# Single-clone and mixed-clone infections versus host environment in *Crithidia bombi* infecting bumblebees

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SUMMARY

Current theories assume that adaptive parasite evolution explains variation in the level of virulence and parasite success. In particular, mixed-genotype infections by parasites should generally be more virulent, and faster multiplying strains more successful, either because fixed strategies have evolved or because parasites facultatively alter virulence in response to co-infecting competitors. We compared several measures of parasite success and virulence between single-clone and mixed-clone infections of 2 strains of the trypanosome *Crithidia bombi* in its bumblebee host, *Bombus terrestris*. Contrary to expectation, we could not find differences between single-clone and mixed-clone infections in parasite prevalence, infection success, duration and clearance rate. However, a clearly significant effect of colony on infection intensity was present, and the colony effect emerged in virtually all other measures. We thus conclude that host environment as defined by the family (colony) genotype and thus host heterogeneity are more important in determining parasite virulence than the parasite characteristics. This does not invalidate modern theories of parasite evolution but suggests that variation in both hosts and parasites must be taken into account in more detail.

Key words: virulence, mixed infections, parasite, host heterogeneity, *Bombus terrestris*, *Crithidia bombi*.

## INTRODUCTION

Modern theories consider parasite virulence, i.e. the damage done to the host and the reduction of host fitness, to be a consequence of parasites adapting to natural populations of their hosts in order to maximize parasite fitness. As several models show, concurrent infection by several competing parasite genotypes will typically lead to a level of host exploitation that is above the optimum for single-genotype infections. As a consequence, mixed infections are predicted to be generally more virulent (Levin & Pimentel, 1981; Bremerman & Pickering, 1983; Nowak & May, 1994; Van Baalen & Sabelis, 1996). Parasites could evolve fixed strategies of host exploitation appropriate for the average level of mixed infections within host populations. Alternatively, conditional strategies could evolve such that parasites adapt their level of exploitation according to how many other parasite genotypes are actually co-infecting the same host (e.g. Frank, 1992; Van Baalen & Sabelis, 1996). Recent studies have shown that mixed-genotype infections of rodent malaria indeed show a different dynamic and often higher levels of virulence than single-genotype infections (Taylor, Walliker & Read, 1997; Taylor, Mackinnon & Read, 1998), as expected.

The intestinal parasite *Crithidia bombi* (Trypanosomatidae, Zoomastigophorea, Lipa & Triggiani, 1980) is common in its bumblebee host *Bombus terrestris* L., (Hymenoptera, Apidae) (Shykoff & Schmid-Hempel, 1991*a*). It infects adult workers, males and queens when it is ingested. *C. bombi* has a simple life-cycle and meets the standard assumptions of many current theoretical models. The parasite multiplies in the mid- and hindgut where cell numbers quickly build up. Transmission starts after a few days when the first cells pass out in the faeces and are subsequently picked up by the next host. This occurs (vertically) within the colony as well as horizontally to other colonies via flower visits (Durrer & Schmid-Hempel, 1994). A high multiplication rate of the parasite within the host leads to many cells shed in faeces and thus, in principle, to a high transmission rate, since infection success depends on dose. *C. bombi* has a number of subtle pathogenic effects, e.g. less developed ovaries (Shykoff & Schmid-Hempel, 1991*a*, *c*). In addition, infected colonies grow more slowly early in the season (Shykoff & Schmid-Hempel, 1991*c*). Infection by non-local parasite genotypes furthermore increases host mortality rate (Imhoof & Schmid-Hempel, 1998). In addition, more than 1 genotype of *C. bombi* can sometimes infect a single host individual, as identified by enzyme electrophoresis (Wu, 1994).

In the present experiment we investigated whether infections by single-genotypes of *C. bombi* differ from infections by mixed-genotypes with respect to infection dynamics and proximate measures of virulence, such as parasite prevalence, infection intensity, and host mortality rate. In particular, we

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infected individual workers of *B. terrestris* with either 1 of 2 different clones of *C. bombi* or a mixture of both. We expected that mixed infections would develop different dynamics and be more virulent, at least for the case where the parasite is able to facultatively change its level of host exploitation in response to other parasites being present.

