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Trap Assays of the Walnut Twig Beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), Reveal an Effective Semiochemical Repellent Combination

Jackson P. Audley[1] · Richard M. Bostock[2] · Steven J. Seybold[1]

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Abstract

Thousand cankers disease (TCD), is an invasive insect-disease complex caused by the walnut twig beetle, *Pityophthorus juglandis*, and fungal pathogen, *Geosmithia morbida*. Semiochemical interruption is a viable option for protecting walnut trees from *P. juglandis* attack. The goal of this study was to test beetle responses to potential repellent compounds. The results of five, flight-intercept assays are reported. Assays 1–3 tested four compounds at variable release rates: (*S*)-(−)-verbenone, (*R*)-(+)-verbenone, racemic chalcogran, and racemic *trans*-conophthorin. Trapping results indicated that the highest release rate tested for each compound was the most effective in reducing the number of beetles caught. (*S*)-(−)-Verbenone was the least effective, reducing *P. juglandis* trap catches by 66%. (*R*)-(+)-Verbenone reduced the number of *P. juglandis* by 84%. Neither enantiomer of verbenone performed as well as chalcogran or *trans*-conophthorin, which both reduced the number of beetles caught by ca. 98%. Following individual assays, the most effective compounds were tested in subtractive-combination assays. Combinations of high release rates for (*R*)-(+)-verbenone, *trans*-conophthorin, and two stereoisomers of limonene (tested in a previous study) were tested in two assays. The subtractive-combination assays were inconclusive in that trap catches were similar across all treatments. All combination treatments were highly effective, achieving approximately 99% reduction in the number of beetles caught. Based on the trapping results, commercial availability, and cost of the semiochemicals tested, we conclude that a combination of (*R*)-(+)-limonene, *trans*-conophthorin, and (*R*)-(+)-verbenone constitutes an effective tool for reducing *P. juglandis* trap catches.

Keywords Bark beetle · Host selection interruption · Semiochemical repellent · *Pityophthorus juglandis* · Thousand cankers disease · Walnut pest

Introduction

The walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), and an associated fungal pathogen, *Geosmithia morbida* Kolařík, Freeland, Utley, and Tisserat (Ascomycota: Hypocreales), comprise the insect-pathogen complex known as thousand cankers disease (TCD) (Kolařík et al. 2011; Seybold et al. 2016; Tisserat et al. 2009). Adult *P. juglandis* attack walnut (Juglandaceae *Juglans* spp.) and wingnut (Juglandaceae *Pterocarya* spp.) trees, subsequently introducing *G. morbida* fungal spores to the phloem tissue in which the beetles feed and reproduce (Hishinuma et al. 2016; Seybold et al. 2016; Tisserat et al. 2009). The feeding activity of the beetles (larvae and adults), combined with the localized phloem tissue necrosis (cankers) surrounding feeding galleries, disrupts nutrient flow within the tree, eventually girdling branches and, sometimes, the main stem. The resulting progressive, top-down dieback is emblematic of TCD (Seybold et al. 2016).

In recent decades, *P. juglandis* and *G. morbida* have expanded well beyond their historic geographic range. The putative native range includes the southwestern states of Arizona and New Mexico in the U.S. and south into northern Mexico, likely associated with Arizona walnut, *J. major* (Torr.) (Cranshaw 2011; Rugman-Jones et al. 2015; Seybold et al. 2016; Zerillo et al. 2014). Currently, *P. juglandis* and TCD have been confirmed from seven additional western states (California, Colorado, Idaho, Nevada, Oregon, Utah, and

Steven J. Seybold is deceased. This paper is dedicated to his memory.

✉ Jackson P. Audley
Jackson.audley@usda.gov; Jackson.audley@gmail.com

[1] USDA Forest Service, Pacific Southwest Research Station, Davis, CA 95618, USA

[2] Department of Plant Pathology, University of California, Davis, CA 95616, USA

Washington) and seven states in the eastern U.S.A (Indiana, Maryland, North Carolina, Ohio, Pennsylvania, Tennessee, and Virginia) (Cranshaw 2011; Fisher et al. 2013; Grant et al. 2011; Hadziabdic et al. 2014; Marshall 2015; Seybold et al. 2012, 2016, 2019; Tisserat et al. 2011). Additionally, the beetle and pathogen have established beyond the North American continent in Italy (Faccoli et al. 2016; Moricca et al. 2019; Montecchio et al. 2016). Walnuts are an important tree species, utilized commercially for nut and timber production (Leslie et al. 2010; Newton and Fowler 2009). Walnuts are also an important urban tree species, planted along roadways for shade (Graves et al. 2009) and an important component in native riparian forests, providing numerous wildlife benefits (Barnes et al. 1998). The continued economic and ecological viability of *Juglans* resources in North America is currently threatened by *P. juglandis* and TCD, particularly in California (Seybold et al. 2019). Despite this pronounced threat, management strategies to control *P. juglandis* populations or to mitigate the impacts of TCD remain limited to sanitation and state quarantines (Newton and Fowler 2009; Seybold et al. 2016, 2019).

Semiochemical interruption of the beetle's host selection behavior may provide an effective strategy for managing this invasive insect-pathogen complex. Previous studies of *P. juglandis* chemical ecology have produced evidence of in-flight recognition of and discrimination of host and non-host cues (Audley et al. 2020b; Hishinuma 2017; Homicz et al. 2020; Lona et al. 2020). Dispersing male *P. juglandis* initiate host colonization and produce the primary aggregation pheromone component, 3-methyl-2-buten-1-ol (MBO), upon successful location of a viable host tree (Seybold et al. 2015, 2016). This signal draws in both females and other males, inducing an aggregation of conspecifics, often culminating in mass attack of host trees. The production of the aggregation pheromone is particularly well known in bark beetle systems; however, evidence suggests that bark beetle species also actively recognize and respond to a complex suite of chemical cues (including host and non-host volatiles and pheromones of heterospecifics) that inform host searching and colonization behaviors (Borden 1997; Raffa et al. 1993; Raffa 2001; Seybold et al. 2018; Silverstein 1981; Wood 1980, 1982). Beetle populations are vulnerable to targeted management efforts during host searching and colonization phases, facilitating effective management strategies for pestiferous bark beetle species (Borden 1997; Seybold et al. 2018; Seybold and Fettig 2020; Silverstein 1981).

