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VIRULENCE AND COMPETITIVENESS: TESTING THE RELATIONSHIP DURING INTER- AND INTRASPECIFIC MIXED INFECTIONS

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Understanding the reasons why different parasites cause different degrees of harm to their hosts is an important objective in evolutionary biology. One group of models predicts that if hosts are infected with more than one strain or species of parasite, then competition between the parasites will select for higher virulence. While this idea makes intuitive sense, empirical data to support it are rare and equivocal. We investigated the relationship between fitness and virulence during both inter- and intraspecific competition for a fungal parasite of insects, *Metarhizium anisopliae*. Contrary to theoretical expectations, competition favored parasite strains with either a lower or a higher virulence depending on the competitor: when in interspecific competition with an entomopathogenic nematode, *Steinernema feltiae*, less virulent strains of the fungus were more successful, but when competing against conspecific fungi, more virulent strains were better competitors. We suggest that the nature of competition (direct via toxin production when competing against the nematode, indirect via exploitation of the host when competing against conspecific fungal strains) determines the relationship between virulence and competitive ability.

KEY WORDS: Entomopathogen, interspecific competition, intraspecific competition, *Metarhizium anisopliae*, mixed infection, *Steinernema feltiae*.

Humans and animals alike are exposed to a variety of parasites at any one time and mixed infections, when the host harbors two or more infections at the same time, are frequent and can be considered the norm in nature (Cox 2001; Read and Taylor 2001). As with other ecological systems where organisms share habitat, mixed infections can mean that parasites interact with each other within the host. These within-host interactions are poorly understood but they are likely to have important effects on within- and between-host population dynamics (Mideo et al. 2008), community structure (Pedersen and Fenton 2006), and parasite evolution. A particularly important evolutionary outcome of mixed infection is thought to be selection on parasite virulence (Stearns and Ebert 2001): a number of theoretical models assume that virulent parasites make better competitors, so that competition among parasites selects for a higher optimal level of virulence than otherwise (Bremermann and Pickering 1983; Bremermann and Thieme 1989; van Baalen and Sabelis 1995; Frank 1996; Gandon 1998; Mosquera and Adler 1998; Gandon et al. 2001a; Brown et al. 2002; Schorring and Koella 2003).

These models of parasite competition are intuitively attractive, but empirical studies suggest that simple competition for resources leading to selection for faster exploiters is not especially common. Only one good example of intraspecific competition favoring strains with higher virulence has been published: more virulent strains of the rodent malaria *Plasmodium chabaudi* suffered less competitive suppression within the host in mixed infections, indicated by both the presence of more parasites in the host's bloodstream, and a greater likelihood of infecting mosquitoes

biting those hosts (de Roode et al. 2005). By contrast, experimentally infecting snails with two different strains of *Schistosoma mansoni* revealed that the less virulent strain was consistently able to outcompete the more virulent strain, indicating that in this case within-host competition would select for lower virulence (Gower and Webster 2005). Cooper and Heinemann (2005) described the effect of competition between bacterial plasmids that behave parasitically. Virulent plasmids were found to be more successful than avirulent derivatives when the environment allowed within-host competition, as predicted by trade-off models. However, modeling the specific interaction showed that the mechanism by which virulence is selected is not a simple trade-off between virulence and exploitation, with interference type competition being the best explanation for the relationship observed between plasmid competitors (Cooper and Heinemann 2005).

Two further studies of bacteriophage systems have also indicated that multiple infection does not necessarily select for virulence, apparently because of a requirement for collective action (Turner and Chao 1999; Brown et al. 2001). These data led Buckling and Brockhurst (2008) in a short review to conclude that the relationship between parasite relatedness and virulence is dependent on the specific within-host interactions ("social behavior") between the parasites in question. The mix of supportive and contradictory empirical evidence regarding the direction of virulence evolution has led theorists to use increasingly complex models in order to describe the intricacies of parasitic interactions within the host. Brown et al. (2002) showed that the direction of virulence selection can depend on the level of collective behavior exhibited by competitors, suggesting that, in certain instances, a reduction in virulence may evolve. Schorring and Koella (2003) modeled selection for virulence as a direct result of lethality and found that whereas lethal parasitic infections conform to the findings of early trade-off models, sublethal infections are predicted to promote reduced virulence.

Recent theoretical work has investigated other effects that could further influence the effects of in vivo parasite interactions on selection for virulence. These include incorporating within-host dynamics, such as host immune effects (e.g., proactive infections; Brown et al. 2008), increased heterogeneity in the host and parasite population due to coinfection (Gandon et al. 2002; Gandon 2004; Brown et al. 2008), and parasite life-history traits such as transmission type, number of life stages, and life cycle (Alizon and van Baalen 2008a). The inclusion of parasite life-history traits that dictate infection dynamics into a mixed infection model has suggested that the selection of optimal virulence is likely to depend strongly on the host–parasite and parasite–parasite interaction biology (Alizon and van Baalen 2008b).