## MATERIALS AND METHODS

To obtain 2 genotypes of *C. bombi* with maximally diverging growth rates, we randomly chose 8 samples out of a stock of cloned cells. These clones, prepared from single cells, were available from earlier studies by Wu (1994) and had been kept in liquid nitrogen before use. In this earlier study, the clones were grown in culture medium (Mattei medium, after Jenni *et al.* 1988) with a gradient of 1–10% gentamycin added to suppress fungal contamination and incubated at 27 °C. For the current study, we put equal doses of different clones in culture medium, stimulated them to multiply and counted the resulting cell concentrations in regular intervals. This provided an estimate of the growth rates of the clones. For the experiment we selected the clone with the maximum ('Fast') and minimum ('Slow') observed growth rate as measured under these conditions. Wu (1994) had originally isolated these 2 particular strains out of the gut of 1 queen of *B. terrestris*, caught near Basel, Switzerland. The genetical distinctiveness of the 2 strains had earlier been ascertained with methods described by Wu (1994) as follows. Cells from the 2 clones were harvested from the culture, lysed and the samples placed into a sample well plate in preparation for acetate cellulose electrophoresis (equipment: Helena Laboratories®). The electrophoresis was run at room temperature and the gels fixed and stained for different enzymes with standard methods (e.g. Richardson, Baverstock & Adams, 1986). The 2 clones in question differed in their genotype for PGM (phosphoglucomutase; EC 2.7.5.1 Richardson *et al.* 1986). The 'Slow' strain showed 2 bands while the 'Fast' strain showed 1 band on the gel.

Workers from 4 different colonies ('W', 'B', 'R', 'Y') of *B. terrestris*, each at least 20 workers strong, served as experimental hosts. Colonies 'W' and 'R' originated from Basel, Switzerland; 'B' from the Netherlands, and 'Y' from Belgium. All were raised in a parasite-free laboratory environment. From each of those colonies, we randomly chose a number of freshly hatched workers and placed them individually in glass jars. When they had reached the age of 4 days, the age at which they normally would have started to forage (Free, 1955; Alford, 1975), we experimentally infected them. A standard dose was prepared for this purpose, either as a single-clone (10000 cells of 'Fast' or 'Slow', respectively) or mixed-clone inoculum (5000 cells each: 'Mixed'). A total of 41 workers (13, 10, 7 and 11 from colonies W, B, R, and Y, respectively) were infected with clone 'Fast'; 40 workers (13, 10, 7 and 10) with clone 'Slow', and 40 workers (12, 10, 7 and 11) received a mixed-clone inoculum ('Mixed'). An additional 41 workers (13, 10, 7, and 11 workers) served as uninfected controls. These treatments were systematically varied among colonies, so that the first hatched worker received treatment 'Fast', the second treatment 'Slow', the third treatment 'Mixed', the fourth 'Control', and so forth.

For the infection, we starved workers for 2–3 h and then offered a droplet of sugar water containing the inoculum. This was readily imbibed and typically led to the establishment of an infection. Every second day we measured the concentration of cells shed in faeces samples to monitor the course of infection. The workers were kept singly and fed with sugar water *ad libitum* for the duration of the experiment, i.e. 22 days, close to the average life-span of a bumblebee worker in the field (e.g. Goldblatt & Fell, 1987; Schmid-Hempel & Heeb, 1991). Workers dying before this time, were immediately frozen for later inspection. Those living until day 22 post-infection were freeze-killed at that time. A few workers had died within 24 h after infection and were excluded from the analysis.

