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Effects of *Beauveria bassiana* on Survival, Blood-Feeding Success, and Fecundity of *Aedes aegypti* in Laboratory and Semi-Field Conditions

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Abstract. The fungus *Beauveria bassiana* reduces *Aedes aegypti* longevity in laboratory conditions, but effects on survival, blood-feeding behavior, and fecundity in realistic environmental conditions have not been tested. Adult, female *Ae. aegypti* infected with *B. bassiana* (FI-277) were monitored for blood-feeding success and fecundity in the laboratory. Fungal infection reduced mosquito-human contact by 30%. Fecundity was reduced by (mean ± SD) 29.3 ± 8.6 eggs per female per lifetime in the laboratory; egg batch size and viability were unaffected. Mosquito survival, blood-feeding behavior, and fecundity were also tested in 5 meter × 7 meter × 4 meter semi-field cages in northern Queensland, Australia. Fungal infection reduced mosquito survival in semi-field conditions by 59–95% in large cages compared with 61–69% in small cages. One semi-field cage trial demonstrated 80% reduction in blood-feeding; a second trial showed no significant effect. Infection did not affect fecundity in large cages. *Beauveria bassiana* can kill and may reduce biting of *Ae. aegypti* in semi-field conditions and in the laboratory. These results further support the use of *B. bassiana* as a potential biocontrol agent against *Ae. aegypti*.

INTRODUCTION

Control of the dengue vector mosquito *Aedes aegypti* continues to rely on chemical insecticides, and growing resistance to these insecticides threatens their continued utility.[1–4] In recent years, entomopathogenic fungi have shown considerable promise as alternative methods of control. Various fungus strains have been shown to be virulent against malaria vectors[5–7] and *Ae. aegypti*.[8–10]

Entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* have been shown to reduce human biting by *Anopheles* spp.,[11] reduce vector competence,[12] and work equally well against insecticide-resistant mosquitoes as against non-resistant strains.[13,14] Infective fungus spores have been applied with preliminary success in the field to sheets of black cloth,[9,15] clay pots,[16] and outdoor resting boxes[17] to reduce mosquito survival, with minimal contribution to airborne spore load.[18]

Most studies that measured entomopathogenic fungus-induced mortality and other related effects on mosquito vectors have been conducted in the laboratory. If one considers the extensive work reported on entomopathogenic fungi for mosquito control, only four studies, two in Tanzania[15,17] (*Anopheles* spp.), one in Benin[19] (*Culex quinquefasciatus*), and one in Brazil[9] (*Ae. aegypti*), have been in field conditions. These experiments exposed mosquitoes to entomopathogenic fungi either on black cotton cloth[9,15,17] or polyester netting.[19] Mosquitoes were then held to observe mortality either in a large cage[9] or in smaller cups[15,19] or plastic tubes.[17] Such small holding containers may introduce artifacts that affect survival or behavior, for example by presenting a shorter distance required to fly to a food source (e.g., sugar/water solution in a small cage), and limit the ability of an insect to thermoregulate in response to environmental cues.[20]

In this study, we report the first laboratory evaluation of the effects of an entomopathogenic fungus on the blood-feeding success of the dengue vector *Ae. aegypti.* We also evaluate the effect of *B. bassiana* on survival, blood-feeding success, and fecundity of *Ae. aegypti* under field conditions in small cages and large, semi-field cages in which mosquitoes were exposed to fluctuating ambient temperatures and allowed a greater range of movement to blood meals and oviposition sites.

MATERIALS AND METHODS

Ethics statement. Human ethics consent for human blood feeding was obtained through the Queensland Institute of Medical Research (project P361) and James Cook University (project H2250), including the provision that any volunteer experiencing signs of discomfort could withdraw from the experiment at any time.

Experiment 1: Effects of fungal infection on blood-feeding success, host-seeking behavior, and fecundity under laboratory conditions. *Mosquitoes. Aedes aegypti* used in this experiment were from an $F_{6–8}$ colony sourced from wild collections in Cairns, Australia. Larvae were reared in five-liter photograph developer trays at a density of 200 larvae in two liters of water. The water used was tap water that had been left out to dechlorinate for 48 hours. Mosquitoes were fed a standardized diet of TetraMin Rich Mix™ fish food (Tetra, Melle, Germany) (0.25 mg/larva/day).[21]

Fungal exposure. Spores of *B. bassiana* isolate FI-277 were obtained from potato dextrose agar slopes according to a standard protocol.[18] Three day-old, adult female mosquitoes in treatment groups were exposed for 24 hours to a surface sprayed with 1.54 mL of *B. bassiana* (FI-277) formulation at a concentration of 2×10^9 spores/mL. This amount was a considered a moderate dose; higher doses would have killed mosquitoes too quickly to enable detection of any changes in behavior.

Spores were mixed in mineral oil (87.5% ShellSol T, 12.5% Ondina EL oil) and sprayed into cardboard drinking cups by using a hand-held artist's airbrush (Badger Air-Brush

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Co., Franklin Park, IL). The air brush had a nozzle width of 0.5 mm and was held at a distance of 30 cm from the target surface. Spraying was conducted with even passes of the air-brush with a hand movement of approximately 12 cm/second. Controls were exposed to oil formulation alone.

Experimental design. Individual female *Ae. aegypti* were placed into four treatment groups: fungus-infected and human blood-fed, non-infected and human blood-fed, fungus-infected and sucrose-fed, and uninfected and sucrose-fed. Each mosquito was held individually in a 164-mL clear plastic container covered with cloth mesh (mesh opening = 0.5 mm) and watered with a moist cotton wick. Sugar-fed treatments contained 100 adult female mosquitoes, and blood-fed treatments contained 120 mosquitoes.

Two days after fungus exposure, all mosquitoes in the human blood-fed treatment groups were offered an opportunity to blood-feed on a human arm (JMD) for 10 minutes and again every two days thereafter. Mosquitoes in the sucrose-fed groups had access *ad libitum* to a cotton wick soaked with 10% sucrose solution.

