

Acoustic Activity Cycles of *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) Early Instars After *Beauveria bassiana* (Hypocreales: Clavicipitaceae) Treatments

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Abstract

Rhynchophorus ferrugineus (Olivier; Coleoptera: Dryophthoridae) is a pest of many crop and ornamental palm tree species in subtropical regions worldwide. Larvae tunnel and feed unseen in the trunks, ultimately causing irreparable harm and killing the palm. *Beauveria bassiana* (Balsamo-Crivelli; Hypocreales: Clavicipitaceae) Vuillemin is under evaluation as a biological control agent for *R. ferrugineus* management but its effects are difficult to monitor under field conditions except by acoustic methods. Older (>30 d) larvae treated with *B. bassiana* display statistically significant reductions in acoustic activity in semi-field studies, but activity of younger larvae has been more difficult to analyze due to their short-duration cycles of increasingly active and then less active feeding and movement, followed by inactive molting. A procedure was developed to characterize effects of *B. bassiana* treatments by subdividing long-term recording periods into activity cycles. Treatment effects were compared within and across cycles. The procedure demonstrated statistically significant differences among acoustic activity rates over time for 15-d-old larvae exposed to control, 10⁴, or 10⁸ conidia ml⁻¹ *B. bassiana* treatments. The results suggest that acoustic technology has considerable utility for monitoring of larval development and for evaluating efficacy of pest management treatments in environments where the targeted insect pests are hidden from view.

Key words: red palm weevil, biocontrol, acoustic technology, molting

The Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier; Coleoptera: Dryophthoridae), an economically important pest of palm trees, has spread invasively from southeastern Asia (Faleiro 2006, Shahina et al. 2009) to all palm-habitable continents except North and South America and is now present in Curacao and Aruba (Fiaboe et al. 2012). It has potential to spread into North and South America, given that a related species, *Rhynchophorus cruentatus* (Fabricius), is native to Florida (Dosunmu et al. 2014). A second related species, *Rhynchophorus palmarum* (L.), is native to South America and has been detected in the southwestern United States (Esparza-Díaz et al. 2013). A third species, *Rhynchophorus vulneratus* (Panzer), difficult to distinguish from *R. ferrugineus*, was discovered in California and eradicated (Hoddle and Hoddle 2016). Adult *R. ferrugineus* can be monitored with pheromones but the larvae, which tunnel into the vegetative apex and injured sections of trunk,

thereafter feeding internally, are difficult to detect until the tree is damaged beyond recovery (Fiaboe et al. 2011).

Integrated pest management strategies against *R. ferrugineus* include chemical control (El-Ezaby 1997, Abo-El-Saad et al. 2001), mass trapping (Faleiro and Chellapan 1999, Hallett et al. 1999), and biological control (Gindin et al. 2006, Llácer and Martínez de Altube 2009, Mazza et al. 2014, Pu et al. 2017). Like many other insects, *R. ferrugineus* has developed resistance to some classes of insecticides (Al-Ayedh et al. 2016). In addition, some of the chemicals are nonselective; hence, they alter natural biodiversity by killing nontarget organisms as well as natural enemies, and they degrade environmental and soil health. For these reasons, early detection of infestations and incorporation of biological control agents into pest management programs have been of continued interest as potential management alternatives. Biological control agents against *R.*

ferrugineus include entomopathogenic fungi such as *Metarhizium anisopliae* (Metchnikoff) Sorokin and *Beauveria bassiana* (Balsamor-Crivelli; Hypocreales: Clavicipitaceae) Vuillemin (Gindin et al. 2006, Dembilio et al. 2010, Güerri-Agulló et al. 2011, Ricaño et al. 2013, Lo et al. 2015), *Bacillus thuringiensis* Berliner bacteria (Pu et al. 2016), and entomopathogenic nematodes such as *Steinernema carpocapsae* (Weiser) (Llácer and Martínez de Altube 2009), *Steinernema abbasi* (Elawad, Ahmad, and Reid) and *Heterohabditis indica* Poinar, Karunakar, and David (Abbas et al. 2001).

