Host modulation of parasite competition in multiple infections

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Parasite diversity is a constant challenge to host immune systems and has important clinical implications, but factors underpinning its emergence and maintenance are still poorly understood. Hosts typically harbour multiple parasite genotypes that share both host resources and immune responses. Parasite diversity is thus shaped not only by resource competition between co-infecting parasites but also by host-driven immune-mediated competition. We investigated these effects in an insect–trypanosome system, combining in vivo and in vitro single and double inoculations. In vivo, a non-pathogenic, general immune challenge was used to manipulate host immune condition and resulted in a reduced ability of hosts to defend against a subsequent exposure to the trypanosome parasites, illustrating the costs of immune activation. The associated increase in available host space benefited the weaker parasite strains of each pair as much as the otherwise more competitive strains, resulting in more frequent multiple infections in immune-challenged hosts. In vitro assays showed that in the absence of a host, overall parasite diversity was minimal because the outcome of competition was virtually fixed and resulted in strain extinction. Altogether, this shows that parasite competition is largely host-mediated and suggests a role for host immune condition in the maintenance of parasite diversity.

Keywords: parasite; diversity; co-infection; competition; trypanosome; immune challenge

1. INTRODUCTION

Parasite genetic diversity is a constant challenge to host immune systems and hampers important health initiatives combating infectious diseases [1–3]. To understand the factors underpinning the emergence and maintenance of parasite genetic diversity is therefore of great importance. Currently, insights into host–parasite interactions are still largely based on studies involving single parasite strains infecting single hosts. Yet, in the real world, hosts are often infected by numerous parasite genotypes of the same or different species simultaneously, and an approach integrating this variation into both experimental systems and models of infection is required [4].

One approach is to consider that co-infecting parasite species or strains will share not only host resources, but are also probably subject to the same host immune responses. Such interactions with the host’s immune system can amplify or reverse inherent differences in competitive ability of co-infecting parasite genotypes. This considerably complicates any attempts to predict the outcome of co-infection. Classical ecological theory provides a useful framework to approach the question [5–9]. In this framework, co-infecting parasites can be seen as either regulated by resource-based (i.e. linked to extraction of host resources; ‘bottom-up’) and/or immune-based (‘top-down’) control mechanisms. In immune-mediated apparent competition, for example, a low-density parasite genotype suffers disproportionately from the presence of a high-density strain in the same host when the latter induces a strong, non-specific immune response [10].

Alternatively, a genotype-specific immune response might primarily affect the high-density parasite genotype that has elicited it, providing an advantage to the low-density strain relative to situations where it infects alone. Various degrees of cross-reactivity of the immune response will obviously modulate this effect in various ways [11]. At the level of the host population, the picture is further complicated by the fact that hosts naturally differ in their immune repertoire and condition.

This work investigates the relative importance of ‘bottom-up’ and ‘top-down’ mechanisms in determining the outcome of co-infection in a natural system. With this aim, we studied experimental infections of the intestinal trypanosome, *Crithidia bombi*, in its bumble-bee host, *Bombus terrestris* L. *Crithidia bombi* shows very high natural genetic diversity: virtually all multi-locus genotypes collected from the field over several years are distinct [12]. Hosts are therefore exposed to a wide range of parasite strains, resulting in the considerable frequency of multiple infections measured in natural populations (more than 40%). Experimental infections with multiple parasite strains have shown, however, that exclusion of one or more of the co-inoculated strains is very common [13]. Interestingly, *C. bombi* has been shown to reproduce mostly clonally (an estimated 84% of cases), even though it is able to recombine and exchange genetic material with co-infecting strains [14]. Furthermore, the parasite population is strongly reduced every year when it is ‘constricted’ in the population of hibernating bumble-bee queens, the only hosts to survive winter. The question thus arises of how strain diversity can be maintained in *C. bombi* in the face of limited recombination, frequent elimination of strains by individual hosts and drastic seasonal population bottlenecks.