Each container was lined with a moist cotton pad that served as an oviposition substrate. Beginning at 5 days post-exposure (dpe) and again every 2 days, each blood-fed mosquito container was inspected for eggs. If eggs were present, the mosquito was temporarily removed while the oviposition substrate was replaced. Eggs were counted and stored in plastic bags for 5 days, then placed into 200 mL of dechlorinated water with a pinch of fish food (50–75 mg) to hatch. After 24 hours, larvae were counted. Adult mortality was monitored daily. All dead mosquitoes were stored on moist filter paper in parafilm-sealed Petri dishes for 5–6 days to observe fungus sporulation.

Data analysis. Mortality data were analyzed as above. Blood-feeding and walking behavior was observed during a 10-minue exposure to a human arm. Observed behaviors during the 10-minute window were characterized as one of four states: blood-fed (i.e., having successfully landed on skin and taken at least half of a replete blood meal), landed (having landed on the skin of a human arm), active (flying or walking inside the cup, but not having landed on skin), or inactive (motionless for the entire 10 minutes). Proportions of mosquitoes in each behavioral state were compared between the control and fungus group by using Z-tests of two proportions.

Three measures of fecundity were calculated. The number of eggs laid per batch (i.e., per mosquito per day) and hatch percentage were analyzed by using PROC MIXED in SAS version 9.1 (SAS Institute, Cary, NC). Treatment (control versus fungus-exposed) was a fixed effect, and mosquito identification number was a random effect (to control for variation between individual mosquitoes). The number of eggs laid per mosquito per lifetime was averaged over all individuals for each treatment, and the means were compared by using a *t*-test.

Experiment 2: Large cage experiments in semi-field conditions. Mosquitoes were released in to semi-field cages to study the impact of *Beauveria* infection under more natural conditions. *Aedes aegypti* eggs were from an F_{2-4} colony originally collected from several suburbs in Cairns, Australia. Larvae were reared and fed as described above. Adults were sorted randomly into cups for exposure to fungus or control formulation (see below).

Two large, semi-field cages were constructed on the Cairns campus of James Cook University, less than 100 meters from a previously described cage.[21] The cage used in this study was an aluminum-framed tunnel house design (13.7 meters × 5.5 meters × 4 meters (Figure 1) with polyester Tentex 72007 cloth internal skin (mesh opening = 1 mm), 90% shade cloth awning, and a concrete floor (Figure 2A). An interior wall served as a partition to divide the structure into two equal-sized cages, hereby referred to as large cages to serve as control and treatment. Cage A was the southernmost half, and cage B was in the northernmost half. Within each large cage was an aluminum garden shed (2 meters × 2 meters × 2 meters) with the door held open to simulate a domicile (Figure 2B). Outside of the shed, the floor within the cage was covered with 10–15 cm of mulch, which was watered for 15 minutes each day to conserve humidity. Flowering, ornamental plants (*Spathiphyllum* sp., *Strobilanthes dyerianus*, and *Crossandra infundibuliformis*) were placed on the mulch in each cage to provide resting surfaces and potential nectar sources. To humanize the shed, plastic chairs and a crate were placed inside, and used towels provided by a local gymnasium were scattered on the floor.

Beauveria bassiana strain FI-277 was obtained from Pennsylvania State University (MBT), where spores were mass-reared as described by Blanford and others.[12] Spores were formulated in a mixture of 87.5% D60 oil (Firesol™) and 12.5% sunflower oil and diluted to 1.0×10^{11} spores/mL. A different formulation than the one described in experiment 1

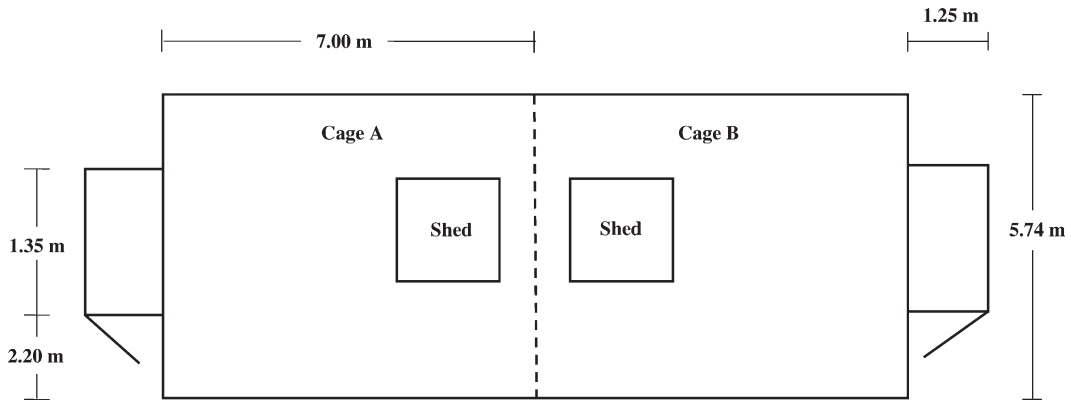

FIGURE 1. Semi-field cage schematic.

FIGURE 2. Semi-field cage at James Cook University, Smithfield Campus, Cairns, Queensland, Australia. **A**, Structure contains two cages bisected by a transverse double-walled partition made of Tentex screening. Cage B is to the north (left in the photograph) and cage A is to the south (right in the photograph). **B**, Interior shed simulating a human-inhabited structure.

was used because ShellSol and Ondina were not available in the required quantities. Both *in vivo* tests on adult female *Ae. aegypti* and *in vitro* sporulation counts of fungal spores on Sabauroud dextrose agar suggested that virulence or germination of *B. bassiana* isolate FI-277 were not affected by substituting oils.