Acoustic technology has been successful in detecting larval and adult stages of *R. ferrugineus* within their host plants (Mankin 2011, Jalinas et al. 2015, Mankin et al. 2016), and has been particularly useful for early detection of *Rhynchophorus* spp. infestations (Herrick and Mankin et al. 2012, Dosunmu et al. 2014), as well as for evaluating the effects of *B. bassiana* treatments on larval health and mortality (Jalinas et al. 2015). However, there are no published records of larval acoustic activity within the host plants when instars with durations less than about 2 wk between molting (Fig. 1) are treated with different dosages of *B. bassiana*. The approximate dates of molting in the laboratory colony were similar to those reported in Mahmoud et al. (2015) for larvae reared on palm heart. To evaluate effects of *B. bassiana* on early instars, 15-d-old *R. ferrugineus* were exposed to 10^4 or 10^8 conidia ml^{-1} treatments of *B. bassiana* and their acoustic activities were compared with untreated control larvae over periods up to 34 d in young palm trees kept in a greenhouse.

Materials and Methods

Insects and Treatments

Larvae (15-d old) from a colony of *R. ferrugineus* maintained at the University of Alicante, Spain (Jalinas et al. 2015) were collected and dipped in conidial suspensions of *B. bassiana* isolated from naturally infected adults (Güerri-Agulló et al. 2010). Dipping treatments included two concentrations of *B. bassiana* (10^8 and 10^4 conidia ml^{-1}) formulated as in Jalinas et al. (2015). The formulations and the control used 0.2% Tween 89 surfactant in distilled water. Twenty-four randomly selected larvae were dipped for each treatment, and two larvae from the same treatment were introduced into 30-mm width by 6-cm depth holes drilled on opposite ends of lower trunk of a 5-y-old potted *Phoenix canariensis* Chabaud palm, approximately 5 cm below the vegetative apex. There were 12 palms for each treatment. Additionally, three palms without larval infestation

were tested on 12 different days at approximately 3-d intervals over the 34-d experiment to ensure that background noise was not being misinterpreted as insect sounds. All palms were kept in a greenhouse maintained at approximately 24°C. To reduce ventilation fan and other background noise interference, each palm was moved temporarily to a nearby storage cabin (also maintained at approximately 24°C) with lower levels of background noise for recording.

Larval sounds were recorded for 180 s from treatment trees on multiple different days during the experiment, with 29 recordings from controls, 30 from 10^4 conidia ml^{-1} treatments, and 15 from 10^8 conidia ml^{-1} treatments. The recordings were obtained using an AED-2010 sensor/amplifier system (Acoustic Emission Consulting [AEC], Sacramento, CA) operated under procedures described previously in Jalinas et al. (2015). Recordings from the 10^8 conidia ml^{-1} treatment were discontinued after the 15th day because larval activity permanently dropped below the threshold for low infestation likelihood by the fifth day of recording (see Signal Processing and Analysis). On the 55th day (40 d after the larvae were inserted into the palms), the palms were dissected and a stereo microscope (MZ6, Leica, Buffalo Grove, IL) was used to observe whether the treated and control larvae showed the presence of *B. bassiana*, were alive or dead, or had metamorphosed into pupae and adults.

Signal Processing and Analysis

Recordings were prescreened using Raven Pro software (Charif et al. 2008) to verify the presence of brief insect sound impulses, 1–10 ms, broadband signals produced during insect movement and feeding activity (Mankin et al. 2011). Although spurious background noise impulses sometimes have frequency spectra similar to those of impulses produced by insects, trains (groups) of seven or more impulses separated by intervals <25 ms, all of whose spectra match insect-specific spectral profiles, typically appear only when insects are present, and consequently are more reliable indicators of an insect-produced sound than the spectra alone (Mankin et al. 2008a,b). The incorporation of temporal pattern analysis into the process of discriminating insect sounds from background noise is similar to methods recently discovered to be used by birds for discriminating calls by conspecifics from other calls in acoustically diverse environments (Araki et al. 2016).

