Journal of Invertebrate Pathology 107 (2011) 179–184

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Comparative growth kinetics and virulence of four different isolates of entomopathogenic fungi in the house fly (*Musca domestica* L.)

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ARTICLE INFO

Article history:
Received 11 February 2011
Accepted 8 April 2011
Available online 21 April 2011

Keywords:
Beauveria bassiana
Metarhizium anisopliae
Quantitative PCR
Fungal growth kinetics
Musca domestica L.

ABSTRACT

Virulence (speed of kill) of a fungal entomopathogen against a particular host insect depends on biological properties of the specific isolate-host combination, together with factors such as fungal dose. How these intrinsic and extrinsic factors affect the actual pattern and extent of fungal growth *in vivo* is poorly understood. In this study we exposed adult house flies (*Musca domestica* L.) to surfaces treated with high and low doses of *Beauveria bassiana* (isolates BbGHA and Bb5344), *Metarhizium anisopliae* (strain MaF52) and *M. anisopliae* var. *acridum* (isolate Ma189) and used quantitative real-time PCR with species-specific primers to examine the relationship between fungal growth kinetics and virulence. At the highest dose, all fungal isolates killed flies significantly faster than controls, with BbGHA, Bb5344 and MaF52 roughly equivalent in virulence (median survival time (±SE) = 5.0 ± 0.10, 5.0 ± 0.08 and 5.0 ± 0.12 days, respectively) and Ma189 killing more slowly (MST = 8.0 ± 0.20 days). At the lower dose, effective virulence was reduced and only flies exposed to isolates BbGHA and Bb5344 died significantly faster than controls (MST = 12 ± 1.36, 15 ± 0.64, 18 ± 0.86 and 21.0 ± 0.0 days for BbGHA, Bb5344, MaF52 and Ma189, respectively). Real-time PCR assays revealed that flies exposed to surfaces treated with the high dose of spores had greater spore pickup than flies exposed to the low dose for each isolate. After pickup, a general pattern emerged for all isolates in which there was a significant reduction of recovered fungal DNA 48 h after exposure followed by a brief recovery phase, a stable period of little net change in fungal sequence counts, and then a dramatic increase in sequence counts of up to three orders of magnitude around the time of host death. However, while the patterns of growth were similar, there were quantitative differences such that higher final sequence counts were recovered in insects infected with the most lethal isolates and with the higher dose. These results suggest that variation in virulence between isolates, species and doses is determined more by quantitative rather than qualitative differences in fungal growth kinetics.

1. Introduction

Individual isolates of entomopathogenic fungi can vary greatly in their virulence to a given insect host but what factors contribute to this variation often remain unclear. Kershaw et al. (1999) hypothesized that differences in isolate virulence can be attributed to the position the phenotype of a particular isolate occupies on a continuum between two main strategies; an isolate may produce a large amount of toxins or may focus their energy into vegetative growth. Both *Beauveria bassiana* and *Metarhizium anisopliae* have been shown to produce metabolites within insect hosts with effects ranging from paralysis to immunosuppression (Gillespie and Claydon, 1989; Hajek and St. Leger, 1994; Hung and Boucias, 1992; Kershaw et al., 1999). While modern molecular techniques have facilitated progress in understanding the biochemistry of fungal metabolites and how they contribute to virulence in insects (Kershaw et al., 1999; Mazet and Boucias, 1996), relatively few studies have attempted to relate virulence to differences in growth kinetics between fungal isolates. That is, in addition to the toxic effects of metabolites, fungi could kill insects via vegetative growth, with death occurring when fungal hyphae penetrate vital organs, block the flow of hemolymph, or sap the nutritive resources from the host (Clarkson and Charnley, 1996).

