

An Herbivore-Induced Plant Volatile From Saltcedar (*Tamarix* spp.) Is Repellent to *Diorhabda carinulata* (Coleoptera: Chrysomelidae)

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Abstract

The leaf beetle *Diorhabda carinulata* Desbrochers (Coleoptera: Chrysomelidae) was introduced into the United States in 1999 for classical biological control of the exotic woody invader saltcedar (*Tamarix* spp. L. [Caryophyllales: Tamaricaceae]). The recent southern expansion of the range of *D. carinulata* in the United States has precipitated conflict between proponents of biological control of *Tamarix* and those with concerns over habitat conservation for avian species. Several semiochemicals that mediate aggregations by this species have been reported, but no repellent compounds have been recorded thus far. We now report a repellent compound, 4-oxo-(*E*)-2-hexenal, induced by adult *D. carinulata* feeding on saltcedar foliage. Collection of headspace volatiles, gas chromatography mass spectrometry, and electroantennographic analyses identified 4-oxo-(*E*)-2-hexenal as an insect-induced compound that is antennally active. Behavioral and exposure assays were conducted to test for repellency and toxicity in adults and larvae. Headspace volatiles were also collected from adult males exposed to 4-oxo-(*E*)-2-hexenal to determine the impact exposure might have on the emission of the aggregation pheromone. 4-Oxo-(*E*)-2-hexenal elicited electrophysiological responses in adults of both sexes. Behavioral responses indicated repellency across multiple doses for reproductive *D. carinulata* adults but not in nonreproductive adults. Exposure assays indicated altered behaviors in first instar larvae and adults, but not in third instar larvae. Collection of headspace volatiles indicated that exposure to 4-oxo-(*E*)-2-hexenal did not alter emission of the *D. carinulata* aggregation pheromone by adult males. The continued development and field deployment of this repellent compound may provide a new tool for the management of *D. carinulata*.

Key words: olfactometer, coleoptera, chrysomelidae, biological control, *Tamarix*

Tamarix spp. L. (Caryophyllales: Tamaricaceae) (hereafter referred to as *Tamarix*) is a complex of invasive weeds that has invaded North American riparian ecosystems since its introduction in the early 1800s, and now occupies as much as 650,000 hectares (Jarnevich et al. 2013). *Tamarix* is considered to be the third most prevalent woody riparian plant in the western United States (Friedman et al. 2005, McShane et al. 2015). The leaf beetle *Diorhabda carinulata* Desbrochers (Coleoptera: Chrysomelidae), formerly *Diorhabda elongata deserticola* Chen (Coleoptera: Chrysomelidae), was tested,

approved, and released in the United States as a biological control agent for *Tamarix* (DeLoach 2003). Both adults and larvae of *D. carinulata* feed on *Tamarix*, rasping and scraping the leaves and photosynthetic stems (Dudley and Kazmer 2005). The high reproductive capacity of *D. carinulata* and its ability to form natural aggregations in the field through the use of an aggregation pheromone has resulted in one of the most effective weed biological control programs in the United States (Lewis et al. 2003; Cossé et al. 2005, 2006; Dudley and Bean 2012).

The distribution of *D. carinulata* was initially expected to be limited to the northern tier of the United States because the original releases did not establish south of the 38th parallel north. *Diorhabda carinulata* was not able to establish in the southwestern United States due to physiological constraints arising from a day-length controlled diapause (Bean et al. 2007a, Lewis et al. 2003). Specifically, when the photoperiod drops below 14 h and 39 min, the insects cease to reproduce, and instead develop fat stores and drop into the litter to overwinter as adults (Bean et al. 2007a, Bean et al. 2012). South of the 38th parallel, day length never exceeds the critical photoperiod that allows for reproduction. It was anticipated that *D. carinulata* would adapt to shorter critical daylengths, allowing it to expand its distribution, but the rate of change was expected to be slow (Bean et al. 2007a). However, rapid adaptation following its U.S. introduction resulted in a significant reduction in *D. carinulata*'s diapause-inducing critical photoperiod (Bean et al. 2012). *Diorhabda carinulata* now occupies vast areas south of the 38th parallel and is impacting *Tamarix* in these areas of the United States.

Although southward expansion of the range of *D. carinulata* may have been welcomed for *Tamarix* management, these burgeoning populations have also drawn *D. carinulata* into conflict over habitat used by the endangered southwestern willow flycatcher, *Empidonax traillii extimus* (Passeriformes: Tyrannidae). The southwestern willow flycatcher was listed as an endangered subspecies in 1995, and in 2005 *Tamarix* was named as a constituent element within the critical habitat for the species (US Fish and Wildlife Service 1995, Federal Register 2005). The southwestern willow flycatcher occupies riparian areas south of 38° N, so interaction between it and *D. carinulata* was not anticipated due to the expectation that the ranges of the two species would not overlap. However, rapid postrelease adaptation to shorter day lengths and expansion of *D. carinulata* southward into the range of the southwestern willow flycatcher raised concerns that *D. carinulata* could negatively affect this avian species. Rapid defoliation of *Tamarix* during the breeding and nesting season could result in the loss of adequate canopy cover, jeopardizing the viability of nesting sites. In addition to concerns over the specific loss of *Tamarix* canopy cover for nesting habitat, the rapid and repeated nature of *D. carinulata* defoliation could locally kill a significant number of *Tamarix* plants before native plant species could recover to reoccupy this ecological niche.

