

# Reduction in fitness of female Asian longhorned beetle (*Anoplophora glabripennis*) infected with *Metarhizium anisopliae*

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## Abstract

Bioassays were conducted to document the effects of *Metarhizium anisopliae* infection on adult female Asian longhorned beetle (*Anoplophora glabripennis*) reproduction before death and subsequent survival of offspring. The effect of infection on fecundity was evaluated for females already laying eggs and for newly eclosed females using *M. anisopliae* isolates ARSEF 7234 and 7711, respectively. Decreased longevity and oviposition compared with controls were observed in females that were already laying eggs when exposed to *M. anisopliae* ARSEF 7234. Newly eclosed females exposed to *M. anisopliae* ARSEF 7711 displayed shortened longevity ( $10.0 \pm 0.7$  days vs  $74.3 \pm 6.8$  days for controls) and decreased oviposition ( $1.3 \pm 0.7$  eggs per ARSEF 7711-exposed female vs  $97.2 \pm 13.7$  eggs per female for controls) compared with controls. Percentages of eggs that did not hatch were greater for both groups of fungal-treated females compared with controls and 60.0% of unhatched eggs contained signs of fungal infection. The percentage of larvae dying within 9 weeks of oviposition was higher for sexually mature females exposed to ARSEF 7234 compared with controls and >40% of dead larvae displayed signs of fungal infection. Thus, for both stages of females and both fungal isolates, fewer surviving larvae were produced after female fungal infection compared with controls. *M. anisopliae* infection affects female fitness by decreasing female longevity, by decreasing female oviposition before death and through horizontal transmission of *M. anisopliae* to offspring.

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## 1. Introduction

The direct effect of pathogens causing acute disease of host insects is death of the host. However, virtually all entomopathogenic fungi do not cause immediate mortality. Delayed death leaves hosts living for variable lengths of time, during which they can feed, mate and reproduce. However, the activity of infected hosts can be altered just before death due to microbial infections. In particular, several studies have indicated that sublethal effects of fungal infections can affect the reproduction of infected adult insects before death (Roy et al., 2006) although infection

does not always have the same effect on reproduction. For example, infection with *Beauveria bassiana* decreased fecundity of the mirid *Lygus hesperus* (Noma and Strickler, 2000) and the chrysomelid *Diabrotica virgifera virgifera* (Mulock and Chandler, 2001) and infection with *M. anisopliae* decreased fecundity of house flies (*Musca domestica*) (Bywater et al., 1994), Mediterranean fruit flies (*Ceratitis capitata*) (Castillo et al., 2000), the mosquito *Anopheles gambiae* (Scholte et al., 2006) and the German cockroach (*Blattella germanica*) (Quesada-Moraga et al., 2004). Fargues et al. (1991) found that *B. bassiana* caused decreased fecundity of *Leptinotarsa decemlineata* at 22 °C but not at 25 °C. The only effect of infection on fecundity of the Russian wheat aphid (*Diuraphis noxia*) (Wang and Knudsen, 1993), the sweet potato weevil *Cylas formicarius* (Rana and Villacarlos, 1991) and the parasitoids *Spalangia*

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*cameroni* (Nielsen et al., 2005) and *Aphelinus asychis* (Lacey et al., 1997) was due to decreased longevity of females. In contrast, El-Tahtaoui (1962) and Bajan and Kmitowa (1972) reported increased fecundity in sublethally infected *L. decemlineata*.

The Asian longhorned beetle, *Anoplophora glabripennis*, is an invasive wood-boring pest in North America, attacking many species of hardwood trees, including maples (*Acer* spp.), poplars (*Populus* spp.), and willows (*Salix* spp.) (Ric et al., 2007). Although native to China and Korea, this beetle has established low density, naturally reproducing, populations in Chicago, IL, New York City and Long Island, New York and several nearby areas of New Jersey, and Toronto, Ont., Canada. *A. glabripennis* has also been reported in Austria, Germany and France (Hajek, 2007). The potential for urban damage due to this insect in the US alone has been estimated at over 600 billion US dollars (Nowak et al., 2001).