To date, many studies evaluating growth kinetics have only considered growth *in vitro* (Fargues et al., 1997; Fargues et al., 1992). However, these methods do not subject the fungus to the same nutritional environment or immune pressure present in an insect host and, therefore, may not be a good representation of natural growth kinetics. Accurate, quantitative *in vivo* measurements of fungal growth are difficult to obtain; most often this

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doi:10.1016/j.jip.2011.04.004

has been done using microscopic methods to examine the concentration of blastospores and/or hyphal fragments in the hemolymph of infected insects (Ouedraogo et al., 1997; Pendland et al., 1993). Unfortunately, these techniques may yield inaccurate results in both early and later stages of infection, as fungal infection may not be evident in the hemolymph for up to 3 days following exposure to spores and blastospores and hyphal fragments may be too numerous to count during later stages of infection (Ouedraogo et al., 2003).

Recently, several researchers have developed quantitative PCR (qt-PCR) protocols to quantify the growth of pathogenic fungi in plants (Boyle et al., 2005; Gachon and Saindrenan, 2004) and insects (Bell et al., 2009), providing a highly sensitive tool to model the dynamics of fungal infection from the point of exposure to the death of the host. Using this application, it is possible to evaluate differences in growth kinetics between fungal isolates and, when paired with bioassays, determine if growth rates can be used to explain the virulence of a particular isolate.

The aim of the current study was to compare the differences in virulence and *in vivo* growth kinetics of four different isolates of entomopathogenic fungi against the house fly (*Musca domestica* L.). Understanding the factors that contribute to the virulence of fungal entomopathogens is important for understanding the ecological and evolutionary basics of insect–pathogen interactions.

2. Materials and methods

2.1. House flies

The house flies used in this study were derived from the Cornell University CS strain, an insecticide susceptible strain. The flies were reared and maintained in an environmental chamber under standard insectary conditions at 27 °C with a 12:12 light: dark photoperiod. Fly eggs were collected by placing a small cup (50 g) of larval diet (wheat bran, Manno-Pro™ calf protein supplement, baker's yeast and water) into fly stock cages for 2 h. Roughly 300 mg of eggs were placed into 1 l of larval medium contained in a 1.5 l plastic container and larval development was monitored daily. Upon pupation, pupae were collected by gently shaking them from the surface of the diet into plastic (~50 ml) soufflé cups. Cups containing pupae were then placed in clean stock cages until eclosion ceased. Adult flies were provided access to food, consisting of a 1:1 ratio of powdered milk and granulated sugar, and water *ad libitum*.

2.2. Fungal isolates

Four isolates of mitosporic Ascomycete entomopathogenic fungi were used: two strains of *B. bassiana* (strains GHA and ARSEF 5344, hereafter BbGHA and Bb5344) and two strains of *M. anisopliae* (F52 and IMI330189, hereafter MaF52 and Ma189) as detailed in Table 1. These isolates were chosen because they represent two different species of fungi that have been shown to vary greatly in their fundamental virulence to range of insect species, including dipterans such as house flies (Lecuona et al. 2005) and mosquitoes (Bell et al. 2009). The fungal isolates used for this study were maintained in long-term storage at −80 °C on microporous beads (Pro-Lab Diagnostics, Austin, Texas, USA) at Penn State University. Fungi were recovered by placing one or two beads onto Potato dextrose agar (Oxoid, UK) or Sabouraud dextrose agar (SDA) (Oxoid, UK) in 9 cm diameter Petri dishes or slopes in 25 ml universal bottles and incubated at 25 °C for 10 days. Spores were then propagated using a diphasic culture system as listed in Jenkins and Goettel (1997). Briefly, conidia were harvested from slopes or plates to make a spore suspension of approximately 1×10^6 conidia ml^{-1} in sterile 0.05% w/v Tween 80 (Sigma) in distilled water. This suspension was then used to inoculate liquid culture medium (4% d-Glucose, 2% yeast extract [Oxoid, UK] in tap water), which was then incubated on a rotary shaker (160 rpm) at 24 °C for 3 days. The resulting suspension was used to inoculate sterile, moistened barley flakes (Bob's Red Mill, Milwaukie, Oregon, USA). Once the sporulate reached <20% moisture content, the conidia were harvested using a Mycoharvester (Acis Manufacturing, Devon, UK) and were placed in glass dishes and dried over silica gel at 24 °C. Upon reaching 5% moisture content, a small sample of conidia from each isolate was taken for quality analysis and the remaining powder was sealed in foil-laminated envelopes with a small sachet of silica gel and stored at 5 °C until use.