Gaffke et al. (2018, 2019, 2020) demonstrated that populations of *D. carinulata* can be directed to and retained in targeted areas through the field deployment of lures emitting an aggregation pheromone produced primarily by adult males. Application of this aggregation pheromone within the shared range of *D. carinulata* and the southwestern willow flycatcher could be used to temporarily minimize interaction between the species. Using a series of strategically placed pheromone lures to concentrate aggregations in specific locations could potentially divert beetles from forming aggregations on *Tamarix* with existing southwestern willow flycatcher nests. However, as local beetle populations increase, all proximate *Tamarix* are likely to be defoliated despite application of aggregating semiochemical treatments (Gaffke et al. 2018, 2019). Therefore, identification and strategic deployment of a repellent compound that could provide a long-lasting deterrent against the formation of aggregations by *D. carinulata* could significantly minimize harmful interactions between the biological control agent and the endangered southwestern willow flycatcher.

Diorhabda carinulata is known to avoid previously defoliated plants, suggesting that herbivore-induced compounds could be repellent to adults (Dudley et al. 2012). Although the behavioral

activity of many feeding-induced compounds has been characterized for *D. carinulata*, only compounds induced within 24 h of feeding have been investigated (Cossé et al. 2006). A starting point of investigation for repellent compounds could be the evaluation of herbivore-induced volatiles produced after feeding is initiated (De Moraes et al. 2001). In the present study, we report the identity of an herbivore-induced volatile compound that elicited antennal responses from *D. carinulata*, and which repelled the beetles in laboratory trials.

Materials and Methods

Insects

Diorhabda carinulata used in this study were sourced from a continuous laboratory culture initiated in the spring of 2016 from adult beetles field-collected in south-central Montana. These were the progeny from established populations originating from releases of beetles introduced from Fukang, Xinjiang Province, China (DeLoach et al. 2003, Lewis et al. 2003). The greenhouse-based cultures of *D. carinulata* were reared on potted *Tamarix* plants enclosed in insect rearing sleeve cages (MegaView Science Co., Ltd, Taichung, Taiwan). Reproductive adults were maintained on a 16:8 (L:D) h cycle at 30°C in a greenhouse while nonreproductive adults were maintained on a 12:12 (L:D) h cycle in a plant growth chamber (model E30B, Percival Scientific Inc., Perry, IN) set at 22°C. Supplemental lighting in the greenhouse was provided with GE MVR1000/C/U multi-vapor quartz metal halide bulbs (100,280 lumens) (General Electric Company, Cleveland, OH). Humidity was allowed to vary. Small cohorts of adults were dissected before experimentation to determine reproductive status. The presence of well-developed ovaries, ovarioles, and accessory glands indicated that individuals were in an active reproductive state, whereas if these structures were small and undeveloped, individuals were considered to be in reproductive diapause (Bean et al. 2007b). All individuals used in experiments were 1–4-wk old and actively feeding on potted plants. Reproductive adults were assumed to be mated as they were sourced from groups of both sexes. All individuals used in experiments were used only once. Laboratory-reared individuals used for behavioral trials represent the progeny of 12–16 generations of continuous laboratory culture.

Collection and Analysis of Headspace Volatiles

Collections of volatiles emitted by mated, feeding adults were conducted in a greenhouse using cylindrical glass collection chambers (inner diameter 95 mm, length 625 mm). Plants were infested with either 20 adult male or 20 adult female *D. carinulata*. Males and females were intentionally segregated to allow for detection of male- or female-produced semiochemicals (Cossé et al. 2005). Ten replicates were collected from male and female-infested plants. Volatiles were also collected from whole, undamaged *Tamarix* plants and from mechanically wounded *Tamarix* plants. The undamaged and damaged *Tamarix* plants were used as a reference source for plant-produced volatile compounds and collections were replicated four times. Mechanical wounding consisted of removing approximately 25% of the apical growing points from the plants with scissors. All *Tamarix* plants had approximate aerial volumes of 0.33 m³. Volatiles were collected for 3 d in a greenhouse with a 16:8 (L:D) h cycle at 30°C with supplemental light from GE MVR1000/C/U multi-vapor quartz metal halide bulbs (100,280 lumens) (General Electric Company, Cleveland, OH). To determine if volatiles were plant-produced or insect-produced, volatiles also were collected

from adults without host plant material. Since *D. carinulata* is known to produce semiochemicals in conjunction with feeding (Cossé et al. 2005), the number of individuals used for collections of volatiles was increased to 250 adults, and the collection time was reduced to 4 h. This was done to ensure that adults had recently fed during the volatile collection period. Adults aerations were started in the mid-morning around 9:00 a.m.

Traps used to collect volatiles contained 30 mg of Super-Q (Alltech Associates, Inc., Deerfield, IL) adsorbent, and were fixed in place at the apical opening of the collection chambers. Purified air was passed through the chambers at a rate of 100 ml min⁻¹. Collected volatiles were eluted from the traps into vials using dichloromethane (200 µl) and the samples were spiked with 10 µl of a 0.74 ng µl⁻¹ solution of 1-nonene in dichloromethane as an internal standard. Volatiles were analyzed using a gas chromatograph (GC, Agilent 6890 instrument; Agilent Technologies, Santa Clara, CA) coupled to a mass selective detector (MSD, Agilent 5973 instrument; Agilent Technologies, Santa Clara, CA). The GC was fitted with a HP-5MS column (Agilent Technologies, Santa Clara, CA) with a helium gas flow of 1.2 l min⁻¹. The starting oven temperature was set a 50°C with a 4 min temperature hold and was increased at 5°C min⁻¹ to 200°C and increased at 25°C min⁻¹ to 280°C. The inlet temperature was set at 250°C and transfer line was set at 230°C. The spectral scan range was set from 5 to 350 *m/z* (EI, 70 eV), and the gain factor was set to 4. Identification of compounds of interest were initially made by NIST mass spectral library and verified by retention time and mass spectra from an authentic standard.

