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ORIGINAL ARTICLE

Oleic acid emitted from frozen *Trogoderma* spp. larvae causes conspecific behavioral aversion

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Abstract

Accumulating evidence in the literature suggests that oleic acid functions as a necromone across widely divergent insect taxa. The prevelance of this phenomenon has not been fully explored, and its application to pest management remains underdeveloped. Khapra beetle (KB), *Trogoderma granarium*, is a pest of stored grains, with larvae that can enter facultative diapause and remain cryptic in warehouses. Here, we examine how death affects oleic acid content of *Trogoderma* spp. cuticular extracts, and whether the compound causes a behavioral response. To assess the generalizability of patterns, many experiments were repeated with warehouse beetle (WB), *Trogoderma variabile*, and larger cabinet beetle (LCB), *Trogoderma inclusum*. Extracts of larvae that were first killed by being frozen had greater oleic acid content than those derived from live insects. Two-choice behavioral assays compared responses of solvent controls to these extracts, at both low (~ 2 µg) and high (68–131 µg) oleic acid content. The natural extracts also contained cuticular hydrocarbons and other unidentified chemicals. High oleic acid in the extracts repelled the larvae of all three species. Lower levels of oleic acid did not affect KB and LCB movement, but were attractive to WB. We also performed the assay using a large range of doses of oleic acid alone. At the lower doses, oleic acid had no effect on movement, but it became strongly repellant at higher doses, beginning at 100 µg. These results indicate that necromones may be an overlooked aspect of stored product insect biology, which if further researched could improve pest management.

Keywords Attractant · Dermestidae · Invasive · Khapra beetle · Repellent · Semiochemical

Introduction

There has been a steady accumulation of information spanning decades that unsaturated fatty acids often serve as necromones in an array of arthropods**.** In particular, oleic acid and linoleic acid are thought to often be the components of dead organisms that induce specific behavioral adaptations (Yao et al. 2009). For example, it has been noted that these acids are emitted by dying or decaying organisms and they may signal alarm to elicit behaviors such as avoidance of predation risk (Abbott 2006; Aksenov and David-Rollo 2017) or colony maintenance in social insects (Wilson et al. 1958; Gordon 1983; Akino and Yamaoka 1996; Chouvenc et al. 2012). Outside of social insects, the phenomenon has often been observed to be a means of avoidance of adverse conditions for arthropods that shelter gregariously, such as isopods and lepidopteran caterpillars (Yao et al 2009), cockroaches (Rollo et al. 1994, 1995), crickets (Shepherd et al. 2018), and springtails (Nilsson and Bengtsson 2004). Yet, this phenomenon remains largely unexplored in some of the most diverse insect taxa such as Coleoptera. Furthermore, we are unaware of any established pest management applications for use of such compounds.

In this study, we seek to uncover whether necromones may exist in *Trogoderma* (Coleoptera: Dermestidae), a genus

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including several pests of stored products such as harvested grains. While true social behaviors have not been described in the genus, they often cluster both within and near food sources. Here, the possibility of inducing necromone production by killing *Trogoderma* larvae through freezing is investigated. Freezing is known to be a mechanism by which oleic acid emission is increased in Hymenoptera (McAfee et al. 2018). Additionally, the behavioral responses of larvae to extracts with elevated levels of the prospective necromone, oleic acid are investigated. Further, the behavioral responses to oleic acid alone are observed to verify that it possesses necromone properties adaptable to pest management applications. Natural repellants, such as necromones, may be particularly effective in stored product settings where commodities are concentrated in controlled environments.

One species of this genus, khapra beetle (KB), *Trogoderma granarium* has an extensive distribution throughout warm and arid regions of Eurasia and Africa. Khapra beetle is a quarantine pest in the United States, with a history of interception at ports and successful eradications at various scales and locations (Armitage 1956). The tendency of larvae to readily enter facultative diapause and survive in adverse environmental conditions further highlights the need for control measures that target larvae. In the diapause state, *Trogoderma* larvae do not exhibit obvious changes in behavioral or morphological characteristics, but simply fail to make progressive molts toward adulthood. These diapausing larvae may remain cryptic in warehouses or in shipping facilities with the potential to reestablish infestations on commodities at a later date, well after detection and remediation efforts have been completed (Athanassiou et al. 2019).

The use of fumigation and contact insecticides has often been critical for remedial actions for KB control (Myers and Hagstrum 2012). Pyrethroid insecticides such as deltamethrin and cyfluthrin (Arthur et al. 2018) or insect growth regulators such as methoprene and pyriproxyfen (Athanassiou et al. 2015) have been developed against KB. However, late instar larvae are often more resistant to such treatments (Ghimire et al. 2016). Fumigation to remediate affected grain had for years relied upon methyl bromide (Cobb 1958). Alternatives to methyl bromide such as phosphine fumigation and heat application have been adopted in the wake of increasing restrictions (reviewed in Fields and White 2002), but their efficacy is highly dependent on sanitation in a facility (Morrison et al. 2019), and increasing worldwide resistance to phosphine complicates its use (Nayak et al. 2020). Thus, novel management options that prevent the need for such remedial treatments would be of value.

Management using semiochemicals has been researched from a few different perspectives. For example, essential oils obtained from certain natural products have been used as repellants (Jood et al. 1993). However, discovering chemicals that attract *Trogoderma* for monitoring purposes has been a longstanding primary objective. Various kairomone attractants have been developed for the species, which are an important component of the current commercially available trapping and monitoring systems. For larvae, it was shown in broad spectrum tests of multiple compounds that carbon dioxide and certain food odors can be attractive, while many short chain (3–7 carbon) alcohols and organic acids can be repellant (Spangler 1965). Additionally, the female-produced, two-component sex pheromone for KB has been identified as (*Z*)- and (*E*)-14-methyl-8-hexadecenal (commonly referred to as trogodermal; Levinson et al. 1978), which is used along with wheat germ as stimuli in cardboard monitoring traps (Barak 1989). It has also been recently determined that the adult pheromone is attractive to older larvae (Morrison et al. 2020). The compounds composing the surface cuticular hydrocarbons of larva (Maliński et al.1986) and adults (Dubis et al. 1987) have been described, but it is not known whether they may affect close-range movement.