Major programs have been undertaken by US and Canadian regulatory agencies to eradicate this invasive species. One means for control that has been investigated is the application of non-woven fiber bands containing cultures of entomopathogenic fungi. This delivery system is used for control of adult lamiine cerambycid pests, particularly *Anoplophora chinensis* (= *A. malasiaca*) and *Psacotha hilaris*, in Japanese orchards with *Beauveria brongniartii* (Higuchi et al., 1997). Fungal bands are placed around tree trunks and branches and adults walking over bands inoculate themselves. Several of the key elements that make this control method effective in Japan include: 1, adult lamiine pests in Japanese orchards are susceptible to *B. brongniartii*; 2, the non-woven bands serve as environments for maintaining viable infective fungal spores for several months (Higuchi et al., 1997; Hajek et al., 2007); and 3, the normal daily activities of lamiine orchard pests in Japan include walking over the bark surface. Adult *A. glabripennis* are susceptible to the fungal pathogen *Metarhizium anisopliae* (Dubois, 2003; Dubois et al., 2007) and they commonly walk on tree branches and trunks (Lance et al., 2000; M.T. Smith, unpublished data). Field trials with fungal bands made with *M. anisopliae* against *A. glabripennis* in China demonstrated faster mortality of beetles in treated plots compared with control plots (Hajek et al., 2006).

Newly eclosed *A. glabripennis* adults require 9–15 days before becoming sexually mature (Keena, 2002; Smith et al., 2002) and average longevity for females at 25 °C is  $78.9 \pm 3.3$  days (mean  $\pm$  SD) (Keena, 2006). During field trials with fungal bands in China, decreased oviposition was recorded for adults caged with bands of *B. bassiana* and *B. brongniartii* (Dubois et al., 2004a) and during uncaged trials testing *B. brongniartii* and *M. anisopliae* against *A. glabripennis* (Dubois et al., 2004b; Hajek et al., 2006). We hypothesized that this effect could be caused by death of reproductive females, but also potentially by decreased oviposition prior to female death.

The present studies were conducted to investigate the effects of *M. anisopliae* infection on reproduction by adult

females and subsequent survival of their offspring. The effect of infection on fecundity was evaluated for both newly eclosed females and sexually mature females that were already laying eggs when infected. We also investigated whether female infection with *M. anisopliae* affected egg hatch and larval survival.

## 2. Materials and methods

### 2.1. Beetles and fungal cultures

*Anoplophora glabripennis* were reared at the USDA, Agricultural Research Service quarantine facility on Cornell University campus, according to methods described by Dubois et al. (2002). Once melanized and mobile, adults were weighed and then fed twigs of sugar maple (*Acer saccharum*) during the ca. 2 week preoviposition period. Each week, saplings of *A. saccharum* at the Arnot Teaching and Research Forest, Van Etten, NY that were ca. 5–7 cm in diameter and 4–8 m tall were felled, cut into ca. 3.0–5.5  $\times$  18.0 cm bolts, and the ends of each bolt were waxed. Males and female adult beetles were placed in 3.8 l glass jars as pairs and supplied with a bolt for oviposition plus twigs for food. Fresh twigs were provided each week throughout the life of each adult. Each week, a new bolt was placed into each rearing jar and the previous bolt was removed. After 21–30 days, a chisel was used to peel the bark and all neonates were removed. Neonates were placed individually into slits cut into the tops of cubes of artificial diet in 58 ml clear plastic cups. Larvae remained in these cups of diet until they had pupated. Pupae were maintained on moistened filter paper and were checked 1–2 times weekly. At eclosion, *A. glabripennis* gradually melanize over numerous days after which the adults are teneral then require additional days to fully sclerotize. Individuals were only considered to be adults when they were completely black and had become mobile. Pupae and adults were reared at 23 °C and 16:8 h (light:dark) but oviposition logs, eggs, and larvae were maintained at 27 °C and 60–80% RH in the dark.

*Anoplophora glabripennis* develop slowly, requiring at least four months from oviposition to adult eclosion. It has been estimated that it costs ca. US\$21 to raise each adult beetle, without overhead costs (Keena, 2005). In our quarantine colony, large, even-aged cohorts were never available for bioassays and studies therefore had to be conducted with limited sample sizes and replication.