Spore formulations were prepared by suspending dry, pure spore powder into a mixture of mineral oils (80% Shel-Sol: 20% Ondina oil). The formulation was homogenized by vortexing for 30 s and sonicating for an additional 30 s to break up aggregates of spores. The concentration of spores for each formulation was counted using an improved Neubauer Hemocytometer and the volume adjusted until the desired concentration was obtained. Concentrations used in this experiment consisted of a high dose (1×10^9 spores/ml) and a low dose (1×10^7 spores/ml). All preparations proved to have germination rates of over 90% as assessed by plating on SDA.

Table 1
Fungal isolates and origins.

Fungal isolate	Geographic location	Source
Beauveria bassiana GHA (BbGHA)	USA	Coleoptera: Chrysomelidae
Beauveria bassiana 5344 (Ba5344)	USA	Diptera: Muscidae
Metarhizium anisopliae anisopliae F52 (MaF52)	Austria	Lepidoptera: Olethreutidae
Metarhizium anisopliae var. acridum IMI330189 (Ma189)	Niger	Orthoptera: Acrididae

2.3. Application of fungal spores to exposure surfaces

Spore formulations were applied to 9 cm circles of HP™ Color-Laser paper using an artist's airbrush sprayer. Paper circles (hereafter referred to as exposure substrates) were taped inside a 0.25 m^2 spray area on the rear wall of a chemical fume hood. The formulation was then loaded into the reservoir of the air brush and sprayed to give an equivalent volume application rate of 20 ml/m^2. Care was taken to keep the airbrush sprayer moving at a constant rate of speed and distance from the exposure substrates during the application process to ensure even coverage. Additionally, four exposure substrates were sprayed with blank oil to serve as positive controls, while another set of four was left unsprayed to serve as negative controls. After spraying, the exposure substrates were removed from the wall of the hood, set into the lids of 9 cm petri dishes and allowed to dry at room temperature overnight.

2.4. Exposing flies to fungal spores

Houseflies were removed from stock colonies using a battery-powered insect aspirator (BioQuip Inc.). All flies were from the same stock cage, and between 2 and 3 days old at the time of exposure. Flies were anesthetized with CO_2 and forty flies of mixed sex were then placed into the bottom of a standard size petri dish. The lid of the dish containing an exposure substrate was then placed over the anesthetized flies. Flies recovered from the CO_2 within 3–5 min and started to walk and groom themselves, at which time the dish was flipped over to encourage the flies to walk and rest on the exposure substrate. Flies remained enclosed in the dishes for

4 h. Eight replicates of forty flies were exposed to each isolate and dose, while four replicates were exposed to positive control (blank oil) and negative control (no oil) substrates.

After the exposure period had elapsed, the flies were removed from the exposure chambers by quickly anesthetizing them with CO_2. Half of the flies from each treatment were placed into 1 l plastic freezer containers that were fitted with mesh tops and used to monitor mortality, resulting in four replicates of 40 flies per container. The remaining flies in each treatment group (160 flies) were combined into $20 \times 20 \times 20$ cm mesh insect cages according to treatment and used as stock populations to monitor fungal growth kinetics. All flies were provided access to sugar cubes and water *ad libitum* for the duration of the experiment.

2.5. Monitoring survival and sampling for fungal growth kinetics

Exposed flies were kept under standard insectary conditions during monitoring as described above. The number of dead flies in each replicate container was recorded daily for 21 days to produce survival curves.

To assess fungal growth kinetics, at least 10 randomly selected live flies were removed from each of the mesh insect cages for qt-PCR analysis immediately after being exposed to treatments (i.e. day 0) and every 48 h thereafter using a clean, battery-powered aspirator. All dead flies (i.e. fresh cadavers prior to any sporulation) were also removed from the survival containers for qt-PCR. Once removed, flies were immediately frozen at −20 °C until they could be processed for qt-PCR analysis.