An aliquot (1.54 mL) of this formulation was spread evenly over the interior of a cardboard drinking cup.[10] Oil formulation without spores was applied to control cups. Twenty-five female mosquitoes and 5–10 males were placed into each cup and held for 24 hours within the shed of the large cage. After 24 hours, mosquitoes were either released into the semi-field cage or into a small cage (see below).

An initial control trial was conducted to measure baseline mortality of uninfected mosquitoes in each large cage. Pupae were sexed by size, sorted by sex into plastic bowls (300 mL) covered with mesh cloth and placed into the large cage. Every 8–12 hours, the sex of eclosed adults was visually confirmed before adults were released.

Fungus-treated mosquitoes were compared with uninfected controls in two separate trials (Table 1). One large cage was randomly designated as the treatment cage. Mosquitoes held in control or fungus-treated cups for 24 hours were released into each respective large cage.

In all experiment 2 trials, females were offered a human blood meal (JMD) daily for 15 minutes. Flowering plants were available for males. Experiments were maintained for 21 days, or until one cage looked to have a population of zero, whichever came first.

Large cages were sampled daily using a CDC backpack aspirator,[22] according to a standardized sampling pattern along the walls that covered 4.75 meter^2 of the inner perimeter of the cage, equating to 5% of the total surface area of the walls, excluding the ceiling. Multiple screened collection cups were used to avoid excessive stress to captured mosquitoes. Mosquitoes were sexed, counted, and released in the cage in the same section where they were collected. When numbers were too high to count quickly, mosquitoes were briefly knocked down with CO_2, counted, and returned to the cage. For the first two collection days, sampled mosquitoes were kept in a 60-cm^3 cage within the field cage overnight and then released back into the large cage to confirm that sampling did not cause additional mortality.

For human landing counts, the number of mosquitoes resting on both legs (between knee and ankle) during daily blood feeding was counted at the six-minute mark.

For egg counts, cloth oviposition strips, affixed to the inside of plastic buckets, were placed in each large cage: two strips per bucket, four buckets per cage. There were two buckets inside the shed of each cage and two buckets outside. Buckets were filled to approximately 25% of capacity with water containing 10% grass infusion. Each day, any strip with eggs on it was removed and replaced. Eggs were counted

TABLE 1
Summary of semi-field experiments*

Experiment	Cage size†	Experimental conditions‡	No. cages	Trial
2	Large	Semi-field	2 × Control	Control (June 2009)
	Large	Semi-field	1 × Control, 1 × Fungus	1 (February 2010)
	Large	Semi-field	1 × Control, 1 × Fungus	2 (April 2010)
3	Small	Laboratory	3 × Control, 3 × Fungus	1 (February 2010)
	Small	Semi-field	3 × Control, 3 × Fungus	1 (February 2010)
	Small	Laboratory	3 × Control 3 × Fungus	2 (April 2010)
	Small	Semi-field	3 × Control 3 × Fungus	2 (April 2010)

*Large cage, semi-field trials (experiment 2) were designed to test virulence of *Beauveria bassiana* in field conditions. Small cage, semi-field experiments (experiment 3) were designed to evaluate effect of limiting mosquitoes ability to thermoregulate in response to environmental conditions by moving to more suitable microclimates. Small cage, laboratory experiments (experiment 3) were designed to evaluate the effect of fluctuating environmental conditions (e.g., temperature, humidity) on *B. bassiana* virulence.

†Large refers to free-flight conditions in a 5 meter × 7 meter × 4 meter semi-field cage (see text for detailed description). Small refers to a 20 cm × 20 cm plastic bucket cage. Each large cage contained 500 females and 500 males. Each small cage contained 50 females and 10 males.

‡Semi-field refers to ambient semi-field conditions. Laboratory refers to laboratory conditions (i.e., 28°C, 12:12 hour light:dark period).

under an S240 dissecting microscope (400×) (Olympus, Tokyo, Japan).

In all trials, data loggers (DS1923 Hygrochron iButton; Dallas Semiconductors, Dallas, TX) were used to record temperature and humidity in the large cages, sheds, and within each small cage. At the conclusion of each experiment, all mosquitoes were removed.

Data analysis. The proportion of surviving mosquitoes was calculated by dividing the number of remaining females by the number of females initially placed into the cage. The survival proportions for each trial were compared using a Z-test for two proportions.

Data analysis for backpack aspirator samples was based on the total number of females collected per cage per day. For human landing rates, analysis was based on the number of mosquitoes resting on the legs after six minutes. For egg collections, data analysis was based on the total number of eggs laid per cage per day. Separate counts were made for eggs laid in buckets inside and outside each shed. Backpack aspirator collections and human landing rates were analyzed by expressing the number of females collected or resting on lower legs (respectively) as a proportion of the number of mosquitoes initially placed into each cage and comparing proportions between both cages by using a Z-test. Tests were made for each day, and we used Bonferroni's method to adjust the *P* value for multiple comparisons.

