE 1. Deterioration of host condition over time in the different infection treatments by the protozoan parasite *Trypanosoma brucei* in the variable-virulence experiment (VVE) and the equal-virulence experiment (EVE).

Measure and treatment	Change (%)†	*t*	df	*P*
A) Variable-virulence experiment				
Erythrocyte loss (anemia)				
Red	−39.3	15.50	9	<0.0001
Mixed	−30.6	9.98	10	<0.0001
Green	−5.0	1.62	10	0.136
Thrombocyte loss (thrombocytopenia)				
Red	−76.1	17.47	9	<0.0001
Mixed	−72.9	23.34	10	<0.0001
Green	−24.7	7.32	10	<0.0001
Glucose loss (hypoglycemia)				
Red	−86.9	29.03	9	<0.0001
Mixed	−52.3	4.29	10	0.002
Green	+3.6	0.62	10	0.552
B) Equal-virulence experiment				
Erythrocyte loss (anemia)				
Red	−27.7	5.91	4	0.004
Mixed	−33.3	8.51	3	0.003
Green	−31.9	4.34	4	0.012
Thrombocyte loss (thrombocytopenia)				
Red	−84.8	19.42	4	<0.0001
Mixed	−84.5	9.46	3	0.003
Green	−86.4	30.08	4	<0.0001

† Average percentage decrease of blood values (erythrocyte, thrombocyte, and glucose concentrations) from the start (average of days 0 and 1 post-infection) to the final measurement (day 5 post-infection in VVE, day 4 in EVE); paired *t* tests.

TABLE 2. Statistical significance of differences in host condition deterioration between the infection treatments in the variable-virulence experiment (VVE).

Measure†	Overall		Contrasts between infection treatments‡						First day§
			Red vs. mixed		Mixed vs. green		Red vs. green		
	$F_{2,19}$	P	t_{19}	P	t_{19}	P	t_{19}	P	
Erythrocyte loss (anemia)	37.03	<0.0001	2.08	0.0511	−6.27	<0.0001	−8.20	<0.0001	4
Thrombocyte loss (thrombocytopenia)	165.99	<0.0001	0.88	0.3878	−15.48	<0.0001	−15.92	<0.0001	3
Glucose loss (hypoglycemia)	166.16	<0.0001	2.83	0.0108	−4.41	0.0003	−18.22	<0.0001	5

Note: In the equal-virulence experiment (EVE) and the density-dependence experiment (DDE), treatments did not differ significantly with regard to the responses measured here (hypoglycemia not assessed).

† Proportional decrease of erythrocyte, thrombocyte, and glucose concentrations relative to starting values (average of days 0 and 1).

‡ The t values and significance level of pairwise differences between treatments on the day of final measurement (day 5); linear mixed-effects models.

§ First day post-infection with significant overall difference between treatments in the respective measures; linear mixed-effects models. Subsequent days always exhibited stronger significance (lower P values).

(Table 1) and in all treatments of the DDE (all $P \leq 0.027$). Mice in the "green" treatment of the VVE ($green_{AVIR}$ alone) did not develop significant anemia and hypoglycemia. Otherwise, all treatments led to significant anemia, thrombocytopenia, and hypoglycemia in all experiments. All three measures were strongly influenced by the presence or absence of the more virulent strain red in the VVE, i.e., the mice were significantly sicker if infected with red (alone or in mixed infections) compared to single infections with the less virulent $green_{AVIR}$ (Table 2). The finding that red was the main driver of host sickness in the VVE was corroborated by the results of the EVE and DDE where the green strain ($green_{VIR}$) had greater virulence and the treatments did not differ with respect to host condition anymore.

In the VVE, hypoglycemia was significantly more severe ($P = 0.0108$) and anemia tended to be more severe ($P = 0.0511$) in the single infection with red than the mixed infection on day 5 post-infection (Table 2). However, in all treatments the glucose levels (as well as mass gain; data not shown) were not affected by infection over most of the experimental period and sharply fell off only shortly before host death, probably owing to a general breakdown of host metabolism and homeostasis. This pattern strongly suggests that the decline in glucose levels is a direct consequence of imminent host death and is not involved in mediating interactions between strains. Glucose concentration is thus also not a good virulence measure in this system. Combining all daily data points per mouse in a single linear mixed-effects model to increase statistical power did not reveal any additional treatment effects in any of the measured variables.

DISCUSSION

This study demonstrates that strong competition between co-infecting strains of the sleeping sickness parasite *Trypanosoma brucei* changes both the parasite's within-host dynamics and the effects of infection on the host. Specifically, our experiments show that (1) strains vary markedly in their growth patterns and virulence, (2) there are interactions between co-infecting strains which can substantially suppress individual strains very early in infection, (3) co-infection with a second, less virulent strain reduces the burden on the host, and (4) the level of suppression of a strain depends on the density of the competitor strain.