Semiochemical repellents have been used effectively to protect individual trees and stands of trees in several systems, perhaps most notably against the mountain pine beetle, *Dendroctonus ponderosae* Hopkins. Verbenone was found to be an anti-aggregation pheromone for both *D. ponderosae* and the closely related *D. frontalis* Zimmerman (Rudinsky et al. 1974), and has been commercialized as a tree protection tool, usually for use against *D. ponderosae*. Various formulations of this compound have been tested in the field and have shown success in protecting trees from attack (reviewed in Seybold et al. 2018). In one such instance, Fettig et al. (2012) reported less whitebark pine mortality when trees were treated with a blend of (*S*)-(−)-verbenone and acetophenone, (*E*)-2-hexen-1-ol, and (*Z*)-2-hexen-1-ol (non-host volatiles).

To date, most studies of semiochemical interruption have focused on conifer-infesting bark beetle systems. In recent decades, investigations of interruption in angiosperm-infesting systems have increased. Much of this work has focused on invasive ambrosia beetles (Coleoptera: Curculionidae: Scolytinae), for example, *Xylosandrus germanus* (Blandford) (see Ranger et al. 2013, 2014; VanDerLaan and Ginzel 2013). Bark and ambrosia beetles differ primarily in their feeding habits within a host tree (Kirkendall et al. 2015); however, they exhibit similar host searching and aggregation behaviors, both of which are primarily chemically mediated (Raffa et al. 2015). Given the evidence of non-host avoidance (Audley et al. 2020b; Homicz et al. 2020) and in-flight host discrimination among *Juglans* (Hishinuma 2017; Lona et al. 2020), the beetle's pest status, and its relatively restricted hosts (only *Juglans* spp. and *Pterocarya* spp.), *P. juglandis* is a good system for investigating semiochemical interruption in an angiosperm-infesting system.

The aim of this study was to evaluate several compounds with a goal of producing a viable semiochemical repellent tool for *P. juglandis*. The first compound was verbenone, 4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one, a bicyclic monoterpenoid. As stated, verbenone is a viable tool for protecting trees against attack from several bark beetle species and has even been considered as a universal bark beetle repellent (Seybold and Fettig 2020). Beyond its well documented repellent activity against conifer-infesting bark beetles, verbenone has been shown to reduce the numbers of angiosperm-infesting, ambrosia beetles caught in baited traps, including *X. germanus*, *X. compactus* (Eichhoff), *X. crassiusculus* (Motschulsky), and *Xyleborinus saxsesni* (Ratzeburg) (Burbano et al. 2012; Dodds and Miller 2010; Dudley et al. 2006; Ranger et al. 2013, 2014; VanDerLaan and Ginzel 2013). Most interruption studies, for both conifers and angiosperms, have tested different purities of (*S*)-(−)-verbenone, which is reflected by verbenone-based commercial formulations for tree protection. Thus (*S*)-(−)-verbenone (95% chemical purity, Bedoukian & ChemTica) is readily available. The (*R*)-(+) enantiomer has not been tested as widely, but has shown repellent activity in some systems, including against *D. valens* LeConte (Zhang et al. 2006). (*R*)-(+)-Verbenone was recently formulated into the proprietary SPLAT® technology (ISCA Technologies Inc., Riverside, California) and is readily available to test in the *P. juglandis* system.

Two other compounds are two spiroketals, chalcogran, 2-ethyl-1,6-dioxaspiro[4.4]nonane, and conophthorin, (*E*)-7-

methyl-1,6-dioxaspiro[4.5]decane. Chalcogran is the main component of the aggregation pheromone of sixtoothed spruce bark beetle, *Pityogenes chalcographus* (L.) (Francke et al. 1977), and has been reported from the bark of willow, *Salix* spp., and aspen, *Populus tremuloides* (Broberg et al. 2005). Conophthorin is a constituent of the volatile profile of the bark of several deciduous tree species (Byers et al. 1998; Francke et al. 1995; Huber et al. 1999, 2001; Zhang et al. 2002). Conophthorin is also a pheromone component in several bark beetle species. Birgersson et al. (1995) collected conophthorin from feeding male and female *Conophthorus coniperda* (Schwarz); however, it was not attractive to either sex but, rather, gave reduced male *C. coniperda* attraction to (+)-*trans*-pityol (DeGroot and DeBarr 2000). Dallara et al. (2000) collected conophthorin from male *P. carmeli* Swain and *P. nitidulus* (Mannerheim). Zhang et al. (2002) measured positive antennal responses to conophthorin in *D. micans* (Kugelann), three species of *Ips*, and five species of *Scolytus*. Behavioral responses to conophthorin among bark beetle species is mixed. While attractive to *P. carniceps* LeConte (Marsham) (DeGroot and DeBarr 2000) and to *X. germanus* (Dodds and Miller 2010; Ranger et al. 2014), conophthorin is repellent to *P. setosus* Blackman (Dallara et al. 2000) and male *P. pubescens* (Marsham) (López et al. 2013).

Both chalcogran and conophthorin have been recovered from the headspace of feeding male and female adult *P. juglandis* and small amounts of conophthorin were recovered from the headspace of walnut branches alone (Seybold et al. 2015). This led to initial screening of both compounds during the elucidation of *P. juglandis* aggregation pheromone components (Seybold et al. 2015). These tests revealed both chalcogran and conophthorin to be repellent to *P. juglandis*, suggesting the potential for either one or both compounds to be components of an anti-aggregation pheromone.

A final compound we were interested in was limonene. Limonene is a common monoterpene found in the volatile profiles of several species of both angiosperm and conifer trees (Shepherd et al. 2007) including species of *Juglans* (Campbell et al. 1998; Blood et al. 2018). Limonene elicits mixed behavioral responses in bark beetles. For instance, Miller (2007) found both enantiomers to be attractive to *C. coniperda*, whereas, Burbano et al. (2012) reported reduced trap catches for *X. compactus* and *X. crassiusculus*. Audley et al. (2020a) measured *P. juglandis* response to stereoisomers of limonene and found a strong repellent effect for both compounds in field trapping assays. *R*-(+)-limonene was also reported to reduce the number of *P. juglandis* caught in funnel traps baited with MBO in a field assay conducted in Tennessee (Blood et al. 2018), further emphasizing the importance of limonene as a potential repellent.