2.6. DNA extraction

The protocol used to mechanically disrupt insects and extract fungal DNA was adapted from instructions included in the DNeasy 96 Plant Kit™ (Qiagen) and is outlined in Bell et al. (2009). Briefly, flies were placed into microtubes along with a 3.2 mm sterile stainless steel ball bearing (BioSpec Products, Inc.) and 0.1 ml of 0.2 mm zirconium beads (OPS diagnostics, Inc.). Flies were mechanically disrupted using a TissueLyzer™ (Qiagen) in two steps. Flies were initially dry-ground without buffer for 1 min at 30 Hz, with all samples repositioned every 15 s to insure uniform disruption. A second grinding cycle, in which 400 μl of lysis buffer was added, completed the disruption process. Fungal DNA was then extracted from the disrupted flies according to the manufacturer's instructions.

2.7. Quantitative PCR

Extracted DNA from fly samples was quantified using specific PCR primers and minor groove-binder probes designed to amplify the ITS2 region of the rRNA gene of *B. bassiana* (GHA) and *M. anisopliae* (189), as outlined in Bell et al. (2009). The primers and probe designed for *B. bassiana* isolate GHA were also used to quantify *B. bassiana* isolate 5344 as sequencing revealed identity for both isolates across the target amplicon. The primer and probe sequences used to amplify the ITS2 region of *M. anisopliae* strain F52 are: F: CAG CCG TCC CTC AAA TCA AT; R: GGG CTC CTG TTG CGA GTG T; Probe: 6FAM-TGG CCC TCC TCT GC-MGB.

Quantification of experimental samples was done by comparing threshold cycle numbers against a standard curves that were generated by running three replicates of serially diluted DNA standards for each isolate (10^6–10^2) in each qt-PCR run. The DNA from 100 spores was considered to be the detection limit, which, considering the volume of DNA in each run, equated to the equivalent of the DNA extracted from a single spore.

2.8. Sporulation of cadavers

Forty dead flies from each bioassay treatment were collected to determine sporulation rates for each isolate and dose. Dead flies were allowed to dry at room temperature for 2 weeks in order to ensure that the internal moisture content was equivalent in all cadavers, and to prevent flies from putrefying due to the growth of bacteria. Cadavers from individual treatments were then placed into plastic cups with a saturated piece of filter paper and sealed with an airtight lid. Cups containing flies were kept at room temperature and assessed for fungal growth after 7 days using a stereomicroscope at 15× magnification. Individual insects were considered to have sporulated if external growth of the appropriate fungus was present on the cadaver.

2.9. Statistical analysis

House fly survival data were analyzed using Kaplan–Meier survival analysis (SPSS, software version 18) with differences in median survival time between treatments at each dose compared using the log-rank test. A univariate general linear model (GLM) was constructed to test for the effects of isolate, dose, time and whether or not the fly was alive or dead at the time of sampling on the number of sequence copies quantified by qt-PCR analysis in flies treated with the high dose of fungus. Maximal models containing all main effects and interactions were fitted first, and, beginning with higher-order interactions, non-significant terms were sequentially removed to generate a minimal model. The values for sequence copies were log-transformed ($\log_{10}(\text{copies} + 1)$) to meet the assumptions of normality and homoscedasticity required for GLM analysis. Live flies that were treated with the low dose of each fungus were found to have a number of sequence copies that were considered below the threshold of the qt-PCR assay (roughly 80% of flies had counts of less than 100 sequence copies on the day of exposure) and were, therefore, excluded from the analysis. Fresh cadavers from the low dose treated flies were found to have quantifiable sequence copies, however, and were analyzed with a GLM to test the effects of dose and fungal isolate on the number of sequence copies detected in dead flies by qt-PCR analysis. The sporulation rate of infected cadavers between isolates and dose was tested using a G test of independence.

3. Results

3.1. Fungal virulence

Each of the isolates tested in this study proved to be pathogenic to house flies, but with virulence varying between isolates and according to dose. The high dose of each fungal isolate killed house flies significantly faster than the controls with no significant difference between the oil or negative controls (Table 2, Fig. 1). At the high dose, both of the *B. bassiana* isolates and MaF52 were similar in virulence, while Ma189 took roughly 60% longer to have the same effect.