It is no surprise that co-infection with a more virulent strain leads to an overall higher virulence of an infection. The finding, however, that co-infection with a less virulent strain actually benefits the host was unexpected. The simple additive model would predict that mixed infections, which received cumulative (i.e., the highest) infecting doses, would be the most detrimental to host fitness because they received the same amount of the more virulent strain as single infections plus additionally the less virulent strain. Indeed, both in rodent malaria (Taylor et al. 1998) and in *Schistosoma mansoni* infections of *Biomphalaria glabrata* (Davies et al. 2002), higher virulence of mixed infections was shown even when the total number of parasites inoculated was kept constant. The tracking of the two co-infecting strains showed that the virulence reduction in mixed infections in this study was due to an active mutual suppression of the strains such that the less virulent strain had an indirect positive effect on the host by suppressing the more virulent strain that ultimately determined the effect of the infection on the host's physical condition. We are aware of one other study showing mutual parasite suppression and increased host survival in mixed infections, also in *T. brucei* but in interspecific mixed infection with the intestinal nematode *Strongyloides ratti* (Onah et al. 2004), and not in response to an intraspecific multiple-strain infection. Interestingly however, different strains of *S. ratti* do not seem to suppress each other in intraspecific mixed infections (Paterson and Viney 2003).

The DDE shows that the level of suppression depends on the quantity of the co-infecting strain (its initial density) and that suppression appears to start soon after

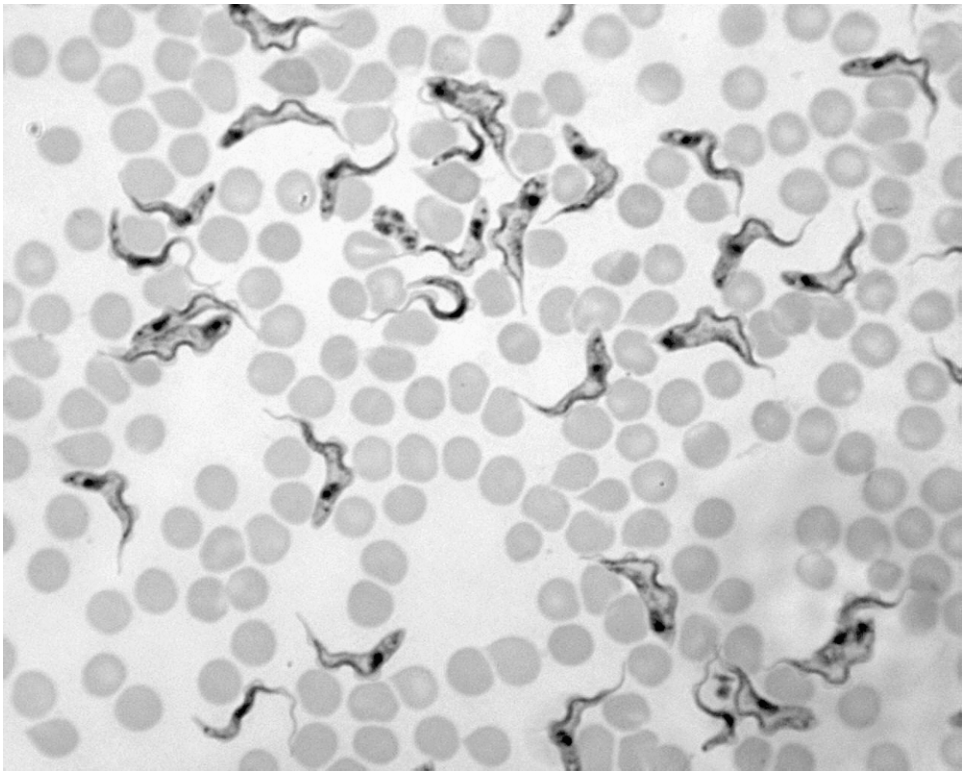

PLATE 1. Light microscopic image of Giemsa-stained *Trypanosoma brucei*, a flagellated single-celled parasite, among mouse red blood cells. *Trypanosoma brucei* is divided into three subspecies: *T. b. rhodesiense* and *T. b. gambiense* cause invariably lethal human African trypanosomiasis (sleeping sickness) in Eastern/Southern and Western/Central Africa, respectively. *T. b. brucei* together with other trypanosome species is non-human infective and causes nagana, a wasting disease of domestic animals, throughout sub-Saharan Africa. Well visible is the distinction between long-slender parasites, which are the replicating stage in the mammalian host, and short stumpy forms, which are in cell cycle arrest and serve as the transmission stage to the tsetse fly (*Glossina* spp.) vector. Photo credit: Tanja Wenzler.

infection. Interestingly, we saw competitive exclusion of a strain only in a minority of cases even in the face of strong suppression and after competition over several weeks in multiple experiments conducted (data not shown). Competition between strains thus appears to be density dependent such that competition pressure on a strain is relaxed when it becomes rare.