To investigate the potential roles of hydrocarbons and necromones for *Trogoderma* spp., we used whole body extracts from late instar larvae of KB and two related stored product pests, the warehouse beetle (WB), *Trogoderma variabile*, and larger cabinet beetle (LCB), *Trogoderma inclusum*. All of these three related species are worldwide pests of stored grains that have similar life histories and present similar concerns for management and quarantine policy (Edde et al. 2012). A number of extraction methods were attempted to determine whether killing the insects by freezing increased or decreased the extraction of certain key compounds, such as oleic acid. We also investigated whether rinsing debris such as frass from the insects affects the chemical contribution to the extracts. Extracts were presented in two-choice behavioral assays such that the number of larval equivalents presented to the insects would be similar to that of a cluster of 4–12 insects. These parameters were chosen to mimic realistic field conditions where small groups of conspecifics might be interacting with each other in the vicinity of moderately to heavily infested host material.

After determining that oleic acid, a common necromone, was present in the extracts and highly correlated with behavioral eversion, additional experimentation was performed to examine the effects of oleic acid more closely for KB specifically. This was accomplished by two-choice assays that examined the movement of larvae with respect to different doses of oleic acid alone compared to a control. This experimental framework allowed us to uncover pathways for both oleic acid emission and response, with the latter explored both with and without the context of other potential semiochemicals, including cuticular hydrocarbons.

Materials and methods

Larval extracts

Extracts were created by collecting and killing larvae using different methods. In this and all experiments, diapause status was unknown for each individual, because it cannot be readily determined from any obvious behavioral or morphological markers. The larvae collected included groups of individuals from all three *Trogoderma* species, KB, LCB, and WB. KB was the only species for which an extraction was performed using each method. For creating the extracts, we selected the largest larvae available (> 5 mm). Semichemically mediated behavior can differ between larval age classes (Morrison et al. 2020), thus for this study, we use older larvae for all experiments. It is thus possible that this method resulted in partial female bias in the extracts, because females tended to be larger in all three species. In every case, regardless of how the insects were killed, extractions were performed by adding hexane at a ratio of 4 ml/g of insect larvae at room temperature. After 5 min, the liquid hexane extract was removed from the larvae by a syringe into a separate vial. Table 1 summarizes the sample sizes and other attributes of the extracts collected.

For all three species, extractions were performed using two methods of freezing to kill specimens. The first, referred to as "slow-frozen" involved placing the insects at − 20 °C for a minimum of 48 h in glass vials or plastic centrifuge tubes. The second method, referred to as "flash-frozen", consisted of placing the insects in centrifuge tubes and then dipping them into liquid nitrogen to rapidly freeze. The very first extracts of all three species created used the "slow-frozen" and "flash-frozen" method, for 1.5–2.8 g of larvae, which were not individually counted. After freezing for the respective time periods, insects were allowed to thaw. They were then rinsed twice with distilled water and strained over metal wire window screen. Afterwards, the insects were allowed to dry on top of the screen and Fisherbrand® Filter Paper (Dia: 12.5 cm) for 3–6 h. After obtaining the initial extracts for these three species, and determining the chemical composition, it was decided to perform two more large-number replicates of WB to confirm results.

Subsequently, more extraction replicates were performed using these freezing methods for the three species, but with smaller numbers of larvae to increase the number of extraction replicates. The numbers of insects and their total mass was recorded for most of these additional extraction replicates, allowing an estimate of the mean weight of individual larvae within each extraction replicate. For the largest species, WB the number of larvae used in each replicate was 80. For LCB and KB, 50 larvae were used in each replicate extract. The weights of all larvae in extracts where the number of larvae was noted and the mean ± SE number of larvae per mg of material was computed. A 100 individual sample of KB, a 100 individual sample of LCB, and a 50 individual

Table 1 Parameters associated with the sampling of larvae for hexane extraction at 4 ml/g

Species	Method	*N*	Larvae	Larvae/mg[a]	Larvae/refuge	Uncounted extracts[b]
WB	Slow-frozen	6	50	0.25 ± 0.02b	6.2	2
WB	Flash-frozen	10	50	0.16 ± 0.01c	3.9	2
WB	Alive (not extracted)	1	50	0.108	–	–
LCB	Slow-frozen	10	50	0.25 ± 0.02b	6.2	1
LCB	Flash-frozen	10	50	0.23 ± 0.01b	5.7	1
LCB	Alive (not extracted)	1	100	0.278	–	–
KB	Slow-frozen	10	80	0.48 ± 0.01a	12.1	6
KB	Flash-frozen	9	80	0.26 ± 0.01b	6.4	7
KB	Alive (not extracted)	1	100	0.242	–	1
KB	Unwashed live	–	–	–	–	15
KB	Pre-rinsed live	–	–	–	–	15
KB	Pre-rinsed flash frozen	–	–	–	–	15

Most replicates (N) consisted of a fixed number of counted larvae ranging from 50 to 100, which allowed estimation of both the number of larvae/mg (mean ± SEM) and the equivalent number of larvae added to 100 µl in the refugia

[a]The different letters in the column describing larvae/mg indicate that the species and freezing treatment combination is significantly different from other treatment combinations with different letters (Tukey–Kramer, $\alpha = 0.05$)

[b]Uncounted extracts ranged from 0.2 to 2.8 g of larvae and were used for GC–MS analysis and behavioral experiments

sample of WB live larvae were collected, counted, and weighed to provide baseline estimates for this parameter.

For KB, three additional extraction methods were used to allow inferences about possible sources of compound production. For one extraction method (referred to henceforth as "unwashed live"), 250 mg of uncounted larvae were taken directly from the colony for each of 15 extraction replicates. Hexane was added to each replicate at a ratio of 4 ml/g, while they were alive but otherwise undisturbed. This was the only method where it was likely that frass and other colony debris was extracted along with the insects in the sample.

