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Moderation of pathogen-induced mortality: the role of density in *Bacillus thuringiensis* virulence

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Virulence in pathogens may be increased or decreased in order to maximize reproduction and transmission. We investigated how reproduction and virulence in the entomopathogen *Bacillus thuringiensis* (*Bt*) changed with bacterial density. We predicted that virulence would be moderated at high pathogen densities because extended time to death allows more growth in hosts. We found that pathogen reproduction (spores produced per cadaver) peaked at an intermediate time to death and was lowest in hosts that die early. Manipulating spore density (spores per unit area of leaf) by combining pathogenic *Bt* spores with a non-pathogenic mutant confirmed our prediction: larval 5-day mortality was reduced at higher pathogen densities. Pathogen reproduction increased with the density of pathogenic *Bt*. We hypothesize that more effective reproduction at high density is a consequence rather than a cause of density-dependent virulence.

Keywords: *Bacillus thuringiensis*; mass action theory; prudence; trade-off; tragedy of the commons; quorum sensing

1. INTRODUCTION

Many opportunistic pathogens upregulate the production of virulence factors according to their density within hosts (Williams *et al.* 2000). The diffusible molecules capable of relaying information about density can have diverse functions. However, microbiologists commonly assert that density-dependent increases in virulence arise because of positive feedback effects on the success in combating host immunity (de Kievit & Iglewski 2000; Williams *et al.* 2000), what ecologists refer to as an Allee (1931) effect. Excessive virulence at low density is hypothesized to elicit an immune response in which bacteria are unable to survive, while coordinated virulence at high density is adaptive.

Evolutionary biology theory suggests a different interpretation of this phenotypic plasticity. Increasingly, microbial virulence factors have been found to be shared social traits (public goods), imposing metabolic costs on individual cells but benefiting groups of pathogens (West & Buckling 2003). If this group-level benefit is proportional to the total production of public goods in a group (number of producers multiplied by *per capita* investment), theory predicts that opportunists will only invest in public goods when the number of producers (the density) is sufficient to ensure that group-level benefits outweigh individual-level costs (Brown 1999; Brown *et al.* 2002). The predictions of this mass action theory fit well with many observed cases of density-dependent virulence.

The applicability of this mass action theory is shown when we consider other ways in which virulence imposes costs upon parasites. If we define virulence as an increase in host death rate, public good models predict that high virulence should impose limits on pathogen growth rate. Conversely, a classical view of parasite virulence is that parasite replication *per se* harms the host, and therefore virulence is positively correlated with parasite growth (Frank 1996). Under the classical virulence hypothesis, competition among strains can lead to a 'tragedy of the commons' (Hardin 1968) and selection for rapid replication (high virulence) may trade off against low transmission if dead hosts are inefficient transmitters of infection. This risk of a 'tragedy' increases as the number of parasites infecting a host increases; therefore, mass action theory predicts that pathogens may moderate virulence as density increases (Brown 1999). This prediction remains largely untested.

In invertebrate pathogens, a tragedy of the commons may operate on host death rate. Many of these pathogens, such as *Bacillus thuringiensis* (*Bt*), must kill their hosts in order to reproduce (Ebert & Weisser 1997). However, these hosts are a rapidly growing resource base, and high virulence can be costly if it leads to rapid host death that produces smaller cadavers releasing fewer infectious particles (Cooper *et al.* 2002; Bonsall *et al.* 2005; Raymond & Hails 2007). Using *Bt*, we tested whether such a tragedy of the commons may exist by investigating the relationship between death rate and bacterial reproduction. We also tested whether *Bt* reproduction was density dependent within hosts, the microbiologists' predicted basis for phenotypically plastic virulence. Finally, we investigated whether virulence is prudently moderated at high density.

2. MATERIAL AND METHODS

(a) *The relationship between death rate and bacterial reproduction*

In order to ensure that pathogens were properly adapted to hosts, we used five rifampicin-resistant strains of *Bacillus thuringiensis kurstaki* (*Btk* rif^{R}), originally isolated from the biopesticide DiPel WP, four of which had recently been selected for improved growth in our invertebrate host, the diamondback moth *Plutella xylostella* (DBM), by three rounds of infection and re-isolation. DBM larvae (LAB-UK strain) were fed on *Brassica pekinensis* leaf discs that had been immersed in inocula of 500 *Bt* spores μl^{-1} using established methods (Raymond *et al.* 2007). For each strain, we dipped seven leaf discs, each leaf disc receiving five third-instar larvae. Cadavers were collected daily and those dying from each replicate on the same day were pooled. *Bt* produces heat-resistant spores when resources for growth are exhausted. Pathogen reproduction was measured by culturing serial dilutions of homogenized, heat-treated cadavers on *Bacillus cereus*-specific agar (*Bc*SA) plates (Oxoid, UK) containing 100 $\mu g\ ml^{-1}$ rifampicin (Raymond *et al.* 2007).