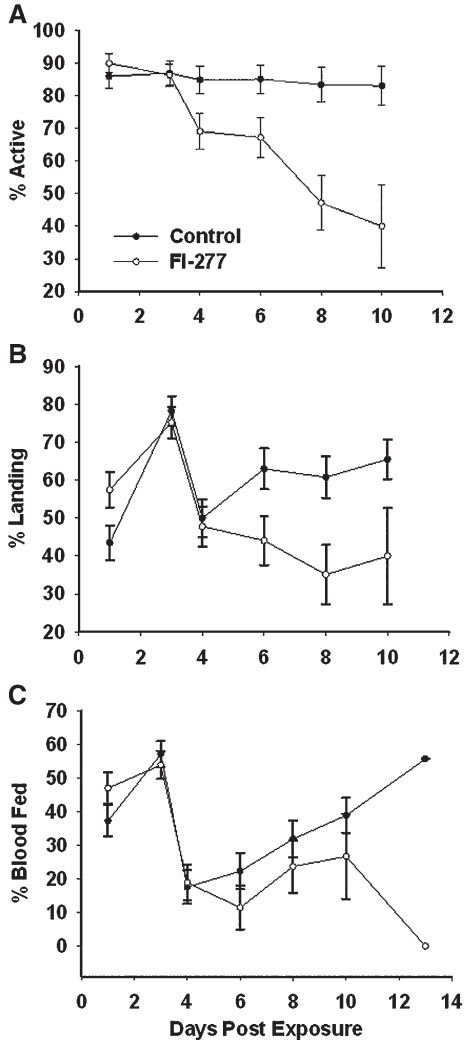

FIGURE 3. Effect of infection with *Beauveria bassiana* on *Aedes aegypti* blood-feeding behavior in the laboratory. **A**, Percent of uninfected and infected mosquitoes displaying activity (e.g., walking, flying) during a 10-minute feeding opportunity. Differences at 4, 6, 8, and 10 days post exposure (DPE) are statistically significant ($P < 0.05$, by Z-test). **B**, Percent of mosquitoes landing on human host (whether feeding or not). Differences at 6 and 8 DPE are significantly different. **C**, Percent of mosquitoes obtaining a blood meal. The difference at 13 DPE is statistically significant. Error bars indicate mean ± SEM.

Experiment 3: Small-cage bioassay in laboratory and semi-field environments. To differentiate between effects of semi-field conditions on fungus-induced mortality and the effects of free flight (as opposed to being confined within a relatively small space), bioassays with 50 three-day-old, adult, female mosquitoes in 60-cm^2 plastic cages (small cages) were carried out by using mosquitoes from the same cohort as experiment 2 and exposed to fungus in the same manner. The bioassays were set up within the shed inside each large cage. Mosquitoes were provided with 10% honey water *ad libitum*. All small cages were monitored daily for mortality for 15 days or until all mosquitoes were dead. Dead mosquitoes were removed and stored on moist filter paper in petri dishes sealed with parafilm to check for sporulation.[10]

To compare fungus-induced mortality in constant conditions (26°C, relative humidity = 80%) to mortality in the semi-field conditions above, some additional small cage trials were carried out in a temperature-controlled laboratory incubator. These bioassays were identical to the ones described above and used the same mosquito cohort and fungus exposure method. All small cages were monitored and dead mosquitoes removed and processed as above.

Data analysis. Median lethal times (LT_{50}) were calculated by using probit analysis (PASW 17, Chicago, IL) and corrected for control mortality by using Abbott's formula.[23] Survival curves were estimated and compared by using Cox regression (PROC TPHREG, SAS version 9.1). Predictor variables evaluated included treatment (control or fungus-exposed) and environmental conditions (laboratory or semi-field). Solely for purposes of comparison with experiment 2, the number of dead mosquitoes in small cage bioassays dead by 10 dpe were pooled and expressed as a proportion ± SEM.

RESULTS

Experiment 1. *Sporulation.* Sporulation of *B. bassiana* was only observed on cadavers of fungus-exposed mosquitoes. All sugar-fed, fungus-exposed mosquitoes sporulated, and all blood-fed, fungus-exposed mosquitoes, except for one, also sporulated.

Effect on blood-feeding behavior. Beginning at 4 dpe, significantly fewer fungus-exposed mosquitoes displayed any activity (84.9% of controls and 69.1% of fungus-exposed; $\chi^2 = 4.16$, degrees of freedom [df] = 1, $P < 0.0413$) during the 10-minute observation window, and this difference increased through 10 dpe (Figure 3A). Likewise, beginning at 6 dpe, fungus-infected mosquitoes were significantly less likely to land on human skin (44% for fungus-infected and 63% for controls; $\chi^2 = 4.19$, df = 1, $P < 0.0407$), and this trend was also observed at 6 and 8 dpe (Figure 3B). Fungus-infected mosquitoes had similar blood-feeding success as controls up until day 13 dpe, at which point no fungus-infected females (n = 6) successfully obtained a blood meal (Figure 3C).

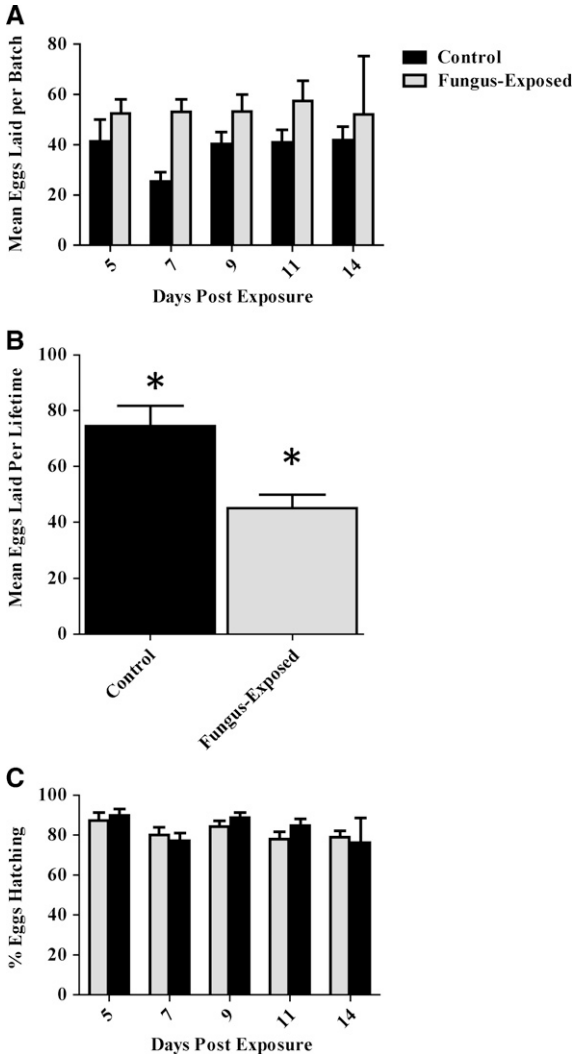

FIGURE 4. Laboratory-measured fecundity of non-infected and *Beauverai bassiana*–infected *Aedes aegypti* **A**, Mean eggs laid per batch during each 48-hour period (see text for general linear model results.) **B**, Average lifetime fecundity per female. **C**, Hatch rate of eggs laid by control and fungus-exposed mosquitoes. Error bars indicate mean ± SEM. Eggs were given five days to embryonate before hatching in 200 mL of water.

