b i o l o g y
letters

Competitive suppression in mixed-clone parasite cultures

Louise H Taylor, Louise Matthews, Darren J Shaw and Daniel T Haydon

References	**This article cites 9 articles, 1 of which can be accessed free** http://rsbl.royalsocietypublishing.org/content/1/1/108.full.html#ref-list-1
Email alerting service	Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click **here**

b i o l o g y letters

Q1

Competitive suppression in mixed-clone parasite cultures

Louise H. Taylor[1,*,†], Louise Matthews[1], Darren J. Shaw[1] and Daniel T. Haydon[2]

[1]*Centre for Tropical Veterinary Medicine, Faculty of Veterinary Medicine, University of Edinburgh, Roslin, Midlothian EH25 9RG, UK*
[2]*Division of Environmental and Evolutionary Biology, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ, UK*
*Author for correspondence (louise.taylor@ed.ac.uk)

Mixed-genotype infections occur frequently in natural populations. Parasite genotypes are expected to interact within a host: competing for shared nutrients and being affected by the host's immune response to each other. Theoretically, competing parasites can be expected to exhibit increased rates of replication. Here, we investigate whether interactions between clones of *Theileria annulata*, a protozoan parasite of cattle, affect clones' replication rates in mixed cultures *in vitro*. Intrinsic replication rates and carrying capacities estimated from single-clone control cultures were used to predict replication rates of mixed cultures under different competitive assumptions. Mixed-culture dynamics deviated significantly from expectations in five out of six different clone combinations tested. Contrary to expectation, mixed cultures often replicated more slowly than predicted from single-clone control cultures. Competition coefficients were then calculated from the mixed-culture data and a competitive hierarchy of clones determined. The results also suggest that inherent competitive ability may be greater in clones with lower carrying capacities—those clones which would otherwise be excluded in a genetically diverse environment. Moreover, significant negative deviations from expected replication rates corresponded with successful out-competing of a higher carrying capacity clone by a lower carrying capacity clone.

Keywords: mixed cultures; *Theileria annulata*; competition; replication rates

1. INTRODUCTION

Microparasite infections are often incorrectly viewed as uniform populations of parasites replicating within a host. In fact, high levels of genetic diversity exist and mixed-genotype infections are common (Day *et al.* 1992; Thompson 2000). Where several parasite genotypes are present in a host concurrently, competition for nutrients and other resources is expected. Theoretically, such interactions should lead to increased replication rates of parasites as prudent parasites lose out to more aggressive competitors (Bull 1994; van

† Current address: Institute for Stem Cell Research, King's Buildings, West Mains Road, Edinburgh EH9 3JQ, UK.

Balen & Sabelis 1995). Interactions between genotypes should also affect disease severity, transmission rates and consequently parasite epidemiology and evolution (Read & Taylor 2001). Adaptive responses to intra-specific competition may evolve as a fixed strategy (replication rate is adaptive for the average genetic complexity of infections) or a facultative strategy (replication rate varies according to the genetic complexity of the current infection). Here, we test whether the latter can occur in culture.

Theileria annulata provides a good model system for investigating interactions between genotypes within a controlled culture environment. It exhibits high genetic diversity, and natural infections are often genetically complex (Ben Miled *et al.* 1994). The parasites live inside and immortalize their bovine host's cells, dividing with them, and consequently can be propagated indefinitely in culture (Brown 1987; Sharma *et al.* 1998). Here we use four genetically distinct *T. annulata* clones to investigate three questions about mixed cultures: (i) do clones growing in mixed culture exhibit higher intrinsic growth rates than when growing alone? (ii) Do mixed cultures produce higher overall parasite loads than single clone cultures? (iii) What are the competitive relationships of the different clones to each other?

2. MATERIALS AND METHODS

The four clones were different parasite genotypes (based on PCR/RFLP and monoclonal antibody analysis) cultured in host cells from the same cow (Taylor *et al.* 2003). Stock cultures of the four clones were maintained separately in exponential growth in RPMI medium (Brown 1987; Taylor *et al.* 2003).

Two 2 ml cultures for each clone and each combination of two clones were set up with an initial density of 1×10^5 cells ml^{-1} in a 24 well plate. Mixed cultures were initiated with a 50:50 mixture of the two component clones. The medium was unchanged for four days and samples taken at exactly 24 h intervals. Three replicates were conducted, providing six cultures of each two-clone mixture and single-clone control. Live cell density was determined by FACScan analysis using 40 μl of the culture and fluorescent beads of a known concentration (Taylor *et al.* 2003).