We hypothesized that in co-infected hosts, parasite competition and consequently parasite diversity are
controlled by: (i) ‘top-down’, immune-based mechanisms
determined by host identity and host immune condition;
and (ii) ‘bottom-up’, resource-based interactions between
co-infecting parasites, determined by resource availability
and parasite identity. In this experiment, the strength of
immune-based control (the ‘top-down’ component) was
modulated by manipulating host immune condition.
Practically, this was achieved by challenging the bees
with an injection of heat-killed bacteria in the haemo-
lymph. Such a bacterial immune challenge, or priming,
whether with heat-killed bacteria or lipopolysaccharide
(LPS) extracted from bacterial surfaces, is routinely
used in experiments and is known to increase antibacte-
rial activity in the haemolymph of insects, including
B. terrestris [15,16]. Bacterial priming provides surpris-
ingly specific and durable protection upon secondary
exposure to related parasites [15,17,18], but becomes
costly when the host faces a mismatched parasite, leading,
for example, to higher infection levels [16]. Bacterial
priming with LPS also decreases host survival under star-
vation [19], but not when hosts are fed ad libitum (the
treatment per se is not detrimental/toxic). These costs
are thus thought to be the result of trade-offs in resource
allocation to defence versus survival (in the latter case)
and/or to different arms of the immune system (in the
former case). Considering the above, we hypothesized
that a bacterial challenge would decrease the host ability
to fight a subsequent exposure to C. bombi. How such a
c change would affect intra-specific parasite competition is,
however, harder to predict. Note that this working hypoth-
thesis does not require the trade-off to be purely
immunological. The expected effects could come about
via a general mismatch between resources that are effec-
tively invested into defence (as a response to the presence
of bacterial elicitors in the haemolymph) and the relevant
parasite infection (by live trypanosomes in the gut).
Parallel to this in vivo experiment, we studied ‘bottom-
up’, resource-based control mechanisms in the absence of
a host immune system with an in vitro competition exper-
iment. Given the homogeneous nature of the resource
(liquid medium), we expected the outcome of in vitro com-
petition to be simpler and less variable than in vivo. However,
because the conditions pertaining to the in vivo and in vitro
situations are obviously different (both in terms of quantity
and quality resources, as well as the presence/absence of
host immunity), more specific predictions could not be
made, and only the qualitative pattern of strain persistence
during experimental infections was used as a signal to
assess the importance of the two hypothesized processes.

2. MATERIAL AND METHODS

(a) Bees, bacteria and trypanosomes

Eight colonies of B. terrestris were started from uninfected
bumble-bee queens collected in spring 2009 from a popu-
lation in western Switzerland (Aesch, Switzerland). All bees
were kept at 26 ± 2 °C under constant red light illumina-
tion, with pollen and sugar water (Apinver, Südzcucker,
Ochsenfurt, Germany) provided ad libitum.

The bacteria used for immune challenges were the
Gram-positive Arthrobacter globiformis (strain no. DSM
20142) and the Gram-negative Escherichia coli (strain no.
DSM 498) obtained from Deutsche Sammlung von Mikro-
organismen und Zellkulturen (Braunschweig, Germany). We
used both Gram types, because it is known that the insect
immune pathways are differentially activated by these two
Gram specificics. Bacteria were cultured separately at 30 °C
(A. globiformis) or 37 °C (E. coli) in medium (10 g bacto-
tryptone, 5 g yeast extract, 10 g NaCl in 1000 ml of distilled
water, pH 7.0). Immediately before use, bacterial cells were
washed three times by centrifugation (3000 r.p.m., 4 °C,
10 min), removal of the supernatant and resuspension in
Ringer saline solution. We determined cell concentration
of both cultures and mixed the two into a single inoculum so as
to reach a final concentration of bacterial cells of 10^8 cells ml^−1
(corresponding to 0.5 x 10^6 cells ml^−1 of each bacterium).
The bacteria were then heat-killed (90 °C, 15 min). Efficiency
of the heat killing was confirmed by plating out samples of
the suspension on agar and checking for an eventual growth of
bacteria (none occurred; data not shown).