In a method referred to as "pre-rinsed live", hexane extracts were performed after rinsing live KB with water over a wire window screen. Again, 250 mg of uncounted larvae were used in each of 15 extraction replicates. The bottom of the window screen was placed against paper towels to wick away additional moisture. It was observed that most larvae remained viable and moving after washing. Thus, in these extractions frass and debris were removed.

In a final method, referred to as "pre-rinsed flash-frozen", extracts were made with KB in which rinsing with water was performed in the same manner as for the "pre-rinsed live" treatment described above. However, instead of extracting from the live insects, they were frozen in liquid nitrogen, as described for the "flash-frozen" larvae. As with many of the uncounted larvae above, approximately 250 mg of larvae were used in each of 15 extraction replicates.

Characterization of extracts of three Trogoderma species

For chemical analyses, all extracts were further concentrated by a factor of 20 by blowing purified nitrogen over 200 µl to force evaporation to 10 µl. Only compounds with low volatility were of interest for the analysis, and thus were not likely to be affected by the procedure. Chemical analyses were conducted using a combined Agilent Technologies 6890 network gas chromatograph (GC) and Agilent 5973 mass spectrometer (MS). The GC was equipped with a DB-5 column (length: 30 m; I.D.: × 0.25 mm; film thickness: 0.25 µm; J&W Scientific Inc., Folsom, CA, USA). Helium was the carrier gas at a constant flow rate of 0.7 ml/min. Injection was splitless at 275 °C. Oven temperature was held at 50 °C for 2 min, then ramped to 280 °C at 10 °C/min and held for 15 min.

In each extract, we confirmed the identities of the four most common peaks in the cuticular hydrocarbon region (Fig. 1) which included *n*-heptacosane (24.5 min., Sycamore Life Sciences, Houston Texas, Sigma Aldrich, St. Louis, MO), *n*-nonacosane (25.9 min., Sycamore), hentriacontane (27.4 min., Sigma Aldrich), and *n*-tritriacontane (29.5 min., Sycamore), which were all previously described for KB larvae and adults (Maliński et al. 1986; Dubis et al. 1987). We also identified oleic acid from a peak that typically eluted at 20.4 min (Sigma Aldrich). The identities of these compounds were confirmed by comparison to mass spectra of standards with > 99% purity. In each case, the entire chromatograph, including the five identified compounds was integrated using MSD ChemStation software (v. E.02.01.11.77 Agilent Technologies, Santa Clara, CA). The abundances of oleic acid and one of the most common hydrocarbons, *n*-heptacosane, were noted and the quantities estimated by comparison to known doses of the analytic standards (0.1, 0.5, 1, 5 and 10 µg oleic acid, 0.01, 0.1 and 1, µg *n*-heptacosane). Analysis of the quantity of *n*-heptacosane was performed to evaluate whether the extraction yield of organic acids such oleic acid is generally correlated to that of hydrocarbons across the different methods. The amounts of oleic acid and *n*-heptacosane that were estimated to be injected into the GC–MS were used to calculate the mass of both compounds extracted from each g of larvae in the original extract.

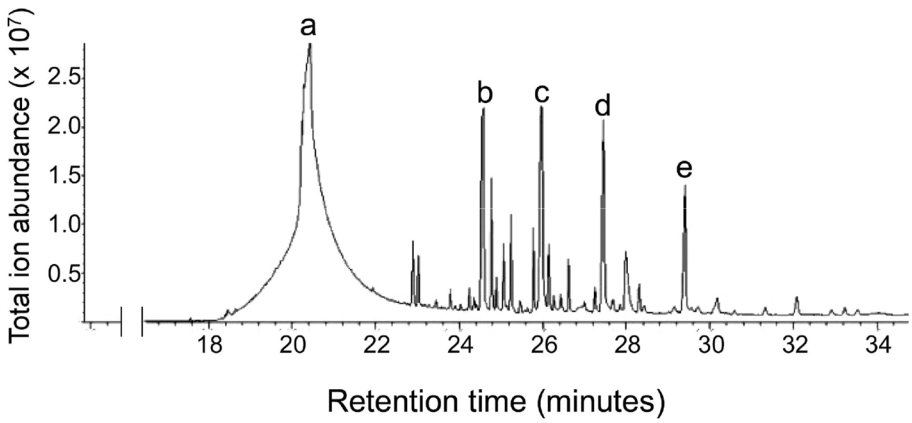

Fig. 1 A "high" oleic acid gas chromatography trace from a WB extract. The major peaks, which are marked by lower case letters, were identified as described in the text: **a** oleic acid, **b** *n*-heptacosane, **c** *n*-nonacosane, **d** hentriacontane, **e** *n*-tritriacontane

Behavioral assay for attraction

For assessing whether preferences for odors affect larval movement, a series of two-choice behavioral assays were performed. For each experiment, filter paper (15 cm, no. 8, Whatman, Bangalore, India) was placed in 15-cm glass petri dish arena to fully cover the bottom surface (Fig. 2). This larger filter paper was folded once along the mid-line, which served to separate the arena into semicircle halves. Within either side, a smaller 2.5-cm diameter filter paper was placed, which was used for presenting a stimulus and providing a possible clustering surface (referred to hereafter as refuges). Each refuge was folded three times in parallel to present a corrugated surface and encourage clustering under the paper or in its folds. For each experimental replicate, each refuge received an aliquot of a hexane control, or other compounds extracted in hexane. HPLC grade hexane (99.9% purity, Fisher Chemical, Fairlawn, NJ) was used for all experiments and was allowed to fully evaporate from the disk for at least 1 hour before beginning experiments.

After assembling the arenas and adding ten late instar larvae (> 5 mm) to the center, the arenas were covered and sealed with parafilm, preventing escape and providing a static air environment. Up to 39 petri dish arenas were replicated per day. They were always placed such that the center lines of all arenas were aligned in parallel, and the position of each refuge was noted (closer to wall 1 or wall 2). The insects were allowed to acclimate overnight in a completely sealed dark room at 28 °C for 16 h. The arenas were covered with a black blanket to further minimize light exposure, particularly when they were checked at the end of the period. The larvae were generally immobile at the end of the exposure period. In each replicate arena, the insects had the opportunity to choose one of the zones, which included the two refuges or the areas adjacent to the refuges on either half of the arena (Fig. 2). We scored the insect as being in the zones of the refuges if any part of its body was less than one body length from the margin (5 mm). A minimum of 36 replications of each treatment of a given experiment was performed, with precise numbers presented on the figures referenced in the results section.

