Virulence and competitive ability in an obligately killing parasite

Farrah Bashey, Courtney Reynolds, Tara Sarin and Sara K. Young

F. Bashey (fbasheyv@indiana.edu), C. Reynolds, T. Sarin and S. K. Young, Dept of Biology, Indiana Univ., Bloomington, IN 47405, USA.

Mixed infections are thought to have a major influence on the evolution of parasite virulence. During a mixed infection, higher within-host parasite growth is favored under the assumption that it is critical to the competitive success of the parasite. As within-host parasite growth may also increase damage to the host, a positive correlation is predicted between virulence and competitive success. However, when parasites must kill their hosts in order be transmitted, parasites may spend energy on directly attacking their host, even at the cost of their within-host growth. In such systems, a negative correlation between virulence and competitive success may arise. We examined virulence and competitive ability in three sympatric species of obligately killing nematode parasites in the genus *Steinernema*. These nematodes exist in a mutualistic symbiosis with bacteria in the genus *Xenorhabdus*. Together the nematodes and their bacteria kill the insect host soon after infection, with reproduction of both species occurring mainly after host death. We found significant differences among the three nematode species in the speed of host killing. The nematode species with the lowest and highest levels of virulence were associated with the same species of *Xenorhabdus*, indicating that nematode traits, rather than the bacterial symbionts, may be responsible for the differences in virulence. In mixed infections, host mortality rate closely matched that associated with the more virulent species, and the more virulent species was found to be exclusively transmitted from the majority of coinfected hosts. Thus, despite the requirement of rapid host death, virulence appears to be positively correlated with competitive success in this system. These findings support a mechanistic link between parasite growth and both anti-competitor and anti-host factors.

Most models of the evolution of virulence assume that the detrimental effect that parasites have on their hosts (virulence) arises as an unavoidable consequence of parasite growth and reproduction. Accordingly, parasites that grow faster or reproduce more within a host are assumed to have a fitness benefit due to higher transmission rates, but pay a cost in terms of reduced longevity (as modelled by Bremermann and Thieme 1989, May and Anderson 1983). However, when two or more parasite strains simultaneously infect a host, the benefit of greater within-host parasite growth can select for increased virulence, if the faster growing parasite is competitively superior within the host (Levin and Pimentel 1981, Bremermann and Pickering 1983). Importantly, the outcome of these models depends critically on the mechanisms of within-host competition and the nature of the correlation of competitive success with virulence, neither of which is well understood in most systems (van Baalen and Sabelis 1995, Mosquera and Adler 1998).

Experimental studies have demonstrated that within-host competitive success is linked to higher virulence in both within- (de Roode et al. 2005, Ben-Ami et al. 2008) and between- (Ishii et al. 2002, Thomas et al. 2003) species mixed infections. Additionally, some studies have shown that this positive correlation is associated with faster within-host growth or higher densities in single infections (Ishii et al. 2002, de Roode et al. 2005). Intuitively, a greater ability to

successfully grow within a host in a single infection should result in a competitive advantage for a parasite in a mixed infection (Inglis et al. 1997, Gruner et al. 2009). However, positive correlations between virulence, within-host growth, and competitive success are not universally found (Hodgson et al. 2004, Gower and Webster 2005). For example, in a study of interspecific competition between fungal pathogens, Hughes and Boosma (2004) found that the less virulent species was able to out-compete the more virulent species in ant hosts simultaneously exposed to both pathogens.

Interactions between the host and parasite may be responsible for a negative correlation between virulence and competitive success (Hughes and Boomsma 2004, Gower and Webster 2005). Many parasites produce immunosuppressant toxins to enable their proliferation within the host. Production of these toxins may be energetically costly, and strains producing less toxin may have a within-host growth advantage in a coinfection, albeit at a cost of lower transmission success when infecting alone (Hughes and Boomsma 2004). These toxins are especially prevalent in obligately killing parasites, as host death is a requirement for transmission, rather than an unavoidable cost of infection (Day 2002, Frank and Schmid-Hempel 2008).