The first step in evaluating the aforementioned compounds is to test variable release rates of individual enantiomers of each compound of interest. We expected to see fewer beetles caught in traps treated with repellent compounds relative to those baited with the aggregation pheromone lure alone. We also expected to observe dose-dependent responses, with fewer beetles caught in traps treated with greater release rates. Following assays of individual compounds, we assessed combinations. We predicted that combinations would have either an additive or synergistic effect, further reducing the number of *P. juglandis* caught.

Methods

Experimental Design Field testing of *P. juglandis* responses were measured via a series of flight-intercept trapping assays. Assays for each of the four compounds individually (except for racemic chalcogran and *trans*-conophthorin, which were combined into a single assay) and for the two subtractive combination assays were conducted at the United States Department of Agriculture (USDA), Agricultural Research Service *Juglans* Collection at the UC Davis Wolfskill Experimental Orchards, Winters, Solano Co., CA (N 38.500335^0, W 121.977565^0). All five assays (Table 1) were conducted using 4-unit Lindgren funnels traps hung from 3 m long, metal conduit poles placed over 1 m long rebar, as per Seybold et al. (2013). A single 15 ml polyurethane screw top bottle, filled with 15 ml of 3-methyl-2-buten-1-ol (MBO) the primary aggregation pheromone component for *P. juglandis*, was attached to the third funnel at the bottom, except on the negative control traps (Seybold et al. 2013). For traps randomly assigned to a repellent treatment, the appropriate release device with the assigned compound was then attached to the same funnel, adjacent to the 15 ml MBO bottle. Traps assigned to the positive control only received the 15 ml MBO bottle.

Trapping blocks with 3–7 trapping stations (based on the number of treatments per assay, see Table 1) were established in the *Juglans* collection prior to each assay, such that each station was located adjacent to a visually declining *J. californica,* and 10 m away from the next trapping station within the block. Blocks were separated by at least 20 m. The collection cup of each trap was filled to a height of approximately 2–3 cm with propylene glycol (Seybold et al. 2013). Baited traps were then randomly assigned to a trapping station within each block, with a single block receiving a single replicate of each treatment. Trap catches were monitored daily. All trap samples were collected if the positive control trap had greater than five *P. juglandis* in at least half of the blocks. Based on this guideline, trap samples were typically collected every 1–3 d. After collecting samples, traps were re-randomized within each block. A minimum of 12 re-randomizations was made for each assay (Table 1). Assays were conducted from late April–mid-June or from August–

Table 1 Descriptions of the five, flight-intercept response assays conducted to evaluate potential semiochemical repellent compounds for the walnut twig beetle, *Pityophthorus juglandis*

Assay	Compound(s)	Dates	Blocks	Treat.*	*N*	Coll.**
1. Commercial verbenone, potentially universal bark beetle anti-aggregation	(*S*)-(−)-verbenone	8/23/16–9/21/16	4	5	340	17
2. SPLAT® Verb (R+) (ISCA), new commercial formula	(*R*)-(+)-verbenone	4/23/18–5/21/18	4	3	144	12
3. Potential anti-aggregation pheromones	Chalcogran & *trans*-conophthorin	9/12/17–10/3/17	4	5	280	14
4. Subtractive-Combination A	(*R*)-(+)-limonene, (*S*)-(−)-limonene, *trans*-conophthorin, (*R*)-(+)-verbenone	5/22/18–6/17/18	2	7	210	15
5. Subtractive-Combination B	(*R*)-(+)-limonene, (*S*)-(−)-limonene, *trans*-conophthorin	5/22/18–6/17/18	2	6	180	15

All assays were conducted during peak *P. juglandis* flight periods in the spring or autumn over a 2-year period, 2016–2018. Assays were performed in the United States Department of Agriculture – Agricultural Research Service National Walnut Germplasm Repository at the University of California Department of Agriculture and Natural Resources Wolfskill Experimental Orchards in Winters, California, *Treat. Indicates the number of treatments included in the assay. This number includes the positive, aggregation pheromone lure only, and negative, no semiochemicals, treatments. **Coll. Indicates the total number of sample collections and trap re-randomizations for each respective assay

October, coinciding with the bimodal annual peak of *P. juglandis* flight periodicity (Chen and Seybold 2014). All trap samples were taken back to the laboratory and sorted using a dissecting stereoscope. *Pityophthorus juglandis* were tallied by sex and preserved in 100% ethanol.

Verbenone Assays Two independent assays were conducted in order to test *P. juglandis* responses to the two enantiomers of verbenone. The first compound tested was (*S*)-(−)-verbenone, (1S,5S)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one. This compound was tested at three different release rates (low, medium, high) utilizing the flight intercept trapping guidelines previously described. Two pre-loaded release devices were purchased from ChemTica (Heredea, Costa Rica), a custom-made bubble cap with a ca. 5 mg/d release rate (rr) and a commercially marketed verbenone pouch (Beetleblock) with a 50 mg/d rr (both loaded with (*S*)-(−)-verbenone at 95% chemical purity, ChemTica). These two devices were the medium and high rr, respectively. To make the low rr device, a 1.5 ml polyethylene tube was loaded with 1.5 ml 95% (*S*)-(−)-verbenone (Bedoukian Research, Inc., Danbury, Connecticut) and 0.01 g of octrizole (Sigma-Aldrich, Inc., St. Louis, Missouri) to stabilize against oxidation (Robert Bedoukian Personal communication). This assay was conducted from 23 Aug– 21 Sept. 2016 with five treatments repeated across four blocks ($N = 340$, Table 1).

The second assay was conducted to test the relatively newly formulated (*R*)-(+)-verbenone, (1R)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one, in SPLAT® Verb (ISCA Technologies Inc., Riverside, CA). Only a single rr was tested in this assay given the relatively restricted commercial availability of the (*R*)-(+)- enantiomer. Per ISCA direction, we doled out 70 g of SPLAT® (7 g AI per 10 g carrier) to achieve approximately the same 50 mg/d rr as the commercial verbenone pouches (Rodrego Silva Personal communication). The 70 g was placed onto a 15 cm^2 piece of cardboard with a hole punched at the top for attachment to the funnel traps with wires. The assay consisted of three treatments repeated across four blocks ($N = 144$) and was conducted from 23 April–21 May 2018 (Table 1).