To explore theoretical predictions of virulence and competitiveness during mixed infections, we use an empirical approach that includes competition between species. Theoretical studies that model mixed infection dynamics generally consider hosts infected with related strains that are likely to exploit host resources using similar strategies (van Baalen and Sabelis 1995; Gandon et al. 2001a; Brown et al. 2002; Alizon and van Baalen 2008a). Relatedness between competing pathogens is thought to regulate the type of within-host competition (Koskella et al. 2006; Buckling and Brockhurst 2008). It is predicted that competition between strains leads to greater rates of host exploitation and hence virulence. Kin selection theory predicts that within-host interference between strains may limit the evolution of pathogen virulence (Frank 1996; Chao et al. 2000). Decreases in within-host pathogen relatedness can potentially lead to a reduction in pathogen cooperation, resulting in a negative feedback on exploitation rates and virulence (Alizon and van Baalen 2008a). Therefore, the comparison we make between the inter- and intraspecific competition is likely to include differences in host exploitation strategies and goes somewhat beyond the model assumptions regarding competing strains. As mentioned above, in natural systems mixed infections usually consist of multiple genotypes and even multiple species (Petney and Andrews 1998; Cox 2001; Read and Taylor 2001; Alizon and van Baalen 2008a). Therefore, it is important to determine the effect such complex "social" interactions have on competitive dynamics, the role of virulence, and the universal applicability of mixed infection models.

The question of how competition between parasites affects virulence is complex, therefore, with theory indicating that many factors could cause selection for increased or decreased virulence in mixed infections. The question remains, however, of how virulence influences competition between parasites in simple systems. Here we test this question using the wax moth *Galleria mellonella* as a laboratory host with the fungus *Metarhizium anisopliae* and the entomopathogenic nematode *Steinernema feltiae* as parasites, with both intraspecific competition between strains of the fungus and interspecific competition between the fungus and the nematode being considered. Both parasites are usually found in the soil and are likely to encounter each other in natural systems, although not in the host used here. *Galleria mellonella* are highly susceptible to both parasites (Dunphy and Thurston 1990; Goettel and Inglis 1997; Kaya and Stock 1997) and their inability to mount an effective immune response benefits the experimental design by minimizing potentially confounding host effects allowing focus to be placed on the parasite interactions. Because these entomopathogenic parasites kill the host after infection and then use host resources to produce infective stages they differ from the conventional parasites considered in most theoretical studies, but the predictions remain the same: virulent parasites that kill the host quickly, and parasites that exploit the host rapidly after death should have higher fitness when in competition with others.

Table 1. Origins of nine *Metarhizium* spp.

Fungal strain	Classification	Collection/ Source	Isolated/Original host	Country of origin
V301	*M. anisopliae* var. *anisopliae*	Swansea Dr T.M. Butt	Isolated from soil	UK
V302	*M. anisopliae* var. *anisopliae*	Swansea Dr T.M. Butt	Isolated from soil	UK
V303	*M. anisopliae* var. *anisopliae*	Swansea Dr T.M. Butt	Isolated from soil	UK
V304	*M. anisopliae* var. *anisopliae*	Swansea Dr T.M. Butt	Isolated from soil	UK
MAM	*M. anisopliae* var. *majus*	ARSEF (2151) Dr R Humber	*Oryctes rhinoceros* [Coleoptera: Scarabaeidae]	Indonesia, Java
M714	*M. anisopliae* var. *anisopliae*	ARSEF (7524) Dr J Enkerli	*Agrigotes* spp. [Coleoptera: Elateridae]	Switzerland, Uri
M500	*M. anisopliae* var. *anisopliae*	ARFEF (7532) Dr J Enkerli	*Melolontha melolontha* [Coleoptera: Scarabaeidae]	Switzerland, Jenaz
M6388	*M. anisopliae*	ARSEF (6388) Dr R Humber	*Anoplophora glabripennis* [Coleoptera: Cerambycidae]	USA, Chicago, Illinois
M1116	*M. anisopliae*	ARSEF (1116) Dr R Humber	*Leucoptera scitella* [Lepidoptera: Lyonetiidae]	Italy, Ravenna

Materials and Methods

EXPERIMENTAL SYSTEM

Nine *Metarhizium* spp. strains were obtained from three sources (Table 1), and grown on Saboraud dextrose agar (SDA) in the dark at 28°C. To prevent the effects of attenuation and the possibility of virulence loss, a common problem in Hyphomycetes (Hall 1980; Butt 2002), the nine fungal strains were passaged once through the host and twice in vitro on SDA prior to experimentation. On the day of inoculation conidia were harvested following full sporulation (approx. 14 days) and five suspensions of different doses created in distilled water. Fungal suspensions were filtered through sterile nylon stockings to remove hyphal fragments and mixed using a magnetic stirrer prior to inoculation. This gave the even distribution of the spores necessary for consistent infection and removed the need for a surfactant.