Chemicals

4-Oxo-(*E*)-2-hexenal, a previously uncharacterized component of the volatiles emitted from *D. carinulata* feeding on *Tamarix*, was evaluated as a possible elicitor of behavioral activity for adult *D. carinulata* (Cossé et al. 2006). 4-oxo-(*E*)-2-hexenal, synthesized according to Moreira and Millar (2005), was diluted with dichloromethane to concentrations of 1, 10, 50, and 100 ng µl⁻¹ to provide a range of concentrations to assay. Aliquots of these four concentrations were used to assess the behavioral responses of *D. carinulata* adults exposed to 4-oxo-(*E*)-2-hexenal in an olfactometer. The compound (*E*)-2-hexenal (Sigma-Aldrich, St. Louis, MO), previously characterized as an attractant by Cossé et al. (2006), was diluted with dichloromethane to a concentration of 100 ng µl⁻¹ and was included as a positive control in electrophysiology studies (see Electrophysiology).

Electrophysiology

Laboratory-reared individuals were used to assess antennal responses of adult male and female *D. carinulata* stimulation by 4-oxo-(*E*)-2-hexenal, using 10 µl of the highest concentration of 100 ng µl⁻¹. Negative controls consisting of 10 µl dichloromethane and positive controls consisting of 10 µl of 100 ng µl⁻¹ (*E*)-2-hexenal in dichloromethane were also tested. The negative control of dichloromethane was included to account for responses to the solvent and to quantify mechanical stimulation of the antennae by moving air. A positive control was included to compare the antennal responses of *D. carinulata* to the previously uncharacterized 4-oxo-(*E*)-2-hexenal to the known behaviorally active (*E*)-2-hexenal (Cossé et al. 2006). Treatments were applied in the following sequence: solvent control, (*E*)-2-hexenal, solvent control, 4-oxo-(*E*)-2-hexenal, and solvent control with approximately 30 s between each treatment and was replicated ten times.

Electroantennography (EAG) was performed by inserting a drawn glass capillary filled with a 0.9% NaCl solution over a silver

electrode into the back of an excised *D. carinulata* head. The distal end of one antenna was connected to the recording electrode, identical to the reference electrode inserted into the back of the head (Supp Fig. 1 [online only]). The head was then positioned via micromanipulator into a glass odor delivery tube flushed with humidified air purified with an activated charcoal filter. The electrodes were connected to a serial data-acquisition interface amplifier, type IDAC-232 (Syntech, Hilversum, The Netherlands), which stabilized the input signal from the antennal preparation and amplified the signal. The airflow and volume of odor stimulus were controlled using standard settings from a CS-55 stimulus controller (Syntech, Hilversum, The Netherlands), with airflow set at 20 ml s⁻¹. The signal was processed and analyzed using EAG software (Syntech NL 2001).

Assays were conducted by applying 10 µl of solution to a 1cm² filter paper slip placed within a glass pipette connected to a controlled, purified air source. The tip of the glass pipette was inserted into the odor delivery tube after the solvent had evaporated from the filter paper, approximately 30 s. Once a stable base line was evident in electrophysiological traces displayed by the acquisition software, a 0.4 s puff of air was initiated, delivering the compound from the glass pipette into the airstream and over the antenna. The resulting signal from the antenna was recorded with the EAG software.

Two-Choice Olfactometer Bioassay

A 4-chambered 30 × 30 × 2.5 cm olfactometer (Sigma Scientific LLC, Micanopy, FL) with a 2-chamber adaptor was used to determine the response of reproductively active or diapausing adults to 4-oxo-(*E*)-2-hexenal. The olfactometer contained a central area with a vertical entry tube for placing test insects in the center of the apparatus. The 2-chamber adaptors were situated in the olfactometer to block two adjacent arms of the olfactometer, effectively creating a Y-tube style olfactometer. The olfactometer body, ports, and adapters were made of ultra-high molecular weight polyethylene and the cover was transparent Plexiglass. The olfactometer was used to assess locomotory responses in test insects, because a consistent flight response is difficult to obtain with *D. carinulata* in a small indoor enclosure. Air purified through activated charcoal filters was supplied to the olfactometer at a rate of 200 ml min⁻¹. The bioassays were conducted in a specifically designed bioassay room located at the Rocky Mountain Research Station Forestry Sciences Laboratory (USDA Forest Service, Bozeman MT), with overhead fluorescent lighting and muted background colors to minimize confounding visual cues. Preliminary trials indicated *D. carinulata* responded more readily with overhead fluorescent lighting versus in a dark room with a single point source light.

Adult beetles 1–4-wk old were starved and stored individually in 1.5 ml microcentrifuge vials for 17–20 h prior to testing. Bioassays were conducted in the late morning to early afternoon. For each bioassay, a 1.5 cm² wedge of filter paper was dosed with 10 µl of the treatment solution and placed in one of the odor source chambers of the olfactometer, while the other arm received 10 µl of dichloromethane on the filter paper wedge as a control. After the paired treatments were placed in their respective odor source chambers, the top of the olfactometer was removed and an individual *D. carinulata* was removed from its vial and placed in the vertical entry tube at the center of the olfactometer. The olfactometer cover was immediately secured and sealed. Each individual was allowed 3 min to respond to odor sources. When an individual entered one arm of the olfactometer, it was removed, and the results were recorded. If the individual did not enter an arm of the olfactometer in 3 min, it was recorded as not responding and was not included in subsequent

analyses. After each individual was tested, the olfactometer was sprayed with hot, soapy water, rinsed and wiped clean. A new treatment paper was added before each new test beetle was introduced. After half the bioassay cohort was tested, the order of placement of the odor treatments in the chambers was reversed. Treatments of 4-oxo-(*E*)-2-hexenal consisted of 10, 100, 500, and 1000 ng in 10 μ l of dichloromethane and the responses of 60 individuals were recorded for each dose.