Effect on fecundity. Egg batches laid by fungus-infected mosquitoes per 48-hour period were larger on average than those laid by controls (Figure 4A). This difference was confirmed by parameter estimates of a mixed model; control mosquitoes laid a mean ± SEM of 36.8 ± 2.4 eggs per egg batch, and fungus-exposed mosquitoes laid 54.8 ± 3.8 eggs per 48 hours ($F_{1,312} = 19.34$, $P < 0.001$). There was no significant difference in day ($F_{6,312} = 1.213$, $P = 0.299$) or a significant component of variation between individuals (estimate = 0.00). Despite laying fewer eggs per batch, control mosquitoes laid more eggs per lifetime (74.5 ± 7.2) than fungus-infected mosquitoes (45.2 ± 4.8 eggs; t = 3.50, df = 205, $P < 0.001$) (Figure 4B), for a difference of 29.3 ± 8.6 (primarily because uninfected mosquitoes survived longer to lay more egg batches). Egg hatch rate was similar for fungus-exposed mosquitoes (85.0 ± 1.9%) and controls (81.6 ± 2.0%) (Figure 4C).

Experiment 2. Overall, temperatures varied between 18°C and 33°C during these trials. Temperatures between cages were largely similar: cage B tended to be 1–2°C warmer than cage A during the hottest period of the day (approximately 2:30 PM–4:30 PM). Shed temperatures between cages tended to be equal. Humidity within the shed tended to be 90–95%. Survival between the two cages with control mosquitoes varied between 20% and 25% after 15 days and did not differ significantly (Z = 1.86, $P > 0.006$) (Table 2).

TABLE 2
Mosquito survival in three large-cage trials in semi-field conditions*

Trial	% Survival (no.) Unexposed	Fungus exposed‡	Adjusted§	Z†	*P* (Z)
Control	24.8 (513)	19.9 (512)	NA	1.86	0.063
1	14.7 (476)	0.8 (484)	5.6	8.96	0.000
2	5.7 (510)	2.4 (509)	61.5	2.70	0.007

*The control experiment ended at 15 days post-exposure (DPE). Treatment –1 and –2 ended at 11 and 10 DPE, respectively.
†Z-test of the difference between the proportions (control versus treatment) surviving in each cage.
‡During the control experiment, both cages were unexposed.

Fungus-exposed mosquitoes exhibited lower survival than controls in both treatment trials, and fungus-associated mortalities rates were 94.4 ± 1.1% and 58.5 ± 2.2%, respectively, after accounting for control mortality (Table 2). Control group survival differed between the two treatment trial replicates (Z = 4.71, $P < 0.0001$).

A total of 1,945 females were collected and rereleased. Individual samples ranged from 0 to 92 females per day, and the greatest overall numbers were counted during control

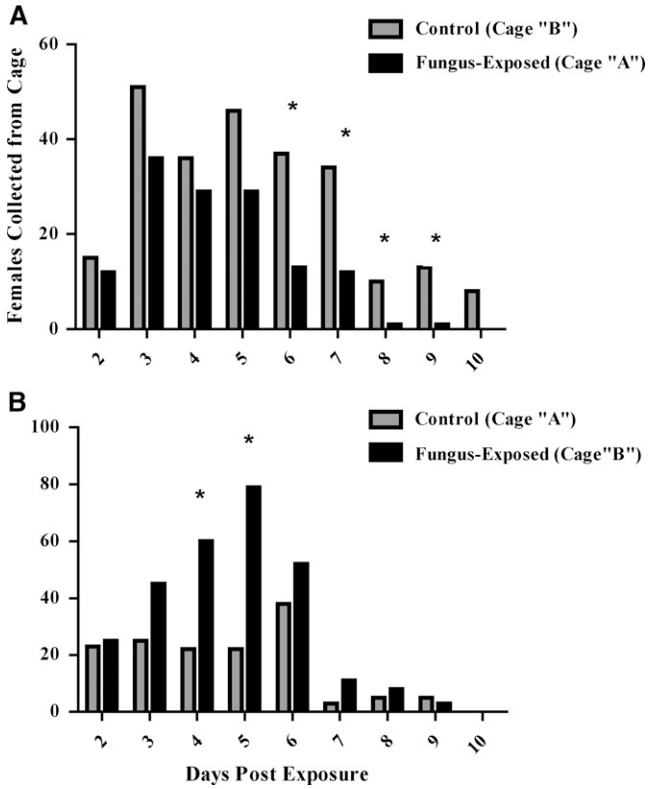

FIGURE 5. Backpack aspirator collections of control and *Beauveria bassiana*–infected *Aedes aegypti* from semi-field cage trials. Pairs of bars marked with an * are significantly different from each other ($P < 0.05$, by Bonferroni-corrected Z-test). **A**, Trial 1; **B**, Trial 2.