Jalinas et al. (2015) identified four spectral profiles that matched well with most of the different types of *R. ferrugineus* larval impulses observed in palm trees, and preliminary analysis using a customized

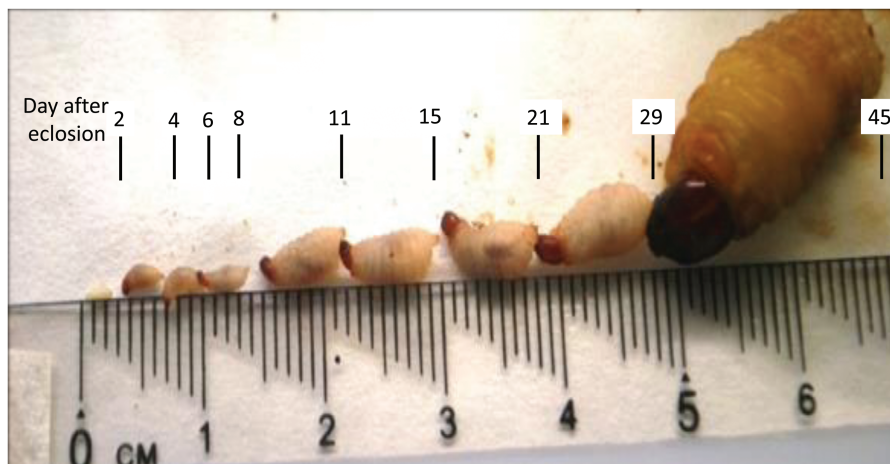


Fig. 1. Examples of the first 9 instars of laboratory-reared *R. ferrugineus*. Bars indicate approximate day after eclosion for molting to next instar (rounded to nearest day).

signal analysis program (DAVIS) (Mankin et al. 2011) confirmed these profiles matched also with sound impulses from recordings in this study, although less successfully with some of the recordings collected in the first few days of testing. Consequently, three additional profiles were constructed to characterize multiple signals from the first 3 d of recording by the same procedures employed for the four original profiles (Jalinas et al. 2015). The seven profiles then were applied by DAVIS to classify each impulse detected in the recordings as a larval or background noise impulse. Trains containing >6 and <200 larval impulses separated by intervals <25 ms were classified as larval sound bursts, with the type of each burst designated as that of the most frequent impulse type in the train.

Bursts of all seven types in each recording were summed together and divided by the recording duration to calculate a total larval burst rate, a commonly used index of larval activity. Also, the impulses in each burst were counted to provide a measure of the timing and energy of the individual mechanical efforts producing the burst, and the mean count of impulses per burst was determined for each recording. The total number of impulses in bursts of each recording was counted and divided by the recording duration to estimate the rate of burst impulses, providing a measure of total larval effort during the recording. The rates of bursts, counts of insects per burst, and rates of burst impulses for each recording were compared in subsequent analyses below. Linear regressions of burst rate on larval age were performed using Proc GLM (SAS Institute Inc. 2012).

Results

Fungal Treatment Effects on Larval Burst Rates

Mean sound burst rates varied considerably in recordings collected on different days after the treated larvae were placed into palm trees (Fig. 2). The mean burst rates in the 10^8 conidia ml^{-1} treatment declined by the 21st day to levels below a previously determined threshold for low infestation likelihood, 0.02 bursts/s (Mankin et al. 2008a), marked at the bottom of Fig. 2. In contrast, the mean rates of larval bursts increased with time after inoculation for both the control and 10^4 conidia ml^{-1} treatments, as seen by the significantly positive slope in the regressions of rate on larval age in Table 1.

Alone however, the regressions of burst rate on larval age do not provide a clear representation of patterns observed in the control

and 10^4 conidia ml^{-1} treatments. For example, the mean rates in both treatments declined below the 0.02 bursts/s threshold of low infestation likelihood near ages 20–21, 30 and 46, while remaining are well above threshold from ages 25–29 and 35–45. For context, it should also be noted that mean burst rates in 36 measurements from untreated trees were 0.0062 ± 0.001 bursts/s, well below the 0.02 bursts/s threshold for low infestation likelihood.

The observed temporal patterns suggest that, due to high variation in the activity levels of larvae as they pass through intervals of molting and feeding, a more representative description of treatment effects might be obtained by evaluating the patterns as cycles of active and quiet periods. Treatment effects then could be compared within cycles or particular treatments could be compared across cycles.