Potential Anti-Aggregation Pheromones Assay Chalcogran was only available as a racemate (96.0%, 2S, 5S and 2S, 5R, Sigma-Aldrich) and in small amounts. Based on its limited availability and high cost, only a single rr was tested, 100 μl chalcogran in a 400 μl polyethylene tube. Conophthorin was commercially available as the commonly occurring *trans* isomer [50% (+) and 50% (−), 95% *trans* and 5% *cis*, ChemTica] formulation. Two rr of *trans*-conophthorin were tested: a low rr, 200 μl *trans*-conophthorin in a 250 μl polyethylene tube inside a small plastic pouch with a reported rr of 0.3 mg/d (Etxebeste and Pajares 2011), and a high rr, 250 μl *trans*-conophthorin in a 400 μl polyethylene tube inside a plastic pouch with a reported rr of 3 mg/d (ChemTica Personal Communication). Given the small number of treatments, we tested both compounds in the same assay. The assay included five treatments (one chalcogran and two *trans*-conophthorin) repeated over four blocks ($N = 280$) and was conducted from 12 Sept–Oct. 2017 (Table 1).

Subtractive-Combination Assays Following the individual assays, we utilized a subtractive-combination method (Byers 1992) as a more efficient way of determining the most effective combination, rather than use a full factorial test or an additive approach. We selected the most effective compounds at the most effective rr from the previously described assays (see results) and two stereo-isomers of limonene from

previous assays not reported here (Audley et al. 2020a). All repellents were presented as a single treatment, then individual compounds were removed systematically for subsequent treatments. If the removal of a compound reduced efficacy (i.e., more *P. juglandis* caught) this was taken as evidence of the importance of the compound. Conversely, if no change in efficacy was observed, the compound removed was considered as not crucial.

Two subtractive-combination assays (henceforth, combination assays) were run concurrently. The first included the highest rr tested (most effective, see results) for (*R*)-(+)-verbenone, *trans*-conophthorin, (*R*)-(+)-limonene, and (*S*)-(−)-limonene (Table 1). (*R*)-(+)-Verbenone was selected over (*S*)-(−)-verbenone based on greater trap catch reduction (see results). Chalcogran was not included as the commercial source (Sigma-Aldrich) as it was no longer available. This experiment was broken into two assays as the amount of (*R*)-(+)-verbenone SPLAT® donated was only enough to replicate across two blocks. Thus, a second assay was run without (*R*)-(+)-verbenone (Table 1). Both assays were conducted 22 May–17 June 2018.

Data Handling and Statistical Analyses *Pityophthorus juglandis* adults from each trapping sample were sorted and tallied by sex using a microscope. Trap catches were normalized by the number of beetles caught per trapping day (rounded to the nearest whole). Data were analyzed for the total number of *P. juglandis* caught/d, male *P. juglandis* caught/d, and female *P. juglandis* caught/d. All analyses were conducted using negative binomial generalized linear mixed effects regression models (glmer.nb). Trap position and block were treated as random effects in all models except for the model for male trap catch in the (*S*)-(−)-verbenone assay, in which the best fit treated trap position as a fixed effect. The best fit models for both combination assays dropped trap position from the model. Final model selections were informed using Akaike Information Criterion (AIC) values and likelihood ratio tests (Zuur et al. 2009). Multiple means comparisons among treatments were made using *post-hoc* pairwise, least squares means (lsmeans) tests with the Bonferroni correction method using the emmeans package. Percent trap catch reduction was calculated for each treatment by comparing the proportion of beetles caught versus the positive control treatment (# per treatment/# per positive control). Finally, a two-sample test for equality of proportions (χ^2 test) with a continuity correction (prop.test) was used to compare the percent trap catch reduction observed in each assay across experiments using the same test. All analyses were conducted using the lme4, emmeans, and R's Base packages with R Statistical Software (version 3.4.4) via RStudio (version 1.2.1335) statistical software (R Core Team 2019).

Results

Verbenone Assays A total of 6793 (1524 males, 5269 females) and 2950 (864 males, 2086 females) *P. juglandis* were caught in the (*S*)-(−)- and (*R*)-(+)-verbenone assays, respectively. *Pityophthorus juglandis* responded to (*S*)-(−)-verbenone with a total trap catch reduction of 66.3%, comparing the 50 mg/d rr (771 beetles) and the positive control (MBO lure) traps (2285 beetles). Trap catch reduction was more pronounced for males than females. The number of males caught in the high rr rate traps was reduced by 93.2% (46 beetles) versus the MBO lure traps (672 beetles). For females, trap catch reduction was 55.1% (725 beetles) compared to the MBO traps (1613 beetles). Multiple means comparisons revealed that both the 5 mg/d and 50 mg/d rr of (*S*)-(−)-verbenone reduced the number of male *P. juglandis* caught compared to the positive control traps (Fig. 1). The number of females caught was only reduced by the 50 mg/d rr treatment (Fig. 1). Neither the mean number of males nor the mean number of females was reduced to a level comparable to the negative control (no semiochemicals) trap.

The total number of *P. juglandis* caught was reduced by 83.5% in the 50 mg/d rr (*R*)-(+)-verbenone traps (416 beetles) compared to the MBO lure only traps (2526 beetles). The number of males caught in the (*R*)-(+)-verbenone treatment traps (98) versus the MBO lure traps (763), constituted an 87.2% reduction. Similarly, only 318 female beetles were caught in the verbenone treated traps compared to 1763 in the positive control traps, an 82% trap catch reduction. Multiple means comparisons revealed that 50 mg/d rr of (*R*)-(+)-verbenone reduced the mean number of both male and female *P. juglandis* (Fig. 2). Despite reductions for both sexes, neither mean catch rate was reduced to the same level as in the unbaited negative control traps.