(a) *Data analysis*

Replication rate characteristics for each single-clone culture were estimated assuming logistic growth. Accordingly, the density of live cells at time *t* (days), $y(t)$, was modelled by

$$\frac{\mathrm{d}y}{\mathrm{d}t} = ry\left(1 - \frac{r}{K}\right). \tag{1}$$

Parameters r (intrinsic replication rate) and K (carrying capacity), and standard deviations were estimated using nonlinear least-squares regression. Parameters were estimated for each culture separately (six per clone), and within each clone for each replicate (three per clone).

The separate culture r and K values were compared using analysis of variance (ANOVA). One outlying K value (for a C2 culture in replicate 1) was excluded after jacknifing justified this, and *post hoc* analysis of differences between groups was carried out using the Bonferroni method.

To assess whether early replication rates of clones growing in mixed culture exceeded those observed in single-clone cultures we divided cell density on day 1 for a mixed culture (e.g. B15+C2) by the average cell density on day 1 for the two single-clone controls (e.g. B15 and C2). One-tailed single sample *t*-tests were then performed for each set of six comparisons to look for ratios significantly more than 1.

To assess whether mixed-clone cultures produced higher overall parasite loads than single-clone cultures we used parameters estimated from single-clone controls to predict cell densities for mixed-clone combinations assuming a mixed logistic process:

$$\frac{\mathrm{d}y}{\mathrm{d}t} = r_i y_i\left(1 - \frac{(y_i + a_{ij}y_j)}{K_i}\right) + r_j y_j\left(1 - \frac{(y_j + a_{ji}y_i)}{K_j}\right) \tag{2}$$

where y is the total cell density (i.e. $y = y_i + y_j$) and a values are the competition coefficients; a_{ij} refers to the impact on population growth of species i of an individual of species j, relative to the impact of an individual of species i (Pianka 2000).

For each of the six cultures of each mixed-clone combination, we generated 1000 simulations of the mixed logistic process. For each simulation, r_i and K_i values were selected from normal distributions using means and standard deviations obtained from the per replicate estimates for single-clone cultures. Deviations of experimental observations from null hypotheses were calculated using the distribution of simulated results for each of days 1–4. Fisher's combined probability tests were used to combine the six probabilities for each clone combination to indicate whether the null hypothesis could be rejected. Two null hypotheses were tested: H_{01}, that $a_{ij} = a_{ji} = 1$ (clones were identical and competitively equivalent to each other), and H_{02}, that $a_{ij} = K_i/K_j$, and $a_{ji} = K_j/K_i$ (clones with higher carrying capacities have less impact on the resource available to the competing clone than a lower carrying capacity clone).

Finally, competition coefficients (a_{ij} values) were estimated by minimizing the sum of squares between the observed mixed-culture data and the growth predicted by the mixed logistic process (equation 2). Replicates were simulated separately using per replicate parameters obtained from single culture growth trajectories. Deviances between data and predicted growth curves were summed over replicates to obtain a total deviance. Values for a_{ij} and a_{ji} that minimized this total deviance were identified.

3. RESULTS

(a) *Single-clone control cultures*

Significant differences between clones were found for both r ($F_{3,20} = 35.773$, $p < 0.01$, figure 1a) and K ($F_{3,19} = 9.266$, $p < 0.01$, figure 1b). *Post hoc* analysis showed that all pairs of clones were significantly different, apart from B15–C6 and C2–C8 for r, and B15–C2, B15–C8 and C8–C6 for K. Significant interactions between clone and replicate for both r and K ($F_{6,12} = 4.920$, $p < 0.01$ and $F_{6,11} = 6.492$, $p < 0.01$, respectively) were found. When these were removed from the model, replicate effects were not significant, however clone effects remained significant.