Here, we examine the relationship between virulence and competitive success using three species of entomopathogenic nematodes. These species are locally sympatric, and therefore,

mixed infections are likely to occur in nature. Unlike many obligately killing parasites, which grow and then kill their host to release propagules into the environment (Ebert and Weisser 1997), these nematodes first kill, then reproduce inside the host carcass (Forst and Nealson 1996), with transmission stage propagules emerging as the carcass becomes depleted (Wang and Bedding 1996). Thus, we predicted that speed in host killing would be decoupled from the competitive outcome of a mixed infection, as nematode reproduction follows, rather than causes, host death. However, these nematodes and their symbiotic bacteria are known to produce insecticidal toxins (Burman 1982, Dunphy and Webster 1988). If toxin production is costly in terms of growth, then, we would predict a negative correlation between virulence and competitive success, with 'cheaters' that invest less in host-killing toxins exhibiting a growth advantage in mixed infections (Brown 2001, West and Buckling 2003, Hughes and Boomsma 2004). Alternatively, a positive correlation between virulence and competitive success would suggest that growth early on in the course of the infection is critical to both processes.

Methods

Study system

Nematodes in the genus *Steinernema* are free-living and non-feeding in the soil as juveniles; they require an insect host for development and reproduction. Each juvenile nematode carries with it symbiotically associated bacteria in the genus *Xenorhabdus*. Once inside the insect, nematodes release their bacteria and resume feeding (Sicard et al. 2004). As a result of the action of both the bacteria (Boemare and Akhurst 1988, Dunphy and Webster 1988) and the nematodes (Goetz et al. 1981, Burman 1982, Simoes 2000), the host dies a few days after infection. The nematodes mature into separate sexes and reproduce for one or more generations inside the host carcass (Wang and Bedding 1996). As the host carcass becomes depleted, the nematodes become colonized by the bacteria, cease feeding, and emerge as infective juveniles (Popiel 1989, Martens et al. 2003). Infective juveniles can survive for several months without encountering a new host (Gaugler 2002).

The nematodes used in this study were isolated from *Galleria mellonella* caterpillars exposed to soil collected in

September 2007 from one hillside in the Indiana University Teaching and Research Preserve at Moore's Creek, as described in Hawlena et al. (2010). Eight parasite isolates were used in this study; each isolate emerged from a different caterpillar. The nematode species composition of each isolate was determined by sequencing 28S rRNA gene (Stock et al. 2001). Additionally, the bacteria species composition of each isolate was identified with 16S rDNA (Tailliez et al. 2006).

Based on the 28S sequences, the isolated nematodes may represent three previously undescribed species in the genus *Steinernema* (S. P. Stock pers. com.). Each species clearly aligns to different clades within the genus, thus, we will refer to them as *S.* sp. C1, *S.* sp. C3, *S.* sp. C4, to denote their clade designations (Uribe-Lorio et al. 2007). BLAST searches of 28S sequences, places *S.* sp. C1 as a sister taxon to *S. affine*, *S.* sp. C3 as sister to *S. kraussei*, and *S.* sp. C4 as sister to *S. costaricense*. Interestingly, *S.* sp. C1 and *S.* sp. C3 were both found associated with *X. bovienii*, which is known to be associated with some *Steinernema* species in Clades 1 and 3 (Lee and Stock 2010). *Steinernema* sp. C4 was found to associate with *X. koppenhoeferi*, which has been previously isolated from a *Steinernema* in Clade 5 (Tailliez et al. 2006).

The natural hosts of these new isolates are not known. *Steinernema* nematodes are able to infect a wide range of insect hosts (Peters 1996). One mechanism that facilitates this killing breadth is that *Steinernema* can suppress the host immune system (Wang and Gaugler 1999, Brivio et al. 2002); although, the ability to do this varies across nematode and insect species (Li et al. 2007). Nevertheless, comparisons of potential host use among sympatric *S. affine* and *S. kraussei* found little differences their abilities to infect a wide range of locally abundant insects (Puza and Mrácek 2010). Moreover, they found consistent differences in the competitive outcome (*S. affine* dominant to *S. kraussei*) across four naturally occurring insect species and *Galleria mellonella*.