We performed preliminary experiments to understand how insects of each species would move in the arena under the experimental conditions, without any potential semiochemicals being present. For all three species, we performed assays in which 100 µl of hexane was added to both refuges and allowed to fully evaporate. This experiment allowed us to assess whether there may have been biases inherent to the experimental environment, such as a preference for the wall on one side of the room versus the other, and whether the three species had different innate clustering tendencies with respect to the refuges. We also performed a control for KB where one filter paper received 100 µl of hexane, and the other received no chemical treatment. The second experiment was adopted to preclude the possibility that there may be some behavioral effect of hexane treatment itself on settlement in the refuges.

Our next experimental objective was to determine whether the presence of oleic acid in the extracts affected the behavior of other conspecific larvae. Extracts were taken from an initial smaller batch of larger volume samples deriving from slow-frozen or flash-frozen larvae of KB, LCB, and WB (Table 1). We selected one extract from each of the three species with > 60% ("high oleic") or < 10% ("low oleic ") oleic acid for separate behavioral assays. For selecting these extracts, percentages were determined by integrating all peaks above the baseline in the chromatographs for the extracts. After comparing the oleic acid content to doses

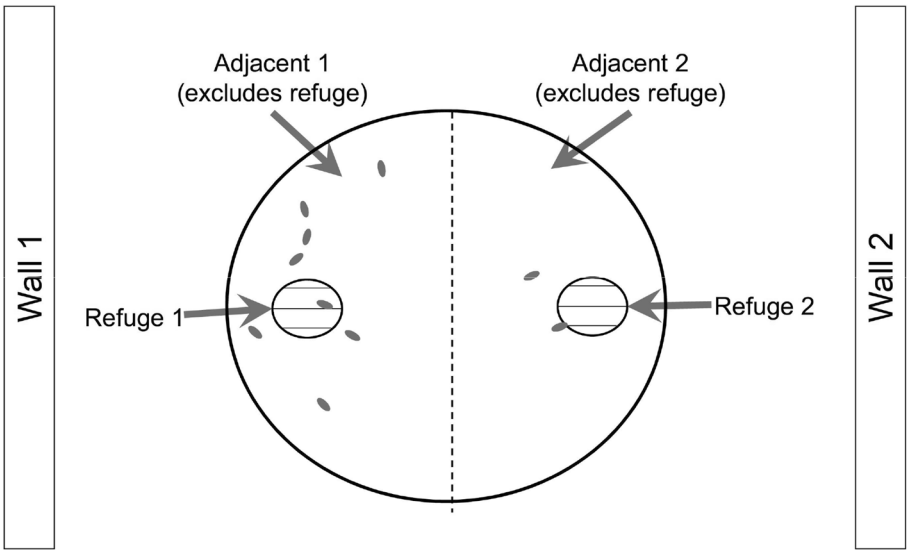

Fig. 2 Petri dish arena assay for extract effect on *Trogoderma* larval movement. Four zones are marked by arrows that include smaller corrugated filter papers (refuges) receiving a chemical treatment and each half of the arena that was adjacent to these smaller zones. The zones adjacent to each refuge were combined with the refuge for certain analyses to compare side 1 to side 2. Ten (grey oval) larvae were always included in each arena. The zone preference of each larva was recorded after acclimation to the dark for 16 h

of synthetic standards, as described above, the amounts of oleic acid in the selected extracts that were applied to each refuge were estimated. The estimated amounts are as follows (extract type in parenthesis): KB high oleic (slow-frozen) = 67.9 µg, LCB high oleic (slow-frozen) = 130.8 µg, WB high oleic (slow-frozen) = 70.6, µg KB low oleic (flash-frozen) = 1.8 µg, LCB low oleic (flash-frozen) = 1.2 µg, WB low oleic (slow-frozen) = 2.6 µg. Furthermore, based on the typical weights of frozen larvae, the extracts had doses ranging from 3.9–12.1 larval equivalents (Table 1), which is suitable to mimic the chemical profile of a small cluster of resting larvae in a natural population such as a warehouse. Other than the obvious differences in oleic acid composition, the extracts had similar typical cuticular hydrocarbon profiles. Only conspecific extracts were presented to the larvae of each of the three study species. One refuge received 100 µl aliquot of a treatment extract, while the other received 100 µl of hexane only as a control. Every time that an experiment was run, half the controls and treatments were aligned to be closer to one wall versus the other. Thus, within room effects were controlled.

A final objective for the behavioral assays was to specifically examine oleic acid alone as a factor affecting movement. We examined this phenomenon for only KB. Dilutions of oleic acid (Sigma-Aldrich) were created in hexane and applied to the corrugated 2.5-cm refuges, such that doses of the compound were 1 µg, 10 µg, 100 µg, 500 µg, 1 mg, or 5 mg in treated refuges. These refuges were used for behavioral assays versus hexane control as described above, also randomizing positions to prevent within room effects.

Statistical analysis

Comparisons among attributes of extracts were performed using JMP15© software, (SAS Institute Inc., Cary, NC, USA). For one analysis involving only KB, five treatments were considered, including unrinsed live, pre-rinsed live, pre-rinsed flash-frozen, flash-frozen, and slow-frozen larvae. For a separate analysis, we also considered flash-freezing versus slow-freezing for each of the three species. The two extract variables we analyzed were the amount of oleic acid (mg/g), and *n*-heptacosane (µg/g). The data for the three variables were not normally distributed, nor could a transformation be found that allowed the presumption of normality (Anderson–Darling, $\alpha = 0.05$). Non-parametric Wilcoxon tests were used to assess rank differences among the treatments with respect to each variable. Pairwise comparisons among the treatments were performed using the Steel–Dwass method.