Final instar larvae of the experimental host, *G. mellonella*, were obtained from the commercial supplier Livefoods Direct (www.livefoodsdirect.co.uk) and kept at 27°C for 24 hours prior to inoculation to allow for acclimatization and reduce the possibility of host mortality as a result of experimental conditions. A commercially available strain of *Steinernema feltiae* (Nemasys Becker Underwood Ltd., Littlehampton, UK) was used to prepare inoculation suspensions. Suspensions were left at room temperature for 2 hours and mixed thoroughly prior to and during infection.

VIRULENCE BIOASSAY

To quantify the intrinsic virulence of each fungal strain we performed a bioassay assessing both the LD (lethal dose) and LT (lethal time) (Table 2). The LT_{50}, an estimate of the time required for 50% of hosts to die at a particular dose of parasites, is considered a standard measure of virulence for *Metarhizium* spp. isolates (Ansari et al. 2004; Shah et al. 2007). For each fungal strain, a dilution series with concentrations of 10^7, 10^6, 10^5, 10^4, and 10^3 conidia mL^{-1} was used. Thirty insects per dose were exposed to the fungus by dipping them in 15 mL of the conidial suspension. Excess moisture was then removed by filtering in a Buchner funnel lined with filter paper (Whatman no. 1, Whatman plc, Maidstone, UK). As a negative control, 30 insects were dipped in distilled water for the same duration (see Goettel and Inglis 1997). Each insect was then placed individually into a well of a 12-multiwell tissue culture plate (Nunc, Thermo Fisher Scientific, Rochester, NY), each lined with a double layer of filter paper (Whatman no. 1). Exposed insects were kept at 27 ± 1°C and 95% RH and monitored daily until death or pupation, a period of 11 days.

INTERSPECIFIC COMPETITION

G. mellonella larvae were randomly assigned to either a control (neither infection), single infection (fungus alone or nematode alone) or a mixed infection treatment with one of the fungal strains and the nematode. Those larvae that were to be exposed to the fungus were given an LD_{90} (dose required to cause 90% host mortality) of one of the nine fungal strains using the exposure protocol described above. Larvae that were not to be dosed with fungus were dipped in distilled water. If the treatment included nematode infection this was followed by the addition of 20 infective juveniles (IJs) of S. *feltiae* suspended in 200 μL of distilled water to the microwell containing the caterpillar either 1 or 3 days afterwards. Other hosts only had 200 μL of distilled water added. Sixty insects were used per treatment.

Table 2. Fungal virulence predictors lethal dose (LD) and dose-lethal time (LT50), including the standard error (SE) of each prediction, for the nine isolates of *Metarhizium* spp.

Fungal strain	Classification	LD25 in conidia/mL	LT50 of LD25 in days	LD50 in conidia/mL	LT50 of LD50 in days	LD75 in conidia/mL	LT50 of LD75 in days	LD90 in conidia/mL	LT50 of LD90 in days
V301	*M. anisopliae* var. *anisopliae*	1.28×10^{4} (0.46)	10.1 (0.14)	7.84×10^{4} (0.46)	7.48 (0.03)	4.81×10^{5} (0.45)	5.52 (0.14)	2.95×10^{6} (0.65)	4.08 (1.62)
V302	*M. anisopliae* var. *anisopliae*	5.69×10^{3} (0.42)	9.34 (0.78)	2.60×10^{4} (0.33)	7.37 (0.32)	1.19×10^{5} (0.39)	5.82 (0.04)	5.41×10^{5} (0.55)	4.60 (1.50)
V303	*M. anisopliae* var. *anisopliae*	8.35×10^{3} (0.46)	8.90 (0.26)	4.83×10^{4} (0.36)	6.99 (0.08)	2.80×10^{5} (0.44)	5.50 (0.03)	1.62×10^{6} (0.63)	4.31 (0.62)
V304	*M. anisopliae* var. *anisopliae*	8.88×10^{3} (0.43)	8.53 (0.45)	4.40×10^{4} (0.34)	6.66 (0.12)	2.28×10^{5} (0.41)	5.19 (0.03)	1.16×10^{6} (0.58)	4.05 (1.05)
MAM	*M. anisopliae* var. *majus*	N/A	N/A	4.48×10^{4} (0.35)	7.74 (0.13)	N/A	N/A	1.3×10^{6} (0.60)	4.31 (0.84)
M714	*M. anisopliae* var. *anisopliae*	4.33×10^{3} (0.48)	9.17 (0.62)	2.51×10^{4} (0.37)	7.27 (0.24)	1.46×10^{5} (0.43)	5.77 (0.01)	8.48×10^{5} (0.62)	4.57 (1.16)
M500	*M. anisopliae* var. *anisopliae*	5.77×10^{3} (0.38)	8.89 (0.84)	2.10×10^{4} (0.31)	7.11 (0.38)	7.63×10^{4} (0.35)	5.70 (0.11)	2.77×10^{5} (0.49)	4.56 (1.57)
M6388	*M. anisopliae*	2.10×10^{4} (0.48)	8.32 (0.44)	1.51×10^{5} (0.39)	6.38 (0.01)	1.09×10^{6} (0.50)	4.89 (0.24)	7.88×10^{6} (0.73)	3.75 (2.11)
M1116	*M. anisopliae*	1.04×10^{4} (0.54)	10.6 (0.80)	9.18×10^{4} (0.41)	7.24 (0.11)	8.09×10^{5} (0.53)	4.95 (0.21)	7.14×10^{6} (0.79)	3.38 (1.96)