4-Oxo-(*E*)-2-Hexenal Exposure Assays

Exposure assays were conducted to evaluate toxicity to both adult and larval *D. carinulata* as 4-oxo-(*E*)-2-hexenal is reported to rapidly cause paralysis in ants and crickets after exposure (Eliyahu et al. 2012, Noge and Becerra 2015). Exposure assays were conducted on two larval stages, first and third, to investigate possible effects of progeny age, size, and mobility on the toxic effects of 4-oxo-(*E*)-2-hexenal. Exposure assays were conducted using glass Petri dishes (9 \times 1.5 cm) containing a circle of Qualitative P5 filter paper (Fisher Scientific, Hampton, NH) treated with 4-oxo-(*E*)-2-hexenal or a solvent control. The edges of the filter paper were folded down to raise the filter paper 0.25 cm off the bottom of the Petri dish, preventing the tested individuals from directly contacting the test compound. A small wedge of filter paper treated with 4-oxo-(*E*)-2-hexenal or the solvent control was placed under the raised filter paper. Cohorts of individuals were collected from the greenhouse-based cultures in the early afternoon using a paint brush and brought into the bioassay room. After an individual was added to the Petri dish, the lid was placed back on top. Behaviors in the Petri dish were recorded every 12 min for 2 h for a total of 10 observations which were subsequently averaged. Preliminary trials indicated that the first and third instars exhibited two behaviors during the assays, with the larvae either walking or quiescent. Preliminary trials with adults determined there were five primary behaviors exhibited during the assay, with the adults walking, cleaning their bodies, cleaning their antennae, opening their elytra, or quiescent. Doses of 4-oxo-(*E*)-2-hexenal treatments consisted of 500 or 1,000 ng in 10 μ l of dichloromethane. The solvent control consisted of 10 μ l of dichloromethane. First instars were exposed to 500 ng, while the larger third instars and adults were exposed to 1,000 ng. Exposure assays were replicated 20 times.

Influence of 4-oxo-(*E*)-2-Hexenal on Pheromone Production

With the discovery of 4-oxo-(*E*)-2-hexenal being repellent to adult *D. carinulata* and from the reports of toxicity from the literature, an additional experiment was conducted to determine the possible effect of exposure to 4-oxo-(*E*)-2-hexenal on pheromone production. If males responded to elevated levels of 4-oxo-(*E*)-2-hexenal by decreasing pheromone production, this could enhance the compounds repellent effect in the field by preventing aggregations. Volatiles were collected from groups of adult males on *Tamarix* plants. Sixty males were placed in each of two of the previously described volatile collection chambers containing whole *Tamarix* plants in the greenhouse under the previously described conditions. Volatiles were then collected over a 4-h period starting at 8:00 a.m., after which the adsorbent traps were eluted for analysis. After the first 4-h collection period, 1,000 ng of 4-oxo-(*E*)-2-hexenal was added to one of the two same volatile collection chambers and volatiles were collected for an additional 4 h, starting at 12:00 noon. The first collection period determined baseline pheromone production for each group of males, while the second collection period determined

the effect of exposure to 4-oxo-(*E*)-2-hexenal on production of the aggregation pheromone by males. The daily rhythm of pheromone emission is not known, and the two collection periods, with and without the treatment will help to control for changes in pheromone emission during the collection periods. Volatile collection chambers containing designated control groups of males were not dosed with 4-oxo-(*E*)-2-hexenal during the second collection period. The same two-step collections of volatiles, pairing control and 4-oxo-(*E*)-2-hexenal treatments in the second step, were replicated seven times. Male *D. carinulata* produce two pheromone components, (2*E*,4*Z*)-2,4-heptadien-1-ol and (2*E*,4*Z*)-2,4-heptadienal (Cossé 2005), of which (2*E*,4*Z*)-2,4-heptadien-1-ol is the primary attractant, with (2*E*,4*Z*)-2,4-heptadienal as a minor component (Cossé 2005). The amounts of both components were quantified.

Statistical Analysis

Paired *t*-tests were used to analyze the EAG data. The maximum deflection in volts from the initial depolarization of the antenna in response to control puffs of solvent delivered immediately before and after puffs of (*E*)-2-hexenal or 4-oxo-(*E*)-2-hexenal were averaged and used as *t*-test controls. The average of the maximum deflection from the control puffs before and after the treatment stimulus accounted for desensitization of the receptor cells in the antennae over the duration of the EAG protocol. The differences in response ratio between (*E*)-2-hexenal: control and 4-oxo-(*E*)-2-hexenal: control were also analyzed using paired *t*-tests to determine whether EAG responses differed. The responses of *D. carinulata* in the exposure assays and olfactometer assays were analyzed using χ^2 tests. Differences in pheromone production after exposure to 4-oxo-(*E*)-2-hexenal were analyzed using a Welch Two Sample *t*-test on the difference in pheromone production between collections 2 and 1. All analysis was conducted using R software version 3.1.2.

Results

Analysis of Extracts of Headspace Volatiles

GC-MS comparisons of the extracts of volatiles identified a compound, unique to collection chambers containing *D. carinulata* and host plants, which had not been reported in previous studies (Cossé et al. 2006) (Supp Fig. 2 [online only]). The mass spectrum of this compound indicated a molecular weight of 112 amu, with significant fragments of *m/z* 83 and 55. A search of the mass spectral library NIST MS Search v.2.0 indicated a close match with 4-oxo-(*E*)-2-hexenal. The mass spectrum and retention time were subsequently found to match those of an authentic standard of 4-oxo-(*E*)-2-hexenal. Feeding by *D. carinulata* adults was correlated with the release of 4-oxo-(*E*)-2-hexenal from affected *Tamarix* foliage and photosynthetic stems at an average rate of 0.7 ± 0.2 ng per individual per day for the males ($n = 10$) and 2.6 ± 1.1 ng per individual per day for the females ($n = 10$). At no point was 4-oxo-(*E*)-2-hexenal detected in the extracts of volatiles from undamaged ($n = 4$) or mechanically damaged *Tamarix* plants ($n = 4$), or in extracts of volatiles from adults with no foliage present ($n = 4$).