The remaining analyses were performed using SAS software (version 9.4, SAS/STAT 15.1, SAS Institute Inc., Cary, NC, USA). For comparing the larvae/mg across the flash- or slow-frozen extracts of the three species, the GLM procedure with the LSMEANS option was used for a 2×3 analysis of variance, with a Tukey–Kramer adjustment performed to correct for experiment-wise error in multiple comparisons of the species and freezing method effects. Both factors were considered fixed effects and there were no random blocking factors within this experiment. The design was unbalanced, with sample sizes shown in Table 1.

For the control experiments involving only the application and evaporation of hexane, simple comparisons were made by obtaining the upper and lower 95% confidence limits of the mean proportions using the MEANS procedure in SAS. Overlap of these 95% confidence intervals of proportions in particular zones were used as a test of significant differences. The extract behavioral assay data, including experiments that involved odor treatments, were analyzed using a multinomial logistic regression model, also known as a generalized logit model; via the GLIMMIX procedure, utilizing a Laplace estimation method to fit a true log-likelihood function. All analyses were performed separately for each species. The multinomial response variable is the frequency count of observed insects in each of the four zones (Fig. 2). The zones are identified as: refuge 1 (hexane control), refuge 2 (odor), adjacent 1 (the half of side 1 that surrounds refuge 1), and adjacent 2 (the half of side 2 that surrounds refuge 2). For the extract experiments odor (high vs. low oleic) was a fixed effect, with the observed responses comparing attraction to the extract versus a hexane control. When using the dosage behavioral assay data, the statistical model and analyses are the same but dose is a classification variable in the multinomial logistic regression.

The subject unit in the model is the replicate plate. For each zone and fixed effect, generalized estimates and standard errors were produced via the SOLUTIONS option, and back transformed proportional means and standard errors of the means (SEM) were produced via the ILINK option. Using the proportional means as true means, 95% confidence intervals were calculated, and differences between the means of the compared refuges or sides were determined.

Results

Extract effects on chemical composition

For KB, most extraction methods tended to yield similar amounts of oleic acid, with the exception of simple rinsing of live insects with water before extraction (Fig. 3a). These samples produced a significantly lower amount of oleic acid at approximately an order of magnitude less of a concentration versus the other methods (Wilcoxon test: $\chi^2 = 30.93$, $4df$, $p < 0.0001$).

All other methods involved either not rinsing the insects, or rinsing away debris before or after freezing the insects to

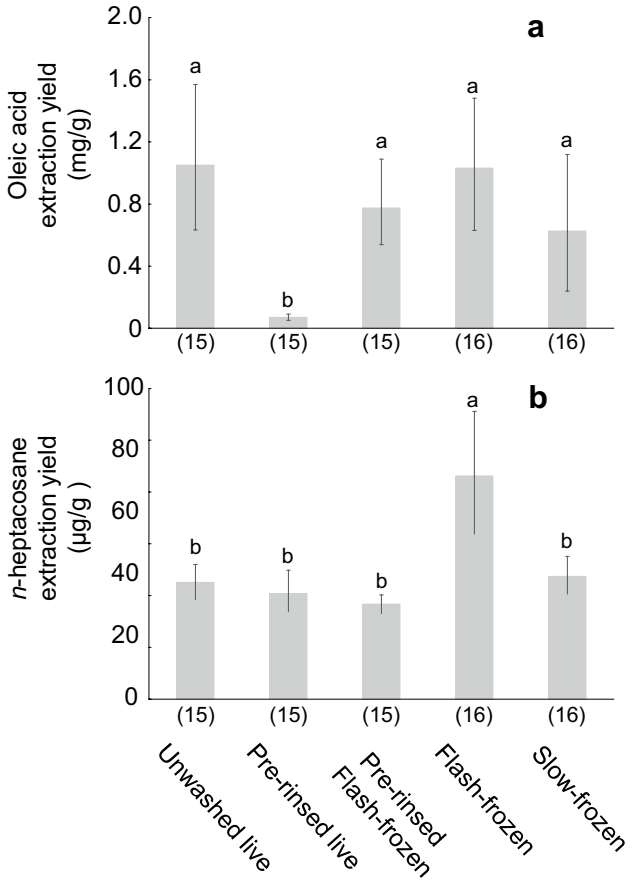

Fig. 3 Chemical composition of KB hexane extracts. Extraction treatments from left to right included unwashed live larvae, pre-rinsed live larvae, pre-rinsed before flash-freezing larvae, flash-freezing before rinsing, and flash-freezing before rinsing. All rinsing refers to the use of distilled water. Flash-freezing was in liquid nitrogen and slow freezing at − 20 °C. Variables analyzed include the per mass extraction yield of oleic acid (**a**) and *n*-heptacosane (**b**). The histogram depicts means and standard errors for each variable. Different letters indicate significant differences between treatments using the Steel–Dwass adjustment of a Wilcoxon test of rank differences. Sample size (number of extracts) are provided below the *x*-axis

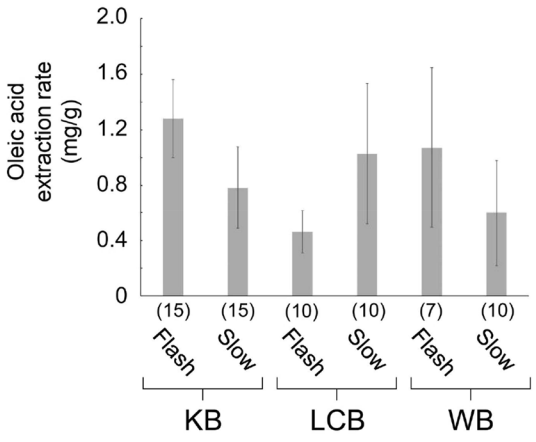

Fig. 4 The amount of oleic acid (mg/g) extracted from the three species KB, LCB, and WB when 4 ml(hexane)/g(larvae) was applied, depending on whether they were flash frozen in liquid nitrogen or frozen at – 20 °C before the extraction. There were no significant differences between treatments using the Steel–Dwass adjustment of a Wilcoxon test of rank differences. Sample size (number of extracts) are provided below the *x*-axis

kill them. While KB produced more oleic acid when flash-frozen rather slow-frozen, the trend was not statistically significant. Furthermore, oleic acid production was not affected by freezing method for any of the species (Wilcoxon test: $\chi^2 = 5.93$, 5*df*, $p = 0.3128$, Fig. 4). While oleic acid extraction was somewhat greater in flash-frozen versus slow-frozen samples of KB and WB, the reverse pattern was true for LCB. We thus generally observed high variability and no significant differences, regardless of whether flash-freezing or slow-freezing was used.