Comparisons by two sample equality of proportion indicated that the proportion of total beetles caught in the (*R*)-(+)-verbenone traps was lower than the proportion caught in the (*S*)-(−)-verbenone traps ($\chi^2 = 191.67$, $P < 0.001$). The results were similar for the proportion of females caught, with a lower proportion in the (*R*)-(+)-enantiomer versus the (*S*)-(−)- enantiomer traps ($\chi^2 = 284.44$, $P < 0.001$). When the proportions of males caught were compared by enantiomers, the number of males caught was lower for the (*S*)-(−)- enantiomer ($\chi^2 = 13.59$, $P < 0.001$). There were differences in proportions between the sexes for both enantiomers of verbenone. The proportion of males caught in the treatment versus control traps was lower than that for females in both cases ($\chi^2 = 306.34$, $P < 0.001$; $\chi^2 = 10.07$, $P = 0.002$ respectively).

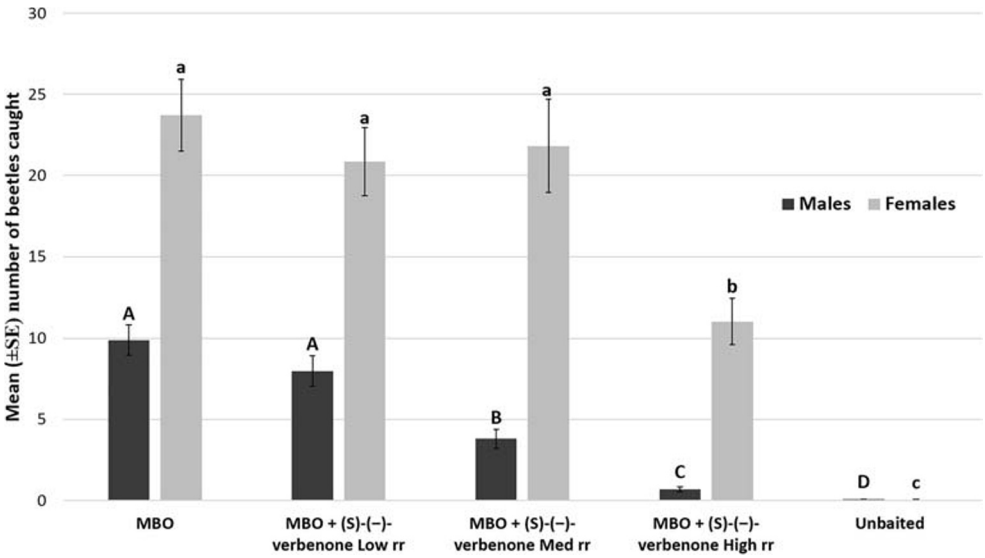

Fig. 1 Response of *Pityophthorus juglandis* to 4-unit Lindgren funnel traps baited with 3-methyl-2-buten-1-ol (MBO) alone, MBO and (*S*)-(−)-verbenone low release rate (rr), MBO and (*S*)-(−)-verbenone medium rr, MBO and (*S*)-(−)-verbenone high rr, or unbaited traps. All analyses were conducted by using negative binomial generalized linear mixed effects regression models in R. Multiple means comparisons were made among treatments by sex using post-hoc pairwise, least squares means tests with the Bonferroni correction method. Means with the same letters are not different ($\alpha = 0.05$)

Potential Anti-Aggregation Assay A total of 1771 adult *P. juglandis*, 519 males and 1253 females, were caught during the trapping period, 12 Sept–3 Oct. 2017. The generalized linear mixed effects regression model indicated differences in the mean number of beetles caught among treatments. Post hoc multiple means comparisons indicated that the chalcogran and both *trans*-conophthorin treatments reduced the mean numbers of male and female *P. juglandis* caught (Fig. 3) to a level similar to that of the unbaited traps. The effects of semiochemical treatments on female trap catches was less pronounced. Female beetle response was different for the two rr of *trans*-conophthorin, with the 3 mg/d rr reducing the number of females caught more effectively than the 0.3 mg/d rr ($z = 3.74$, $P = 0.002$; Fig. 3). No difference in female beetle response was found between the high rr of *trans*-conophthorin and chalcogran; however, neither compound reduced female trap catches to the same level as unbaited traps (Fig. 3).

The chalcogran and high rr of *trans*-conophthorin treatments both reduced the total *P. juglandis* caught by 98% (32 and 29 total beetles respectively) compared to the MBO lure traps (1625 beetles). The responses of male and female beetles were similar for both compounds. The chalcogran treatment conferred reductions of 98.6% (7/495 beetles) and 97.8% (25/1134 beetles) for males and females, respectively. The high rr of *trans*-conophthorin yielded similar reductions, 99.2% (4/498 beetles) for males and 97.9% (24/1134 beetles) for females. Two sample equality of proportion comparisons