Experimental design

We performed a total of four infection assays. In each assay, three different infection treatments were used, such that caterpillars were exposed to nematodes of one of two species singly, or to a 50:50 mixture of the two species simultaneously. In two assays, species C1 and C4 were used and in two assays species C3 and C4 were used. Unique isolates were used in each assay, for a total of eight isolates (Table 1). Coinfections between C1 and C3 were not performed, as we had no

Table 1. Results of proportional hazards regression of the time of host death for each of the four assays (shown in Fig. 1a, c, 2a, c). In each assay, unique parasite isolates (identified in parentheses) were used in single and mixed infections. Single infections show significant differences between species in the timing of host death. Host survival in the mixed infections more closely matches the faster killing species, than the slower killing species.

	Host survival when infected with competitor (C1 or C3) alone relative to C4 alone			Host survival in mixed infection relative to faster killing parasite in single infections		
	Hazard ratio	χ^2	p	Hazard ratio	χ^2	p
C1 versus C4						
Assay 1 (B94 vs K60)	0.399	38.47	< 0.0001	0.830	1.8181	0.1775
Assay 2 (B66 vs K43)	0.218	96.67	< 0.0001	0.759	3.0866	0.0759
C3 versus C4						
Assay 1 (B47 vs K45)	1.50	14.89	0.0001	1.048	0.1800	0.6713
Assay 2 (B59 vs K65)	2.04	33.00	< 0.0001	0.743	6.4276	0.0112

reliable marker to distinguish their bacterial symbionts and mismatches between nematode and bacterial species have been known to occur in mixed infections (Koppenhoefer et al. 1995). Subsequent fingerprinting of the bacterial genotypes (Hawlena et al. 2010) indicated that each assay compared a unique combination of bacterial genotypes. Additionally, sexual reproduction of nematodes within the caterpillar host potentially made each nematode isolate unique.

For each treatment, 70–80 caterpillars of the greater wax moth *Galleria mellonella* were individually exposed to a dose of 50 nematodes. Thus, each assay was comprised of 210–240 caterpillars. Each caterpillar was placed in a 60×20 mm petri dish, lined with filter paper (Whatman no. 1) moistened to a total volume of 0.5 ml of dH_2O. To infect each caterpillar, a drop of dH_2O containing 50 nematodes was placed on the dorsum of the caterpillar. Infected hosts were kept at 18°C and censused at intervals for mortality. Caterpillars were scored as dead, if they did not move in response to being touched by a probe. Seven days post-infection, caterpillars were transferred to White traps (White 1927, Bashey et al. 2007) for maintenance of the infection and collection of emerging nematodes. Nematodes were allowed to emerge from the host cadaver for 38 days post-infection at which time the caterpillars were discarded. Nematodes were stored in dH_2O at 8°C until analyzed for nematode and bacteria species identification.

Identification of nematode species

PCR-RFLP was used to determine the nematode species composition resulting from the mixed infection (Sicard et al. 2006). For each sample tested, DNA was extracted from 1000 infective juvenile nematodes using the spin column protocol of the DNeasy Blood and Tissue Kit. The 28S rRNA gene was amplified using the PCR cycling parameters as described in (Stock et al. 2001). One µl of the 922 bp PCR product was incubated at 37°C for 2 h with 2 µl EcoRI, 2.5 µl NE enzyme buffer, and 19.5 µl Millipore water. The fragments were separated on a 1.5% agarose gel in 1% × TBE at 60 V for 2 h. The fragments were visualized by ethidium bromide staining and then photographed. The species of nematode was assessed based on the banding patterns. EcoRI cuts *S.* sp. C4 at 661 bp, resulting in two fragments, 661 bp and 264 bp, but does not cut the other two nematode species. Known mixtures of the nematode species showed that a mixture containing greater than 10% either species could be detected.