INTRASPECIFIC COMPETITION

In order to quantify the effects of intraspecific competition, we took advantage of a unique morphological feature of one fungal isolate, *M. anisopliae* var. *majus* (Petch 1931). This strain has elongated conidia, approximately 10 μm long on average, whereas the conidia of the remaining eight isolates are all approximately 5 μm long. This means that the conidia of *M. anisopliae* var. *majus* are easily distinguished from those of the eight others, so this isolate can be used as a reference strain and the performance of the other eight strains assessed following a mixed infection. For this experiment, host larvae were exposed to an LD_{50} dose of the reference strain, *M. anisopliae* var. *majus* and either an LD_{75} or an LD_{25} dose of one of the eight conspecific strains of *M. anisopliae* (Table 2), giving a total of 16 mixed (two-way) infections plus single infection treatments of the nine fungal strains. Fungal inoculation, as before, was via direct immersion for 30 seconds and for the fungal formulations each suspension contained both doses in a 1:1 ratio. In the mixed inoculations, the initial conidial concentrations were doubled before mixing, giving final doses of each strain that were equal to the single dose concentrations. Immediately after dosing insects were placed in 12 microwell plates (Nunc) lined with a double layer of Whatman no. 1 filter paper. Sixty insects were dosed per treatment.

VARIABLES MEASURED

Following exposure to parasitism, larvae were monitored every 24 hours and the period from dosing to mortality recorded. Upon mortality insect cadavers were placed on modified White traps (Kaya and Stock 1997), made from an inverted 35-mm petri dish lid within a 90-mm petri dish containing 20 mL of distilled water. A single 70-mm diameter piece of filter paper (Whatman no. 1) was placed over the smaller lid and immersed in the water around its edges. The white traps served two purposes: they maintained the relative humidity required for sporulation while also providing the facility to capture emerging nematode juveniles following lysis. Monitoring continued and the period until the first sign of mycosis or the first evidence of emergence of nematode IJs (maturation time) was noted.

Once the cadavers were fully mycosed conidial production was estimated in 10 randomly selected hosts from each treatment. The cadavers were washed (Goettel and Inglis 1997), by being placed into 2 mL Microcentrifuge tube with 1.5 mL distilled water containing 0.01% Tween 80 and shaken using a vortex for 30 seconds to dislodge the conidia from the cadaver. The insect was then removed and conidial concentration estimated by counting in an Improved Neubauer hemocytometer under a light microscope (Zeiss Stemi 2000-C, Carl Zeiss AG, Oberkochen, Germany). Four counts per insect were taken in order to give an accurate estimate of spore load.

For those hosts that were producing nematode IJs, lysis was allowed to proceed for 5 days after which IJs were removed using a 10 mL glass pipette and placed into 25 mL universal containers. The volume of nematode suspension in each universal tube was made up to 20 mL. Using three 1 mL dilutions of this suspension, nematode concentrations were counted under a light microscope to give an estimated IJ count per insect. IJ counts were made for all lysing hosts unless more than 10 lysed in a particular treatment, in which case 10 randomly selected hosts were used for counts.

ESTIMATING COMPETITIVE ABILITY—SUPPRESSION

Using the spore load and IJ counts from the sampled insect cadavers competitive suppression estimates were calculated for each fungal strain in a mixed infection following Bell et al. (2006), as the proportional reduction of the fecundity of the strain in the presence of a competitor, defined as:

1. [(Spore or IJ production following competition)/(Spore or IJ production in a single infection control)].

This measure of competitive ability therefore has a maximum of 1, when the parasite strain is completely suppressed, equals 0 when the competitor has no impact on respective spore load or IJ count, and is negative when performance of the parasite is enhanced by the presence of the competitor (Bell et al. 2006).