(b) *Mixed-clone cultures*

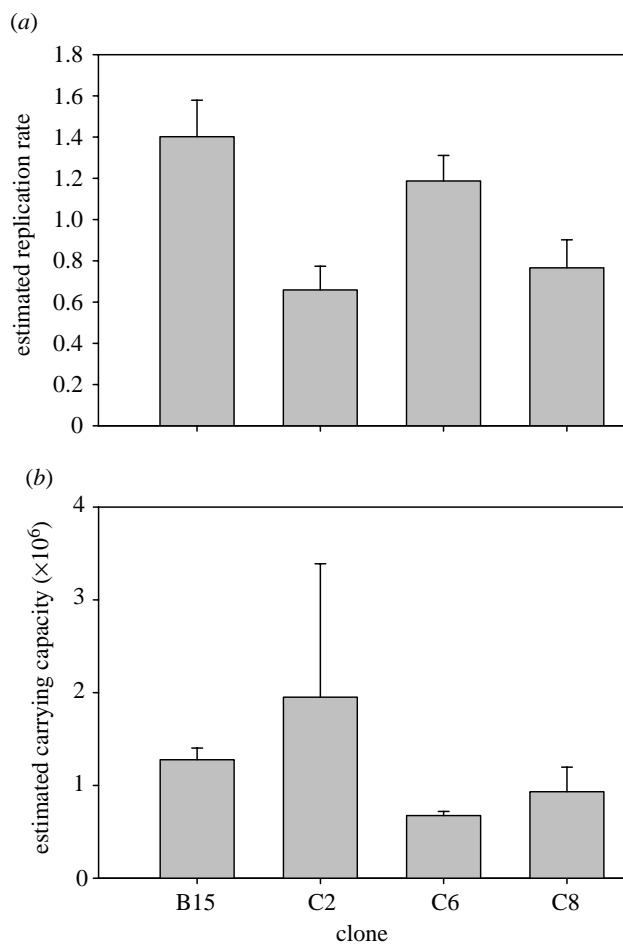The initial replication rate (assessed by cell density on day 1) of a two-clone mixed culture was never

Figure 1. Estimated values of (*a*) intrinsic replication rates and (*b*) carrying capacities for clones grown alone (+ standard error). The outlier K value from C2 is excluded (see §2).

significantly more than the average replication rate of the two component single-clone controls (*p* always > 0.4). For each of the six clone-pair comparisons, mixed-culture cell densities only exceeded average cell density of the relevant controls on 0/6, 1/6, 3/6, 3/6, 3/6 and 4/6 occasions. Thus, there was no evidence of increased replication rates in the face of competition.

From the predictions of a model that assumed ecological equivalence (H_{01}), the resulting reduction in overall population size was most evident on days 1 and 4 where 4/6 and 2/6 of mixed-clone combinations were significantly less populous than expected (day 1: B15+C2, $p < 0.05$; B15+C6, $p < 0.01$; B15+C8, $p < 0.05$; C6+C8, $p < 0.01$; day 4: B15+C6, $p < 0.001$; B15+C8, $p < 0.05$; figure 2a, table 1). Assuming that inter-clone competition was proportional to the ratio of the competitors' carrying capacities (H_{02}), the results remain largely unchanged (day 1: B15+C2, $p < 0.05$; B15+C6, $p < 0.01$;

C6+C8, $p < 0.01$; day 4: B15+C8, $p < 0.05$). Only one mixed-clone combination on one day was significantly larger than expected (C2+C6 on day 3, $p < 0.05$) under both hypothesized schemes (H_{01} and H_{02}). When the same models are used to predict the growth rate of single-clone cultures, the data do not deviate significantly from model predictions, i.e. the models are a satisfactory fit to the single-clone culture data (figure 2a).