For bioassays with sexually mature females, *M. anisopliae* ARSEF 7234 (VD 1) was used. This isolate originated from the cadaver of an adult *A. glabripennis* that had emerged from infested wood cut in Chicago, Illinois in 1999. The adult beetle emerged from the wood and died in the USDA, Forest Service quarantine in Ansonia, CT (Dubois, 2003; M. Keena, pers. comm.). After our sexually mature female bioassays with ARSEF 7234, when there were once again enough beetles in the colony to continue bioassays, our laboratory had changed emphasis and was focusing efforts on a *M. anisopliae* isolate that is registered

for pest control, ARSEF 7711 (F 52). This isolate originated from *Cydia pomonella* in Vienna, Austria (G. Zimmermann, pers. comm.) and is registered with the US Environmental Protection Agency as Tick-Ex G (EPA Registration Number: 72098-12) (Novozymes Biologicals, Salem, VA, formerly Earth Biosciences Inc, New Haven, CT). We found no significant difference in pathogenicity or virulence between ARSEF 7234 and ARSEF 7711 when challenging adult *A. glabripennis* from our quarantine colony (Dubois et al., 2007). Therefore, we continued our studies using ARSEF 7711 with newly eclosed females. Both fungal isolates were grown on full strength Sabouraud dextrose agar plus yeast (SDAY; Goettel and Inglis, 1997) in 100 mm Petri dishes at 20 °C in the dark.

## 2.2. Sexually mature females exposed to ARSEF 7234

### 2.2.1. Producing fungal inoculum

We did not have the expertise to produce fungal bands made with ARSEF 7234 when beginning this study so we exposed females to conidial lawns on cultures grown on SDAY. We considered this substitution valid because for both fungal bands and Petri dish cultures, females would be exposed to a lawn of conidia on the fungal culture that produced them. Cultures of ARSEF 7234 were grown for approximately 2 weeks, until surfaces of Petri dishes were covered with conidia. To count conidial densities on Petri dishes, three 4 mm diameter cores were removed from each plate, homogenized individually in 0.05% Triton-X and conidial concentrations were quantified using a hemocytometer to calculate conidia/cm<sup>2</sup>. To estimate conidial densities, samples were quantified from 2–3 Petri dishes for each of the three repetitions of this study. To evaluate conidial viability, three 90 mm diameter Petri dishes containing SDAY were each inoculated with 1 ml of a suspension of  $1 \times 10^7$  conidia/ml in 0.05% Tween. Petri dishes were maintained at 23 °C for 12–14 h and germination was recorded for 100 conidia at three different locations on each plate. Densities of viable conidia were calculated using conidial density and percent germination. Cultures of ARSEF 7234 averaged  $2.11 \times 10^8 \pm 4.68 \times 10^6$  viable conidia/cm<sup>2</sup> (mean  $\pm$  SE).

### 2.2.2. Bioassay methods

For studies with sexually mature females exposed to *M. anisopliae* ARSEF 7234, a mating pair was placed in a 3.8 l (=1 gallon) glass jar, closed with a lid, for at least two weeks prior to fungal inoculation. This step was required to document the ability of beetle pairs to produce fertile eggs and only pairs producing eggs were included in the study. Females included in the study had melanized and were mobile for >16 days but <50 days before exposure to the fungus. Immediately before exposures, two 1.6 cm diameter cores were removed from Petri dish cultures and placed next to each other in a 60 mm Petri dish. A female beetle was held on top of these two conidial-covered cores for 30 s and then removed to a clean oviposition jar con-

taining their mate, *A. saccharum* twigs and a new bolt. Females averaged  $31.9 \pm 1.1$  days old (range: 16–49 days) when entering this study and males they were paired with averaged  $29.4 \pm 1.4$  days old. Each pair of conidial-covered cores used for exposures was only used once. Control beetles were held for 30 s on two 1.6 cm diameter cores of uninoculated SDAY. Control adult females were then placed individually in 3.8 l glass jars with their mate, a bolt and twigs.

After exposure to *M. anisopliae*, each week females were provided with a fresh bolt and fresh twigs. Females were checked daily for the rest of their lives. Dead beetles were removed and placed individually in 58 ml clear plastic cups containing a saturated cotton ball and were checked weekly for *M. anisopliae* outgrowth. For bioassays with sexually mature females, the numbers of females used varied between 15 and 18 for the three repetitions of this study, with a total of 50 control females ( $n = 18, 17, 15$  females per repetition) and 49 fungal-treated females ( $n = 18, 16, 15$  females per repetition).