EAG Responses

Significant antennal responses were elicited in EAG experiments using excised *D. carinulata* heads puffed with 1,000 ng of 4-oxo-(*E*)-2-hexenal (Fig. 1) or (*E*)-2-hexenal (Supp Fig. 3 [online only]) impregnated filter paper wicks. For the male beetles, greater depolarizations were elicited on stimulation with 4-oxo-(*E*)-2-hexenal (paired *t*-test $P = 0.002$, $n = 10$) and (*E*)-2-hexenal (paired *t*-test

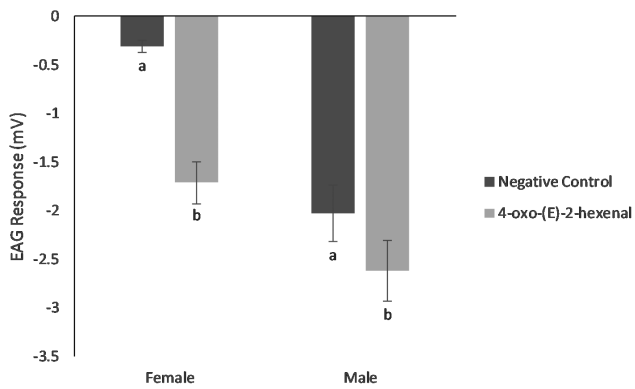


Fig. 1. Electroantennogram (EAG) responses of *D. carinulata* to a negative control (dichloromethane) and 1,000 ng of 4-oxo-(*E*)-2-hexenal. Within each sex, different letters below error bars denote statistical differences between the treatments.

$P = 0.052$, $n = 10$) than from stimulation with the dichloromethane control. Responses of antennae of males assessed using the 4-oxo-(*E*)-2-hexenal:control ratio and the (*E*)-2-hexenal:control ratio did not differ (paired t -test $P = 0.241$, $n = 10$) (Supp Fig. 4 [online only]), indicating similar responses to each of the two compounds. Antennal responses of females were analogous, with significantly greater depolarization when exposed to 4-oxo-(*E*)-2-hexenal (paired t -test $P < 0.001$, $n = 10$) and (*E*)-2-hexenal (paired t -test $P < 0.001$, $n = 10$) compared with the control. In addition, antennae of females responded similarly to 4-oxo-(*E*)-2-hexenal and (*E*)-2-hexenal (paired t -test $P = 0.58$, $n = 10$) (Supp Fig. 4 [online only]). Males had the greatest antennal depolarizations to 4-oxo-(*E*)-2-hexenal, but females had a much larger depolarization ratio when compared with the solvent control.

Two-Choice Olfactometer Bioassay

Reproductive male *D. carinulata* exhibited a preference for the control arm versus the treatment arm at the 500 ng and 1,000 ng doses of 4-oxo-(*E*)-2-hexenal, which indicated that the compound was repellent ($\chi^2 = 4.01$, $P = 0.045$, $n = 60$; $\chi^2 = 8.9$, $P = 0.003$, $n = 60$) (Fig. 2). Of the tested individuals at the 500 ng and 1,000 ng doses, 24 and 22% were nonresponders. There were no significant differences in responses between the 10 ng and 100 ng doses of 4-oxo-(*E*)-2-hexenal and the controls ($\chi^2 = 0.11$, $P = 0.74$, $n = 30$; $\chi^2 = 0$, $P = 1$, $n = 60$) (Fig. 2), and 14 and 23% of individuals were nonresponders. Only one dose of 4-oxo-(*E*)-2-hexenal was tested with nonreproductive adult males (1,000 ng), and the males displayed no preference between the treatment and the control ($\chi^2 = 0.11$, $P = 0.74$, $n = 30$) (Fig. 3). Of the tested nonreproductive males tested, 12% were nonresponders.

Reproductive female *D. carinulata* also exhibited a preference for the control arm of the olfactometer at the 100, 500, and 1,000 ng doses of 4-oxo-(*E*)-2-hexenal, indicating that the compound was repellent to both sexes ($\chi^2 = 14.2$, $P < 0.001$, $n = 60$; $\chi^2 = 10.6$, $P < 0.001$, $n = 60$; $\chi^2 = 10.6$, $P < 0.001$, $n = 60$) (Fig. 2). Of the tested individuals at the 100, 500, and 1,000 ng doses, 13, 8, and 20% were nonresponders. Reproductive females exhibited no preference at the 10 ng dose of 4-oxo-(*E*)-2-hexenal ($\chi^2 = 0.11$, $P = 0.74$, $n = 30$) with 11% being nonresponders. Only one dose (1,000 ng) of the 4-oxo-(*E*)-2-hexenal was tested for the nonreproductive adult females, and the females displayed no preference at this dose ($\chi^2 = 0.64$, $P = 0.42$, $n = 30$) (Fig. 3) with only 9% of individuals being nonresponders.