(b) *Density-dependent pathogen growth within hosts*

Third-instar DBM were inoculated by dipping 4.8 cm leaf discs of *B. pekinensis* Lour. (var. One Kilo S.B.) into purified spore

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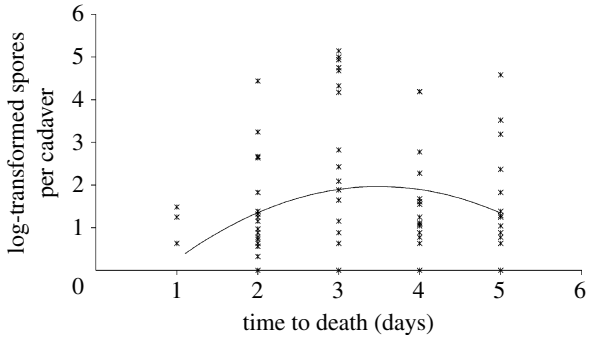

Figure 1. The relationship between time to death and reproductive rate in *Bt*. Data are log-transformed spore counts per cadaver. The plotted line (log counts $= -1.38 + 1.92$ day $- 0.27$ day^2) represents the fitted values for the fixed effects in a linear mixed model ANOVA.

suspensions of *Btk* rif^R as described previously. The leaf discs were inoculated with six doses of inoculum, six leaf discs were dipped for each dose and five third-instar larvae confined on each Petri dish for 7 days before the collection of cadavers.

(c) *Within-host density and virulence*

We manipulated spore density in two experiments, without altering the initial toxin content, by combining *Btk* rif^R with a near-isogenic mutant that fails to produce crystal endotoxins (*Btk* rif^R Cry-). This mutant was isolated after three transfers of LB broth culture of *Btk* rif^R grown at 37°C and inspection of crystal production via microscopy. These toxins are the major virulence factor in *Bt*; they induce pore formation in the insect midgut and determine access to host resources (Schnepf *et al.* 1998). Crystal toxin production occurs late in the bacterial growth cycle, and is packaged within an exosporium (and therefore physically distinct from the host in which it is produced); this toxin must be cleaved within the midgut before being active and is therefore targeted at spore-induced infections in different (subsequent) hosts rather than having a role in virulence during vegetative growth (Schnepf *et al.* 1998).

By keeping the quantity of crystal toxin-producing bacteria constant in low- and high-density treatments and increasing density by adding Cry- mutants, we manipulated density without affecting the virulence resulting from crystal toxin production. We conducted two experiments. In experiment 1, we measured daily mortality for 5 days; in experiment 2, we conducted a larger bioassay with *Btk* rif^R alone and two higher density treatments, using a 1 : 1 or 1 : 3 ratio of *Btk* rif^R: *Btk* rif^R Cry-. Experiment 2 was replicated twice but mortality was scored only on day 5. Both experiments used 30–35 larvae per dose.

(d) *Statistical analysis*

Statistical analysis was carried out in R (http://www.r-project.org) using generalized linear modelling and mixed model ANOVA. Bacterial counts were log transformed and mortality logit transformed, and analysed with binomial errors. Model assumptions were confirmed with graphical analyses. Mixed model analysis of the death/reproduction experiment used linear and quadratic terms as fixed covariates (day and day^2), strain as a fixed factor and days nested within Petri dish as a random factor. Selection of minimal adequate models proceeded by model simplification.

3. RESULTS

(a) *The relationship between virulence (time to death) and reproduction*

We tested whether *Bt* reproduction would be reduced in more virulent infections that killed hosts more quickly. Reproduction was reduced in insects that died early (figure 1), but peaked at day 3, an intermediate time to death. A quadratic function was the most appropriate description of the relationship between spore counts and time to death (linear term: likelihood ratio $= 7.08$, d.f. $= 1$, $p = 0.0078$; quadratic term: likelihood ratio $= 6.60$, d.f. $= 1$, $p = 0.0102$). There was no significant difference between the five strains (ancestral and host-adapted) on *Bt* reproduction (likelihood ratio $= 4.07$, d.f. $= 4$, $p = 0.396$).