## 2.3. Newly eclosed females exposed to ARSEF 7711

### 2.3.1. Producing fungal inoculum

For newly eclosed females exposed to ARSEF 7711, females were exposed to conidial lawns on fungal bands made with *M. anisopliae* ARSEF 7711. Fungal bands were produced as described by Shanley (2007) with 238.6 g/m<sup>2</sup> Soft & Bright 100% polyester batting (The Warm Company, Lynnwood, Washington) as a substrate. Bands were stored at 4 °C for <1 month before use.

Three representative bands were selected for quantification of conidial densities. From each band three 5 cm<sup>2</sup> sections were cut and these were blended individually in 0.2% Tween. The blended suspensions were filtered through a fine mesh sieve and the conidial densities of the resulting suspensions were quantified. Percent germination was evaluated as above to calculate the density of viable conidia. Densities of viable conidia on bands averaged  $1.30 \times 10^8 \pm 1.48 \times 10^7$  conidia/cm<sup>2</sup>.

### 2.3.2. Bioassay methods

For studies with newly eclosed females, pupae were monitored daily to observe when melanization was complete and females started moving, at which time they were considered adults. We waited one more day, during which time we hypothesized that females would have emerged from trees and would begin walking on tree trunks and branches where they could contact a fungal band. For inoculation, one day old females were held on top of a 5 cm<sup>2</sup> piece of fungal band for 30 s. Each piece of band was used only once. Control beetles were held for 30 s on a 5 cm<sup>2</sup> section of non-inoculated band material. Adult females were then placed individually in 3.8 l glass jars with a male, a bolt and twigs and were treated in the same manner as sexually mature females. For bioassay with newly eclosed females, 30 treatment and 30 control females were used.

#### 2.4. Methods for quantifying effects on larvae and eggs

All larvae and unhatched eggs were removed from bolts 21–30 days after the week that bolts had been exposed to ovipositing females. The neonate larvae extracted from wood were reared in the same manner as the laboratory colony larvae (see above). By 21–30 days after oviposition, the majority of eggs had hatched (Keena, 2006). However, in case unhatched eggs found beneath the bark were stragglers that would still hatch, extracted eggs were placed in 60 mm Petri dishes on top of wet filter paper and were checked daily for 14 days. If they still did not hatch, they were considered dead. Any larvae hatching from these eggs were reared using the same methods as the laboratory colony larvae. Four–five weeks after larvae were provided with artificial diet, they were checked for death; larvae were not checked before this time because they tunnel inside of the cube of artificial diet and checking them is very disruptive. For sexually mature females exposed to ARSEF 7234, unhatched eggs and dead larvae were placed on wet filter paper in Petri dishes and were checked weekly for outgrowth of *M. anisopliae*. After 2–4 weeks, all unhatched eggs and dead larvae without fungal outgrowth were dissected and examined microscopically at 400× for fungal structures. Unfortunately, for newly eclosed females exposed to ARSEF 7711, we did not monitor fungal growth in or on unhatched eggs or dead larvae.

#### 2.5. Data analysis

Bioassays with the newly eclosed females and sexually mature females and the two isolates were conducted at different times and were not designed for statistical comparison. To analyze female time to death for ARSEF 7234 bioassays with sexually mature females, female age was included. A general linear model was used with treatment and age as fixed effects (PROC GLM; SAS Institute, 2004). Because the variable age is continuous and interactions with this variable needed to be explored, beetles were split into two categories with relatively equal numbers: beetles <30 days and beetles ≥30 days old at time of exposure. Least square means tests were used for post hoc comparisons.

For analyses of fecundity of sexually mature females exposed to ARSEF 7234, only females producing at least one viable egg were included because we knew that these females had successfully mated; for females not laying any viable eggs we could not tell whether their lack of viable eggs was due to their own infertility, mating with an infertile male or a treatment effect. To compare the numbers of eggs produced by sexually mature females exposed to *M. anisopliae* ARSEF 7234, a general linear model with a negative binomial distribution was used to evaluate treatment and age categories (PROC GENMOD; SAS Institute, 2004).