We used the following quantities as measures of virulence and to assess the dynamics of the infection. (1) Prevalence: the proportion of infected workers per treatment and colony. (2) Growth rate of *C. bombi*, estimated from the number of cells shed over time by a worker. (3) Infection intensity: the cell number shed in a worker's faeces averaged over the 22 days of the experiment. (4) Duration of the infection: the time-period between the first day post-infection when cells were observed in the faeces of a worker and the day after which no more cells could be found. (5) Mortality rate: for each treatment and colony, we calculated the proportion of workers per colony dying before day 22 post-infection. (6) Clearance rate: the proportion of infected workers in a colony that lost the infection before the end of the experiment. In addition, we used logit analyses to test for the effects of colony and treatment on the success (yes/no) of infection, host survival (alive/dead) until the end of the experiment, and clearance (infection cleared/not cleared) by the host; factorial ANOVA was used to identify treatment and colony effects on infection dynamics. All analyses were done with SPSS 6.1. Where necessary, data were transformed to normalize residuals. Welch's approximate *t*-test was used whenever the checks indicate unequal variances in the two groups to be compared (Zar, 1984).

Table 1. Synopsis of results

| | Prevalence (*n*) % | Intensity* (±S.E.; *n*) cells/$\mu$l | Duration*, ‡ (S.E.; *n*) days | Clearance*, ‡ (*n*) % | Mortality†, ‡ (*n*) % |
|---|---|---|---|---|---|
| Treatment | | | | | |
| 'Fast' | 74·4 (39) | 343·22 (±98·02; 29) | 9·53 (±1·25; 17) | 58·6 (29) | 10·3 (29) |
| 'Slow' | 84·2 (38) | 214·45 (±43·45; 32) | 6·67 (±0·75; 21) | 65·6 (32) | 12·5 (32) |
| 'Mixed' | 67·6 (37) | 256·26 (±67·90; 25) | 8·21 (±1·09; 19) | 76·0 (25) | 8·0 (25) |
| Control | — | — | — | — | 10·8 (37) |
| Colony | | | | | |
| 'W' | 91·9 (37) | 438·43 (±92·78; 34) | 9·33 (±1·07; 21) | 61·8 (34) | 15·9 (44) |
| 'R' | 67·9 (28) | 243·53 (±49·39; 19) | 7·33 (±1·14; 12) | 63·2 (19) | 3·4 (29) |
| 'B' | 66·7 (18) | 79·27 (±26·99; 12) | 6·00 (±0·94; 9) | 75·0 (12) | 22·2 (18) |
| 'Y' | 67·7 (31) | 130·35 (±26·20; 21) | 8·00 (±1·29; 15) | 71·4 (21) | 3·1 (32) |

* For infected workers only; (cells/$\mu$l).
† For treatments fast, slow and mixed: infected workers only.
‡ Before end of experiment (day 22).

## RESULTS

Reliable cell counts were obtained from 114 of the 121 inoculated workers and of those 86 (75·4%) developed an infection. We found that prevalence per colony (% successfully infected workers) was highest for treatment 'Slow' (84·2%), intermediate for 'Fast' (74·4%) and lowest for 'Mixed' (67·6%), but did not differ significantly among treatments nor among colonies (Table 1) (logit model: all parameters N.S.). The same result was obtained with a logit-analysis for infection success in individual hosts (infected/not-infected: for all main effects and interaction terms, $|z| < 1{\cdot}48$, N.S.). However, when only main effects were considered, the colony effect (highest prevalence in colony 'W': 91·9% infected workers) was significant ($\chi^2 = 8{\cdot}013$, D.F. = 3, $P = 0{\cdot}045$), while treatment was not ($\chi^2 = 2{\cdot}839$, D.F. = 2, $P = 0{\cdot}24$).