STATISTICAL METHODS

Lethal dose and lethal time estimates for each fungal strain were analyzed from cumulative mortality data via logistic regression with binomial errors to give virulence estimators for each strain in terms of LT (lethal time) and LD (lethal dose). Linear mixed effects models (LMMs) were used to explore the effects of time to sporulation and LT_{50} on the levels of competitive suppression. The LMMs used strain identity as a random effect because of the nonindependence of repeated measures from the same fungal strain. The models were fitted using a maximum-likelihood algorithm to allow simplification via likelihood ratio tests, and terms significant at $P < 0.05$ were retained, leaving a minimal adequate model (Crawley 2007). To test for model goodness of fit, residuals were plotted against fitted values. Although, in the interspecific dataset not all relationships were completely linear further evaluation of residual distribution and reanalysis with extreme datapoint removal showed the models to be robust and a good depiction of the data. Error structure was also close to normal when plotting standard normal quantiles for each fungal strain. All models were run using R version 2.7.1. (R Foundation for Statistical Computing, Vienna, Austria).

Results

COMPETITION BETWEEN NEMATODE AND FUNGUS

The success of both pathogens in mixed infection depended on the timing of the application of the nematodes. When the nematodes were applied one day after fungal infection significantly more hosts lysed and produced nematode IJs than when they were applied three days after (41% and 13%, respectively, paired *t*-test comparing proportions lysing per strain for the two treatments, $t = 6.078$, df $= 8$, $P < 0.001$). Conversely, significantly fewer hosts sporulated when the nematodes were applied one day after the fungus than when the nematodes were applied three days after (23% and 71% respectively, two-sided paired *t*-test, $t = -8.494$, df $= 8$, $P < 0.001$). The proportion of negative outcomes (no mycosis and no nematode infection) was also different, but not significantly so between these treatments (35% when the nematodes were applied one day after fungal inoculation, 29% when they were applied three days after, two-sided paired *t*-test, $t = 1.705$, df $= 8$, $P = 0.127$). In no hosts did both parasites (nematode and fungal isolate) successfully coinfect and complete their respective life cycles.

In mixed infections where nematodes were applied a day after the fungus a significant interaction between virulence (LT_{50}) and sporulation time determined the level of suppression of the fungus (Table 3A). Strains with a short LT_{50} and a short sporulation time were more suppressed (less competitive). Conversely, strains with a long LT_{50} and a longer sporulation time were less suppressed (more competitive) (Fig. 1A). Neither fungal LT_{50} nor sporulation time significantly predicted nematode suppression (Table 3B). When the nematodes were added three days after the fungus none of the explanatory variables were significantly related to competitive suppression (Tables 3C and D).

COMPETITION BETWEEN FUNGAL STRAINS

When the competing fungal strain was used at an LD_{25} dose neither of the fungal traits were significant predictors of competitive suppression for either the competing isolates or the reference competitor *M. anisopliae* var. *majus* (Table 4). The inclusion of LD_{25} as a covariate show it to be significant ($P = 0.016$), this indicates that at the lower doses the difference in size of spore inoculums between strains could have a contributing effect. The estimated coefficient is small ($>1.00E+-03$), but with a small variability in the response (suppression) relative to the predictor (LD_{25}) its effect may be important. Through the inclusion of LD as a covariate in the LMMs, it is likely that any influence due to inoculum size (conidia/mL) does not unduly effect the significance of the effects seen in the LMMs (Table 4B).

When the competing strain was added at an LD_{75} dose the time to sporulation had a significant effect on the suppression of the eight competing *M. anisopliae* isolates by the reference strain (Table 4C, Fig. 2): the longer a competing isolate took to sporulate following host death in a single infection, the less competitive (more suppressed) it was.

The virulence of the competing fungal strains also affected the degree of suppression of the reference strain when the

Table 3. Linear mixed effects models of fungal competitive suppression and nematode competitive suppression following, from hosts infected with nematodes on day one and day three. Estimated effect values and their standard errors shown for fixed variables. †Nonsignificance based on term deletion (see Statistical Methods). Model remains nonsignificant when simplified to individual terms. LD_{90} included as a covariate to remove any effect due to size of dose.