For newly eclosed females and ARSEF 7711, general linear models were used to compare days to death and

numbers of eggs produced by females for treatment versus control beetles (PROC GLM; SAS Institute, 2004). For newly eclosed females, because the chance that no fertile eggs would be laid was much greater due to the preovipositional period, we included all females in fecundity analyses.

For each fungal isolate/female stage combination, to compare total numbers of eggs, unhatched eggs and surviving larvae produced by fungal-treated versus control females at 1 and 2 weeks after fungal inoculation, one-sided Wilcoxon two-sample tests were used (PROC NPAR1WAY). For analyses of egg and larval survival and hyphal growth,  $\chi^2$  and Fisher's exact tests were used (PROC FREQ; SAS Institute, 2004).

### 3. Results

#### 3.1. Sexually mature females

For sexually mature females exposed to *M. anisopliae* ARSEF 7234, the interaction between treatment and age category had a significant effect on the length of time before adult females died ( $F_{1,87} = 10.43$ ;  $P = 0.0017$ ). Regardless of age group, fungal-treated females always died much more quickly than control females and control females >30 days old when entering this experiment died earlier than control females <30 days old when entering this experiment ( $P < 0.05$ ) (Fig. 1). After death, fungal outgrowth was evident on 87.8% of the cadavers of fungal-treated females while significantly fewer control cadavers produced fungal outgrowth (28.6%) ( $\chi^2 = 36.18$ ;  $P < 0.0001$ ).

Sexually mature females treated with fungus produced eggs for a maximum of 2 weeks after treatment, while control females produced eggs for up to 9 weeks. For females exposed to *M. anisopliae* ARSEF 7234, the average total number of eggs laid before female death was lower for

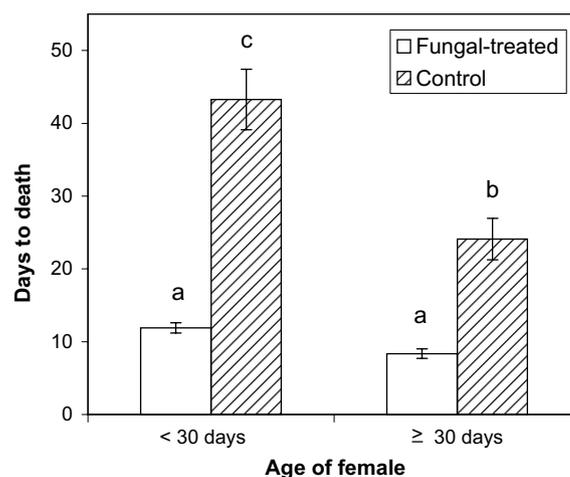


Fig. 1. Mean days to death ( $\pm$ SE) for *Anoplophora glabripennis* females of different ages that were already laying eggs when exposed to *M. anisopliae* ARSEF 7234.

infected females after inoculation ( $5.2 \pm 0.9$  eggs per female) than for control beetles ( $21.9 \pm 3.3$  eggs per female) ( $\chi^2 = 22.28$ ,  $P < 0.0001$ ). The age category of adult females was not associated with egg production ( $\chi^2 = 1.53$ ,  $P = 0.2206$ ). A higher percentage of fungal-treated females (33.3%) produced no eggs after fungal inoculation, compared with control females (14.0%) ( $\chi^2 = 4.31$ ,  $P = 0.04$ ). Control females produced up to a maximum of 74 eggs while the maximum number of eggs per fungal-treated female was 20.

Since no adult females laid eggs after 2 weeks post fungal inoculation, we specifically compared egg production for weeks 1 and 2. For the first week after inoculation of treated beetles, the number of eggs produced by fungal-infected females ( $4.9 \pm 0.9$  eggs/female) did not differ from controls ( $4.5 \pm 0.6$ ) ( $S = 1565.5$ ,  $P = 0.4027$ ). However, the number of unhatched eggs from fungal-treated females was significantly greater than controls ( $S = 1281$ ,  $P = 0.0025$ ). For the second week, the mean number of eggs produced per female was lower for treated beetles than controls and the mean number of unhatched eggs per fungal-treated female were greater than controls (Wilcoxon two-sample tests,  $P < 0.05$ ). Throughout the lives of females after inoculation with *M. anisopliae* ARSEF 7234, among the eggs that hatched, 72.4% of the larvae from fungal-treated females died within a month (Table 1). In contrast, among control females, not all eggs hatched or larvae survived but significantly more than for fungal-treated females ( $\chi^2 = 102.60$  and  $67.90$ ; both  $P < 0.0001$ , respectively). Fungal hyphae were seen in or on more than half of the eggs from treated females that did not hatch (60.0%) and larvae that died (47.4%), while fungal hyphae were associated with very few unhatched eggs or dead larvae from controls. For 90.9% of unhatched eggs with signs of fungi, hyphae were observed only by dissection and microscopic examination while for 94.6% of dead larvae with fungal signs, fungal hyphae grew outside of cadavers.