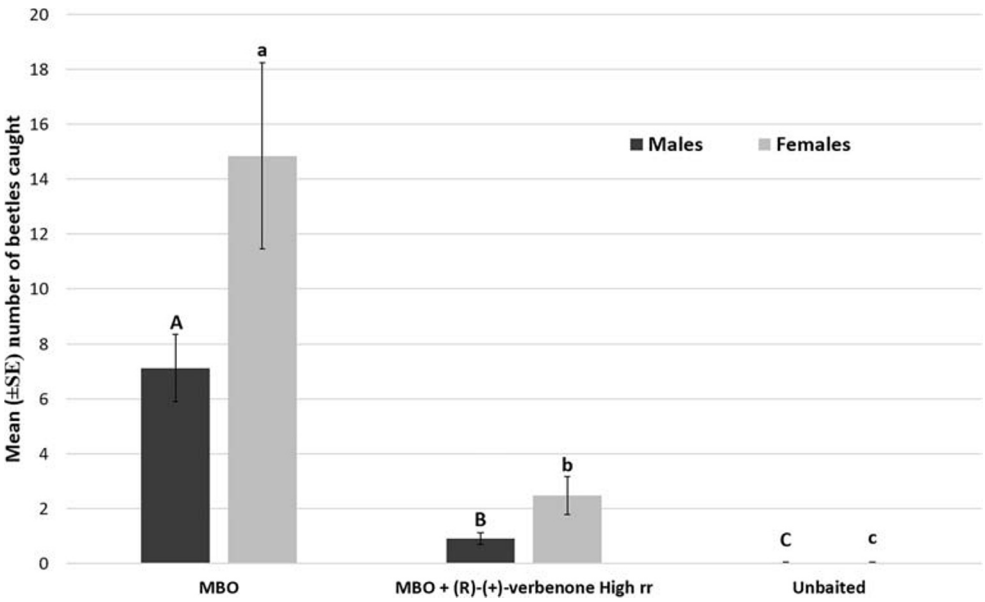

Fig. 2 Response of *Pityophthorus juglandis* to 4-unit Lindgren funnel traps baited with 3-methyl-2-buten-1-ol (MBO) alone, MBO and (*R*)-(+)-verbenone SPLAT® high release rate (rr), or unbaited traps. All analyses were conducted by using negative binomial generalized linear mixed effects regression models in R. Multiple means comparisons among treatments by sex were made using post-hoc pairwise, least squares means tests with the Bonferroni correction method. Means with the same letters are not different ($\alpha = 0.05$)

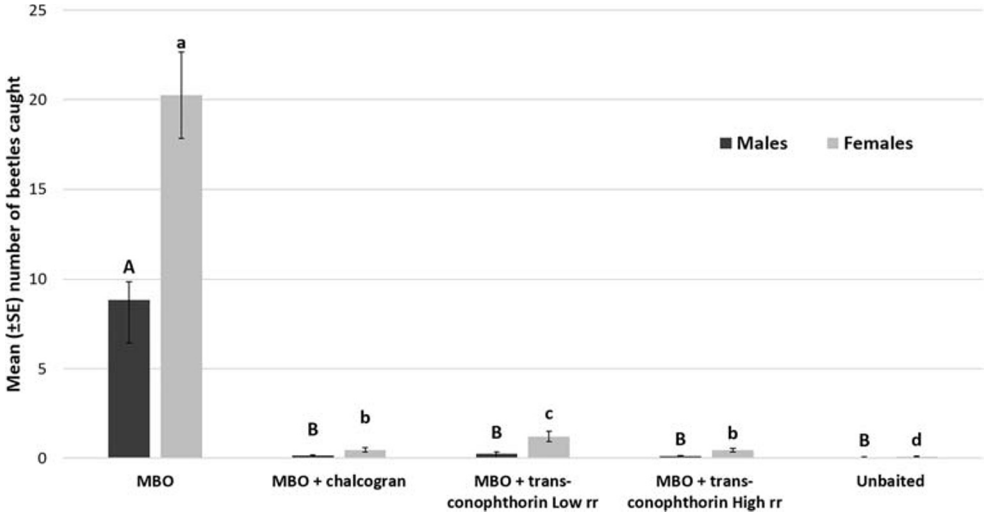

Fig. 3 Flight-intercept response of *Pityophthorus juglandis* to 4-unit Lindgren funnel traps baited with 3-methyl-2-buten-1-ol (MBO) alone, MBO and racemic chalcogran, MBO and racemic *trans*-conophthorin low release rate (rr), MBO and racemic *trans*-conophthorin high rr, or unbaited traps. All analyses were conducted by using negative binomial generalized linear mixed effects regression models in R. Multiple means comparisons among treatments by sex were made using post-hoc pairwise, least squares means tests with the Bonferroni correction method. Means with the same letters are not different ($\alpha = 0.05$)

indicated that the proportions of total, male, and female *P. juglandis* caught across the two semiochemical treatments were not different (total beetles: $\chi^2 = 0.07$, $P = 0.796$; males: $\chi^2 = 0.37$, $P = 0.544$; females: $\chi^2 = 0.0$, $P = 1.0$). Similarly, when male and female responses were compared within each compound, no differences were found (chalcogran: $\chi^2 = 0.75$, $P = 0.398$; *trans*-conophthorin: $\chi^2 = 2.76$, $P = 0.097$).

Subtractive-Combination Assays During the first subtractive-combination assay (Combo A), a total of 791 *P. juglandis* were caught, comprising 242 males and 549 females. The GLMM model indicated that the MBO lure (positive control) treatment was the only significant treatment variable for total beetle catch, male beetle catch, and female beetle catch analyses. The positive control traps caught an average of 25.7, 7.7, and 17.97 beetles for each respective category. Conversely, all other treatments averaged fewer than a single beetle across categories. Post hoc multiple means comparisons indicated that the MBO lure treatment caught more male and female beetles than all other treatments (Fig. 4). Conversely, no

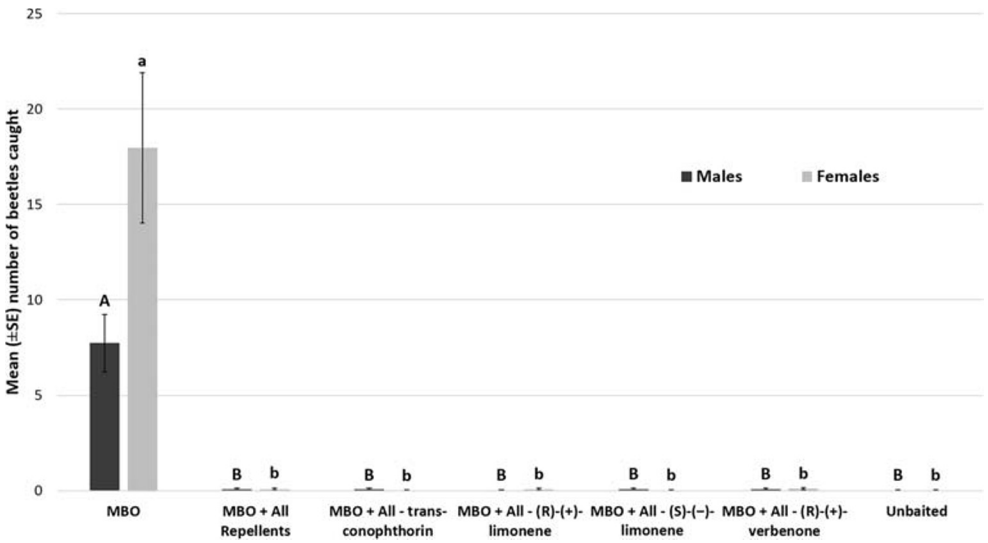

Fig. 4 Combination A assay of flight-intercept response of *Pityophthorus juglandis* to 4-unit Lindgren funnel traps baited with 3-methyl-2-buten-1-ol (MBO) alone, MBO plus (*R*)-(+)-limonene, (*S*)-(−)-limonene, racemic *trans*-conophthorin, and (*R*)-(+)-verbenone all at a high release rate (rr; All repellents), MBO plus All except for racemic *trans*-conophthorin, MBO plus All except for (*R*)-(+)-limonene, MBO plus All except for (*S*)-(−)-limonene, MBO plus All except for (*R*)-(+)-verbenone, or unbaited traps. All analyses were conducted by using negative binomial generalized linear mixed effects regression models in R. Multiple means comparisons among treatments by sex were made using post-hoc pairwise, least squares means tests with the Bonferroni correction method. Means with the same letters are not different ($\alpha = 0.05$)