This same pattern of relative virulence was also seen in flies treated with the low dose but with survival times over twice as long (Fig. 2). Both *B. bassiana* strains killed flies significantly faster than controls, while MaF52 differed from the negative control treatment, but not the blank oil control (in spite of the fact that negative and blank oil controls were not themselves significantly different from one another). Survival of flies exposed to the low dose of isolate Ma189 did not differ from either of the controls.

3.2. Fungal growth kinetics

In vivo fungal growth was found to vary according to isolate, the time point at which the fly was sampled and whether the fly was

Table 2
Effect of selected strains and dose of fungal isolates on survival time of adult *M. domestica* median survival time in days (±SE) and 95%CI as determined by Kaplan–Meier survival analysis. Different letters indicate significant differences in survival ($p < 0.05$).

Strain	High dose			Low dose		
	MST (±SE)	95% CI		MST (±SE)	95% CI	
BbGHA	5.00 (±0.10)	4.81–5.12	a	12.00 (±1.36)	9.34–13.65	a
Bb5344	5.00 (±0.08)	4.85–5.15	a	15.00 (±0.64)	13.76–16.24	ab
MaF52	5.00 (±0.12)	4.76–5.24	b	18.00 (±0.86)	16.32–19.68	bce
Ma189	8.00 (±0.20)	7.62–8.38	c	21.00[a]	[a]	def
Oil control	19.00(±0.98)	17.08–20.92	d	19.00(±0.98)	17.08–20.92	cdef
Neg control	20.00 (±1.11)	17.83–20.17	d	20.00 (±1.11)	17.83–20.17	df

[a] No error or 95%CI is presented for this value because the survival of all flies in this dose was calculated at exactly 50% at the end of the experiment.

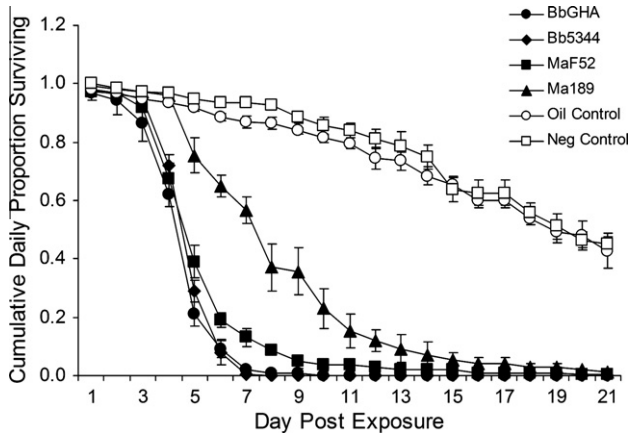

Fig. 1. Mean cumulative daily percent survival of house flies infected with the high dose (1×10^9 spores/ml) of each fungal isolate. Control flies were exposed to substrates sprayed with sterile oil (oil control) or unsprayed substrates (neg control). Bars represent ±1 SEM.

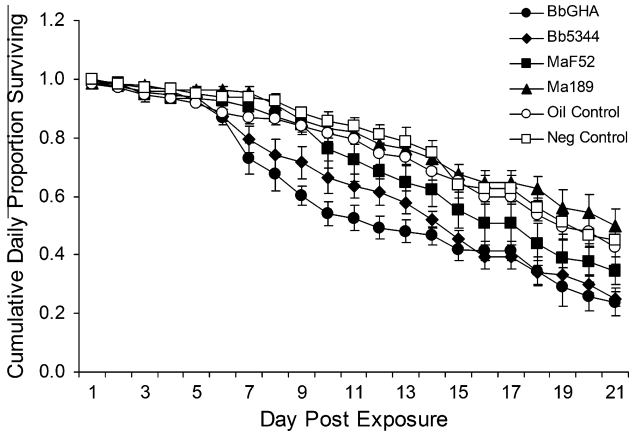

Fig. 2. Mean cumulative daily percent survival of house flies infected with the low dose (1×10^7 spores/ml) of each fungal isolate. Control flies were exposed to substrates sprayed with sterile oil (oil control) or unsprayed substrates (neg control). Bars represent ±1 SEM.