	Fungal competitive suppression			Nematode competitive suppression		
Addition of nematodes day 1	**(A) 87 hosts**			**(B) 89 hosts**		
Random effect:						
	SD			**SD**		
Strain identity	1.163×10^{-5}			1.840×10^{-5}		
Residual	0.227			0.733		
Fixed effects:						
	Value	**SE**	***P***	**Value**	**SE**	***P***
Intercept	6.949	2.638	0.0102	−0.823	0.079	0.922
LD90	-9.291×10^{-8}	$<1.0\times10^{-7}$	0.0242	1.0×10^{-6}	1.0×10^{-6}	0.23
LT50	−1.357	0.627	0.042*	N/S		
Time to sporulation	−1.692	0.692	0.025*	N/S		
LT50 : Time to sporulation	0.386	0.169	0.034*	N/S		
Addition of nematodes day 3	**(C) 89 hosts**			**(D) 89 hosts**		
Random effect:						
	SD			**SD**		
Strain identity	2.09×10^{-1}			3.60×10^{-2}		
Residual	0.372			0.288		
Fixed effects:						
	Value	**SE**	***P***	**Value**	**SE**	***P***
Intercept	0.971	2.347	0.680	0.025	0.937	0.241
LD90	-7.490×10^{-7}	$<1.0\times10^{-7}$	0.508	5.336×10^{-8}	$<1.0\times10^{-7}$	0.876
LT50	−0.113	0.476	0.808†	0.32	0.192	0.26†
Time to sporulation	0.078	0.152	0.602†	−0.061	0.061	0.308†
LT50 : Time to sporulation	N/S			N/S		

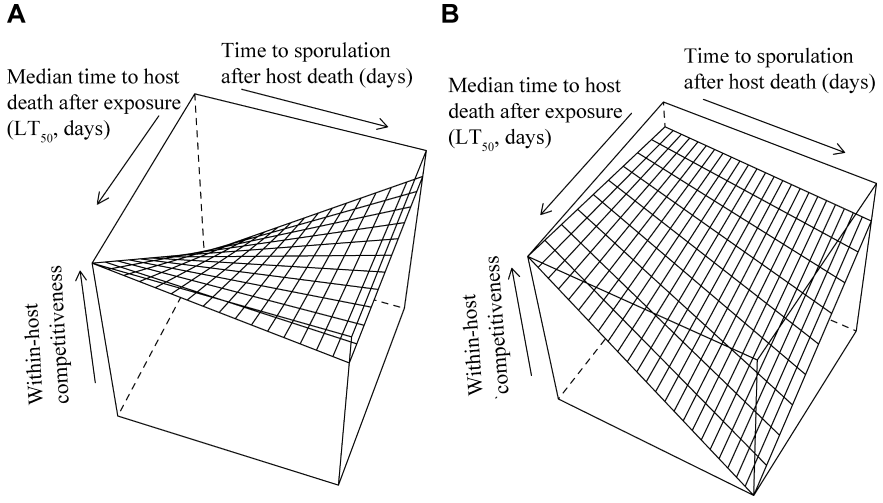

Figure 1. The effects of LT50 and the period from death to sporulation on competitiveness of the fungus in inter- and intraspecific competition. (A) Interspecific competition: the most virulent strains (i.e., the lowest LT50 values and the shortest postmortem time to sporulation) are the worst competitors. The surface shown is the response surface from the interaction term between the two predictor variables from the minimal adequate model when the fungal isolates are in competition with the nematode *Steinernema feltiae*. (B) Intraspecific competition: the most competitive strains are the most virulent. Again the response surface shows the interaction term between the two predictor variables from the minimal adequate model, this time when the fungal isolates at an LD_{75} dose were in competition with the reference strain *M. anisopliae* var. *majus* (MAM). Summaries for both LMMs; see Tables 3 and 4.

Table 4. **Minimum adequate linear mixed effects models showing competitive suppression of the 8 competing *Metarhizium anisopliae* isolates and that of the reference isolate *Metarhizium anisopliae* var. *majus* when at a high and low dose in a total of 80 sampled hosts. Estimated effect values and their standard errors shown for fixed variables, N/S $P > 0.05$ and term dropped from model. *Significant effect, <0.05; †Nonsignificance based on term deletion (see Statistical Methods). LD50 for each competing *M. anisopliae* isolate included as a covariate to allow for effect irrespective of spore load.**

	Suppression of 8 competing strains			Suppression of reference strain (MAM)		
Competing fungal strains at LD25	**(A)**			**(B)**		
Random effect:						
	SD			**SD**		
Strain identity	1.67×10^{-1}			9.80×10^{-2}		
Residual	0.297			0.462		
Fixed effects:						
	Value	**SE**	***P***	**Value**	**SE**	***P***
Intercept	−2.066	1.424	0.151	2.064	0.674	0.003
LD25	2.74×10^{-5}	1.46×10^{-5}	0.133	-4.67×10^{-5}	1.30×10^{-5}	0.016*
Time to sporulation	0.379	0.209	0.089†	−0.349	0.182	0.075†
LT50	0.131	0.103	0.21†	N/S		
LT50 : Time to sporulation	N/S			N/S		
Competing fungal strains at LD75	**(C)**			**(D)**		
Random effect:						
	SD			**SD**		
Strain identity	1.65×10^{-1}			1.24×10^{-1}		
Residual	0.372			0.203		
Fixed effects:						
	Value	**SE**	***P***	**Value**	**SE**	***P***
Intercept	−0.457	0.409	0.268	−8.05	6.339	0.208
LD75	1.0×10^{-6}	1.0×10^{-6}	0.977	-2.179×10^{-7}	1.0×10^{-6}	0.709
Time to sporulation	0.246	0.096	0.027*	3.234	1.219	0.021*
LT50	N/S			1.933	1.109	0.099†
LT50 : Time to sporulation	N/S			−0.676	0.22	0.011*