In studies of *Plasmodium chabaudi* that investigated competitive effects over a range of virulences, the virulence of a strain was found to correlate with the strength of suppression between strains (de Roode et al. 2005, Bell et al. 2006). In our variable-virulence experiment (VVE) and equal-virulence experiment (EVE), suppression of red is similar despite greatly increased virulence of $green_{VIR}$ in the EVE. But this result is based on a single comparison. We can therefore not draw strong conclusions about the effect of virulence on the strength of suppression in trypanosomes. However, virulence affects host mortality, as the difference between the VVE and EVE shows. The differences between the two experiments are due to qualitative difference between $green_{AVIR}$ and $green_{VIR}$, and cannot be explained by higher inocula in EVE, as $green_{VIR}$ was also more virulent (as virulent as red) at the lower inoculation dose used in the VVE (data not shown).

A common way to explain (Begon et al. 1996) or model (Antia et al. 1996) competition between strains is to invoke a global "carrying capacity," i.e., a total population number that can not be exceeded due to resource or space limitation, such that a high abundance of one genotype automatically reduces the maximum attainable abundance of the other. However, the suppression we observe in our VVE cannot be explained by such numerical effects because the combined population size of red and $green_{AVIR}$ in the mixed infection actually reached significantly lower levels than that of the single red infection. This is in contrast to results in rodent malaria (Read et al. 2002, de Roode et al. 2003), where parasite densities and virulence of mixed infections are indistinguishable from or even higher than those of the most virulent single infections. The suppression of parasite population growth in our VVE must thus be the result of an active inhibition, and not a mere numerical response caused by a limitation of the maximal attainable population size.

Three mechanisms could plausibly account for the observed mutual competitive suppression: resource

competition, direct allelopathic interference competition, or immune-mediated apparent competition. Blood glucose is the most likely resource to be limiting because trypanosomes rely entirely on glycolysis for their energy metabolism and have an inactivated Krebs cycle in the vertebrate host (van Hellemond et al. 2005) and therefore use 19 times more glucose per ATP produced than other eukaryotic organisms. However, glucose levels remained constant in mice in all treatments except on the last day before host death, when they abruptly decreased to near zero. Glucose therefore is unlikely to mediate an interaction between strains earlier in infection. We cannot exclude further resources (e.g., transferrins, purines) in our study, but competition between strains started so early in infection, long before any resources are plausible to become limiting, that resource competition is unlikely in general. A further point that speaks against resource competition is that any mechanism invoked must be strain-specific. Otherwise we would expect suppression to depend on total population density only and would thus expect no difference between single and mixed infections.

Direct competition between strains through allelopathic interference is plausible via excreted factors either limiting cell growth of the other strain or killing parasites of the other strain. Growth suppression in trypanosomes has been demonstrated in vitro by pyruvate, a waste product of glycolysis (Brun et al. 1981), and has been implicated in vivo based on sequential infection experiments (Turner et al. 1996). The transformation of dividing slender parasite forms to nondividing stumpy forms, which is induced in a density-dependent manner by an as yet uncharacterized "stumpy induction factor" (Matthews et al. 2004), can be excluded as a relevant factor here, as the number of stumpy forms detected was negligible. Allelopathic antagonism between conspecific genotypes via toxins is a well-known mechanism in bacterial systems (e.g., colicins in *Escherichia coli* (Chao and Levin 1981)) but has so far not been described in trypanosomes.

Immune-mediated apparent competition could involve innate (nonspecific) or adaptive (acquired, specific) immunity. The early onset of competition implies a response of the innate immune system, while the required specificity to distinguish between conspecific strains points to the adaptive immune system. However, recent evidence suggests that the innate immune response can be more specific than traditionally thought (Takeda and Akira 2005, Gazzinelli and Denkers 2006), so that strain-specificity does not rule out innate immunity as a mechanism. The involvement of the immune system in mediating competition between co-infecting strains has been shown in rodent malaria (Raberg et al. 2006), and in a microbial system specific effector cells of the innate immune response were identified that mediate competition between *Haemophilus influenzae* and *Streptococcus pneumoniae* co-colonizing mucosal surfaces (Lysenko et al. 2005). Immune-mediated apparent competition is thus plausible. If mutual suppression is mediated by specific immunity, the strength of suppression may depend on the identity of the dominant cell surface molecules expressed by the involved strains. However, antigenic switching itself is unlikely to play an important role in this study because the responses we show happen considerably faster than the rate at which dominant cell surface molecules are exchanged.