The average intensity of the infection in successfully infected workers was 270·03 (±41·68 S.E.) cells/$\mu$l of faeces. Intensity did not differ among treatments but varied among colonies (Table 2; also with 1-way ANOVA for factor 'colony': $F_{3,57} = 3{\cdot}92$, $P = 0{\cdot}011$) (Fig. 1). Similar results were found when the 2 clones Fast and Slow were compared with one another, or when Single infections (Fast or Slow) were compared with Mixed infections (Table 2). Interestingly, we also found significantly higher infection intensities in colonies 'W' and 'R' (originating from Switzerland (Table 1; ln-transformed mean intensity = 5·141 ± 0·194 S.E. cells/$\mu$l, $N = 53$ workers), than in colonies 'B' and 'Y' originating from the Netherlands and Belgium (mean = 4·227 ± 0·190, $N = 33$) (Welch's $t' = 3{\cdot}36$, D.F. = 80·08, $P = 0{\cdot}001$). All other measures were not different with respect to this grouping, however. We also found no difference among colonies or treatments for the following variables: duration of infection, clearance rate, or time to clearance (Tables 1 and 2). For example, an average proportion of 66·3% of all workers were able to clear the infection before the experiment ended on day 22, but this was similar for all colonies and treatments (Table 1). However, an interaction effect of colony treatment on clearance day was found when Single infections were compared with Mixed infections (Table 2). This suggests that colonies differed in how they cleared single or mixed infections, although given the data of Table 2 a cautionary note as to the reality of this effect is due.

On average, 12·8% of all the workers included in the analysis ($N = 156$; 6 of $N = 162$ perished within 24 h and were excluded) died before the end of the experiment. When all workers per treatment group were averaged (i.e. regardless of whether the infection was later confirmed as successfully established or not), then for treatments 'Fast' and 'Slow' mortality rates were the same: 12·5% ($N = 40$) and 12·5% ($N = 40$), respectively. Similarly, in treatment 'Mixed' 15·4% ($N = 39$) of the workers died and 'Control' mortality was 10·8% ($N = 37$). Hence, although mixed infections seemed to cause the highest mortality in their treatment group, the effect was not significant ('Mixed' *vs* 'Single', $\chi^2 = 0{\cdot}187$, N.S.), nor did mortality differ among all 4 treatment groups ($\chi^2 = 0{\cdot}37$, N.S.). The same result was found

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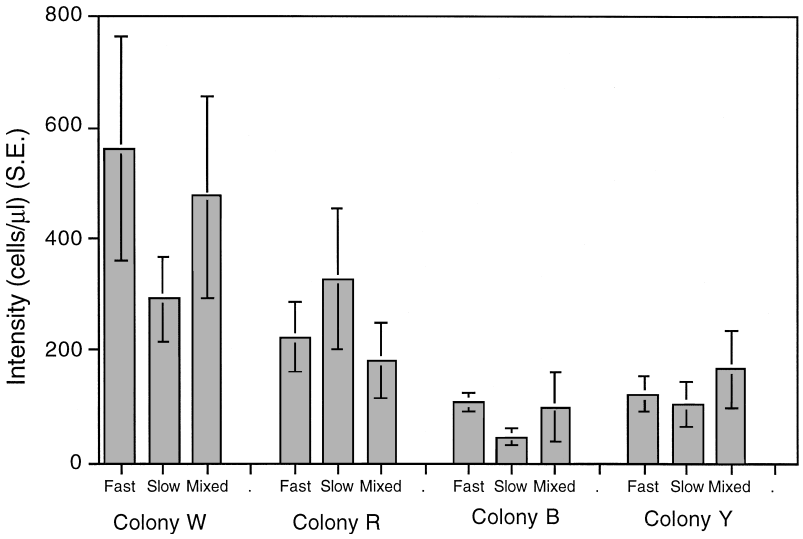


Fig. 1. Infection intensity, estimated from cells shed in faeces. For statistics, see Table 2.