(c) Competition in mixed-clone combinations
Estimated pair-wise competition coefficients (from equation 2) are reported in table 1. Assuming a classical '2-species Lotka–Volterra' competition model (Pianka 2000), the coefficients can be used to predict the long-term outcome of competition. When the competition coefficients are both equal to 1, the clone with the higher carrying capacity will eventually exclude the other. When competition coefficients
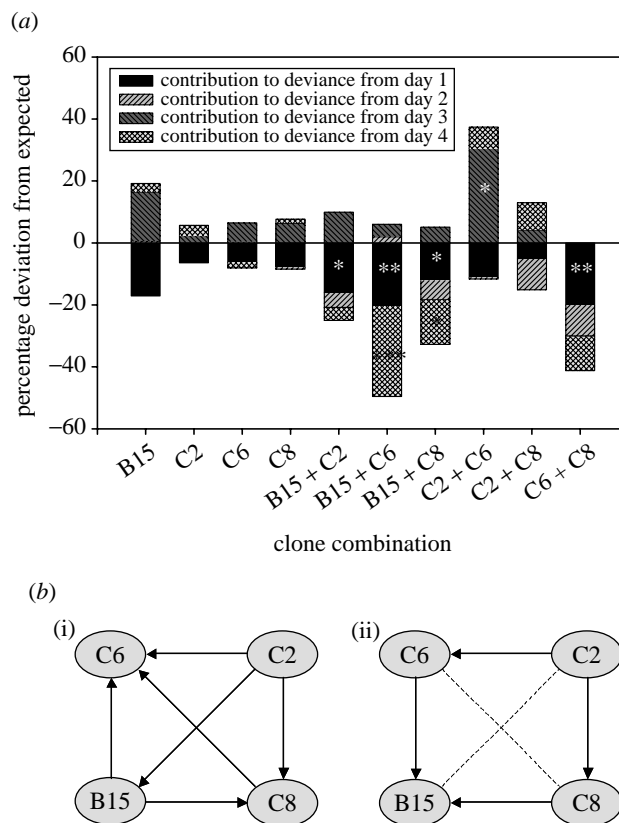

Figure 2. (*a*) Percentage differences comparing populations of cultured clones with expected population sizes. Expectations are calculated for each pair of replicates, and per cent deviations are averaged over (2×3) six realizations. Deviations are calculated for days 1–4 of the replication process, and their relative contributions to the overall deviance indicated by shaded bars. The first four bars indicate deviances of single-clone cultures from those predicted by the single-clone models (equation 1). The other six bars indicate deviations from predictions of the mixed-clone model (equation 2) assuming clone ecological equivalence. Significant deviations are indicated by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (*b*) Dominance hierarchy of the four clones. (i) Assuming two-species Lotka–Volterra dynamics with a_{ij}s all equal to 1; (i) assuming a_{ij}s estimated in table 1. Arrows from *i* to *j* indicate *i* to be dominant to *j*. Dashed lines indicate a conditional interaction (i.e. the outcome is determined by the initial parasite densities).

Table 1. Competition coefficients estimated from single-clone cultures.

population	a_{ij}	a_{ji}	predicted winner
B15+C2	1.0	2.0	conditional
B15+C6	2.4	0.2	C6
B15+C8	1.6	0.7	C8
C2+C6	0.8	0.9	C2
C2+C8	0.0	1.4	C2
C6+C8	1.1	4.0	conditional

deviate from 1, other scenarios are possible: the clone with the lower carrying capacity may exclude the other; the two clones may coexist; or the clone which prevails may be determined by initial densities (conditional dependence). The outcome of competition, however, cannot be predicted from competition coefficients alone; it depends also on the carrying capacities. For example, a C6 individual 'uses up' $2.4/K_{B15}$ of B15's carrying capacity, but a B15 individual uses up only $0.2/K_{C6}$ of C6's carrying capacity, and thus it is not surprising that C6 prevails.

The resulting competitive hierarchy is illustrated in figure 2b. Figure 2b(i) shows the competitive relationships that would arise solely from consideration of different carrying capacities and assuming the competition coefficients were all equal to 1. Figure 2b(ii) shows the competitive relationships assuming the estimated competition coefficients in table 1. There are no circular dominance relationships (such as; A dominates B, which dominates C, but C dominates A), but there are two conditionally dominant relationships. Overall, C2 is the most successful competitor and B15 the least.

The four largest competition coefficients in table 1 all correspond to the competitive impact of a lower carrying capacity clone on a higher carrying capacity clone. Moreover, these are clone-pair combinations for which (i) owing to competition there has been a reversal of fortune and the higher carrying capacity clone does not necessarily prevail and (ii) the largest negative deviances from expectation were observed.

4. DISCUSSION

This experiment investigates whether genetic complexity in cultures of *T. annulata* can lead to facultative changes in replication rates. Virulence theory suggests that in the face of competition, replication rates should either stay the same (a fixed evolutionary strategy, optimal for the average genetic complexity of infections encountered) or increase (a facultative strategy; Bull 1994). However, our results suggest that *T. annulata*'s replication rates generally fall in mixed cultures, and that mixed cultures maintain lower overall parasite loads.