Treatment Effects During Larval Activity Cycles

To consider the potential utility of analyses of treatment effects within and across activity cycles, the experiment was subdivided into sections based on larval ages at which the rates of bursts in the control treatment quickly fell toward and then rose above the threshold for low infestation likelihood. Three activity cycles resulted from the subdivision process, indexed as: 1) 18–20 d, 2) 25–29 d, and 3) 35–46 d (Fig. 2). The mean burst rates in each activity cycle (Table 2 and Fig. 3A) showed a pattern of treatment effects more representative of the pattern observed in Fig. 2 than the simple linear regressions as shown in Table 1. For example, the mean burst rate in the 10^4 conidia ml^{-1} treatment decreased slightly from the first to the second cycle,

Table 1. Parameters for regressions of larval burst rate on age in 10^4 *B. bassiana* conidia ml^{-1} and control treatments

Parameter	10^4 conidia ml^{-1}	Control
Model (error) SS	0.155 (4.04)	0.253 (7.661)
Root MSE (r^2)	0.1062 (0.0369)	0.1488 (0.0319)
F ($P > F$)	13.71 (0.0002) ^a	11.41 (0.0008) ^b
Slope (SEM)	0.00219 (0.00059)	0.00283 (0.00084)
Slope t ($P > t$)	3.70 (0.0002)	3.38 (0.0008)

SS, sum of squares; and MSE, mean square error.

^adf = 1, 359.

^bdf = 1, 347.

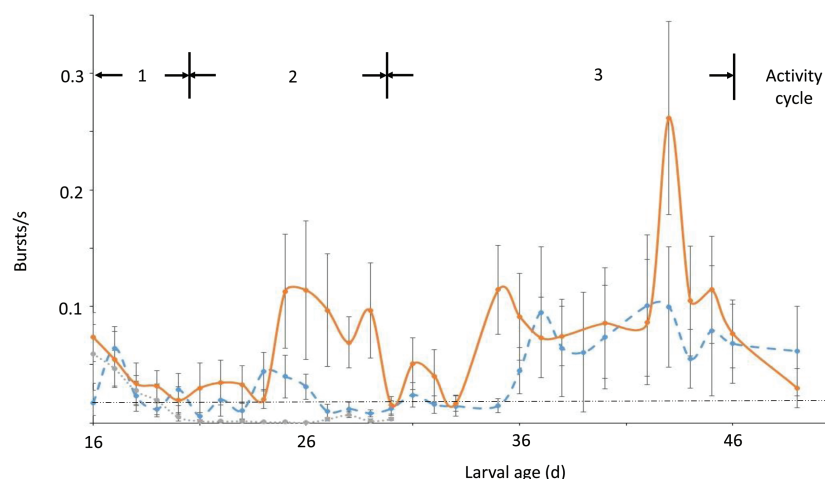


Fig. 2. Mean \pm SEM rates of larval bursts at different ages after treatment and insertion into palm tree at age 15 d for treatments: control, solid; 10^4 conidia ml^{-1} , dashed; and 10^8 conidia ml^{-1} , dotted lines. Threshold of low infestation likelihood is designated by dash-dot-dot line. Beginnings and ends of larval activity cycles 1–3 are designated by bars and arrows.

Table 2. Differences among mean burst rates for 10^8 , 10^4 conidia ml^{-1} , and control treatments during activity cycles 1) 18–20 d, 2) 25–29 d, and 3) 35–46 d