Identification of bacteria species

Bacteria were isolated from nematodes that had emerged from an insect host. For each sample tested, bacteria were isolated from approximately 2000 infective juvenile nematodes. Nematodes were spun down and the pellet was surface sterilized for 3 min in 2% NaOCl. Sterilized nematodes were spun down again and rinsed three times with sterile water. A small amount of sand was added to the remaining pellet and ground using a disposable pestle. Three-hundred µl of LB was added to the pellet and 10^{-1} and 10^{-2} dilutions were made. One hundred µl were taken from each dilution and plated on NBTA plates. After incubation for two days at 28°C, bacteria species composition was determined.

For the C3 versus C4 infections colony color was used to determine the bacterial species composition from the mixed infection giving a detection limit of approximately 1%. In the C1 versus C4 infections, colony color was less distinct and species identity was confirmed with 16S rDNA sequencing (Tailliez et al. 2006). A minimum of two clones were sequenced per host to establish that subtle color differences were correctly interpreted.

Statistical analyses

To determine differences in mortality rate among infection treatments, we performed Cox (proportional hazards) regressions using the TPHREG procedure in SAS. The proportion of hosts with successful parasite emergence was compared across infection treatments using a χ^2-analysis in the FREQ procedure. The parasite species composition released from hosts in the mixed infection treatment was tested against the null hypothesis of a random binomial using the FREQ procedure.

Results

In the C1 versus C4 assays, C4 parasites killed the insect hosts at a significantly faster rate than C1 parasites (Fig. 1a, c, Table 1). The hazard ratio of C1 infected hosts relative to C4 infected hosts indicates that the rate of host death when infected with C1 alone was only 20–40% of that when infected with C4 alone (Table 1). In contrast, hosts infected with a mixture of C1 and C4 died at a rate that did not differ significantly from hosts infected with C4 alone (Table 1).

The probability of parasite emergence differed significantly across infection treatments (assay 1: $\chi^2 = 41.75$, DF = 2, p < 0.0001, Fig. 1b; assay 2: $\chi^2 = 90.30$, DF = 2, p < 0.0001, Fig. 1d). In both assays, hosts infected with C4 alone showed greater than 90% emergence, hosts infected with C1 alone showed less than 50% emergence, and hosts in the mixed infection treatment showed an intermediate level of emergence. In the mixed treatments, only one species of nematode or bacteria was found to emerge successfully from an individual host. In all cases, the bacterial and nematode species identification matched, indicating the specificity of the symbiosis. The C4 parasite was dominant in each assay, as more hosts with parasite emergence released C4 than would be expected by a random binomial distribution (assay 1: observed percent = 88.00, 95% CI = 75.26–100, p = 0.0001, Fig. 1b.; assay 2: observed percent = 92.86, 95% CI = 79.37–100, p = 0.0013, Fig 1d.).