All of the specimens yielded statistically similar amounts of the most common hydrocarbon compound, *n*-heptacosane, with the exception of those flash-frozen in liquid nitrogen and rinsed afterward (Wilcoxon test: $\chi^2 = 21.76$, 4*df*, $p = 0.0002$, Fig. 3b). These specimens had nearly twice the amount of this hydrocarbon as those in other treatments, which all had nearly identical amounts. The variation in the concentration of *n*-heptacosane also increased when liquid nitrogen was used before rinsing with water. We do not report the corresponding data for *n*-heptacosane with respect to freezing technique among the three species, because there were no significant differences in oleic acid extraction amounts that could potentially be correlated.

Freezing effects on body weight across the species

There were differences in the average number of larvae in a gram of material with respect to both species and how the samples had been frozen. An analysis of variance showed a significant overall effect of the species and freezing technique ($F_{(5,49)} = 83.2$, $p < 0.0001$), as well as the main effects for species ($F_{(1,49)} = 94.2$, $p < 0.0001$), freezing technique ($F_{(1,49)} = 116.6$, $p < 0.0001$), and the interaction of the effects ($F_{(1,49)} = 35.5$, $p < 0.0001$). After performing a Tukey–Kramer adjustment, it was apparent that the flash-frozen WB were larger, and KB slow-frozen larvae the smallest, respectively, having lowest and greatest numbers of larvae/mg (Table 1). For all three species, the flash-freezing tended to result in the number of larvae per mg being lower than when frozen at − 20 °C, and closer to that of the live sample. This result indicates that more mass was being retained in the flash-frozen specimens, while mass was being lost during the slow-freezing process. However, the effect was clearly much greater for the KB and WB larvae, which both lost approximately a 50% of their mass when slowly frozen, while CB larvae lost very little mass.

Behavioral assay hexane controls: room and solvent effect

When two hexane solvent control refuges were provided to the larvae, there were no preferences for larvae to prefer the refuge on one side of the room versus the other (Fig. 5). There also was not a preference to move to one particular side of the entire arena. This was true for all three of the species, with all of the confidence intervals overlapping. The only trend observable within the data is that WB tended to be in the refuges more often than the other species. If we consider the two refuges together (not shown graphically), the confidence intervals for the probability of being in a refuges are as follows: (KB: 0.192, 0.325), (LCB: 0.274, 0.415), and (WB: 0.431, 0.575).

Likewise, there was no effect on movement of KB when hexane treatment was applied to one refuge while the other was left untreated (right side of Fig. 5). Individuals were equally likely to be in either refuge or on either side of the arena. Furthermore, the probabilities of presence in the refugees were similar to what was observed for the hexane versus hexane control.

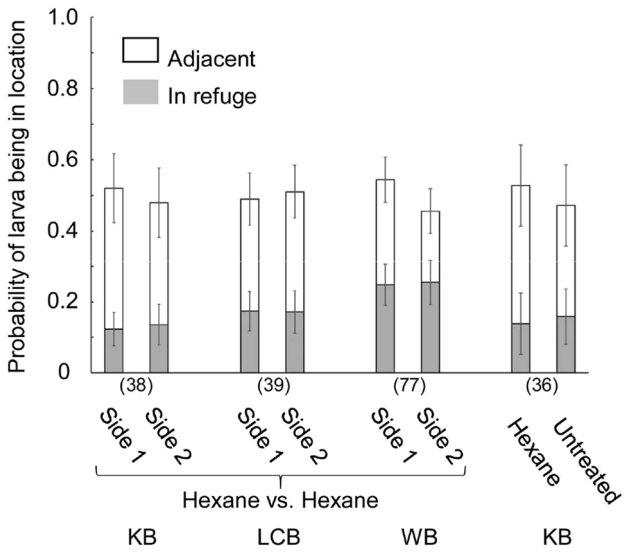

Fig. 5 Probability of larvae settling in each of the bioassay zones when two hexane refuges are provided for KB, LCB, and WB, or a hexane versus an untreated refuge are provided to KB. Side 1 versus Side 2 indicates consistent orientation of the refuges compared to the opposite walls of the room. For each comparison the percentages found within the refuges are indicated by grey shading with those in the adjacent half of each arena stacked above in white. Thus the entire bar represents the percentage on one side of the arena. The 95% confidence intervals are provided for the probability insects on the refuges (lower) and the combined number on the refuges and adjacent area (upper). All of the investigated intervals within the experiments overlapped, indicating no significant effects. Sample size are provided below the *x*-axis, between the histograms

Behavioral experiment 1: effects of oleic acid in whole body extracts

Larvae of all three species were more likely to be found on the side of the petri dish arena with the hexane control, and less likely to be on the side with the conspecific larval extract, only if it had a high oleic acid content (Fig. 6). For these same high oleic acid content extracts, the larvae were also more likely to be on or near the control refuge