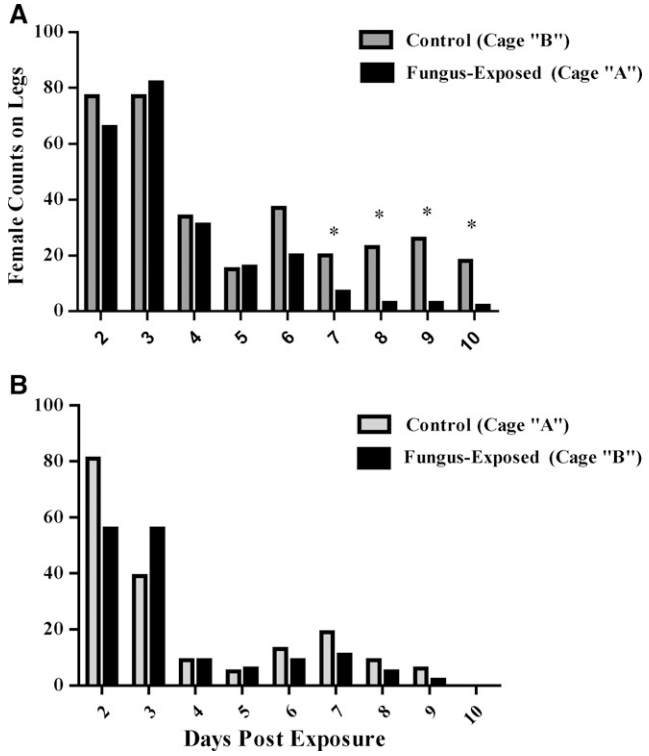

FIGURE 6. Landing rates of control and *Beauveria bassiana*–infected *Aedes aegypti* females after six minutes in semi-field cages. Bar heights represent number of females counted on lower legs (between knee and ankle). Pairs of bars marked with an * are significantly different from each other ($P < 0.05$, by Bonferroni-corrected Z-test). **A**, Trial 1; **B**, Trial 2.

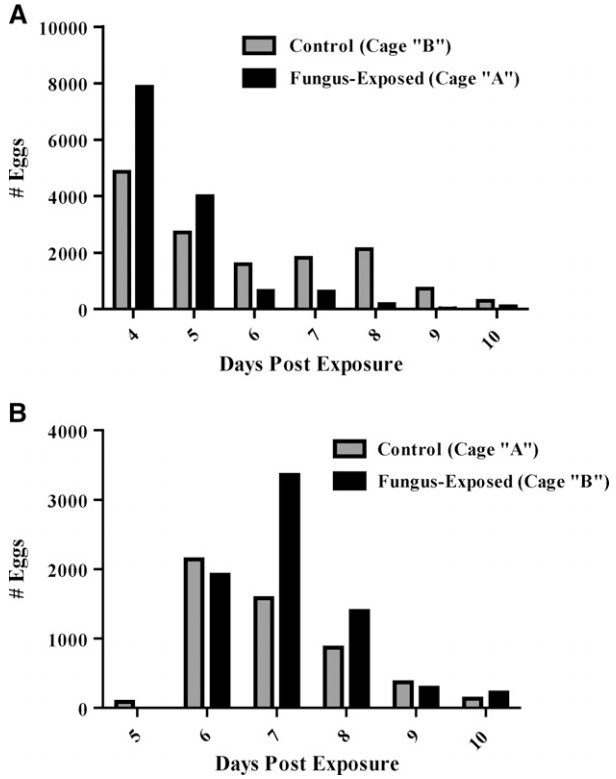

FIGURE 7. Fecundity of control and *Beauveria bassiana*–infected *Aedes aegypti* in large-cage trials. Fecundity (eggs laid) in large cage trials. **A**, Large cage trial 1. **B**, Large cage trial 2. No difference in total fecundity was detected (t = 0.715, degrees of freedom = 2, $P = 0.54$).

experiments (Figure 5). There was no consistent association between mosquito collections and fungus exposure. However, on all days when significantly more mosquitoes were collected from one cage, that cage was always cage B.

All females that landed on human legs were observed to blood-feed. No interrupted feeding or partial blood meals were observed, although this experiment was not designed to measure interrupted feeding. Landing counts ranged from 0 to 83 (Figure 6) and tended to be higher during the warmer months (i.e., treatment trials 1 and 2, as opposed to the control trial). After 6 dpe, significantly fewer (approximately 80%) fungus-infected mosquitoes fed on blood during the first treatment trial (Figure 5). There were no detectable differences in blood-feeding during the control trial. During the second treatment trial, more control mosquitoes tended to feed during the last four days, but this trend was not significant. No cage positional effects impacting blood-feeding were detected.

A total of 39,952 eggs were collected and counted: 27,592 during the first treatment trial and 12,360 during the second experiment. Each oviposition strip yielded 0–1,225 eggs. There was no obvious difference between number of eggs laid inside or outside the shed for either treatment. During the first treatment trial, more eggs tended to be laid by fungus-exposed mosquitoes during the first two collection days, after which the control mosquitoes laid more eggs (Figure 7A). During the second treatment trial, control mosquitoes laid more eggs 6 dpe, after which fungus-exposed mosquitoes laid more eggs 7 and 8 dpe, until finally egg numbers from both treatments decreased to negligible levels (Figure 7B). No difference in total number of eggs laid was detected (t = 0.715, df = 2, $P = 0.54$).

Experiment 3. Control mosquitoes in small-cage trials had an LT_{50} of 18–22 days, and fungus-exposed mosquitoes had an LT_{50} of 8–10 days (Table 3). Laboratory and semi-field bioassays in small cages demonstrated similar survival between respective control and fungus-exposed groups (Figure 8A) (Cox hazard ratio [HR] = 0.94, $P = 0.6$), suggesting no detectable effect of field conditions versus laboratory conditions during this period. As expected, exposure to fungus in these assays exerted a strong overall reduction in survival (HR = 34.4, $P < 0.0001$).

Mosquito survival was similar between the two small-cage, semi-field trials (HR = 1.1, $P = 0.43$) (Figure 8B), and fungus had the greatest effect on mortality (HR = 54.4, $P < 0.0001$).

