Serial passage of the parasite *Crithidia bombi* within a colony of its host, *Bombus terrestris*, reduces success in unrelated hosts

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In the wild, *Bombus* spp. bees may contract infections of the trypanosome parasite *Crithidia bombi* from their nestmates or from others while foraging on contaminated flowers. We expected that as *C. bombi* is transmitted repeatedly among related workers within a colony, the parasite population would become more successful in this relatively homogeneous host population and less successful in individuals from unrelated colonies of the same or different species. To test our prediction, we serially passaged cocktails of *C. bombi* strains through workers from the same colony, taking the intensity of infection in related versus unrelated workers as a measure of parasite success at each step in the serial transfer. Using a repeated measures ANOVA, we found the ability of *C. bombi* to exploit *Bombus* spp. hosts did not increase within a colony, but did decrease for infections in workers from unrelated colonies. This reduction in success is most likely due to a gradual loss of appropriate *C. bombi* strains from the infecting the population as the cocktail is ‘filtered’ during the serial passage within a given colony, without a corresponding increase in overall intensity of the surviving strains.

Keywords: experimental evolution; virulence; host–parasite co-evolution; host heterogeneity; serial passage

1. INTRODUCTION

Parasite virulence has been loosely defined as the damage caused to a host by the growth and propagule production of a parasite, usually measured in terms of host fitness or mortality (Poulin & Combes 1999; Galvani 2003). Theory predicts that the costs and benefits of virulence are traded-off in order to maximize the fitness of the parasite (Ewald 1983; Bull 1994). Parasite fitness, therefore, takes the centre stage in these considerations. Understanding what costs and benefits shape parasite fitness is vital to making predictions about how a parasite population might change as its environment changes either naturally or due to anthropogenic factors. These costs and benefits will in turn depend on the properties of the host population.

For example, the genetic heterogeneity of the host population, which can vary considerably within and among parasite–host systems, is expected to have an effect on the evolution of parasite virulence, given that parasite fitness varies with both host and parasite genotype (Schmid-Hempel & Ebert 2003). In a genetically homogeneous population, each host is essentially identical to the previous one. Thus, an adaptation that makes a parasite strain successful in one host will make it just as successful in the next. The parasite could, therefore, evolve to an optimal virulence that maximizes its fitness on this host type and as predicted by the standard virulence evolution models (Frank 1996). In a heterogeneous host population, by contrast, each host is a new environment to

the parasite. Since the parasite is selected by the environment represented by the current hosts, its success in the next host will not be the same and thus its virulence will probably be sub-optimal (Ebert & Hamilton 1996). Not only might adaptations to the current host have little benefit in a new host, genes that increase a parasite’s fitness in one host may even decrease it in another, a process termed antagonistic pleiotropy (Gould 1979; Fry 1990). Alternatively, genes that are not under selection in the current host but that are important in other hosts may accumulate mutations that can become fixed by drift, leading to a loss of fitness in other host types (Reboud & Bell 1997; Cooper *et al.* 2002).

These hypotheses have been used as possible explanations for the attenuation of parasites observed in serial passage experiments: a population of parasites passaged through hosts of a type different from their original host becomes less successful and less virulent in the original host while generally becoming more virulent in the new host (Ebert 1998). Note that in such passage experiments, the consequences of virulence for the damaging effects within a host and the consequences for subsequent transmission are typically decoupled, since the parasites are experimentally transmitted with a standard dose. Hence, passage experiments test only for the effect of within-host evolution, in effect, how successful the parasite can become at exploiting a given host for its own replication.

In normal circumstances, given more than one host type, a parasite population is faced with adapting to many hosts at the same time. This may lead to a situation where one host is maximally exploited at the expense of fitness in the other, or alternatively to a generalist strategy resulting

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in sub-optimal exploitation, and thus virulence, in both (Regoes *et al.* 2000; Ganusov *et al.* 2002). Since genetic heterogeneity of hosts varies across populations, so will the co-evolutionary pressures on parasites. The parasites must continually balance the costs of tracking either the local host population or the global host population. Here, we experimentally test whether a parasite population transmitted within a homogeneous host population will become more successful within that population and whether this increase comes at a cost, namely decreased success in dissimilar hosts.