alive or dead at the point of collection. There was a significant main effect of isolate on the number of fungal sequence copies recovered ($F = 8.74_{3220}$, $p < 0.001$, Fig. 3). The Tukey post-hoc test revealed that the least virulent fungal isolate, as determined by the survival bioassay (Ma189), produced fewer sequence copies ($p < 0.001$) than either of the two *B. bassiana* isolates or MaF52 which produced roughly equivalent numbers of sequence copies.

There was a significant interaction between the time at which the fly was sampled and the condition of the fly at the time of sampling (i.e. alive or dead), on the number of fungal sequence copies

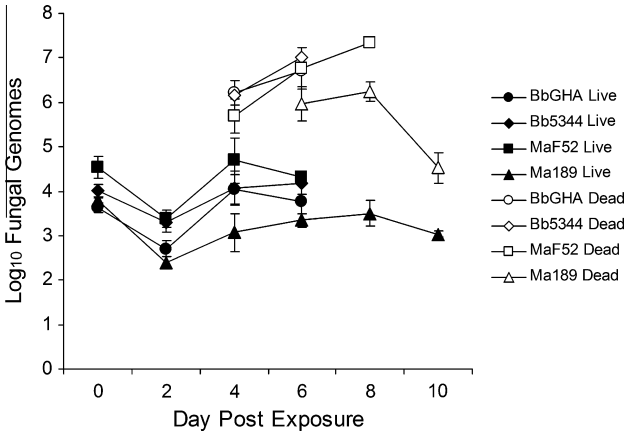

Fig. 3. Genome counts recovered from live and dead house flies treated with the high dose (1×10^9 spores/ml) of each fungal isolate over the course of infection. Bars represent ±1 SEM.

recovered from a fly ($F = 3.14_{3220}$, $p < 0.011$). This result indicates that live flies had significantly lower sequence copies than dead flies that were sampled on the same day. After picking up spores from the exposure substrate, fungal counts dropped significantly after 48 h. At day four, there was then a significant increase in the number of sequence copies, with counts rebounding to levels that were similar to the number counted at the initial pickup and remained at this level as long as the fly was alive (i.e. once a fungus had fully established within a fly, there was no evidence of continued fungal replication in live flies up until the point of death). Once a fly died, however, the number of recovered sequence copies increased significantly ($F = 114.80_{3220}$, $p < 0.001$) to 10–1000-fold the level seen in live flies within 24–48 h. This result was consistent regardless of how long the fly lived; the first flies to die following infection had roughly the same counts as flies that had died at the end of the experiment.

Although live flies that were treated with the low dose of each isolate had sequence copy counts that were too low to include in the analysis of growth kinetics from the outset, counts rose to measurable levels once the flies in this group had died. Similar to the results seen in high-dose treated flies, the greatest number of fungal sequence copies was detected in flies infected with the most virulent isolates (BbGHA, Bb5344 and MaF52), with significantly fewer sequence copies recovered from cadavers of flies infected with the least virulent isolate, Ma189 (Fig. 4).

In dead flies, there was found to be a significant main effect of isolate on sequence copy counts ($F_{3170} = 11.455$, $p < 0.001$). The Tukey post-hoc test revealed that sequence copy counts from the most virulent isolates (BbGHA, Bb5344 and MaF52) were significantly higher than the least virulent isolate, Ma189 ($p < 0.001$).

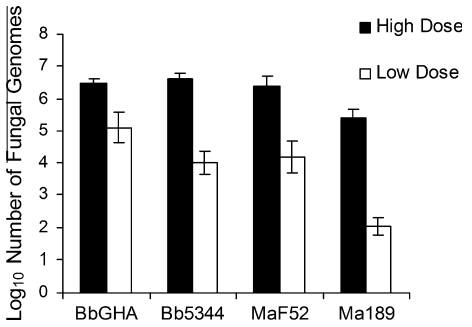

Fig. 4. Genome counts recovered from dead house flies treated with either the high dose (1×10^9 spores/ml) or low dose (1×10^7 spores/ml) of each isolate. Bars represent ±1 SEM.