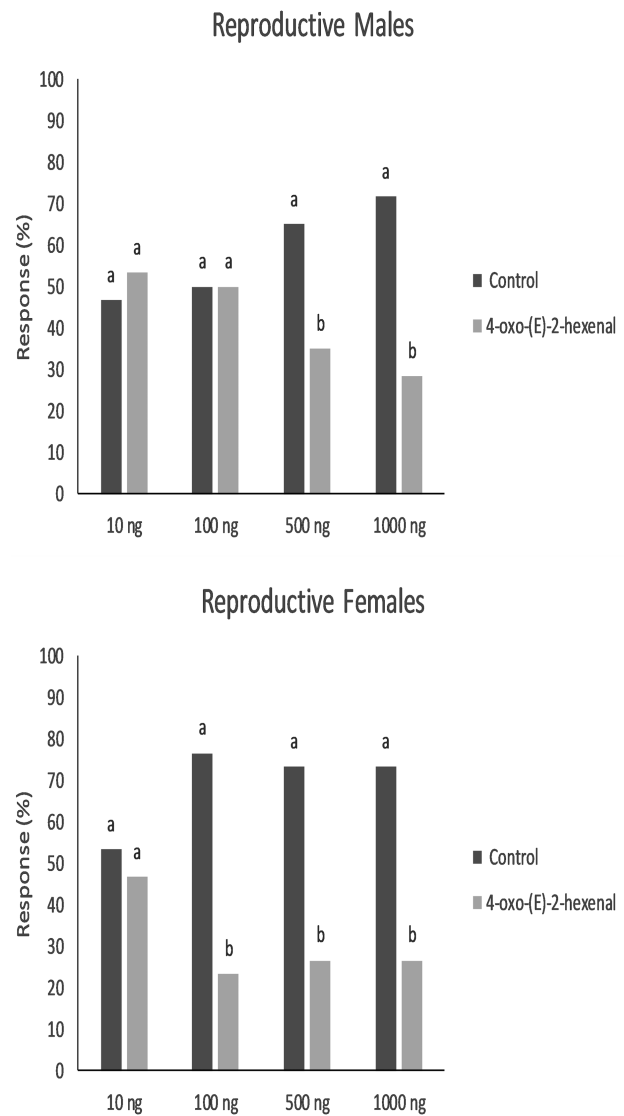


Fig. 2. Proportion behavioral response (%) of adult *D. carinulata* in a two-choice olfactometer to 4 doses of 4-oxo-(*E*)-2-hexenal versus dichloromethane (control). Response was recorded as percentage of individuals choosing the treatment or control arms in a 2-choice olfactometer. Different letters above the bars denote statistical differences between the treatments.

4-Oxo-(*E*)-2-Hexenal Exposure Assays

Adults exposed for 2 h to 1,000 ng of 4-oxo-(*E*)-2-hexenal exhibited significantly different behaviors compared with control adults ($\chi^2 = 15.35$, $P = 0.004$, $n = 20$) (Fig. 4a). Specifically, antennal grooming and elytral opening behaviors increased when the adults were exposed to 4-oxo-(*E*)-2-hexenal. Adults exposed to the compound cleaned their antennae and opened their elytra 7 and 9.5% of the time in the treated Petri dishes, compared with 2 and 0.5% of the time in untreated dishes. Two-hour exposure of first instars to 500 ng of 4-oxo-(*E*)-2-hexenal resulted in the individuals being quiescent 39.5% of the time compared with 19% of the time for control larvae ($\chi^2 = 5.36$, $P = 0.02$, $n = 20$) (Fig. 4b). In contrast, 2-h exposure of third instars to 1,000 ng of 4-oxo-(*E*)-2-hexenal did not result in noticeable differences in behavior compared with control larvae ($\chi^2 = 0.01$, $P = 0.92$, $n = 20$) (Fig. 4c).

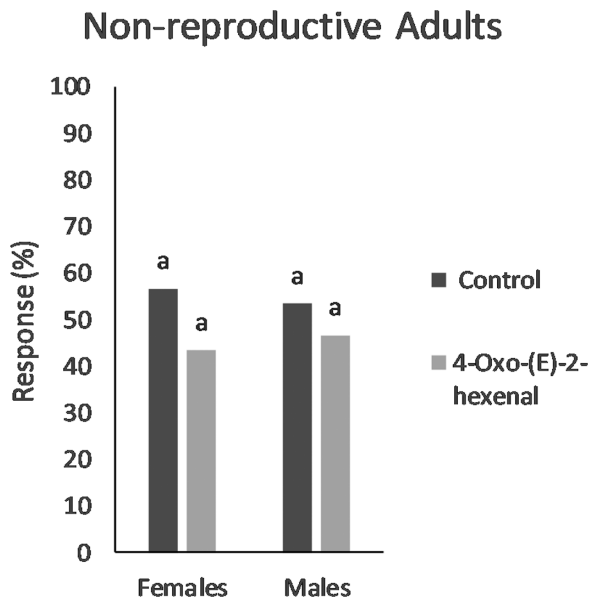


Fig. 3. Proportion behavioral response (%) of nonreproductive adult *D. carinulata* in a two-choice olfactometer to 1,000 ng of 4-oxo-(E)-2-hexenal versus dichloromethane (control). Response was recorded as percentage of individuals choosing the treatment or control arms in a 2-choice olfactometer. Within each sex, there were no significant differences between the treatments and control.