competing strains were applied in an LD_{75} dose. There was a significant interaction between fungal LT_{50} and time taken to sporulation, indicating that strains with both a short LT_{50} and a brief period before sporulation caused the greatest suppression of *M. anisopliae* var. *majus* (Table 4D, Fig. 1B).

Discussion

The majority of theoretical models predict that virulent parasites will be more competitive during a mixed infection and traits that aid in rapid host exploitation will be preferred and selected for over time (van Baalen and Sabelis 1995; Frank 1996; Mosquera and Adler 1998; Gandon et al. 2001a). The present study offers support for these ideas when fungi are competing with conspecific strains: those strains that sporulated faster were suppressed less by the presence of the reference strain, and fast sporulation together with a low LT_{50} led to greater suppression of the reference strain. In the case of interspecific competition, however, the opposite was found: the fungal strains with high virulence (low LT_{50}) and fast exploitation of the host following host death (short period to sporulation) were the most suppressed by the nematode competitor.

A likely explanation for the difference in outcome between inter- and intraspecific competition is that the type of competition within the host may differ between these two cases. The nature of interspecific competition between entomopathogenic nematodes and Hyphomycete fungi is not well known, but both scramble competition (indirect) and interference competition (direct) via toxin production have been suggested (Roberts 1980; Kaya 2002). Kaya (2002) suggests that the competitive interaction is predominately mediated by resources, despite a suggestion from Roberts (1980) that the antimicrobial metabolites produced by both parasites would be antagonistic. However, Kaya and Koppenhofer (1996) suggest that the possibility of direct antagonism due to antimicrobials is an area to be explored. The extent of fungal and nematode suppression found in this study and the absence of coinfection during interspecific competition indicates that direct competition via antibiosis is likely to be important. This finding is

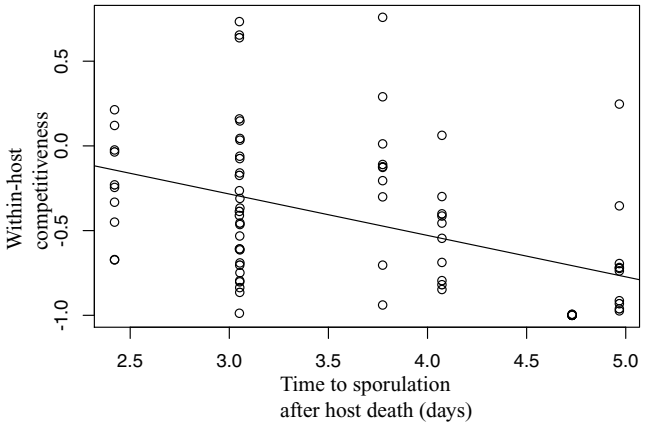

Figure 2. The relationship between postmortem exploitation speed and competitive ability. This plot shows isolate competitiveness plotted against the time taken by an isolate to sporulate following host death, when the competing strains are at a higher dose (LD_{75}) than the reference competitor MAM (LD_{50}).

further supported by the occurrence of negative infections where even upon host death neither parasite can successfully reproduce. A further possibility is that the interaction is an indirect one mediated by host immune effects but we consider this unlikely for two reasons: first, as discussed in the introduction, *G. mellonella* is not known to be able to produce an effective immune response to either of these parasites in single infections (Dunphy and Thurston 1990; Goettel and Inglis 1997; Kaya and Stock 1997) and second the significant effect of the postmortem exploitation speed of the fungus on competitive ability which is unlikely to be influenced by host immune effects.

Given that competitive fungal strains (those causing the most nematode suppression) tend to kill the host slowly and to take longer to sporulate, it is possible that these strains gain their advantage from an increased opportunity to produce toxic metabolites, and a fitness trade-off between the benefits of rapid resource monopoly and a fungal strain's ability to produce antagonistic metabolites would explain why certain isolates performed better than others. Kershaw et al. (1999) suggest that *Metarhizium* spp. isolates have two virulence strategies, rapid host utilization (growth) or increased toxicity, with most strains being located somewhere on a continuum between these extremes. This idea is supported by a study of fungal biomass (A. K. Charnley and J. Graystone, unpubl. data) which found that a strain that produces a toxin, destruxin, that is associated with high virulence (isolate ME1) grew slowly when compared to one that did not invest heavily in metabolite production (isolate 703). The competitive differences observed between fungal isolates in the current study may well be due to their individual strategies and relative position within this proposed virulence scale.