There was also a significant main effect of dose on the number of sequence copies recovered ($F_{1170} = 81.087$, $p < 0.001$) indicating that dead flies that were exposed to the high dose of fungus had significantly higher sequence copy counts than flies exposed to the low dose for each isolate (Fig. 4). The interaction between the isolate to which the flies were exposed and the dose to which they were exposed was not significant ($F_{3170} = 2.453$, $p < 0.065$), indicating that the isolates produced comparable sequence copy counts in each isolate relative to dose.

The sporulation of dead flies from the bioassays revealed that flies that were exposed to the low dose of each isolate sporulated at a lower frequency than flies that had been exposed to the high dose treatment ($G = 26.186$, d.f. = 3, $p = <0.0001$). Flies treated with the high dose of BbGHA and Bb5344 showed sporulation of 100% and 97.5%, respectively, while 73% of flies exposed to Ma189 sporulated. In the low dose exposures, these rates were reduced to 67.5%, 65% and 0%, respectively. Interestingly, none of the flies exposed to MaF52 sporulated in either the high or the low dose treatments.

4. Discussion

All four fungal isolates were found to infect houseflies and cause significant mortality depending on dose. *B. bassiana* 5344 was originally isolated from *M. domestica* so its infectivity to this species was not surprising. *B. bassiana* GHA and *M. anisopliae* MaF52 are well characterized fungal isolates and have been shown to exhibit high levels of virulence against a wide variety of insect pests, including house flies (Blanford et al., 2005; Castrillo et al., 2010; Liu and Bauer, 2008; Lohmeyer and Miller, 2006; Shapiro-Ilan et al., 2008; Wraight et al., 2010). Ma189 proved to be the least virulent isolate to house flies and agrees well with previous experiments that have used this isolate to challenge other hosts (Arthurs et al., 2003; Peveling and Demba, 1997) including dipterans (Blanford et al., 2009). Ma189 may have evolved as a relatively specialist pathogen of acridid grasshoppers and could have lost some plasticity to successfully parasitize non-orthopteran hosts.

Although the initial pickup of spores by flies varied among the different isolates tested, the general pattern of fungal growth was qualitatively similar among fungal isolates over time. Following initial pickup of spores from the treated substrates, the number of sequence copy counts decreased after 48 h. At 4 days post-exposure, fungal sequence copy counts rebounded to levels comparable to the number of fungal sequence copies initially picked up from the exposure substrate and remained relatively stable at this level as long as the fly was alive. Once the fly died, another phase of growth occurred, with sequence copy counts increasing 10–1000-fold. These data suggest rapid fungal growth either at the point of death, or shortly following death, as no live insects (even those expected to die within 24 h) showed the high numbers of fungal sequence copies observed in the fresh cadavers.

This same general pattern of fungal growth was reported in a study by Bell et al. (2009) investigating infection of *Anopheles* mosquitoes with *B. bassiana* isolate GHA (referred to as IMI 391150 in Bell et al. 2009). In this recent study, mosquitoes picked up similar numbers of fungal spores to the houseflies during exposure and then exhibited a drop in fungal sequence copy numbers over the following 2 days. There was then a slight increase in sequence copy counts, followed by a relatively stable phase until around the time of mosquito death, at which point sequence copy counts increased dramatically.

A possible mechanistic explanation for the observed growth kinetics is that insects pickup a set number of spores during exposure but then experience a loss in fungal sequence copies as spores get dislodged through grooming and/or succumb to effects of immune responses during initial stages of fungal penetration and infection (Butt et al., 1988; Gillespie et al., 2000). An earlier study by Pendland et al. (1993) reported that blastospores of *B. bassiana* injected into the hemolymph of *Spodoptera exigua* larvae were rapidly phagocytosed by circulating hemocytes. Once in the hemocyte, however, major components of the cell wall, which are known elicitors of insect immune responses, are shed, allowing the cells to replicate. This replication is consistent with the recovery of sequence copy numbers observed after a few days. During this phase, the fungus also produces immunosuppressive metabolites, which have been shown to prevent granulocytes from recognizing and destroying circulating hyphal bodies (Hung and Boucias, 1992; Huxham et al., 1989). After sufficient time has elapsed to disable the cellular immune response, walled hyphal bodies are produced and released from the hemocytes to complete the infection process. This latter stage is expected to result in extensive multiplication of the fungus, as observed.