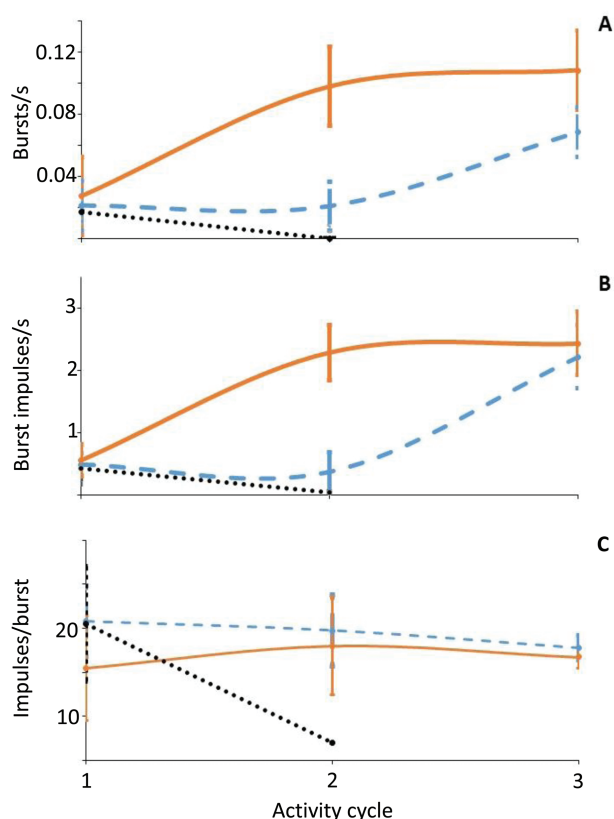
Cycle period (d)	Treatment mean rate \pm SEM (No. of bursts/s)			Test value	P
	10^8	10^4	Control		
18–20	0.0174 ± 0.0039^a	0.0213 ± 0.0068^a	0.0275 ± 0.0096^a	0.56 ^a	0.5740
25–29	0.0027 ± 0.0167^b	0.0202 ± 0.0161^b	0.0978 ± 0.0118^a	18.43 ^b	<0.001
35–46	—	0.0687 ± 0.0109^b	0.108 ± 0.0143^a	4.02 ^c	0.0460

Means in a row followed by same letter are not significantly different ($P > 0.05$). HSD, Tukey Honest Significant Difference.

^aF-test, $df = 2, 106$. Means subjected to HSD test.

^bF-test, $df = 2, 178$. Means subjected to HSD test.

^cWelch's t -test, $df = 1, 250$.

**Fig. 3.** Mean \pm SEM of (A) rates of larval bursts, (B) rates of larval impulses, and (C) No. of impulses per burst within activity cycles for treatments: control, solid lines; 10^4 conidia ml^{-1} , dashed lines; and 10^8 conidia ml^{-1} , dotted lines.

and was found to be significantly lower than the control during the second cycle. The larvae appeared to recover from the 10^4 conidia ml^{-1} treatment in the third cycle, during which the mean burst rate was no longer significantly different from that of the control.

Enhanced temporal pattern perspective is also evident in statistical comparisons of burst rates for the control and the 10^4 conidia ml^{-1} treatment in cycles 2 and 3. The difference between cycle 2 and 3 mean burst rates was not significant in the control ($t = 0.169$, $df = 1, 178$, $P = 0.682$), but was significant in the 10^4 conidia ml^{-1} treatment ($t = 2.50$, $df = 1, 190$, $P = 0.0133$), which suggests that the activity cycle analysis has greater power than the linear regression analysis detecting differences among treatments.

The rates of impulses in bursts showed a similar pattern as burst rates across activity cycles, except that the ratio of the control to 10^4 conidia ml^{-1} rate was greater than for burst rates during the second

cycle, 6.2 compared with 4.7, and lower during the third cycle, 1.1 compared with 1.6 (Table 3 and Fig. 3B). In contrast, the mean counts of impulses per burst had a different pattern across activity cycles, with no significant differences among treatments except for the 10^8 conidia ml^{-1} treatment, in which the mean decreased significantly after the first cycle (Table 4, Fig. 3C).

Larval Development and Mortality in Different Treatments

After 55 d, the palms were dissected, and no pupae were recovered from the 10^8 conidia ml^{-1} treatment due to 100% larval mortality. There was 50% pupation and 50% larval mortality in the 10^4 conidia ml^{-1} treatment, while 75% of untreated larvae had metamorphosed into pupae, 5% remained in the larval stage, and 20% had died. All of the dead larvae in the 10^8 and 10^4 conidia ml^{-1} treatments exhibited hyphal growth and other visible signs of *B. bassiana* infection (Fig. 4). Pupae found in the control and 10^4 conidia ml^{-1} treatments did not exhibit signs of *B. bassiana* infection.