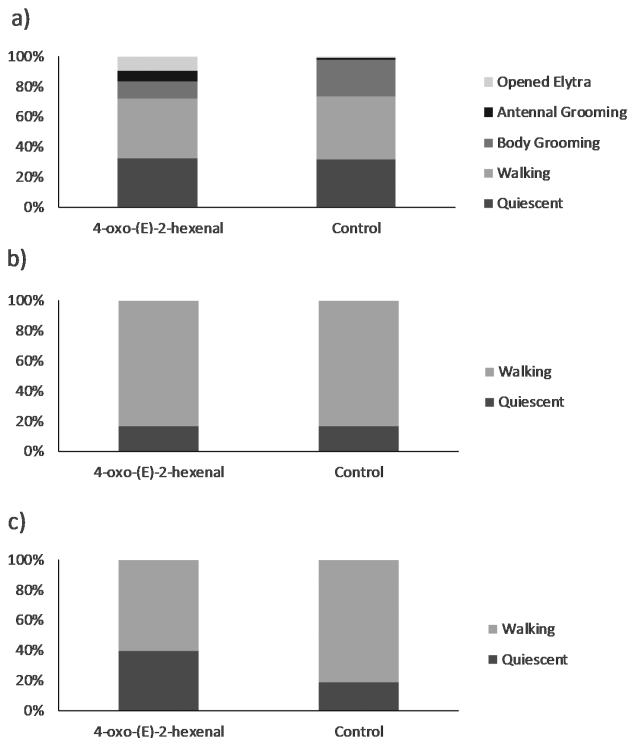


Fig. 4. Average percentage of time behaviors were observed over a 2-h period for (a) adults, (b) third instars, and (c) first instars exposed to 1,000 ng (a,b) or 500 ng of 4-oxo-(E)-2-hexenal (c) ($n = 20$).

Influence of 4-oxo-(E)-2-Hexenal on Pheromone Production

No differences in emission of (2E,4Z)-2,4-heptadien-1-ol or (2E,4Z)-2,4-heptadienal between the control and treatment were detected during

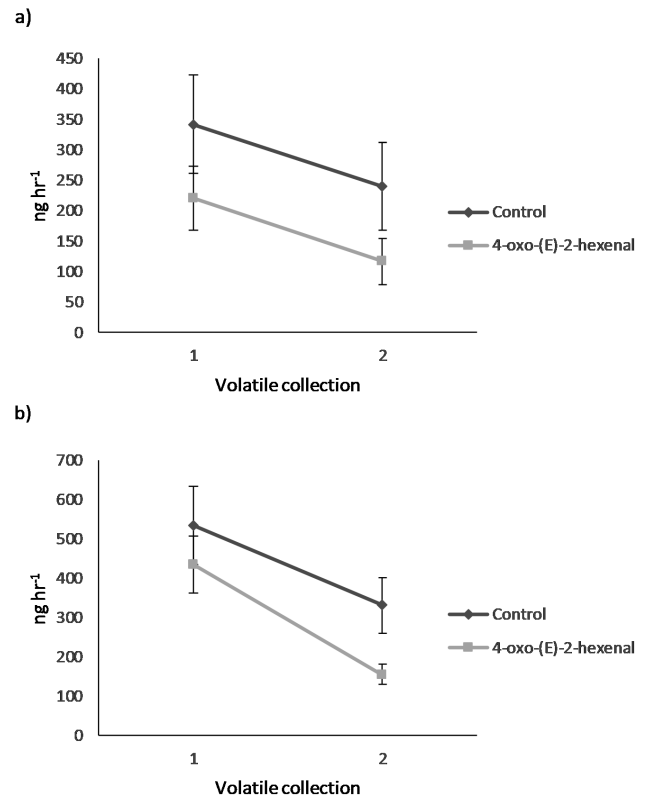


Fig. 5. Emission rates \pm SE (ng h^{-1}) of the aggregation pheromone components (a) (2E,4Z)-heptadien-1-ol and (b) (2E,4Z)-heptadienal by adult male *D. carinulata* before exposure to 1,000 ng of 4-oxo-(E)-2-hexenal (volatile collection 1) and after exposure (volatile collection 2). The control was not exposed to 4-oxo-(E)-2-hexenal during the second volatile collection. There was no statistical difference between emission rates of the pheromone after exposure to 4-oxo-(E)-2-hexenal compared with the control ($n = 7$).

the trials ($P = 0.98$, $t = 0.026$; $P = 0.53$, $t = 0.65$) (Fig. 5). However, there was a trend for lower emission of pheromone during the second collection period compared with the first collection for both the control and 4-oxo-(E)-2-hexenal exposed males. This result suggests a possible diurnal emission pattern for the pheromone, with greater amounts of the pheromone being produced in the morning versus the afternoon.

Discussion

The objective of our study was to identify semiochemicals repellent to adult *D. carinulata*. The compound 4-oxo-(E)-2-hexenal, apparently produced by *Tamarix* fed upon by adult *D. carinulata*, was confirmed as a repellent. This compound was repellent across multiple doses for reproductive males and females, but did not repel nonreproductive adults, even at the highest dose. Diapause-destined males and females both aggregate and remain on trees where they feed and eventually drop to over-winter in the litter. Reproductive insects generally remain on the move and do not linger on trees where feeding is heavy. Differential responses to 4-oxo-(E)-2-hexenal could play a role in this behavioral difference. First-instar larvae exposed to the compound exhibited different behavior compared with controls, spending significantly more time quiescent.

4-oxo-(E)-2-hexenal is a well-documented defensive compound of true bugs (Hemiptera: Heteroptera) (Aldrich 1988, Noge et al. 2012), and an herbivore-induced green leaf volatile produced from polyunsaturated fatty acid oxidation when plants are damaged by insect feeding (Long and Picklo 2010, Matsui et al. 2012, Silva et al.

2017). 4-oxo-(E)-2-hexenal is reported to be an insect paralytic, with paralysis being observed in ants and crickets after exposure to this compound (Eliyahu et al. 2012, Noge and Becerra 2015). Exposure to as little as 2 ng of 4-oxo-(E)-2-hexenal vapor was reported to paralyze and kill juvenile house crickets (*Acheta domesticus* L.) (Noge and Becerra 2015). Exposure to 500 ng or 1,000 ng of 4-oxo-(E)-2-hexenal was expected to result in paralysis of larval or adult *D. carinulata*. However, at the end of the exposure assays, none of the treated individuals exhibited signs of paralysis. While the first instars were significantly more quiescent upon exposure than controls, at the end of the trials, all first instars were capable of coordinated movement when stimulated by a soft bristle paint brush. Thus, our results indicate that *D. carinulata* have a high tolerance to 4-oxo-(E)-2-hexenal vapor in comparison to other insect species (Eliyahu et al. 2012, Noge and Becerra 2015).