The five C. bombi strains (labelled A, B, C, D, E) used
for the experimental infections were obtained from faeces
of naturally infected queens collected in spring 2008
(Neunform, Switzerland). Single infective cells were isolated
using a fluorescence-activated cell sorter and subsequently
maintained clonally in liquid medium at 27 °C and 3 per
cent CO₂ (R. Salathé 2007, unpublished data). The strains
had distinct multi-locus genotypes at four polymorphic
microsatellite loci (see below) and could thus be readily dif-
fentiated by genetic markers in a mixture.

(b) Bacterial challenge and in vivo inoculations

Workers were collected as callows (freshly hatched workers)
from the eight experimental colonies during three to four
consecutive days and kept in groups for three additional
days (i.e. until they were 3 to 6 days old). Individual workers
were then chilled on ice and randomly assigned to the chal-
denged (C) or naive groups (N). Immune challenge of the
workers was performed by injecting 2 μl of the inoculum
containing 10^8 cells ml^−1 heat-killed bacteria between the
first and second abdominal tergites. Naive bees were sham-
manipulated in the same way but not injected because the
wounding associated with injection itself is known to induce
an immune response [20]. Thus, we compared bees given a
general immune activation (Gram-positive/Gram-negative
bacterial challenge and wounding) with naive bees. All bees
were kept individually from that point.

On day 9, all bees were exposed to C. bombi. For this, bees
were starved for 4−5 h before being presented with 10 μl of
sugar water containing the inoculum of live C. bombi cells.
We administered the five C. bombi strains either alone
(‘single exposure’, with strains A, . . . , E, at 10 000 cells each),
or in any of the 10 possible pairwise combinations (‘double exposure’,
with strain combinations AB, . . . , DE, at 5000 cells per strain to
keep the overall inoculum constant). On days 4, 6 and 8 post-
infection, faeces were collected and kept in glass micro-
capillaries until further assessment of infection status. All bees were
frozen on 8 days post-infection. In order to attain sufficient
sample sizes while ensuring that all bees were the same age,
the above procedure was repeated several times during colony
development (from two to six times, depending on colony pro-
ductivity), so that bees from each colony were infected in
successive batches.

(c) Transmission speed, infection status, composition
and intensity

To assess infection status, all collected faeces (three samples
per individual) were microscopically checked for the presence

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of the parasite (infected/uninfected). For each infected individual, transmission speed was coded as ‘fast’, ‘medium’ or ‘slow’, depending on whether the faeces contained the first C. bombi cells from day 4, 6 or 8 post-infection, respectively. All infected individuals had their gut dissected out and homogenized in 100 μl Ringer solution. DNA was extracted from these individual gut preparations using a Qiagen DNeasy 96 tissue kit. The gut extracts were then genotyped at the C. bombi microsatellite markers Cri4, Cri1B6, Cri4G9 and Cri2F10 following Ulrich et al. [13] to identify the strains present in each infected individual. Previous work shows that the method is sensitive enough to detect very low concentrations of different C. bombi strains in mixed infections [21]. Note that while this method provides reliable information on the presence/absence of strains, it does not allow strain-specific quantification in mixed infections. Instead, the total relative infection intensity was measured from gut extracts using a quantitative PCR (qPCR) reaction amplifying a portion of the C. bombi 18sRNA gene [13].

(d) In vitro inoculations
The same five C. bombi strains used for in vivo infections were also assayed in vitro (that is, with living cells maintained in axenic cultures in media). For this purpose, 10,000 cells of the same 10 pairwise strain combinations as above were inoculated in 2 ml of liquid medium housed in 24-well culture plates and incubated at 27°C and 3 per cent CO₂ for 8 days. Each strain pair was cultivated in six replicates; furthermore, two replicates each of single strain inocula were used as positive controls. After removal of the culture medium, the cells were washed by resuspension in 1× PBS, centrifugation (10,000 r.p.m., 5 min) and removal of the supernatant. DNA was extracted from the cell pellets by adding 10 μl Viagen direct PCR lysis reagent (Viagen Biotech Inc., Los Angeles, CA), 90 μl H₂O and 1 μl proteinase K to each sample, followed by incubation at 55°C for 45 min and 85°C for 40 min. These cell culture extracts were then genotyped as above.