Discussion

The cyclic variation in activity patterns observed in this study is similar to that found by Dosunmu et al. (2014) with *R. cruentatus* (Fabricius) larvae, including the presence of three clearly discernable activity cycles in both species, one of which ended near age 21 d. The multiple falls and rises in activity are consistent with occurrences of instars in *Rhynchophorus*, reported in studies such as Shahina et al. (2009), 9 instars over a 50- to 80-d period; Dembilio and Jacas (2011), 13 instars over a 40- to 160-d period; Norzainih et al. (2015), 9 instars over a 70-d period depending on temperature; and Mahmoud et al. (2015), 12 instars over a 126-d period. Larval cyclic activity also was observed by Shade et al. (1990) during acoustic monitoring of *Callosobruchus maculatus* (Fabricius), by Pittendrigh et al. (1997) monitoring *Sitophilus oryzae* (L.) (Pittendrigh et al. 1997), and by Mankin et al. (2004) monitoring *Cephus cinctus* Norton and *Metamasius callizona* Chevrolat. In Pittendrigh et al. (1997), the molting and feeding activities of *S. oryzae* instars were observed acoustically and visually by cracking open the wheat kernels before the recordings began. The three cycles shown in Fig. 2 correspond well with ages reported for seventh to ninth *R. ferrugineus* instars feeding on palm heart (Mahmoud et al. 2015), which strongly suggests that the observed activity cycles in this report correspond to *R. ferrugineus* instars.

The reduced rates of bursts, burst impulses, and counts of impulses per burst observed in treated larvae compared with control larvae (Tables 2–4) may result from increased mortality (Gindin et al. 2006), weaker movements, and reduced feeding (Ekesi 2001,

Table 3. Differences among mean rates of burst impulses for 10^8 , 10^4 conidia ml^{-1} , and control treatments during activity cycles 1) 18–20 d, 2) 25–29 d, and 3) 35–46 d

Cycle period (d)	Treatment mean rate \pm SEM (No. of impulses/s)			Test value	P
	10^8	10^4	Control		
18–20	$0.425 \pm 0.256a$	$0.491 \pm 0.181a$	$0.561 \pm 0.256a$	0.56 ^a	0.870
25–29	$0.050 \pm 0.017b$	$0.372 \pm 0.315b$	$2.288 \pm 0.446a$	15.15 ^b	<0.001
35–46	—	$2.217 \pm 0.505a$	$2.431 \pm 0.505a$	0.0896 ^c	0.765

Means in a row followed by same letter are not significantly different ($P > 0.05$).

^aF-test, df = 2, 106. Means subjected to HSD test.

^bF-test, df = 2, 178. Means subjected to HSD test.

^cWelch's *t*-test df = 1, 214.

Table 4. Differences among mean No. of impulses/burst for 10^8 conidia/ml, 10^4 conidia/ml, and control treatments during activity cycles 1) 18–20 d, 2) 25–29 d, and 3) 35–46 d

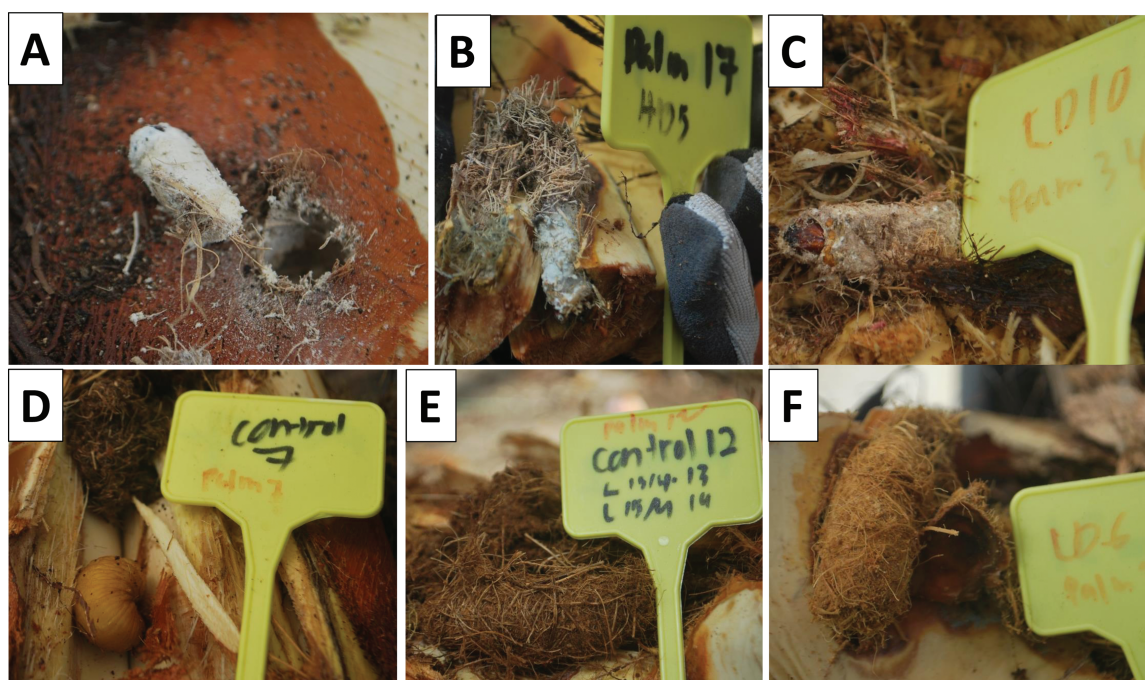
Age period (d)	Treatment mean \pm SEM (No. of impulses/burst)			Test value	P
	10^8	10^4	Control		
18–20 ($N = 60$)	$20.470 \pm 6.57a$	$20.770 \pm 4.023a$	$15.494 \pm 5.953a$	0.45 ^a	0.637
25–29 ($N = 84$)	$7.0 \pm 0b$	$19.744 \pm 4.130a$	$18.028 \pm 5.564a$	14.93 ^b	<0.001
35–46	—	$17.767 \pm 1.381a$	$16.724 \pm 1.269a$	0.299 ^c	0.586