Our results also agree well with an *in vivo* growth model proposed by Kershaw et al. (1999), which argues that virulent isolates of *M. anisopliae* produce large amounts of toxic metabolites that suppress the cellular response of the immune system. During this time, *M. anisopliae* masks the epitopes on cell walls by producing a collagenous coat around hyphal fragments (Wang and St. Leger, 2006). The period in which relatively little fungal growth was detected in living house flies may thus be explained by the fungus "waiting" until the fly was sufficiently immunosuppressed before initiating rapid vegetative growth.

Live flies treated with the low dose of each isolate had initial sequence copy counts that were too low to be analyzed reliably. This result was unexpected but subsequent to the experiments conducted in the current study, Bell et al. (2009) explored the efficiency of the spray technique used to treat our exposure substrates and reported that >95% of spores were lost during application, resulting in much lower doses than anticipated on the treated surfaces. Nonetheless, with the most virulent isolates, significant increases in mortality were still observed relative to controls. Moreover, all fly cadavers analyzed from the low dose treatments revealed positive infections using PCR and many exhibited external sporulation (with the exception of MaF52, which we discuss below), indicating that exposures of <100 conidia per fly were sufficient to cause infection.

As with the high dose exposures, fresh cadavers from the low dose treatments showed high sequence copy counts, consistent with rapid fungal proliferation at, or immediately following death. However, the numbers of fungal sequence copies were much lower than those recovered from the high dose flies for all of the isolates. The reason for this difference in absolute numbers is unclear. It is possible that the fungi would have gone on to achieve the same total sequence copy number but because they were starting from a lower level, needed more than 48 h post-death to do so. However,

these data might also indicate that fungal replication is not simply limited by the available resources in the host, and that the number of spores picked up during the initial infection stage has an ongoing influence on overall growth. Alternatively, speed of kill could play a role since the low dose flies all took longer to die. In particular, we found significantly fewer sequence copies produced in flies infected with Ma189, which also proved to be the least virulent isolate. The one inconsistency in this regard is MaF52, which although relatively virulent and yielding high sequence copy counts, completely failed to sporulate at either dose. The reason for this lack of sporulation is again unclear, but could possibly result from cytotoxic effects of fungal destruxins on the insect midgut epithelium, allowing the release of saprophytic bacteria that compete with the developing fungus (Vey and Quiot 1989).

In summary, all of the isolates used in this study proved to be pathogenic to houseflies, but virulence (i.e. effect on survival time) was shown to vary between isolates and to depend on the dose the insect received upon initial exposure. Both strains of *B. bassiana* showed equivalent high virulence, with *Metarhizium* strain MaF52 slightly less virulent and Ma189 least virulent. More rapid speed of kill correlated with more extensive fungal replication; greater 'intrinsic' virulence or higher doses increased fungal sequence copy numbers within the insect at death. In spite of variations in virulence and absolute sequence copy numbers, the growth kinetics of the different fungal species/isolates were qualitatively similar. While the exact virulence mechanisms (e.g. variation in growth forms and production of toxic metabolites) are likely to differ between isolates, the conserved patterns of growth are consistent with some kind of 'sit and wait' strategy, whereby extensive fungal proliferation is constrained until the host is either sufficiently compromised or even dead. Extension to other host-isolate combinations would be valuable to further explore the generality of these *in vivo* growth kinetics.

Acknowledgments

We thank J. Scott from Cornell University, Ithaca, NY for supplying the house flies. We also thank N. Jenkins for the production of fungal spores and the Pennsylvania State University for funding.

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