In the C3 versus C4 assays, C3 parasites killed the insect hosts at a significantly faster rate than C4 parasites (Fig. 2a, c, Table 1). The hazard ratio of C3 infected hosts relative to C4 infected hosts indicates that the rate of host death when infected with C3 alone was 1.5 to 2 times as great of that when infected with C4 alone (Table 1). In assay 1, hosts infected with a mixture of C3 and C4 died at a rate that did not differ significantly from hosts infected with C3 alone (Fig. 2a, Table 1). However, in assay 2, the mixed treatment resulted in an intermediate rate of host death (Fig. 2c, Table 1). Even so, host survival rate in the mixed treatment more closely matched the survival rate
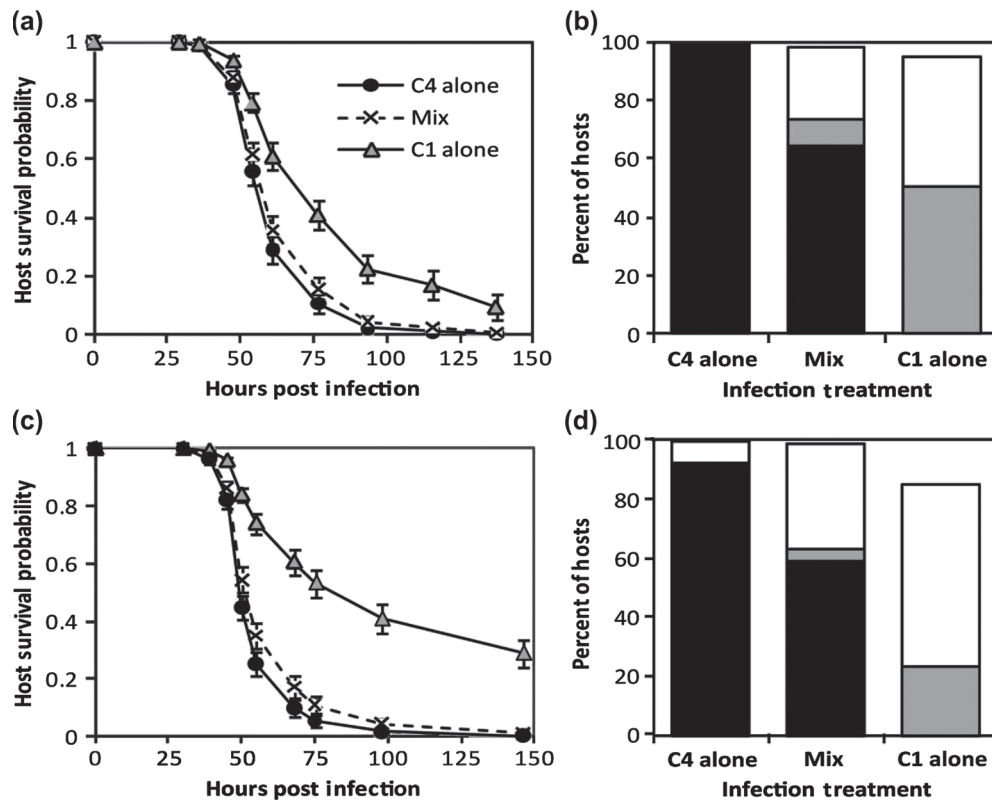
Figure 1. Outcomes of single and mixed infections in the two assays conducted comparing parasites C1 and C4. Unique parasite isolates were used for each assay and > 210 caterpillars were infected in each assay. Host survival probability (± 1 SE) as a function of hours post infection for hosts infected with parasite C4 alone (black circles), C1 alone (gray triangles) or 50:50 mixture of the two (dashed line with x) in (a) assay 1 and (c) assay 2. Percent of infected hosts killed and showing: no parasite emergence (white fill), emergence by C4 (black fill), or emergence by C1 (gray fill) as a function of infection treatment in (b) assay 1 and (d) assay 2.

of C3 alone than of C4 alone as the hazard ratio of hosts infected with C4 alone was 0.66 of those infected with the mixed treatment ($\chi^2 = 14.07$, p = 0.0002), while the hazard ratio of the mixed treatment versus C3 was 0.74 (Table 1).

The effect of infection treatment on the probability of parasite emergence differed between the two C3 versus C4 assays. In assay 1, the probability of emergence was > 73% in all treatments, with the only significant difference occurring between the C4 alone and the mix infection treatments ($\chi^2 = 7.12$, DF = 1, p = 0.0076, Fig. 2b). In assay 2, C4 alone and mixed infection treatment showed approximately 80% emergence, while the C3 alone showed less than 50% ($\chi^2 = 26.88$, DF = 2, p < 0.0001, Fig. 2d). Nevertheless, in both assays the C3 parasite emerged from a majority of hosts in the mixed infection treatment. As in the C1 versus C4 mixed treatments, only one species of nematode or bacteria was found to emerge successfully from an individual host; and in all cases, the bacterial and nematode species identification matched. In assay 1, all of the hosts sampled from the mixed infection treatment released only C3 parasites (95% CI = 85.75–100, Fig. 2b). While in assay 2, approximately 60% of the mixed-treatment hosts with parasite emergence released only C3 parasites (95% CI = 40.59–81.20, Fig. 2d). Interestingly in assay 2, the total percent of infected hosts releasing C3 parasites did not differ between the C3 alone and the mixed treatment (Fig. 2d). This result suggests that C4 emergence from the

mixed treatment occurred only in hosts where C3 was not successful.