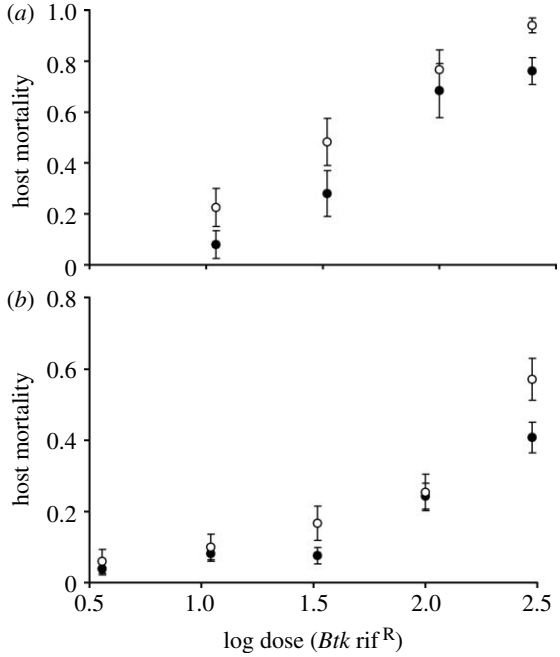

Figure 2. Density-dependent effects on virulence within hosts. Spore density was manipulated by infecting hosts with *Btk* rif^R alone (open symbols) or with an additional dose of a near-isogenic crystal-free mutant *Btk* rif^R Cry- (filled symbols). Experiments (*a*) 1 and (*b*) 2 have been plotted. Data are mean mortality $\pm$s.e.

(b) *Density-dependent pathogen growth within hosts*

Bacterial reproduction within insects was positively density dependent. At doses of 11, 33, 100, 300, 900 and 1800 spores μl^{-1} in inoculum, we recovered increasing densities of bacteria from cadavers. Log-transformed pooled means (s.e.) per dose were, respectively, 0.39 (0.31), 0.23 (0.15), 0.11 (0.11), 1.37 (0.83), 1.12 (0.81) and 2.58 (1.03). We compared linear mixed-effect models with dose nested within replicate as a random effect and either log-transformed dose as a covariate or a two-level treatment fixed factor (high dose >100 spores μl^{-1} and low dose ≤ 100 spores μl^{-1}). There were significant effects of dose on bacterial reproduction. The two-factor model produced a better fit (covariate model—AIC $= 332.9$, d.f. $= 90$, ln (dose) $t = 2.144$, $p = 0.035$; two-level factor model—AIC $= 326.8$, d.f. $= -90$, two-level dose treatment $t = -3.04$, $p = 0.0047$).

(c) *Density-dependent virulence within hosts*

We tested how increasing the density of spores with a Cry- mutant would affect mortality rates. Larvae in the higher density treatment (with additional Cry- spores) had reduced 5-day mortality relative to the lower density *Btk* rif^R-only treatment ($\chi^2 = 12.4$, $p < 0.001$; figure 2*a*). Larvae in the high-density treatment also tended to die later than those in the lower density treatment (arithmetic means 5.9 and 5.5 days,

respectively), although survival analysis indicated that this difference was not significant ($\chi^2=3.49$, $p=0.0616$). Increasing the dose of crystal-producing *Btk* rif^R shortened time to death ($\chi^2=38.7$, $p<0.0001$). In experiment 2, increasing density with *Btk* rif^R Cry- spores also reduced mortality ($\chi^2=5.86$, $p=0.015$; figure 2), although the treatments using different ratios of Cry- spores behaved similarly ($\chi^2=0.01$, $p=0.91$). Log-transformed dose and experimental replicate also affected larval mortality ($\chi^2=30.5$, $p<0.0001$ and $\chi^2=4.44$, $p=0.035$, respectively).

4. DISCUSSION

We predicted that the bacterial pathogen *Bt* would prudently reduce virulence at high density so that faster time to death would not curtail bacterial reproduction. Our experiments supported this hypothesis. Reproduction was reduced at the shortest time to death, supporting the prediction that high virulence could curtail reproduction, in a manner analogous to a tragedy of the commons. However, insects that died latest were not the best resources for growth. This could result from the onset of developmental immunity seen in many Lepidoptera, in which lower pathogenicity and poorer pathogen growth occurs in the last instar (Raymond & Hails 2007).

Bacterial quorum sensing provides a mechanism whereby investment in virulence can change with density. Quorum-sensing pathways can affect the expression of vegetative virulence factors in *Bt* (Gominet *et al.* 2001) and pathogens can use quorum sensing to downregulate virulence (Zhu *et al.* 2002). Other explanations for our results are possible. Antagonism may reduce virulence in mixed infections of distantly related bacteria. However, the near-isogenic strains in the Cry- experiment should have identical production of and resistance to antagonists. Nevertheless, we cannot entirely exclude the role of more subtle differences between the strains used. For instance, Cry- mutants can dominate growth in mixed infections with endotoxin producers (B. Raymond 2008, unpublished data), and selection for rapid growth in broth (used to produce this mutant) may lead to the downregulation of other virulence traits.

At this stage, it is also unknown whether plastic virulence in *Bt* results from variable investment in virulence factors or moderation of replication rates. Phenotypic plasticity at high density could lead to increased overall reproductive success because resources are diverted from public goods into growth, or because slower replication allows for increased investment in interspecific competition. Antagonistic interactions with resident gut flora in the cadaver are known to limit the growth of *Bt* in the cadaver (Takatsuka & Kunimi 2000). Thus, in this system, it is possible that Allee effects or density-dependent reproductive success in *Bt* is not a cause of density-dependent virulence but rather a consequence of density-dependent plasticity.

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