(e) Data analysis and statistics
(i) Determinants of parasite establishment, infection intensity and transmission speed
All statistical analyses were performed in R v. 2.8.1 [22]. Owing to the overall low infection rate (see §3), we performed separate analyses for parasite establishment success (uninfected/infected) using the entire dataset, and infection intensity (as measured with qPCR) using the subset of infected individuals. Determinants of parasite establishment success and infection intensity were investigated using binomial and Poisson generalized linear-mixed models (GLMMs), respectively (lmer function in lme4 library in R). The models used immune-treatment (challenged versus naive), and infection type (one of 15 double or single exposures: A, E, and AB, DE) as independent fixed variables, and ‘batch’ and host type (one of eight host genetic backgrounds) as random variables. Additionally, whenever overdispersion was detected, it was taken into account by incorporating individual-level variability as a random effect in the model. We evaluated the significance of fixed effects and their interaction by comparing models using log-likelihood ratio tests (LRTs) following deletion of terms (starting with the interaction). Terms for which deletion did not significantly decrease model fit were omitted, until only significant terms remained in the model (α < 0.05). The above models were also tested with a two-level infection variable for the multiplicity of exposure (single versus double exposure) in place of the 15-level infection type variable. However, the minimized models were identical in both cases because they did not retain the infection variable (see §3) and we thus present the results obtained only with the first approach (15-level infection variable) in the text. Effect sizes for the significant terms of the final models are reported in the text; the effect sizes for all terms (significant and non-significant) and for both types of models (15-level and 2-level infection variables) can be found in the electronic supplementary material.

An ordinal logistic model for transmission speed (slow, medium and fast) with infection type and immune-treatment as predictors was fitted (and minimized by removing non-significant terms) using the brm function from library design R.

(ii) Determinants of coexistence and diversity
We used a binomial GLMM to analyse determinants of parasite coexistence (i.e. both strains maintained) versus exclusion (i.e. one strain maintained) in the subset of infected individuals that had received a double exposure. Again, immune treatment and infection type (one of 10 double exposures AB to DE) were used as fixed factors and their interaction investigated, whereas ‘batch’ and host type were used as random factors. Models were minimized as above.

In cases where one of the strains was excluded, we used simple binomial tests to link strain performance in single inoculations (infection intensity) to their success in co-infections (frequency of maintenance versus exclusion), for naive and challenged hosts separately.

Furthermore, we tested whether the frequency distribution of double infections (e.g. observed prevalence of AB) differed from what would be expected if the strains infected independently (e.g. prevalence of A in single infections × prevalence of B in single infections) with Kolmogorov–Smirnov tests, for naive and challenged hosts separately.

To investigate strain-specific effects of host immune challenge and co-inoculation, prevalence data (infected/uninfected) were analysed separately for each strain using a GLMM with the multiplicity of exposure (single/double exposure), and immune treatment as fixed factors, and colony and ‘batch’ as random factors. The second-order interaction between fixed effects was investigated, and models were minimized as above. Note that the strain-specific dose differed between single exposures and double exposures: because the total inoculation dose was kept constant across all treatments, the dose of any strain in a double exposure is half that of a single exposure. However, from previous unpublished work on this system, it is known that both prevalence and infection intensity increase with dose to reach a plateau at doses considerably lower (approx. 1000 cells) than those used in this study (see the electronic supplementary material, figure S1).

Finally, we computed Simpson’s inverse index of diversity, \( D = (\sum p_i^2)^{-1} \) (where \( p_i \) is the frequency of strain \( i \) in the parasite population), on the subset of double exposures in the naive and challenged hosts, as well as in the in vitro parasite population.

3. RESULTS
(a) Parasite establishment, infection intensity and transmission speed
Overall infection success was low, at around 29 per cent (135 infected out of 472 exposed individuals). This is partly
attributable to two colonies, which produced a large number of workers (n = 204) and were almost completely resistant to the C. bombi strains used (19 infected individuals).