Means in a row followed by same letter are not significantly different ($P > 0.05$).

^aF-test, df = 2, 58. Means subjected to HSD test.

^bF-test, df = 2, 82. Means subjected to HSD test.

^cWelch's *t*-test df = 1, 151.

**Fig. 4.** Examples of mortality and hyphal growth observed for larvae exposed to (A–B) 10^8 treatment and (C) 10^4 treatment. Live larvae with no signs of *B. bassiana* infection were found in (D) control palms, and no signs of infection were observed in pupae from the (E) control and (F) 10^4 treatments.

Nussenbaum and Lecuona 2012). Weaker movements, for example, could shorten the durations of individual sound bursts, thereby reducing the numbers of impulses per burst. Reduced feeding could reduce the rates of feeding-induced sound bursts as well as the rates

of burst impulses. In this study as well as other reports (Gindin et al. 2006, Dembilio et al. 2010), *R. ferrugineus* larvae that received $>10^7$ conidia ml^{-1} doses of *B. bassiana* had 100% mortality. In this study, even lower doses (10^4 conidia ml^{-1}) temporarily reduced the activity

of early instars, although the activity appears to have renewed in later instars. This is consistent with a previous study by Zhang et al. (2014), which found that *B. bassiana* often is melanized in molting fluid, delaying conidia germination. The rate of mortality in the 10^4 conidia ml⁻¹ treatment was similar to what had been observed in a previous study of older larvae (Jalinas et al. 2015).

The activity cycles displayed in Fig. 2 suggest that mortality of larvae in the 10^8 conidia ml⁻¹ treatments occurred primarily during the first cycle after treatment (estimated as seventh instar), with a small fraction surviving until the second cycle (estimated as eighth instar). The palm dissections confirmed that all larvae in this treatment died, in agreement with the results in Fig. 2, where the burst rate decrease below threshold by the fourth day after treatment.

The results suggest that larvae in the 10^4 conidia ml⁻¹ treatments suffered some mortality during the first and second cycles after treatment (estimated as seventh and eighth instars). However, the activity of 10^4 treatment survivors was similar to the control by the third cycle after treatment.

The results of these experiments suggest that aspects of *R. ferrugineus* larval development, including feeding and molting of instars, and effects of management treatments can be acoustically monitored over multiple instars in semi-field tests. Acoustic technology is a promising method to help explore insect development and to determine the efficacy of pest management treatments in environments where the targeted pests are hidden from view. This is particularly important for *R. ferrugineus* because the alternative method for assessment of treatment effectiveness in the field is palm dissection, which is time-consuming, expensive, and destructive. Combined with geospatial analysis (Mankin et al. 2007), the acoustic technology can be of considerable utility in field studies to evaluate effects of entomopathogenic fungi and other *R. ferrugineus* management options in heritage- or conservation-protected areas.

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