Here, we measured only total cell density in mixed-clone cultures, so slight increases in replication rate of one clone could have been masked by a decrease for the other. In future, monoclonal antibody analysis could elucidate the fine details of interactions occurring in mixed-clone combinations.

However, this does not detract from the overall negative impact of competition shown here. Such interactions may be stronger between faster growing clones than between slower ones (B15+C6 cultures show the largest deviation from predictions and C2+C8 cultures one of the smallest); further experiments would be needed to confirm how general this is.

Assuming classic two-species Lotka–Volterra dynamics, a clear competitive hierarchy emerges from analysis of mixed cultures with clone C2 as dominant and B15 at the bottom. That C2 is the most successful clone is in part attributable to its high carrying capacity; it will naturally exclude other clones even when competition coefficients equal 1. Inspection of the competition coefficients alone reveals that the strongest competitive interactions are in each case those between a lower carrying capacity clone and a higher carrying capacity clone (i.e. B15 on C2, C6 on B15, C8 on B15 and C6 on C8). This makes intuitive biological sense; in the absence of competitive interactions, clones with lower carrying capacities tend to lose out in a mixed environment. Moreover, these clone-pair combinations (for which competition affects the clone which prevails) also correspond to cultures in which the greatest negative deviance from expected was observed. Thus, the outcome of competition is an overall reduction in the number of cells present compared with expectation.

What might mediate such negative interactions is unknown. Host immunity, perhaps the most obvious mediator of negative interactions, is clearly absent *in vitro*. Direct interference has not been demonstrated in parasites, but pathogenic bacteria can produce allelopathic substances that actively suppress competitors (Riley & Gordon 1999), and competing viruses can produce interference molecules (Hart & Cloyd 1990), so a similar effect cannot be ruled out here.

If virulence to the host is directly related to parasite loads, then mixed infections of *T. annulata* might lead to reductions in virulence, the opposite of theoretical predictions. Whether negative interactions between replicating stages of *T. annulata* extend to decreased transmission rates between hosts will depend on how production of sexual transmission stages relates to asexual replication rate. Competitive suppression of replicating parasites could translate into increased production of transmission stages, increasing the spread of disease.

Ben Miled, L., Dellagi, K., Bernard, G. *et al.* 1994 Genomic and phenotypic diversity of Tunisian *Theileria annulata* isolates. *Parasitology* **108**, 51–60.

Brown, C. G. D. 1987 *Theileriidae*. *In vitro* methods for parasite cultivation (eds. A. E. R. Taylor & J. R. Baker), pp. 230–253. London: Academic Press.

Bull, J. J. 1994 The evolution of virulence. *Evolution* **48**, 1423–1437.

Day, K. P., Koella, J. C. *et al.* 1992 Population-genetics and dynamics of *Plasmodium falciparum*—an ecological view. *Parasitology* **104**, S35–S52.

Hart, A. R. & Cloyd, M. W. 1990 Interference patterns of Human Immunodeficiency Virus HIV-1 and HIV-2. *Virology* **177**, 1–10.

Pianka, E. R. 2000 *Evolutionary ecology*, 6th edn. San Francisco: Addison Wesley Longman, Inc.

Read, A. F. & Taylor, L. H. 2001 The ecology of genetically diverse infections. *Science* **292**, 1099–1102.

Riley, M. A. & Gordon, D. M. 1999 The ecological role of bacteriocins in bacterial competition. *Trends Microbiol.* **7**, 129–133.

Sharma, G., Sharma, R. D. & Nichani, A. K. 1998 Successful long-term *in vitro* cultivation of *Theileria annulata* schizonts in media supplemented with homologous and heterologous sera. *Vet. Parasitol.* **79**, 135–141.

Taylor, L. H., Katzer, F., Shiels, B. R. & Welburn, S. C. 2003 Genetic and phenotypic analysis of Tunisian *Theileria annulata* clones. *Parasitology* **126**, 241–252.

Thompson, R. C. A. 2000 *Molecular epidemiology of infectious diseases*. London: Arnold.

van Balen, M. & Sabelis, M. W. 1995 The dynamics of multiple infection and the evolution of virulence. *Am. Nat.* **146**, 881–910.

Author Queries

JOB NUMBER: 0256

JOURNAL: RSBL

Q2   In figure 2 'i' and 'j' mentioned in the figure legend do not feature on the figure itself. Please check.

Q1   Please check the inserted short title.