The question of how interspecific competition might differ from intraspecific competition in selecting for or against parasite virulence has not been addressed theoretically, to our knowledge. As mentioned in the introduction, existing models of coinfection that specify the relationships between parasite strains have considered different strains of the same species (van Baalen and Sabelis 1995; Gandon et al. 2001a; Brown et al. 2002; Alizon and van Baalen 2008a), but the conclusions from these are likely only to apply to certain interspecific interactions: the results obtained here demonstrate the potential for interspecific interactions to lead to very different outcomes to intraspecific ones. In cases such as this there is clearly a need to consider the balance between intra- and interspecific coinfections if we wish to understand selection for virulence in host-parasite systems.

In all of the intraspecific mixed fungal infection treatments spore types from the eight competing *M. anisopliae* spp. and those of the larger *M. anisopliae* var. *majus* were present. This evidence of coinfection in all treatment lines, as opposed to a scenario where there is competitive exclusion, suggests that these fungal strains compete indirectly in a race for the host resource, a conclusion strengthened by the fact that when the competing fungal isolates are at a higher dose (LD_{75}), they are much more competitive.

Intraspecific competitive ability, and hence the eventual outcome of mixed fungal infections, depends on the speed at which the competing strains are capable of producing infective conidia following host death. It has been suggested by a number of studies involving coformulated fungal infections that the most virulent isolate (lowest LT_{50}) will eventually outcompete other strains, driving them toward extinction within the host (Leal-Bertioli et al. 2000; Wang et al. 2002; Thomas et al. 2003; Hughes et al. 2004). Suppression of the reference strain *M. anisopliae* var. *majus* by competitors at a higher dose (LD_{75}) in the current study supports this prediction but our data indicate that the rate at which hosts are exploited postmortem plays a more significant role.

Empirical evidence suggests that it is naive to think that current virulence models hold all the answers (Read and Taylor 2001). Bull (1994) warns: "Thus far, there is but a shallow foundation toward our understanding of microparasite virulence"; although this statement was made some time ago, the situation remains largely unchanged. Bull (1994) argues that in order to make use of generalities that may exist concerning virulence, knowledge of within-host dynamics should be sought. A major component of interactions within the host between parasites is, of course, the type of competition. The empirical findings presented here suggest that if mixed infection persists then the type of competition (direct and/or indirect), occurring within the host, can play a major role in determining a parasites evolutionary fitness and is an important factor determining the direction of selection on virulence.

Competition between parasites is important from an ecological as well as an evolutionary perspective. The strong competitive interaction described here between *S. feltiae* and *M. anisopliae*

is similar to some of the stronger interactions described between other parasite species, being highly asymmetric and also showing a priority effect (Poulin 2001). How important such interactions are in structuring parasite communities is unclear, with competition probably playing an important role in some communities (Poulin 2001; Pedersen and Fenton 2006) but phenomena such as parasite aggregation reducing species interactions in others (e.g., Morand et al. 1999; Krasnov et al. 2006). We know little of how species interact in the soil-dwelling entomopathogenic parasite community where these two parasites both originate, but both *M. anisopliae* (Purwar and Sachan 2006) and especially *S. feltiae* (Georgis et al. 2006) are used as biopesticides for the control of a variety of pest insects. For control purposes both are applied by inundative release and this addition of large quantities of conidia or infectious nematode juveniles might be having negative effects on other components of the community and could ultimately have serious knock-on effects on both the host and parasite communities.

Understanding virulence evolution is an important goal with clear benefits including understanding virulence changes in extant and novel diseases (e.g., Knell 2004; Pulkkinen et al. 2010), predicting virulence evolution as a consequence of management interventions (Gandon et al. 2001b) and possibly applying Darwinian methodology to medicine with the aim of directing the evolution of less harmful parasites (Williams and Nesse 1991; Bull 1994). It is evident from our comparison of inter and intraspecific mixed infections that the role played by virulence on the outcome of competition is dependent on a number of factors including the timing of inocula, the doses administered and importantly the specific competitive interaction that occurs between parasites within the host.

In general, the dynamic nature of mixed infections, together with the number of possible parasite-host combinations, complicates the problem of describing a universal theoretical model of virulence selection. Current mathematical models are incorporating some of this biological complexity (Alizon and van Baalen 2008a, 2008b; Brown et al. 2008), but a great deal more empirical data are needed to validate these models before meaningful theoretical progress can be achieved.

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