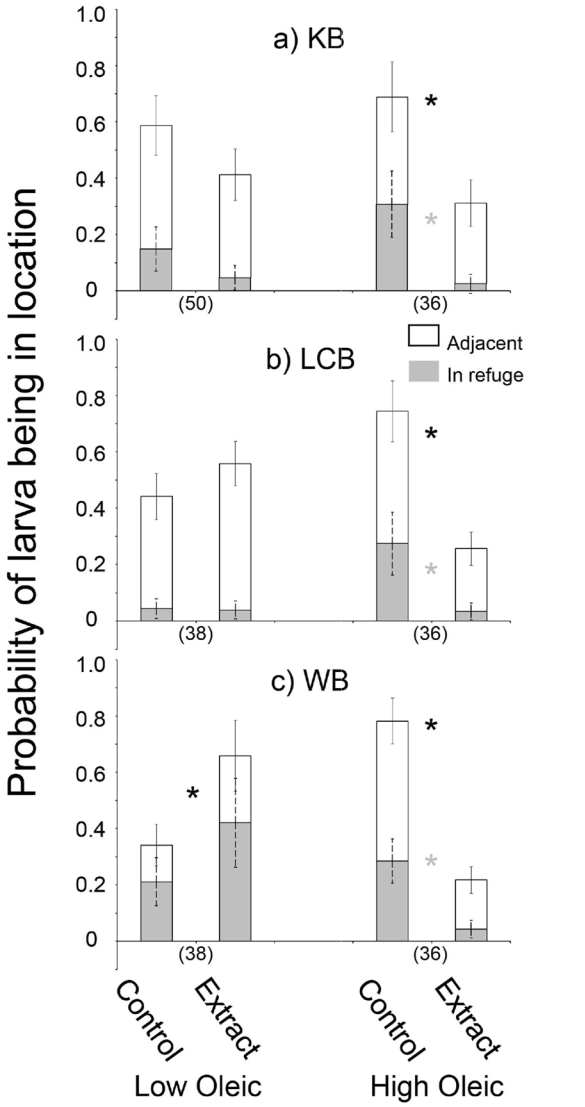

Fig. 6 Probability of larvae settling in each of the bioassay zones in the behavioral assays for **a** KB, **b** LCB, and **c** WB, depending on whether a low or high oleic acid extract was used as a choice. For each species and extract type, the zones included the control and treated refuges (grey shade) and adjacent half of each arena housing that refuge (white). The 95% confidence intervals are provided for the probability of insects being in the refuges (lower) and the refuge plus the adjacent area (upper). Grey and black asterisks are used to highlight non-overlapping intervals that indicate significant differences. Sample size are provided below the *x*-axis

in comparison to the refuge treated with the larval extract. Thus, the high oleic acid conspecific extract repelled the assembly of the larvae of all three species.

For both KB and LCB, there was no effect of the extract when it had a low oleic acid composition. Both the filter papers and the adjacent sides of the arenas had similar probabilities of attracting larva, regardless of whether or not the low oleic acid extract or a control dose of hexane was applied (Fig. 6a, b). However, WB was attracted to the low oleic acid extract-treated refuge and also had a greater likelihood of being on the associated side of the arena (Fig. 6c).

Behavioral experiment 2: effects of increasing doses of oleic acid

At the lowest dose at which synthetic oleic acid was provided to KB (1 µg), there were no significant differences in the probability of insects being located on the oleic acid-treated refuges than on the control refuges (Fig. 7). When considering entire sides including the refuges, there was very high variability in the preference, which was not statistically significant. As the concentration of oleic acid increased, there were typically more individuals on the control refuges than the oleic acid-treated refuges, with the exception of the highest dose of 5 mg, where the preference for the control was no longer significant. As the doses increased, there were also consistently significantly more insects on the control halves of the arena. At the very highest dose of 5 mg, an extremely high proportion preferred the control side to the extract side despite the control disk itself not being significantly preferred over the oleic acid-treated disk. Thus most insects still seem to have been repelled away from the treated refuge at this high dose, but were not sheltering in and around the control disk.

Discussion

The natural extracts obtained from *Trogoderma* did not, in most cases, cause attraction of larvae in our assays, but rather behavioral aversion. The only attraction we could document was for WB, when extracts had a low amount of oleic acid in them. This attraction was significant when considering the entire side of the arena, but not when the refuges alone were considered. It is plausible that other compounds in the extracts such as cuticular hydrocarbons may have elicited this attraction. The role of cuticular hydrocarbons as insect pheromones has been known for some time (Howard 1982), with ever increasing knowledge of the importance of such communication channels (Blomquist et al. 2018). It is also noteworthy that WB was more likely to enter the refuges than the other species in the control experiments, even when only hexane was used to treat the two refugia. This observed difference in the behavioral attributes of WB from KB larvae is consistent with other differences in larval responses to attractants previously reported in other bioassays (Morrison et al. 2020). While further research would be needed to explore these differences and investigate the potential role of cuticular hydrocarbon to cause attraction in *Trogoderma*, it is clear that high oleic acid concentrations repel all three species.

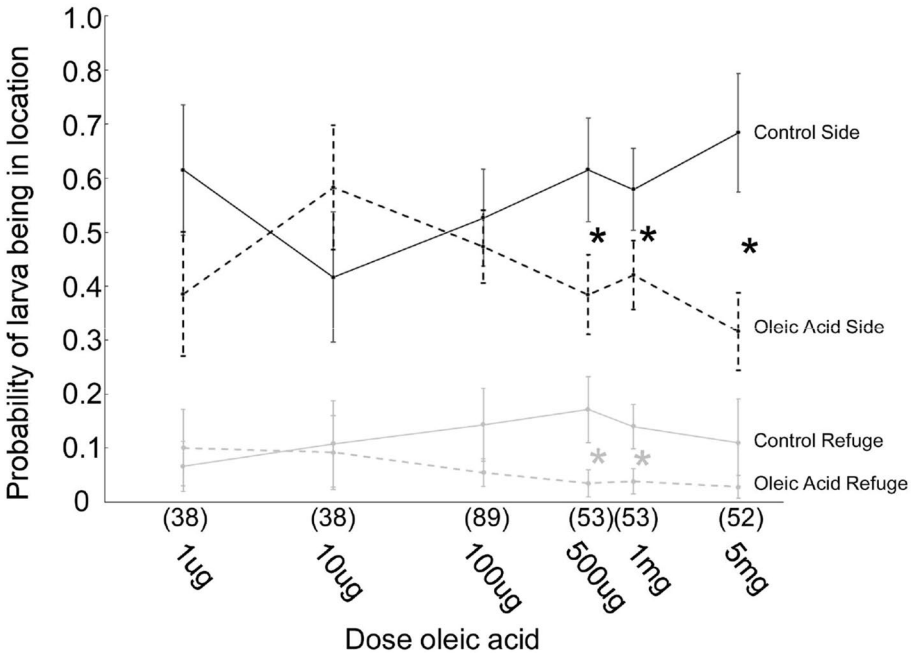

Fig. 7 Probability of larvae settling in each of the bioassay zones for increasing doses of oleic acid. The probability insects are on the control refuges (solid grey line) or odor-treated refuges (dashed grey lines) are provided with 95% confidence intervals. Likewise, the probabilities of being on either respective side of the arena (black soled and dashed lines) are provided with 95% confidence intervals. Asterisks of the respective colors highlight non-overlapping intervals that indicate significant differences. Sample sizes for each dose are listed on the *x*-axis