Bombus terrestris L. and *Bombus lucorum* L. are both annual, primitively eusocial species whose distributional ranges and seasonal activity overlap. The intestinal parasite *Crithidia bombi* (Trypanosomatidae, Kinetoplastida, Zoomastigophorea; (Lipa & Triggiani 1988) resides in the hind gut and infects all castes when they ingest cells. Infections can be picked up within the nest or from flowers contaminated by an infected bee (Durrer & Schmid-Hempel 1994). *C. bombi* cells can be seen in the faeces approximately 5 days post-infection, increasing in numbers until 10–14 days post-infection at which point the numbers of cells in the faeces level-off (Schmid-Hempel & Schmid-Hempel 1993; Logan *et al.* 2005). When queens emerge from hibernation in the spring, a proportion of them harbour *C. bombi* infections. These infected queens may or may not be successful at founding a colony (Brown *et al.* 2003). However, once they emerge, they begin to forage and may infect other queens. An infected queen that manages to found a colony will almost certainly infect her workers. As the season progresses, the colonies grow and *C. bombi* is transmitted both within the colonies and between colonies through the contamination of flowers by foraging workers. As autumn approaches, males are produced in many colonies but only a fraction of colonies produce new queens. The queens will mate once and then find a suitable hibernation site to spend the winter.

Females (queens) of *B. terrestris* and *B. lucorum* are singly mated and, as with all hymenopteran insects, the males are haploid. Thus, the relatedness among the non-reproductive worker caste of a single colony is high (with $r=0.75$). Such a colony of full sisters, therefore, represents a genetically homogeneous, high-density patch of hosts, both of which should favour the evolution of high virulence in *C. bombi*. However, there are limits to how specialized to the current colony *C. bombi* can become if it comes at the expense of success in unrelated colonies. For example, host reproductive performance and thus the opportunity for the parasite to overwinter (*C. bombi* only survives in infected queens) depends on colony size, which is negatively affected by parasitism (Muller & Schmid-Hempel 1992a,b). Furthermore, recent work shows that *C. bombi* reduces the success of colony founding by infected queens as well as the performance of established colonies of *B. terrestris* (Brown *et al.* 2003; C. P. Yourth & P. Schmid-Hempel, unpublished data). Failure to start a colony or slow growth of an established colony deprives the parasite of opportunities to spread. In fact, queens leaving their natal nests in the fall rarely forage and thus must be infected while still in the nest (Alford 1975). As colonies founded by infected queens produce fewer daughter queens, the infection must spread to neighbouring colonies late enough in the season that the infection does not interfere with queen production, yet early enough

that the infection has time to spread within that colony and infect the new queens. *C. bombi* is thus under selection to infect and spread within a given colony, but also to be able to successfully spread to another colony.

We, therefore, wanted to know if *C. bombi* adapts to a given host colony by becoming more successful at exploiting that colony and if this in turn results in a reduced success in other colonies. In this experiment, we used *B. terrestris* and *B. lucorum* hosts to test whether a serial passage of the *C. bombi* infection among related worker bees resulted in an increase in infection intensities in the related hosts, and a concomitant reduction in infection intensities in unrelated hosts. In a reciprocal design, we infected bees from pairs of colonies with a cocktail of *C. bombi* strains and after each passage, infected other workers from the same colony as well as workers from the unrelated paired colony.

2. MATERIAL AND METHODS

We collected newly emerged queens of *B. terrestris* and *B. lucorum* in spring 2002 from populations in northern Switzerland. The queens were reared to colonies and those with *C. bombi* infections were used as sources for experimental infections while uninfected colonies were used for the experimental tests. We collected faeces from the workers of seven infected colonies, combined them and then diluted the raw mixture with sugar water so that a 20 µl inoculum contained 10 000 *C. bombi* cells. Since paired groups of bees were infected at different times, a total of five infection cocktails (drawn from the same sources and thus containing the same strains) were used in blocks over the course of the experiment.

To ensure that the colonies were at a similar stage of development, 20 unrelated colonies were paired (a total of 10 pairs) on the basis of numbers of workers and amount of brood. For the tests, three workers were haphazardly selected from each of the paired colonies and infected with the *C. bombi* cocktail by feeding them a 20 µl inoculum after starving them for approximately 3 h. The bees were then kept individually in a plastic box (10×13×6.5 cm), where they had access to pollen and a sugar water mixture (approximately 1 : 1 by volume) *ad libitum*. After 7 days, we collected faeces from each of the three bees, combined it, and used this mixture to create another inoculum stock for further infections (to create the serial passage). We used this stock to infect another three bees from the same colony (within-colony transmission), plus another two bees from the unrelated, paired colony (between-colony transmission). The same was done in a reciprocal fashion for the other colony of the pair (figure 1). This process was repeated until there had been three to five passages within each colony. Once faeces had been collected for the next infection, each donor bee was frozen at -80°C until they could be dissected and their infection intensities scored. The total time from first passage until the end of the fifth passage was 35 days. To achieve the full set of five within-colony and four between-colony passages, it was necessary that each colony produced enough workers such that three workers could be taken for the first infection, and another five every 8 days. If any of the colonies did not have enough workers when they were needed, the chain of infection was broken and thus the pair of colonies was eliminated from the remainder of the experiment.