Two fundamentally different mechanisms could account for the effects of multiplicity of infection on the host: the host–parasite interaction may remain unchanged but the interaction among strains changes the total and/or relative densities of the strains so that the host is affected by different numbers of the parasite. In this case, the host experiences qualitatively the same interactions with the parasite but on a different quantitative level. Alternatively, a strain could qualitatively change the way another strain interacts with the host. This has been demonstrated to happen in *P. falciparum*, where cp26 and cp29, two variants of a malaria cytotoxic T-cell epitope carried by different strains, mutually interfere with the immune response of the host toward the other variant (Gilbert et al. 1998, Plebanski et al. 1999). In this case, there is a direct interference by the parasite strains with the host response to infection and the host–parasite interaction is qualitatively changed in multiple infections. We have no indication for the latter in our system. The changes in parasite growth patterns we observe in both strains indicate that in our case the first mechanism is at play.

The infecting doses used in our experiment are higher than in nature, so extrapolations to the natural situation must be made with caution. Also, the effects we show occurred early in the infection and may not expand over the whole infection in natural infections. However, the main point is that our results show (1) that mechanisms exist in *T. brucei* by which strains interact and (2) that multiple-strain infections can change both parasite dynamics and effects on the host. The use of outbred mice strengthens our results because significant effects were observed despite host variability, which increases inter-individual variability in the data and makes the results more realistic.

Implications of intraspecific competition

Multiple-strain infections are common in nature in *T. brucei* (Balmer and Caccone 2008) and most other parasite species (e.g., Sharp et al. 1997, Schmid-Hempel and Reber Funk 2004, Warren et al. 2004, Keeney et al. 2007), and virulence and growth patterns vary greatly across strains (O. Balmer and R. Brun, *personal observation*). The competition scenarios investigated here should thus be common in nature. Our results therefore have important implications on several levels.

First, our findings demonstrate that intraspecific variation introduced by multiple strains can alter both the behavior of the parasite population and its effects on

the host. In our system, multiple-strain infections are profoundly different from the respective single-strain infections. To fully understand host–parasite dynamics it is therefore necessary to understand the differences between strains and the interactions both among the strains and between the host and the strains. Neglecting intraspecific variation can lead to false predictions of population dynamics or to misinterpretations of observed patterns. Parasites can regulate host population dynamics (Dobson and Hudson 1992, Vandegrift et al. 2008). Intraspecific interactions between parasite strains could thus change the regulation of the host population dynamics by relaxing (or aggravating) parasite effects, fundamentally altering the outcome of host–parasite interaction.

Second, the mutual suppression may affect transmission dynamics because it changes absolute and relative concentrations of parasite strains taken up by the vector. In *P. chabaudi* it has been shown that increased relative population size in a host can indeed lead to increased transmission success of a strain (de Roode et al. 2005) and that strains are better transmitted from multiply than from singly infected hosts (Taylor et al. 1997). In *T. brucei*, however, it has been shown that decreased population size (of a single parasite strain) in the host does not seem to reduce transmission success (Van den Bossche et al. 2005). Therefore, a second process may be of greater importance in *T. brucei*. Transmission dynamics may be affected primarily by the reduced fitness effects of the more virulent strains on their hosts in mixed infections, because they lengthen the residence of infected hosts in the infective pool, leading to an increased transmission potential and a longer persistence of the more virulent parasite strains in the host population. This would set up a conflict between different levels of selection (Keller 1999): what is good for an individual host may be detrimental for the host population as a whole.

Third, intraspecific competition between strains can lead to the selection of parasite traits enhancing strain performance in two ways: (1) for the individual parasites, competition will lead to the evolution of life-history traits, e.g., to an increase in virulence. There is well-established theory on this (Frank 1996) but very limited empirical evidence yet (but see de Roode et al. 2005). Our findings clearly demonstrate that competition between strains is intense. The potential for rapid evolutionary change is therefore given (Reznick et al. 1997, Hairston et al. 2005). (2) If competition is mediated by a cross-reactive strain-specific immune response, we would predict that the parasite populations segregate into immunologically distinct strains with nonoverlapping repertoires of cell surface antigens to elicit minimal cross-reactivity (Gupta et al. 1998), as has been demonstrated in *Neisseria meningitidis* (Gupta et al. 1996) and dengue virus (Kawaguchi et al. 2003). This prediction could be tested by competing groups of strains from within different regions. We would predict the least cross-reactivity between strains from areas with the highest prevalence of mixed infections.

Fourth, our findings may have applied relevance. Trypanosomes continue to put an intolerable burden on the African continent (Barrett et al. 2003). Despite the scale of the problem and over a century of research, there are still no satisfactory treatments for human African sleeping sickness, especially for late-stage patients (Brun and Balmer 2006). The identification of the exact mechanisms responsible for the mutual competitive suppression reported here may be exploitable to find new ways to fight the disease.

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