We determined that *Trogoderma* larvae can be repelled by the amount of oleic acid emitted from the equivalent of 4–12 frozen larvae. Larvae are often observed in clusters of hundreds, suggesting that freezing under natural conditions could trigger the aversive behavior. It remains to be determined if any other methods of killing can lead to the emission of comparable levels of oleic acid. Oleic acid is a very common component of the tissues of many animals and plants, including some that are likely to interact with these *Trogoderma* larvae. Prior work had noted that there was some attraction to oleic acid in adult KB, along with other long-chain fatty acids (Cohen et al. 1974). Oleic acid is also a component of the free fatty acids of KB frass and of wheat (Ikan et al. 1970). It is, therefore, very likely that the source of oleic acid in the unrinsed KB specimen treatment was from frass, because the colony jars from which they were removed had accumulated large amounts of frass from several generations of feeding and reproduction. However, it is also clear that freezing increased oleic acid production when such debris was rinsed off the specimens.

It was also previously determined that the exposure to fecal material could be a determining factor for causing diapause in KB (Karnavar and Nair 1969) and that the oleic acid in such material may play an important role in it induction (Ikan et al. 1970). Our results, considered in this context, suggest that oleic acid may also play a complex role with respect to mediating not just initiation of diapause, but also movement within areas where khapra beetle aggregate, such as infested material. It would be of interest to perform more extensive experiments that examine the effects of diapause upon both the production and response to oleic acid, using the extraction and behavioral assays described here. However, it is not possible to verify diapause status for any given individual based on visual observations. Such experiments would require controlled manipulations of rearing conditions to make the triggering of diapause more or less likely in groups of individuals. It has long been known that many factors contribute to larval diapause in KB (Burges 1963), and accumulated knowledge has evolved into established protocols for triggering diapause in KB (e.g., Gothi et al. 1984).

Despite the uncertainty of the diapause status of the test subjects in the present study, the emission of oleic acid and its repellency in all of the three *Trogoderma* species examined would suggest that diapause has little effect. While diapause in KB larvae is known to be induced by adverse environmental conditions and overcrowding (Hinton 1945; Hadaway 1955; Burges 1962; Wilches et al. 2016), it is not caused by overcrowding in WB (Loschiavo 1960; Partida and Strong 1975) and LCB (Strong 1975; Klein and Beck 1980). Thus, WB and LCB are less likely to enter diapause in colony conditions where overcrowding occurs, but other environmental conditions remain favorable. Following this expected pattern, there were relatively few WB and LCB larvae compared to adults in our colony jars (< 20%). Likewise, the vast majority of KB (> 80%) in our colonies are larvae at any given time, and this proportion always appears to be even higher in colony jars where there has been overcrowding after the production of several generations of offspring.

The use of larger larvae and the possible resultant bias toward females may also influence the results. There is behavioral evidence that the response of larger and smaller KB and WB larvae to semiochemicals can differ depending on whether smaller or larger larvae are used in assays (Morrison et al. 2020). For example larger, older larvae respond positively to the adult pheromone in a wind tunnel, while smaller younger larvae do not. As discussed above for diapause status, the only way to determine sex and precise instar number of larvae is to follow the development of each individual. Such research could be of interest, however, because the female bias of older larvae could have affected production and response to oleic acid. Moreover, any information about how diapause, larval age, or sex specificity affect behavior could become important for management considerations. For example, it has already been determined that long diapause periods make adult males less likely to respond to pheromones (Gothi et al. 1984).

The different freezing methods employed indicated that the stress of the larvae adapting to freezing is not a factor that ultimately contributes to oleic acid production. Flash-frozen insects, which were killed quickly without any time to react neuro-physiologically to the adversity, were just as likely to produce larger amounts of oleic acid. Thus, it is not currently possible to explain the high variation in the extractible amounts of oleic acid in most of the treatments. Additional exploration of the precise conditions under which the samples are created and stored would need to be further manipulated on a more granular scale. For example, slight variations in extraction time or rinsing procedures may have disproportionately large effects on the composition of the extracts. However, the different freezing methods did indicate some other differences in physiological properties of the three species that may be relevant. There was a significant loss of mass when WB and KB were slowly frozen, instead of flash-frozen, indicating that they may be exerting metabolic activity in an effort to attempt to survive the freezing procedure, or are prone to desiccation. However, LCB did not tend to lose much weight while being slowly frozen.

The potential applications for the demonstrated behavioral effects of oleic acid upon larvae remain a topic for further exploration. While the compound appeared to have little effect at the lower doses in this experiment, it may still be attractive to certain life stages in particular contexts (Cohen et al. 1974). However, the repellency described for older larvae here suggests the potential for adaptation into management strategies, such as creating push–pull

systems (e.g. Khan et al. 2010), or treated storage bags that more effectively protect commodities. Likewise, our study suggests that different cuticular hydrocarbons may have opposing net behavioral effects on larvae. Such behavior is inferred by the attraction of WB larvae to extracts with low oleic acid. Additionally, cuticular hydrocarbon attraction cannot be precluded in the other species, because an extensive array of doses of the relevant compounds was not tested. Thus, research that uncovers the complete array of behavioral mechanisms of short-range olfactory behavior in *Trogoderma* will be necessary to produce effective management tools.

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Author contributions MD, WM, and SM conceived and designed research. MD conducted experiments. KY and MD analyzed data. MD wrote the manuscript. All authors read, revised, and approved the manuscript.

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