In summary, total numbers of surviving larvae differed between treated females, averaging  $0.7 \pm 0.2$  surviving larvae/female, and control females, averaging  $13.1 \pm 2.2$  surviving larvae/female ( $S = 1969$ ;  $P < 0.0001$ ) (Fig. 2). Fewer surviving larvae were produced by treated females compared with controls during both the first and second week after fungal inoculation (Wilcoxon two-sample tests;

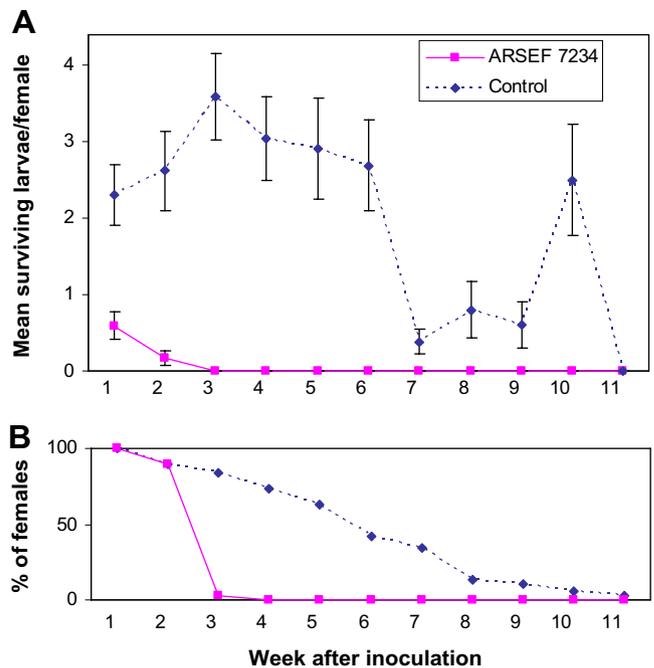


Fig. 2. (A) Mean numbers of surviving larvae ( $\pm$ SE) produced per reproductively active *Anoplophora glabripennis* female exposed to *M. anisopliae* ARSEF 7234, compared with controls. (B) Percentage adult females alive during the study.

$P < 0.05$ ). By the third week after inoculation, no surviving larvae were produced by treated females.

### 3.2. Newly eclosed females

Control females lived an average of  $74.3 \pm 6.8$  days (range: 14–139 days) while females exposed to *M. anisopliae* ARSEF 7711 lived an average of  $10.0 \pm 0.7$  days (range: 2–19 days); longevity of these two groups was significantly different ( $F_{1,57} = 97.40$ ;  $P < 0.0001$ ). Most cadavers of fungal-exposed females produced *M. anisopliae* outgrowth (82.8%). Although a few cadavers of control beetles also produced *M. anisopliae* outgrowth (17.2%), the percentage was significantly lower than for treated cadavers ( $\chi^2 = 24.90$ ;  $P < 0.0001$ ).

For newly eclosed females, the numbers of eggs produced were vastly different between treatment and control

Table 1

Fates of eggs and larvae from female *Anoplophora glabripennis* exposed to two isolates of *Metarhizium anisopliae*

	Total eggs	% Eggs unhatched <sup>a</sup>	% Unhatched eggs with fungal hyphae <sup>a</sup>	% Larval mortality <sup>a,b</sup>	% Dead larvae with fungal hyphae <sup>a</sup>
<i>Sexually mature females</i>					
ARSEF 7234	205	48.8 b	60.0 b	72.4 b	47.4 b
Control	827	15.8 a	0.0 a	30.9 a	0.5 a
<i>Newly eclosed females</i>					
ARSEF 7711	39	41.0 b	—	52.2 a	—
Control	2772	22.9 a	—	51.0 a	—

<sup>a</sup> Pairs of numbers in columns followed by different letters are statistically significantly different ( $P < 0.05$ ). Chi-squared tests were used for ARSEF 7234 % unhatched eggs and % larval mortality. Fisher's exact tests were used for all other comparisons.