TABLE 3

Median lethal times for *Beauveria bassiana* FI-277 small-cage bioassays in laboratory and semi-field conditions

Environment	Treatment	LT_{50} (95% CI) (days)	Slope ± SEM	χ^2	$P(\chi^2)$
Laboratory	Control	17.9 (15.7–20.7)	0.06 ± 0.01	36.4	0.50
	Fungus	9.7 (8.2–11.1)	0.29 ± 0.01	102.4	0.00
Semi-field (trial 1)	Control	21.5 (17.2–26.6)	0.05 ± 0.03	26.4	0.90
	Fungus	9.2 (7.5–10.8)	0.37 ± 0.02	111.2	0.00
Semi-field (trial 2)	Control	18.3 (16.2–21.0)	0.11 ± 0.01	39.9	0.73
	Fungus	8.5 (7.2–9.9)	0.47 ± 0.02	107.2	0.00

LT_{50} = median lethal time; CI = confidence interval.

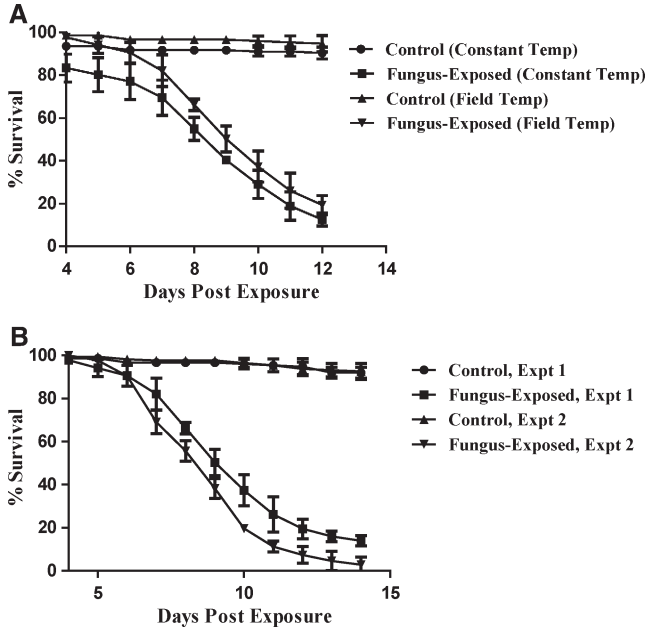

FIGURE 8. Reduced survival of *Beauveria bassiana*–infected *Aedes aegypti* in small-cage bioassays. **A**, Control and fungus-infected survival in small-cage bioassays in laboratory conditions (constant temperature) and semi-field conditions (field temperatures). **B**, Small-cage bioassays in semi-field conditions in February (trial 1) and April (experiment 2). Error bars indicate mean ± SEM.

Overall survival was higher in these trials than for the large-cage trials in experiment 2 (Figure 9). Control-adjusted mortalities in small-cage bioassays were 68.2 ± 2.6% and 61.8% at 10 dpe.

DISCUSSION

We exposed female *Ae. aegypti* to *B. bassiana*-treated substrate and ran simultaneous bioassays: in small cages in constant conditions (i.e., temperature, humidity), in small cages in semi-field conditions, and in large (5 meters × 7 meters × 5 meter) cages in semi-field conditions. The effect of fungus on mosquito survival in small cage bioassays was consistent between laboratory-controlled and field conditions. Recent studies have shown that in addition to mean temperature, daily fluctuation of temperatures can affect transmission of dengue virus[24] and malaria parasites.[25] Our results here suggest that *B. bassiana* induces similar virulence in its insect host at constant temperature and a daily temperature range of approximately 15°C. Although additional research needs to be conducted at larger temperature ranges and different fungus species and strains, the results so far are encouraging for future application of entomopathogenic fungi for mosquito control.

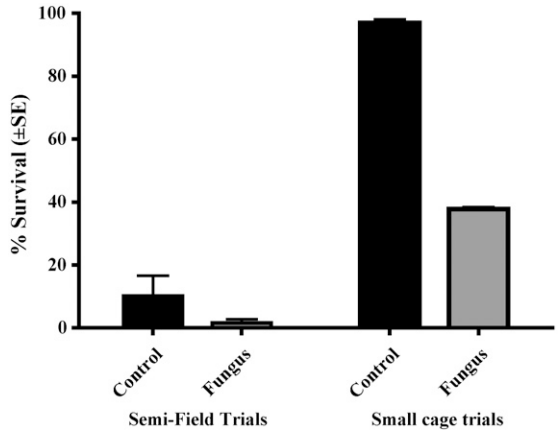

FIGURE 9. Survival of *Aedes aegypti* exposed to *Beauveria bassiana* for 10 days in semi-field conditions in small cages and large cages (n = 2). Error bars indicate mean ± SEM.

Fungus-exposed mosquitoes in the two large cage trials had standardized mortalities of 94.4% and 39.5% after 10 days, compared with 61% and 63% in small cages. One advantage of carrying out bioassays in cages of this size is the presence of microclimates. Grasshoppers infected with *M. anisopliae* have higher survival if they are able to move to warmer areas where fungal growth is reduced,[26] but the current study detected no strong evidence that *Ae. aegypti* displayed analogous behavior. Similarly, in another study, although *Anopheles stephensi* were found to distribute themselves non-randomly on a thermal gradient, there were no detected infection-dependent differences in distribution.[27]

Control mortality was considerably higher in our large cage trials than in small cage trials. The reasons for this finding are not clear. One potential explanation is diet: although mosquitoes in small cages had access to sugar *ad libitum*, free-flying mosquitoes were forced to search for nectar or human blood to obtain energy. Small cages inside the field cage also retained humidity better, never decreasing below 84% relative humidity, and humidity in the semi-field cages sometimes decreased to 70%. Finally, although temperatures in each small cage tended to be approximately 1°C lower than the rest of the cage (outside of the shed) during the day and approximately 1°C higher at night, it is questionable that this would make such a substantial difference in adult survival.