We found a general, positive effect of a general immune challenge in the host for C. bombi (figure 1). Immune condition had a positive effect on C. bombi establishment (LRT for models with versus without the variable ‘immune-treatment’: $\chi^2 = 4.33$, $p = 0.037$; see electronic supplementary material, table S1a), with 33 per cent of bees infected when challenged versus 25 per cent in the naive group. A similar analysis performed on the subset of infected individuals revealed that immune-challenged hosts also had higher infection intensities ($\chi^2 = 4.90$, $p = 0.027$; see electronic supplementary material, table S2a), with 923 ± 113 cells μl$^{-1}$ of gut extract (mean ± s.e.) in challenged hosts versus 537 ± 89 cells μl$^{-1}$ in naive hosts.

In contrast, neither infection type (one of 15 double or single exposures: A, ..., E, and AB, ..., DE) nor multiplicity of exposure (single/double) affected C. bombi establishment or infection intensity, as indicated by the fact that these variables were not retained in the respective models, so that the two approaches ‘converged’ to the same minimal model (see electronic supplementary material, tables S1a,b and S2a,b).

Finally, the results from an ordinal logistic model showed that transmission speed was faster in challenged hosts ($\chi^2_{111} = 7.59$, $p = 0.006$).

**Figure 1.** The outcome of infection by pairs of Crithidia bombi strains. For each strain combination (AB–DE), bar length and pie chart area represent the frequency of cases where the two strains coexisted (black), or one of the two strains infected alone (white, the letter indicates the infecting strain), after co-inoculation in (a) naive hosts, (b) challenged hosts and in vitro (pie charts). Grey bars show, for each strain combination, the expected frequency of double infections if both strains infect independently from the other, i.e. the product of their respective prevalences following single exposure: $f_{exp}(AB) = f(A) \times f(B)$. One infected host (as determined by faeces check and qPCR) whose infection genotype could not be established is not represented in this figure.

(b) **Parasite coexistence and diversity**

The outcome of co-inoculation in naive hosts varied across infection types such that it depended on the particular strain combination sharing a host (figure 1a): in two cases (combinations BC, CD), a single strain was consistently maintained, whereas the other was excluded; in four cases (AB, AC, AD, BE), either one or the other strain was maintained but coexistence was never observed; in three cases (BD, CE, DE), a strain was present in all infections, either alone or in coexistence with the other; all three possible outcomes were observed in only one case (AE). Globally, 19 of the 30 possible outcomes were observed (considering three possible outcomes—exclusion of one or the other strain, or coexistence—for each of the 10 different combinations in double exposures).

In challenged hosts (figure 1b), the variability in the outcome of co-inoculation was even greater, with 8 out of 10 strain combinations (all but CD and DE) showing all three possible outcomes. Globally, 28 of the 30 possible outcomes were observed. Thus, the challenge often seemed to allow the otherwise less competitive C. bombi strain to coexist with the other strain, or even to infect alone. To formally test the hypothesis that the challenge disproportionately benefited the less competitive strain, we compared the average increase in the prevalence of the dominant strain—defined for each pair as the strain that more frequently infected alone in naive hosts—with
that of the weaker strain in naive versus challenged hosts (in one case where both strains had equal records, we took the dominant strain to be the one with overall higher prevalence and infection intensity in naive hosts). Although the average increase in the prevalence of the weak strain of each pair was higher (mean ± s.e.: 0.097 ± 0.031, n = 10) than that of the dominant strain (mean ± s.e.: 0.054 ± 0.023, n = 10), this difference was not significant (Wilcoxon–Mann–Whitney test: \( W_1 = 35, p = 0.27 \)). Note that in naive hosts, performance in double inoculations appeared not to be associated with success in single infections: in about half of the cases of exclusion (18 out of 30 possible outcomes observed). Competitive exclusion was relatively rare (42%) and appeared to follow a simple rule: in all but one case, competitive exclusion was the one-sided binomial test: \( p = 0.035 \). Thus, the success of a strain in single infections appeared to be a better predictor of its success in double infections for challenged hosts when compared with naive hosts.