Discussion

The ecological dynamics and evolution of sympatric parasites may be shaped by within-host competition (Poulin 2007). We performed mixed infections where one focal parasite species was inoculated simultaneously into a host with one of two different, congeneric parasites. Our goal was to determine the outcome of within-host competition and the extent to which infection dynamics of single parasite infections predict this outcome. In each competition, only one parasite species was found to emerge successfully from an individual host. Moreover, by examining parasite success (percent of hosts releasing each parasite) in the mixed-species relative to the single-species infections, we saw that competition was asymmetric, such that one parasite was more negatively affected by competition than the other. Interestingly, the competitive success of the focal parasite (C4) varied between its two sympatric competitors: C4 was dominant over C1 (Fig. 1b, d), but was outcompeted by C3 (Fig. 2 b, d). While many factors may explain the outcome of within-host competition, the experimental infections performed here point to a link between more rapid host death and competitive success, as C3 killed hosts significantly faster than C4 (Fig. 2a, c) while C1 killed hosts markedly slower (Fig. 1a, c).
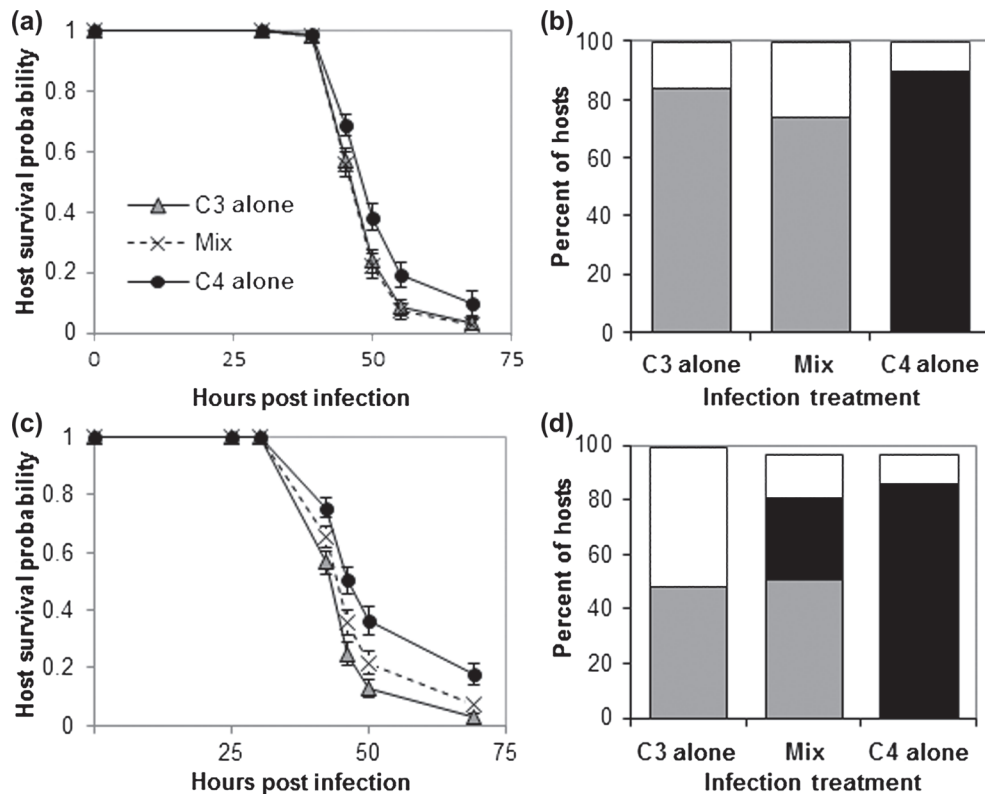
Figure 2. Outcomes of single and mixed infections in the two assays conducted comparing parasites C3 and C4. Unique parasite isolates were used for each assay and 240 caterpillars were infected in each assay. Host survival probability ($\pm$ 1 SE) as a function of hours post infection for hosts infected with parasite C4 alone (black circles), C3 alone (gray triangles) or 50:50 mixture of the two (dashed line with x) in (a) assay 1 and (c) assay 2. Percent of infected hosts killed and showing: no parasite emergence (white fill), emergence by C4 (black fill), or emergence by C3 (gray fill) as a function of infection treatment in (b) assay 1 and (d) assay 2.