Table 2. ANOVA results

| | | Treatments Fast, Slow, Mixed | | | Treatments Fast *vs* Slow | | | Treatments Single *vs* Mixed† | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Measure | Source | D.F. | F | *P* | D.F. | F | *P* | D.F. | F | *P* |
| Intensity* (cells/$\mu$l) | Colony | 3 | 2·85 | 0·043 | 3 | 2·01 | 0·123 | 3 | 3·31 | 0·024 |
| | Treat | 2 | 0·50 | 0·581 | 1 | 1·12 | 0·294 | 1 | 0·01 | 0·975 |
| | Colony Treat | 6 | 0·34 | 0·916 | 3 | 0·34 | 0·795 | 3 | 0·36 | 0·784 |
| | Error | 74 | | $r^2 = 0{\cdot}156$ | 53 | | $r^2 = 0{\cdot}155$ | 78 | | $r^2 = 0{\cdot}137$ |
| Duration (days) | Colony | 3 | 1·17 | 0·332 | 3 | 2·18 | 0·111 | 3 | 0·70 | 0·554 |
| | Treat | 2 | 1·88 | 0·164 | 1 | 3·55 | 0·069 | 1 | 0·47 | 0·496 |
| | Colony Treat | 6 | 0·96 | 0·464 | 3 | 0·69 | 0·566 | 3 | 1·34 | 0·272 |
| | Error | 45 | | $r^2 = 0{\cdot}233$ | 30 | | $r^2 = 0{\cdot}297$ | 49 | | $r^2 = 0{\cdot}143$ |
| Clearance (day) | Colony | 3 | 1·27 | 0·296 | 3 | 3·00 | 0·046 | 3 | 0·98 | 0·411 |
| | Treat | 2 | 1·34 | 0·272 | 1 | 2·47 | 0·126 | 1 | 0·15 | 0·700 |
| | Colony Treat | 6 | 1·85 | 0·110 | 3 | 0·13 | 0·941 | 3 | 3·61 | 0·020 |
| | Error | 45 | | $r^2 = 0{\cdot}292$ | 30 | | $r^2 = 0{\cdot}297$ | 49 | | $r^2 = 0{\cdot}240$ |

* Analysis with $\ln(1+x)$-transformed values, and fixed effects.
† Single = Fast or Slow.

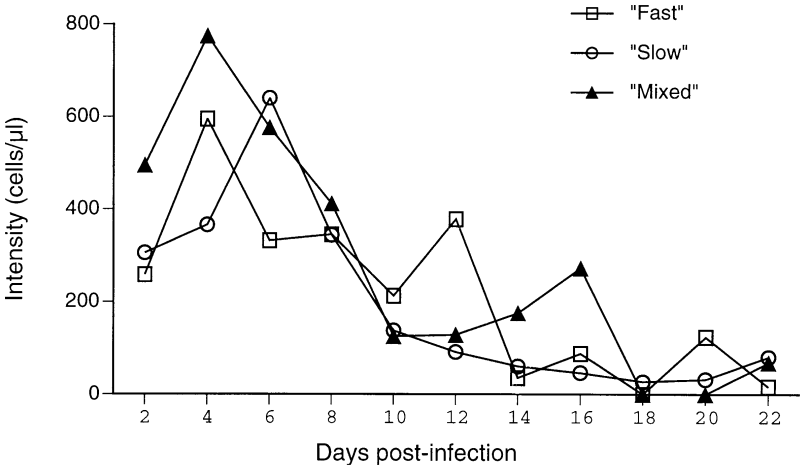


Fig. 2. Course of infection for treatments 'Fast', 'Slow' and 'Mixed'.

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when only those workers per treatment group were included that were confirmed as successfully infected (i.e. the figures given in Table 1). On the other hand, the survival of successfully infected workers appeared to vary among colonies ($\chi^2 = 7{\cdot}472$, $P = 0{\cdot}058$; Table 1). Within colony, mixed-clone infections tended to inflict higher mortality than single-clone infections in 3 out of 4 colonies (in colony 'W', the opposite was observed) but the effect was again not significant ($\chi^2 = 1{\cdot}098$, D.F. = 4, N.S.).