A binomial GLMM on the coexistence/exclusion data showed that the outcome of co-infection differed according to the infection type (LRT for models with versus without the variable ‘infection type’: \( \chi^2_0 = 18.84, p = 0.027 \); see the electronic supplementary material, table S3) and immune treatment (LRT for models with versus without immune treatment: \( \chi^2_4 = 4.50, p = 0.034 \)), with exclusion of one of the strains being less frequent in challenged (57%) compared with naive hosts (76%; figure 1). Kolmogorov–Smirnov tests did not detect any significant difference between the observed and expected distributions of double infection prevalence in challenged hosts (\( D = 0.4, p = 0.401 \); figure 1). In naive hosts, the difference between the two distributions was more pronounced, but failed to reach statistical significance (\( D = 0.6, p = 0.055 \)).

As illustrated in figure 2, strain-specific mixed-effects models detected a negative effect of co-inoculation on the prevalence of strain A (LRT for models with versus without the variable infection multiplicity: \( \chi^2_2 = 4.27, p = 0.039 \); see electronic supplementary material, table S4) and strain C (\( \chi^2_2 = 7.55, p = 0.006 \)), as well as a positive effect of the immune challenge on the prevalence of a strain C (LRT for models with versus without the variable immune treatment: \( \chi^2_1 = 8.33, p = 0.004 \)).

The outcome of competition \textit{in vitro} showed great repeatability: in contrast to the \textit{in vivo} situation, the outcome of competition between a given pair of strains was virtually constant (see pie charts in figure 1), with only 11 out of 30 possible outcomes observed. Competitive exclusion was relatively rare (42%) and appeared to follow a simple rule: in all but one case, competitive exclusion was observed towards strain D, which consequently disappeared from the \textit{in vitro} parasite population. Note that this cannot be due to a general failure of this particular strain to grow in liquid medium, because single \textit{in vitro} inoculations with strain D were successful.

Finally, Simpson’s inverse index was lowest in the \textit{in vitro} parasite population (\( D = 3.97 \)), intermediate in naive hosts.
4. DISCUSSION

In line with our prediction, a general immune challenge affected the ability of hosts to defend against the trypanosome *C. bombi*. This resulted in more infected hosts, which carried higher parasite loads than their naive counterparts and started transmitting infective cells earlier. These results mirror those of a study by Sadd & Schmid-Hempel [16], where bumble-bee queens that had received a bacterial challenge produced offspring that were more susceptible to *C. bombi* (but did not incur a survival cost under starvation). Here, we show that a similar infection cost also materializes in the short term, within the lifetime of an individual. Both cases illustrate, we believe, the negative consequences of an immune ‘mismatch’: because immune activation is costly, responses to immune challenges that are distinct in type (wounding and bacterial versus trypanosomal) and/or location (haemocoel versus gut) cannot necessarily be simultaneously optimized, but must often be traded off against each other [23,24]. These costs could come about directly (via resource allocation trade-offs between different arms of the immune system) or indirectly (if, for example, immune-challenged hosts are generally weaker and more susceptible to new parasites).

Here, the immune challenge was performed under controlled conditions, but immune mismatches are probably not uncommon in nature, where hosts routinely encounter a range of different parasites. Because of such encounters, hosts in natural populations are unlikely to have naive immune systems, and thus the trade-off between defences against different parasites is relevant in the wild, too. The magnitude of its effects is likely to be more dramatic when resources are limited, as must be the case in the field. Our experiment also makes the point that individual differences in the strength and repertoire of immune responses against a particular parasite arise not only from genetic variation in resistance, but also from ‘immune history’. Indeed, we expect that different hosts or categories of hosts (e.g. sex, age, population of origin) encounter different parasites and carry a record of their immune history, either in the form of ‘resident’ live parasites or, if the infection has been cleared, in the form of an altered immune condition. Here, this history is recent, as the exposure to *C. bombi* occurs shortly after challenging the immune system with bacteria. However, the existence of corresponding trans-generational effects of priming [18,25,26] and the fact that insects such as *B. terrestris* possess an individual immune memory lasting for weeks [15] suggest that immune history will be relevant even over longer time periods. Recently, Telfer *et al.* [27] used time-series data from a wild vole population to show very large (positive or negative) effects of some infections on the susceptibility to other parasite species—sometimes even after clearance of the first infection—and invoked immune-mediated mechanisms as one potential explanation. Here, we find an infection cost associated with an unrelated immune challenge involving no live parasite. The situation is thus analogous to a putative vaccine that would affect host susceptibility to unrelated diseases, such that immunization against a parasite would be traded off against protection to other pathogens. This risk has not received much theoretical or empirical attention and could add to the detrimental effects of imperfect vaccines on virulence evolution [28–30].