Figure 1. Diagram of the paths of infection of one half of a reciprocal pair of colonies. (a) A cocktail of *Crithidia bombi* strains is prepared by collecting faeces from workers of greater than 5 infected colonies. (b) Inocula of the cocktail standardized to 10 000 cells in 20 μ l are used to infect three workers from the first colony. (c) The infected workers are kept individually and fed sugar water and pollen *ad libitum* for 7 days while the infection develops. (d) After this period, faeces containing *C. bombi* is collected from all three bees, standardized as above, and used to infect another three workers from the same colony as well as (e) two workers from the paired colony. (f) The within-colony infections produce the *C. bombi* for the next infections, but the between-colony infections are not used for further infections. The infection cocktail is passed through five groups of workers within the first colony and is transferred out into the paired colony four times. The same process is performed concurrently with another set of workers, reversing the roles of the two colonies but using the same initial infection cocktail, thus completing the reciprocal design.

The success of the *C. bombi* infection was estimated as the infection intensity observed in a worker. For this purpose, we removed the hindgut of a worker, then triturated and vortexed it in 200 μ l of Ringer solution. We then scored the density of *C. bombi* cells in the resulting mixture using a counting chamber (Neubauer system). We present our results in terms of these counts but the total number of cells per millilitre of the prepared guts can be calculated by multiplying the counts by 5×10^4 . The mean of the cell counts for the groups of the three within-colony workers and for the two unrelated between-colony workers from the paired colony were used for the analysis. In both cases, a repeated measures analysis of variance (ANOVA) was used. We only included data from those colonies with the full five within-colony and four between-colony passages.

C. bombi DNA was extracted from the hindgut samples and PCR amplified using three different primers for polymorphic microsatellite loci following the techniques outlined in Schmid-Hempel & Reber Funk (2004). For each locus, electrophoresis of the samples from all workers in the infection sequence was performed on the same gel, allowing for easy comparison of the alleles present.

3. RESULTS

A complete set of five within-colony passages was achieved for ten colonies, which included one pair of *B. lucorum* colonies and one mixed species pair. Only four passages were achieved in eight other colonies and only three within-colony passages were achieved in the remaining two colonies. The between-colony passages of the paired colony could not be continued once the chain of within-colony passages was broken, but within-colony passages were not dependent on the between colony passages. As such, the number of complete four-passage series is the same as the within-colony infections with one exception; two colonies were excluded from the between-colony infections analysis because one pair of workers in each series received the wrong inoculum, leaving only eight colonies with the full four between-colony passages.

Colonies could only be positively identified as *B. terrestris* or *B. lucorum* once they had produced males, which did not occur until midway through the experiment. We chose to include these cases in the analysis because the key question of the experiment was the effect of passage within the same colony as compared to transmission to a foreign colony from the perspective of the parasite. Additionally, we only compare the infection intensities of individuals within each of the two colonies, and not intensity differences across species. However, excluding this pair does not change the outcome of the analyses.

Of the five infection cocktails, only three are represented in our analysis with one cocktail infecting two colonies and the other two cocktails infecting four colonies each. There was no effect of the different cocktails on the overall intensity of infection either within colonies ($n=10$, $F_{2,7}=0.792$, n.s.) or between colonies ($n=8$, $F_{2,5}=0.208$, n.s.). However, in the within-subjects contrasts for the within-colony passages, there was an interaction between cocktail and colony ($n=10$, $F_{2,7}=5.171$, $p=0.042$) meaning that the slopes representing the changes in intensity of infection differed between the groups of colonies receiving the different cocktails. This is probably a consequence of the strong genotype–genotype interaction between *C. bombi* strains and *Bombus* spp. colonies. A given *C. bombi* strain will produce different infection intensities in different colonies and vice versa (Mallon *et al.* 2003).

Counts of *C. bombi* cells present in a fixed volume of prepared gut samples for a total of 211 workers were included in this analysis. One worker was not included because it died before the end of the experiment, and two were excluded because they received the wrong inoculum. In each of these cases, the mean of the two remaining workers in the treatment group was used; in all other cases, the mean of all three workers was calculated and used for the analysis.

Contrary to our expectations, the mean *C. bombi* counts (our index of infection intensity) overall did not increase with passages through related workers ($n=10$, $F_{1,9}=0.018$, n.s.; figure 2) even though the overall infection intensity after the second passage was maximal (figure 2). As predicted, increasing numbers of passages among related workers resulted in decreasing mean *C. bombi* cell counts when the parasite population was transmitted to unrelated workers ($n=8$, $F_{1,7}=10.68$, $p=0.01$; figure 3). Excluding the mixed species colony pair did not change this result ($n=6$, $F_{1,5}=6.83$, $p=0.05$).