The growth rates of the 2 clones of *C. bombi* were different in culture medium. But this difference did not result in drastic differences of estimated growth rates or the overall dynamics in host workers, albeit 'Slow' reached its first peak parasitaemia somewhat later than 'Fast', and 'Mixed' reached higher levels than either of the single-clone infections (Fig. 2). Independent of treatment, however, the infection was eventually cleared, on average 16–18 days post-infection (Fig. 2).

## DISCUSSION

Summary measures of parasite success such as prevalence and intensity indicate the capability of the parasite to infect the host, but also the capability of the host immune system to resist or suppress the infection. On the host side, resistance to infection is influenced by many factors such as host age, body size, nutritional status, infective dose, past experience of infections, and gender. In our experimental paradigm, these factors were randomized and females only were used. We thus expected higher prevalences, higher infection intensities and consequently higher host mortality rates for the mixed-clone infection as compared to single-clone infections, and/or the 'Fast' infection as compared to 'Slow' only. Although mortality rates inflicted by the mixed-clone infection seemed to be somewhat higher, no clear effect emerged. We also found no clear difference in the respective, average dynamics which would indicate a superiority of the faster, i.e. more competitive strain. Instead, for the mixed-clone infection prevalence was lowest and proportion of clearance of infection highest while infection intensity and duration was intermediate. With this result it seems unlikely that the 1 clone (e.g. 'Fast') always could dominate the other inside the host, or that the parasites can change their growth pattern in a way that corresponds to presence of other clones inside the same host, as expected from the conceptual framework of parasite evolution described above. It remains a possibility though that *C. bombi* have fixed strategies suitable for the average level of mixed infections within bumblebee host populations and which cannot be detected in our experiment.

On the other hand, the most prominent uncontrolled effect in this experiment was host genotype, determined by the colony (i.e. 'family') where the test worker originated. In fact, it is well known that variation in host resistance has a strong genotypic component in diverse groups such as mammals (Wakelin, 1978), plants (Thompson & Burdon, 1992), and also social insects (Kulincevic, 1986; Schmid-Hempel, 1998). Similarly, in almost any measure considered here, a colony effect was found, although it was not always significant. That the parasites achieved higher intensities in host colonies from the same geographic area (colonies 'W', 'R') as compared to colonies from distant areas ('B', 'Y') concurs with the earlier finding that host and parasite interact differently according to where each party comes from (Imhoof & Schmid-Hempel, 1998). However, only 4 colonies could be tested here and thus the respective conclusions must be viewed with some caution. We, at least tentatively, conclude that the success and virulence of an infection, single-clone and mixed-clone, must be strongly overshadowed by 'colony genotype', the most important difference among colonies under standard laboratory conditions. Some support comes from the observation of effects of host genotype on infection success from earlier findings in the same system. For example, Shykoff & Schmid-Hempel (1991*b*) infected groups of workers of *B. terrestris* assembled from different colonies and showed that hosts from colonies that were naturally infected were again more likely to become infected with the experimental inoculum. Similarly, Schmid-Hempel & Schmid-Hempel (1993) found that workers from different colonies varied in their susceptibility to *C. bombi* passed on at different times post-infection from an infected source worker.

Host variability in the response to infection is therefore probably more important in determining the level of virulence than adaptive changes brought about by parasite evolution as it is usually advocated (e.g. Ewald, 1994; Ebert & Herre, 1996). Note that this does not *a priori* invalidate the basic logic of how parasite strategies of multiplication and transmission should evolve. But it indicates that the intrinsic variability of hosts is a major constraint for this process. In fact, several studies suggest the same conclusion. For example, Taylor *et al.* (1998) testing the virulence of single- and mixed-clone infections by rodent malaria in mice came to identical conclusions. There is also increasing observational and experimental evidence that host heterogeneity is crucial in driving the dynamics of an infection, and that this cannot be separated, by implication, from the resulting spectrum of disease severity (e.g. Gupta & Hill, 1995; Thompson & Lymbery, 1996; Dwyer, Elkinton & Buonaccorsi, 1997).

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