differences in mean number of beetles caught were found. Each of the combination treatments in the Combo A assay reduced *P. juglandis* catch by greater than 99% compared to the MBO lure traps.

A total of 2193 *P. juglandis*, comprising 660 males and 1533 females, were recovered from traps in the two trapping blocks during the second subtractive-combination assay (Combo B). The MBO lure and "No *trans*-conophthorin" tretaments were highly significant in the models for both total beetle and female beetle catches. All treatments were significant in the model for male catches. Most of the beetles were recovered from the MBO lure traps. These traps averaged 73.1, 22.0, and 51.1 beetles for total, male, and female catches respectively. As reported for Combo A, all combination treatments in Combo B averaged fewer than a single beetle across categories. Multiple means comparisons for the total beetle catches indicated a slight increase in the number of *P. juglandis* recovered from traps when *trans*-conophthorin was removed compared to the combination treatment including all semiochemicals ($z = 2.99$, $P = 0.042$). The "No *trans*-conophthorin" treatment was not different from either the "No (*R*)-(+)-limonene" or "No (*S*)-(−)-limonene" treatments. When analyzed by sex, however, all variables were different for male trap catch and only the MBO lure treatment was different from all other treatments for female beetle responses (Fig. 5). Similar to Combo A, all the semiochemical treatments in Combo B reduced trap catch by ca. 99% compared to the positive control.

Two sample equality of proportion comparisons were conducted for the "All" treatments from both combination assays. No differences in proportions were found for total beetle catch ($\chi^2 = 2.1$, $P = 0.148$), male beetle catch ($\chi^2 = 0.9$, $P = 0.343$), or female beetle catch ($\chi^2 = 0.27$, $P = 0.6$). Both combination treatments within each respective assay also elicited a similar reduction for male and female *P. juglandis* (Combo A: $\chi^2 = 0.105$, $P = 0.746$; Combo B: $\chi^2 < 0.001$, $P = 1.0$).

Trap Catch Reductions across Assays As a final measure of repellent efficacy, the percent trap catch reductions for the most repellent treatment (i.e., that which conferred the greatest reduction in trap catch relative to the positive control) from each of the five assays were compared across the three levels (total, male, female). All of the equality of proportions comparisons are summarized in Fig. 6. The two combination treatments had the greatest amount of trap catch reduction, achieving reductions >99% across all levels (Fig. 6). Both *trans*-conophthorin and chalcogran also had trap catch reductions ranging from 97.9–99.2% and 97.8–98.6%, respectively. Both enantiomers of verbenone reduced trap catches by a significantly lesser percent (Fig. 6). (*S*)-(−)-Verbenone achieved a significantly greater reduction for male *P. juglandis* catch (93.2%) compared to those of all three levels for (*R*)-(+)-verbenone; however, the (*R*)-(+)- enantiomer was far more consistent with reduction percentages ranging from 82 to 87.2%. Conversely, the (*S*)-(−)- enantiomer

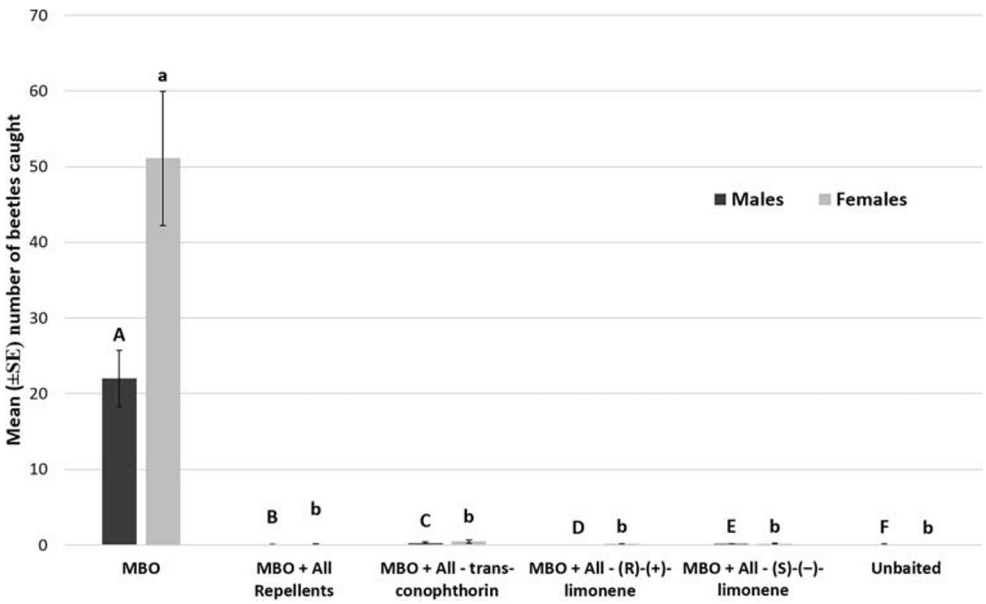

Fig. 5 Combination B assay of flight-intercept response of *Pityophthorus juglandis* to 4-unit Lindgren funnel traps baited with 3-methyl-2-buten-1-ol (MBO) alone, MBO plus (*R*)-(+)-limonene, (*S*)-(−)-limonene, and racemic *trans*-conophthorin all at a high release rate (rr; All repellents), MBO plus All except for racemic *trans*-conophthorin, MBO plus All except for (*R*)-(+)-limonene, MBO plus All except for (*S*)-(−)-limonene, or unbaited traps. All analyses were conducted by using negative binomial generalized linear mixed effects regression models in R. Multiple means comparisons among treatments by sex were made using post-hoc pairwise, least squares means tests with the Bonferroni correction method. Means with the same letters are not different ($\alpha = 0.05$)