Figure 2. Mean counts of *Crithidia bombi* cells in gut samples from infected workers in relation to passage number within colonies.

For all steps, the genotypes in the parasite population were typed with micro-satellite markers (Schmid-Hempel *et al.* 1999; Schmid-Hempel & Reber Funk 2004). The banding patterns of the microsatellite loci were visually inspected. Two of the loci produced three bands while the third produced eight, although no individual had more than five bands at that locus (theoretically if each of the seven infecting strains were heterozygous and unique at the microsatellite locus, there could be as many as 14 bands for each locus). For the majority of groups of infected bees, the same bands that were present in the population at first infection were also present after the final transmission in the experiment. In two cases, i.e. for two transmission series, two out of three initially present bands at one locus were missing from the final transmission step suggesting the strain or strains carrying the alleles represented by these bands were no longer present in the final infection. However, this does not exclude the possibility that strains were also lost in the other transmission series, as different strains may share alleles at some loci and thus losses would not be resolved unless a unique allele/band is lost.

4. DISCUSSION

Contrary to expectation, repeated transmission among related workers did not result in an increase in the success of the *C. bombi* infections in those workers, but did result in a reduced success in unrelated workers. Taken on its own, the first result would suggest that there has been no change in the *C. bombi* population in terms of its ability to exploit host resources as it passes through the related hosts. However, the second result indicates that the parasite population has indeed evolved, reducing its ability to exploit the resources of other host types. Our initial prediction is thus only partially supported, and evolution did not follow the simple theoretical expectations.

We can exclude any explanations involving host adaptation to the *C. bombi* infections on the part of the colonies because all hosts used were naive and the genetic background of an established colony cannot change over the course of a season. In addition, neither the individual hosts, nor their source colony were exposed to *C. bombi*

Figure 3. Mean counts of *Crithidia bombi* cells in gut samples from infected workers in relation to passage number between colonies.

before or during the experiment. Only the hosts used for the treatment came into contact with the parasite. The increasing age of the colony over the course of the experiment might explain changes in infection patterns. We expect that the average age of a random worker (as used here) will increase as the colony ages. However, as Doums *et al.* (2002) have shown, the workers' ability to mount immune responses decreases with age; as such, infection intensities should increase over the course of the experiment rather than decrease or remain constant as observed here.

We may also have failed to observe an increase in the degree of host exploitation because the parasite could have quickly reached its optimal level of virulence in each host genetic background, that is, within the first passage. This explanation is unsatisfactory because to achieve an optimal virulence requires feedback based on host mortality (Bull 1994; Lenski & May 1994). As we are essentially decoupling virulence and transmission by transmitting the parasite experimentally, such feedback does not exist; thus there is no cost to extremely high virulence. This lack of feedback is why serial transmission is believed to select for higher degrees of host exploitation and thus virulence (Ebert 1998). Theoretically, *C. bombi* could continue to exploit the host without any form of negative feedback until the hosts no longer survive the 7 day infection period. Strains with such a high virulence would not be transmitted within the parameters of this experiment. As few hosts died in our experiment, this feedback was probably not a factor.

It is reasonable to assume that there is a maximum infection intensity, being limited by nutrients in the host gut or simply by the available space for cells to attach to the gut wall (although no correlation between body size and infection intensity has been found; unpublished data). Selection would favour those *C. bombi* strains that can best compete for the limited resources. These strains gradually exclude the other strains not as suited to the infected colony, but the adaptations that make them suited to that colony may result in reduced success in another colony. Thus, after three or four passages within a colony, only the best competitors within that colony remain and are able to

generate the same overall infection intensity. At the same time, these strains are incapable of maintaining high infection intensities if transferred to an unrelated colony. If strains were being eliminated in this way, this would be visible in the differences of the genotypic composition of the gut sample of both the infected within-colony and between-colony workers. We checked for this by using microsatellite markers to identify the strains present in each within-colony and between-colony infection but only two out of ten showed a loss of genetic diversity and at only one locus.

Our results suggest that parasite populations adapt to host populations without showing increased infection intensities. These adaptations have costs to the parasite under certain circumstances, such as in the event of exposure to a different host population. Thus, isolation of a parasite within a particular host population, and limiting its exposure to other host types, may be an effective strategy for controlling long-term spread. At the same time, the host *B. terrestris* does not seem to carry a large cost of parasite adaptation that would increasingly threaten the survival of the colony as its life cycle unfolds.

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