<sup>b</sup> Numbers of dead larvae 4–5 weeks after being placed in cubes of artificial diet.

beetles. Among fungal-treated females, only 13.3% laid any eggs at all, while 93.3% of control females laid eggs. Control females produced an average of  $97.2 \pm 13.7$  eggs (mean  $\pm$  SE) (range: 0–229) while fungal-exposed females produced an average of  $1.3 \pm 0.7$  eggs (0–15). A higher percentage of eggs from fungal-treated females did not hatch compared with control females ( $\chi^2 = 7.06$ ;  $P = 0.0079$ ) (Table 1). No difference was seen in survival of larvae from treated females compared with control females ( $\chi^2 = 0.01$ ;  $P = 0.91$ ).

Because females did not die during the first week after inoculation and not all females died during the second week we specifically compared reproduction and offspring survival by week for this interval. For both weeks 1 and 2 after fungal inoculation, the numbers of eggs and numbers of surviving larvae from fungal-treated females were significantly lower than controls (Wilcoxon two-sample tests;  $P < 0.05$ ). During the third week after treatment, only three of the fungal-treated females were still alive and none of them produced any larvae that survived.

In total, control females produced an average of  $37.0 \pm 6.0$  larvae that survived which was far greater than the  $0.8 \pm 0.5$  surviving larvae per fungal-treated female ( $S = 1296$ ;  $P < 0.0001$ ) (Fig. 3).

#### 4. Discussion

Studies with both fungal isolates and both stages of females demonstrated that infection reduces fitness of adult female beetles. Newly eclosed females exposed to ARSEF

7711 were nearly prevented from laying any eggs and  $>40\%$  of eggs that were laid did not hatch. Females that were already ovipositing when infected by ARSEF 7234 laid fewer eggs than healthy females,  $>40\%$  of their eggs did not hatch and  $>70\%$  of larvae died, resulting in far fewer surviving larvae than controls. Results from a bioassay of ARSEF 7711 with sexually mature females (A.E. Hajek, unpublished data) were also consistent with this trend. Thus, we have documented that while exposure of new females (the age that would just have emerged from within trees) to *M. anisopliae* severely limits successful production of offspring, *M. anisopliae* exposure also has an important negative impact on fitness of females that are already ovipositing when exposed to this fungal pathogen.

Fungal infections can affect fitness by both decreasing longevity and decreasing fecundity. While longevity is always affected by acute fungal infections, in some systems fecundity is not. In other host systems, fecundity did not decline before death of fungal-infected Russian wheat aphid (*Diuraphis noxia*) (Wang and Knudsen, 1993) and green peach aphid (*Myzus persicae*) (Liu et al., 2003). In these examples, fungal infection of these short-lived aphids only had an impact on fecundity by affecting longevity. In contrast, *A. glabripennis* females are long-lived and they lay their eggs slowly over many weeks (see controls in Fig. 3). In this study, healthy, newly eclosed females lived as long as 19 weeks, laying eggs yielding surviving larvae during up to 18 of those weeks. Among newly eclosed females, few of either treatment or controls laid many eggs during the first week after we considered that they were adults, as would be expected due to the normal preovipositional period after eclosion; however, fungal-treated females still laid fewer eggs during this interval when compared with controls. For females already laying eggs when infected, during the first week after fungal inoculation, egg production did not decrease, although egg survival did. Results from our study demonstrated that even during the first week after becoming adults, for both stages of females, fungal-treated females produced fewer surviving larvae than controls. Thus, for these long-lived beetles, *M. anisopliae* infections affected fitness, decreasing fecundity due to sublethal effects of infection as well as by decreasing longevity.