Many theoretical models assume that faster within-host growth causes both higher virulence and greater competitive success. More virulent clones have been found to be superior competitors in genetically diverse malarial infections, but even in this well-studied system, the causal mechanism that links virulence and competitive success has still not been determined (Bell et al. 2006). Additionally, several 'grow-then-kill' parasites have shown a positive correlation between within-host competitive success and virulence (Ishii et al. 2002, Thomas et al. 2003, Ben-Ami et al. 2008). In these parasite systems, a faster growing species can numerically dominate within the host, fully exploit the host, and induce host death, thereby excluding any slower growing parasites that have yet to increase in density (Ishii et al. 2002). However in our system, host death precedes parasite reproduction, so we predicted that the rate of host death would be decoupled from competitive outcome. Alternatively, we predicted a negative association between virulence and competitive success, if the growth of a species investing less in host-killing toxins could be facilitated by the presence of a more virulent species (Hughes and Boomsma 2004).

So, why might faster host killing be positively correlated with competitive success in our system? In the initial stage of infection, the nematode must switch from its non-feeding, free-living stage to its feeding, parasitic stage. As part of this switch, nematodes release their symbiotic bacteria into the insect hemocoel. Between-species differences in virulence and competitive ability could be due to differences in the

speed at which nematodes switch between stages, in the initial dose of bacteria released, or in the bacterial growth rate within the insect. Moreover, species could differ in the production of toxins which disable the insect immune system or of factors used to acquire nutrients from the host. Little is known about any of these mechanistic aspects of the infection, especially in the specific parasite isolates used in this study. The current model of gene expression in the closely related bacterium *Xenorhabdus nematophila* invokes a temporal regulatory progression, such that genes involved in suppressing the insect immune system are activated first, followed by genes involved in accessing host nutrients (Richards and Goodrich-Blair 2009). Thus, it appears that a link between virulence and competitive success in this system may arise by how quickly this temporal progression begins, with a faster species colonizing the host and somehow precluding the development of the other. It is important to note, however, that both C3 and C1 carry the same species of bacteria, which suggests that the nematodes rather than the bacteria may be central to the differences we observed.

In summary, we find that within-host competition and virulence appear to be linked even in a system where parasites kill, then grow. Since host killing occurs early in the course of the infection and is necessary for successful transmission, it is likely that these parasites face strong selection for quickly overcoming the host immune system (Frank and Schmid-Hempel 2008). The species we studied varied in how quickly they killed the host, which may be due to

variation in responsiveness to the host environment (Wille et al. 2002, Hodgson et al. 2004). While differences in host specificity might explain competitive outcomes, we found that in one of our pairings, the faster killing and competitively dominant species was in fact less successful (i.e. had a lower percent emergence from hosts) on its own than the competitively weaker species (Fig. 2c, d). Given the prevalence of coinfections in nature (Cox 2001, Read and Taylor 2001), studies such as this one, which challenges the assumed mechanisms linking pathogenesis and within-host competition, will broaden and strengthen our view of the evolution of virulence and the ecological context of host–parasite interactions.

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