An additional result of our study was the discovery of the difference in behavioral responses between reproductive males and females to 4-oxo-(E)-2-hexenal. Reproductive females were repelled by all but the lowest dose (10 ng), while reproductive males were only repelled by the two highest doses (500 ng and 1,000 ng). The increased sensitivity of females to this compound might be expected. Because *D. carinulata* are gregarious and aggregate in the field, there is a constant trade-off between increased competition for food and the benefits of being a member of an aggregation. This tradeoff has been demonstrated in a related species, the gregarious blue willow leaf beetle (*Phratora vulgatissima* L.) (Stephan et al. 2015). A meta-analysis for herbivorous insects has demonstrated that reproductive females select the habitat and hosts that are most advantageous for successful rearing of offspring (Gripenberg et al. 2010). When depositing egg masses, females benefit from their ability to assess host cues. A greater ability of females to perceive this herbivore-induced compound would allow for better oviposition choices if the compound indicated reduced host quality (Raitanen et al. 2013). Although reproductive males do not experience the same pressure to assure survival of offspring, the reproductive success of the males indirectly depends on resource quality. Male mating success is dependent on the sensory responses and behaviors of females, which should select for males that respond to cues similar to females (Ryan and Keddy-Hector 1992, Sakaluk 2000).

Non-reproductive males and females were not repelled by 4-oxo-(E)-2-hexenal, even at the highest dose tested. Considering that nonreproductive diapausing adults are seeking hosts to consume for development of metabolic reserves to overwinter and are not seeking potential mates, it is not surprising that they were not repelled by herbivore-induced odors because they are competing for limited resources as winter approaches (Rohr et al. 2002, Bean et al. 2007a). The nonreproductive adults could experience a compromised ability to overwinter if they were deterred from food resources that were previously fed upon but not fully depleted, as long as there is no cost to fitness from exposure to 4-oxo-(E)-2-hexenal (Bean et al. 2007a).

The 1,000 ng dose of 4-oxo-(E)-2-hexenal elicited different maximum antennal depolarizations according to the sex of the test beetles. Depolarizations in response to the 1,000 ng dose were greater for males than females. The difference in the magnitude of the depolarization was surprising, especially given the greater sensitivity of the females to low doses of 4-oxo-(E)-2-hexenal in the olfactometer bioassays. Males had the greatest antennal depolarizations, but females had a much larger depolarization ratio when compared with the solvent control. These differences in the magnitude of the depolarization and the greater ratio of the response to 4-oxo-(E)-2-hexenal versus the solvent control suggests a potential difference in the number or

the distribution of olfactory sensillae, because there are no obvious differences in the antennal morphologies between sexes.

Although the results of this study do not demonstrate field activity of 4-oxo-(E)-2-hexenal, the identification of this compound and our bioassay results suggest that definitive field experiments should be performed, possibly in conjunction with the male-produced aggregation pheromone, as well as with the known attractive host-plant volatiles (Cossé et al. 2005, 2006; Gaffke et al. 2018, 2019). It would be of particular interest to determine whether 4-oxo-(E)-2-hexenal could effectively inhibit the attractiveness of these semiochemicals, or if the compound is repellent only in the absence of other semiochemicals which typically elicit positive responses. While exposure of the males to 4-oxo-(E)-2-hexenal did not result in males down-regulating production of their pheromone, neither does it indicate that the repellent compound will lose its repellent effects in the presence of attractant semiochemicals. Before large-scale deployment of this compound is considered, the dosage required to elicit significant repellence in the field should be assessed. This compound is an effective defensive compound for true bugs and is toxic to some other insects, and its electrophilic structure suggests high doses might be broadly toxic to many species. Even though this compound is likely widely present in the environment, especially in stands of *Tamarix* infested with *D. carinulata*, elevated levels of the compound locally may have indirect negative effects on a variety of organisms. In addition to concerns about toxicity, studies to develop economical large-scale methods of synthesizing and formulating this rather unstable compound would be required before it could be considered for areawide deployment in IPM programs.

The field application of semiochemicals repellent to adults could have broad implications for the management of *D. carinulata* in the United States. Repellents could be used to resolve conflicts resulting from the deployment of *D. carinulata* for biological control of *Tamarix*, specifically conflicts arising over southwestern willow flycatcher critical habitat requirements and unintended *D. carinulata* defoliation of that habitat. The potential interaction of *D. carinulata* and the southwestern willow flycatcher repeatedly delayed the broad implementation of the *Tamarix* biological control program, and eventually resulted in the suspension of the program (Deloach et al. 2004, APHIS 2010, Bean and Dudley 2018). Although this suspension may reduce the potential risk that *D. carinulata* poses to the southwestern willow flycatcher, it also reduces the protection of natural ecosystems and native biodiversity that *Tamarix* biological control could have provided (Dudley and Deloach 2004, Dudley and Bean 2012). The purposeful deployment of the repellent compound, if deemed safe for avian species, within the critical habitat for the southwestern willow flycatcher could effectively 'push' *D. carinulata* out of specific locations, minimizing their damage to flycatcher nesting habitat while promoting defoliation of nearby nonnesting areas to facilitate recovery of suppressed native vegetation. Furthermore, strategic integration of the repellent compound with compounds known to stimulate aggregation could produce a 'push-pull' system that might allow for even more effective manipulation of *D. carinulata*'s spatial distribution (Cook et al. 2007; Gaffke et al. 2018, 2019, 2020).

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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