To expose new females to fungal pathogens, we needed to estimate how long it would take new females to chew out from inside of trees. We wanted to expose new females to *M. anisopliae* only after they would be outside of trees after eclosion. Our methods for rearing do not provide information on emergence of adults from wood because we rear using artificial diet. For our study, we waited until females were completely melanized and sclerotized and were moving before considering that they might be ready to emerge from the wood. We estimated that females at this stage might already have chewed part of their way out from within wood but would require one more day to complete their emergence; therefore, we exposed new females to *M. anisopliae* one day after they had become active. Recent studies have demonstrated that our estimation of the tim-

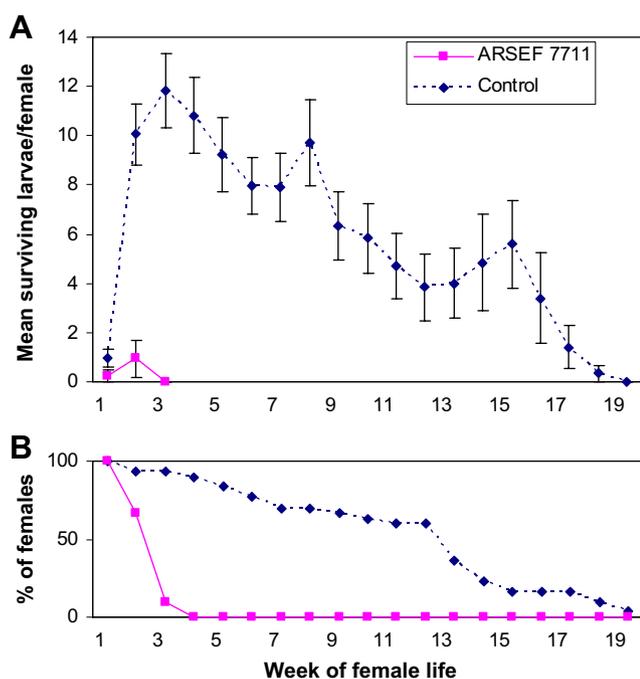


Fig. 3. (A) Mean numbers of surviving larvae ( $\pm$ SE) produced per *Anoplophora glabripennis* female exposed to *M. anisopliae* ARSEF 7711 one day after becoming an adult, compared with controls. (B) Percentage adult females alive during the study.

ing of beetle emergence from trees agrees with other estimates (M. Keena, personal communication).

During bioassays challenging sexually mature females with ARSEF 7234, we unexpectedly found outgrowth of *M. anisopliae* on 28.6% of cadavers of control females. The control cadavers with fungal outgrowth were all from the same repetition of the experiment. We consider that some contamination must have occurred during this repetition of the study within the small quarantine facility where these studies were conducted. Controls with outgrowth from cadavers still required >3 times longer before dying than fungal-treated females, which supports our hypothesis that contamination occurred. Shanley (2007) found that with progressively lower doses of ARSEF 7711, time to death progressively increases. We hypothesize that the cadavers from controls that subsequently displayed outgrowth were probably exposed to very low doses of *M. anisopliae* as an environmental contaminant at some time during the study.

We demonstrated that female infection was associated with decreased egg hatch. In bioassays with sexually mature females, infection was also associated with decreased larval survival. For both unhatched eggs and dead larvae, fungal growth was often found in or on unhatched eggs or dead larvae. Quesada-Moraga et al. (2004) suggest that mortality of offspring from fungal-treated females without subsequent fungal outgrowth may have been caused by reduced immune reactions and nutrient depletion instead of fungal infection. In this study many of the eggs from treated females that did not hatch and larvae that did not survive had been invaded by *M. anisopliae*. We hypothesize that when females laid their eggs, either *M. anisopliae* spores externally contaminating the female could have been deposited on the egg when it was placed under the bark or it is possible that eggs that were laid had already been invaded by the fungus within the female before oviposition. At the time of oviposition, conidia could have been deposited in egg sites under bark and this inoculum eventually infected eggs and neonates. Adult female *A. glabripennis* chew bark to create their sites for oviposition under tree bark so it seems likely that females might contaminate these newly-formed sites with conidia themselves. Therefore, females most probably vector the fungus that subsequently infects and kills some eggs and larvae under the bark. Horizontal transmission of *M. anisopliae* during oviposition has also been documented for the Mediterranean fruit fly (Castillo et al., 2000).

In summary, for adult female *A. glabripennis* exposed to *M. anisopliae*, decreased longevity, decreased oviposition and egg survival prior to female death and horizontal transmission of the fungus to offspring act together to decrease female fitness.

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