The increase in parasite load in challenged hosts was associated with an early onset of transmission, suggesting that rapid within-host growth accelerates transmission. Bumble-bees live in dense social groups of frequently interacting individuals, where parasite transmission easily occurs between nest-mates. Accelerated transmission might thus increase within-colony prevalence at the crucial step of parasite transmission to queens, who are the only hosts to transmit the infection from one year to the next (workers and males do not survive winter) and whose fitness is severely reduced by *C. bombi* infections [31].

Our findings suggest that the immune challenge acted to increase the host ‘space’ available to the *C. bombi* population (figure 3). This increase benefited not only the dominant, more competitive *C. bombi* strain, but also the otherwise weak strain of the co-inoculated pair. The increase in available host space also translated into a more variable outcome of co-infection and more multiple infections. The frequency of multiple infections is of importance for pathogen evolution because it is thought to select for more virulent strains [6,32–35]. In the *Bombus–Crithidia* system, the multiplicity of infection in workers is associated with the probability of transmission to queens, and therefore linked to host fitness [13].

The consequences of host immune challenge and co-inoculation showed considerable variation across *C. bombi* strains (figure 2), so that no strain was consistently more successful than the others. For example, strain A performed well alone (with the highest prevalence and infection intensity in both naïve and challenged, singly exposed hosts) but suffered in double exposures. Strain C also did poorly in double exposures but this detrimental effect was partially offset in immune-challenged hosts. These strain-specific effects might be at the basis of the lack of a general effect of the multiplicity of exposure on infection characteristics in this study, as well as in a previous experiment using only two strains [36].

The importance of host-mediated effects is reflected by the difference between *in vitro* and *in vivo* co-inoculations. *In vivo* co-inoculation was characterized by frequent exclusion of one of the two strains, whereas *in vitro* competition occurring in the absence of host-mediated immune effects more often resulted in strain coexistence. This might intuitively lead to the conclusion that host immunity acts to decrease parasite diversity. However, this is not the case. In fact, the variability in outcome and the diversity of the parasite population (as calculated with Simpson’s inverse index) were lowest *in vitro*, intermediate in naïve hosts and maximal in challenged hosts. *This is because in vitro competition appeared to follow very simple rules, with a virtually fixed outcome per strain combination. One strain was outcompeted to extinction despite the overall infrequent competitive exclusion, resulting in low overall diversity. The host environment thus appears to play a role in maintaining parasite diversity. Furthermore, maximum diversity was...*
found in challenged hosts whose immune condition was altered, which is probably the situation closest to the environment parasites naturally encounter.

Although the numerical differences between the outcomes of in vivo and in vitro co-inoculations cannot be taken as directly representing the difference between immune-mediated competition and resource competition (for instance, because resource competition could also take place in vivo), a qualitative comparison provides insight into the relative importance of ‘bottom-up’ and ‘top-down’ control mechanisms on the diversity of parasite populations. ‘Bottom-up’, resource-based competition alone explained only the simple interactions between parasite strains occurring in the absence of a host. ‘Top-down’, immune-mediated mechanisms associated with the host environment thus appear to be at the source of the complex and diverse parasite population observed in vivo in this study, as well as in the wild. In a recent study on a mouse–trematode system, Beltran et al. [37] showed that the protective effect provided by a repeated light infection with a parasite strain decreased with increasing genetic distance with the later infecting strain, a mechanism by which the vertebrate protective immunity drives parasite genetic diversity. A similar explanation has been proposed in a bovine parasite showing high genetic diversity in the field despite frequent bottlenecks [38].

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