The range of control mortalities in large cage trials was 75–95%. The trial with the lowest control mortality, the control trial comparing two non-fungus exposed cages, took place in June, when temperatures ranged from 18°C to 30°C. Fungus trials took place in February and April, when temperatures were higher (23–34°C). Higher mosquito survival at the lower (June) temperatures likely accounted for some of the variability between the two treatment trials and the control trial. Furthermore, the presence of predatory geckos, known to consume as many as 76 *Ae. aegypti* per gecko per day,[28] in the control cage of the second trial (there were no geckos detected at any other time), may have accounted for that cage having the lowest control survival (6%).

Despite consistent survival rates for uninfected mosquitoes, mosquito density estimates using CDC backpack aspirator samples and egg counts displayed a positional bias, and cage B consistently showed more mosquito activity and eggs, regardless of treatment. The mechanism of bias is presumably environmental and should be further explored.

Comparison between previous semi-field studies and the current study and others is difficult because studies differed by mosquito species and geographic location. In addition, previous studies evaluated adult mosquitoes trapped in the wild and did not control for factors such as age, physiologic status, larval diet, or larval-rearing density. Larval diet is reported to affect mosquito larval survival in the presence of entomopathogenic fungi,[29] and mosquitoes blood-fed before

being exposed to fungus have been observed to live longer than non-blood fed, fungus-exposed mosquitoes.[30,31] However, little more is known about the influence of other factors.

Because overall arbovirus transmission potential is determined by biting propensity in combination with mosquito density, if fungal infection reduces blood-feeding, it could potentially reduce vectorial capacity well before the death of the mosquito. In the laboratory (experiment 1), we found that a moderate dose of *B. bassiana* reduces landing rate on a human hand by approximately 30%, and this difference increases over time. Scholte and others reported reductions in blood-feeding success of 50–65% for *An. gambiae* infected with *M. anisopliae*.[11] Interestingly, despite reductions in the landing rates in fungus-infected mosquitoes, there was no difference in the proportion of blood-fed mosquitoes. Thus, there were fewer mosquitoes that landed on a human hand (perhaps probing) but did not feed. It may be that fungus-infected mosquitoes are hungrier and more persistent, consistent with a scenario where fungal infection reduces their energy or nutrition reserves.

In semi-field conditions (experiment 2), we found evidence that a high dose of *B. bassiana* reduces host-seeking after six days. The trend was statistically significant in only one trial (Figure 6B), but lower control survival in the second trial (Figure 6C) (possibly caused by unwanted predatory geckos, present only in the control cage and only during the final treatment trial) may have affected these results. The semi-field study provides support of laboratory results and is the first to report any host-seeking reduction of *Ae. aegypti* in semi-field conditions, although a similar effect has been reported with *Cx. quinquefasciatus* in Benin.[19]

Fungus-exposed mosquitoes in the laboratory laid fewer eggs over a lifetime than control mosquitoes, most likely caused by reduced lifespan. A similar effect was found in *An. gambiae* infected with *M. anisopliae*.[11] In *Ae. aegypti*, however, we found that fungus-infected mosquitoes laid relatively more eggs initially. This trend was also seen in desert locusts (*Schistocerca gregaria*) infected with *M. anisopliae*[20] and may represent an adaptation for infected individuals to maximize reproductive output. Another possibility is that because fungal infection increases blood meal size and therefore egg batch size, had fungus-infected mosquitoes lived longer they would have laid cumulatively more eggs. Our study did not measure blood meal size or count the number of unlaid eggs. However, Scholte and others[11] found that *An. gambiae* infected by *M. anisopliae* took fewer and smaller blood meals and laid fewer eggs per batch. Garcia-Munguia and others[32] found that *B. bassiana* infection reduced *Ae. aegypti* fecundity by 69–95%, depending on fungus strain.

The laboratory oviposition assay is limited in the sense that mosquitoes would be able to lay eggs on the substrate even if they would be unable to fly sufficient distances to seek out oviposition sites. Examination of fecundity data in a more realistic setting is critical to determine if fungus-infected mosquitoes would be able to contribute to subsequent generations in the wild. Sample size limitations of semi-field fecundity data prevented formal analysis, but it is nevertheless evident that despite blood-feeding less, fungus-exposed mosquitoes are able to reach oviposition sites and lay eggs. In trial 2, fungus-exposed mosquitoes even appeared to lay more eggs than controls, certainly in comparison to trial 1, although this finding could be accounted for by the relatively higher survival of fungus-exposed mosquitoes in trial 2 (Table 2) and also by observed higher activity levels of cage B mosquitoes during each trial (see above). In any case, *B. bassiana* infection reduces longevity while permitting oviposition, making its use a potentially long-lasting method of mosquito vector control if continued reproductive output weakens selection pressure for fungus-resistance genes.

Beauveria bassiana is already known to reduce longevity in *Ae. aegypti* populations in the laboratory[8–10] and in small (> 0.6 meter^2) cages.[9] These studies do not necessarily predict how the fungus will perform in realistic conditions, in which mosquitoes move within a larger area and are subject to microclimatic effects. We have shown that the fungus reduces mosquito survival in semi-field conditions found in northern Queensland, an area to which dengue virus transmission is endemic.

Although our results are promising for use of *B. bassiana* as a potential dengue vector control agent, one limitation of the current study is that mosquitoes were exposed to spores in a laboratory environment. Thus, a major challenge will be designing targets to bring wild *Ae. aegypti* in contact with infective fungus spores. Previous trials have shown some success exposing wild, free-flying *Anopheles* mosquitoes to spores using treated cloth[15] and resting boxes.[17] Although resting boxes successfully exposed *An. arabiensis* to *M. anisopliae* spores in rural Tanzania, any resting boxes designed to attract *Ae. aegypti* in northern Queensland (i.e., in and around residential structures) would be subject to visual competition[33] from household containers. For this reason, treated cloth (e.g., curtains or other visual targets) may represent the more likely strategy in Australia.

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