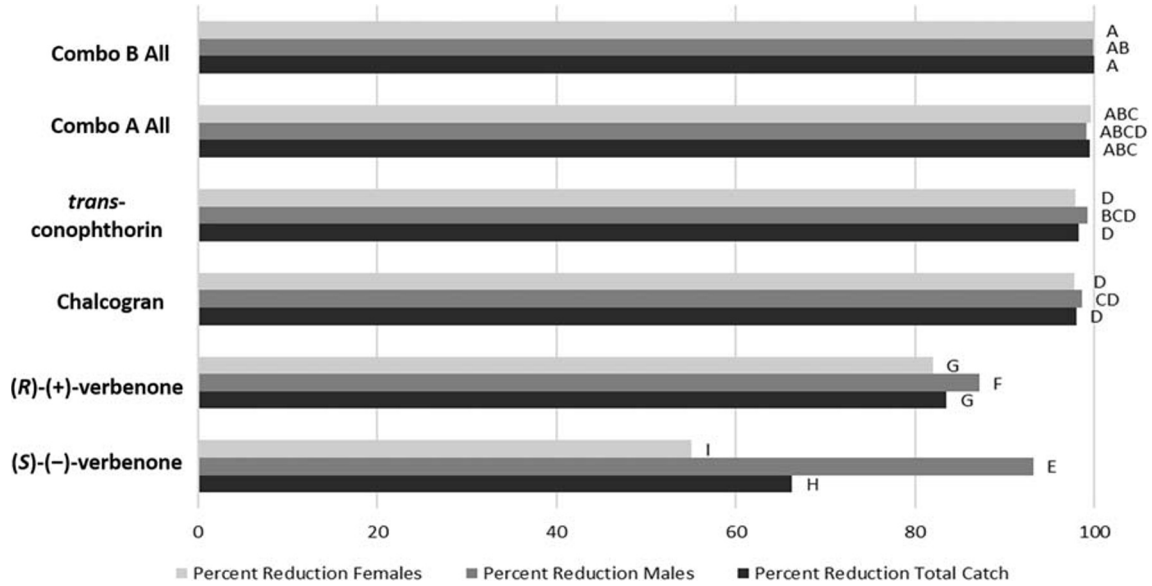

Fig. 6 Percent trap catch reduction comparisons across five assays measuring *Pityophthorus juglandis* flight-intercept responses to Lindgren funnel traps baited with potential repellent volatile compounds. Percent trap catch reductions were determined as number of beetles caught in the most effective repellent treatment traps/number of beetles caught in the 3-methyl-2-buten-1-ol (MBO) traps. Percent trap catch reduction was determined for total, male and female beetle catch for each compound. Pairwise comparisons were made using a two-sample test for equality of proportions with a continuity correction (prop.test) in R. Means with the same letters are not different ($\alpha = 0.05$)

performed relatively poorly for female beetle responses, reducing catch by only 55.1% (Fig. 6).

Discussion

These data provide evidence for the use of a semiochemical repellent as a viable strategy to manage *P. juglandis* populations. The least effective compound tested was (*S*)-(−)-verbenone. Perhaps the most interesting observation about this compound was the difference in effectiveness between male and females. Male beetle trap catch, compared to positive control traps, was reduced by 93%, whereas female catch was only reduced by just over half. The relative ineffectiveness of (*S*)-(−)-verbenone on female *P. juglandis* impacted the overall performance of this compound as a potential repellent. (*R*)-(+)-Verbenone, however, performed well. Although male trap catch reduction to this compound was not reduced as much (87% versus 93%) as by (*S*)-(−)-verbenone, female trap catch was reduced more, 82% versus 55%.

Given that male *P. juglandis* initiate new attacks and produce the aggregation pheromone (Seybold et al. 2013, 2015, 2016), it may be sufficient to optimize repellent semiochemicals based on male beetle responses. Both sexes are known to carry *G. morbida* fungal spores (Hishinuma 2017). Therefore, if *P. juglandis* attacks have occurred prior to semiochemical repellent treatment, interrupting subsequent male beetle colonization may not provide protection for a tree. Thus, we opted for compounds that elicited similar trap catch reduction efficacy for both sexes. Based on the results reported here, we determined that the (*R*)-(+)- enantiomer of verbenone was the better choice.

Interestingly, we did not see a dose-dependent response as we predicted. Unlike the observed male and female responses to both enantiomers of limonene (see Audley et al. 2020a), only a single dose-dependent response in male beetle catches to (*S*)-(−)-verbenone was observed (Fig. 1). The limited supply of chalcogran and (*R*)-(+)-verbenone precluded the possibility of observing dose dependent responses to these compounds, as only a single rr of each was tested. It seems unlikely that we would have observed a dose-dependent response to chalcogran, had we been able to test other release rates, because both males and females responded dramatically to low doses of both chalcogran and *trans*-conophthorin. This result adds evidence to the hypothesis that either or both of these compounds may be components of an antiaggregation pheromone for *P. juglandis*.

Both combinations improved the percent trap catch reductions observed in individual compound assays, especially Combination B (Fig. 6). Unfortunately, the subtractive-combination assays did not reveal the most important components in either combination. For both combinations, all treatments effectively shut down trap catch for both sexes (Figs. 4

and 5). We did observe a slight increase in the total trap catch when *trans*-conophthorin was removed. This increase was only significant for total and male trap catch, although the difference was marginal. We took this as evidence that *trans*-conophthorin is a critical component for a *P. juglandis* repellent.

The lack of statistical separation among other treatments in both combination assays allowed for other considerations, such as cost and commercial availability, for moving forward with repellent tool development. Based on our data and that of Audley et al. (2020a), we determined that both *trans*-conophthorin at a 3 mg/d rr and limonene at ca. 700 mg/d rr were important components to the final repellent. Based on the current cost per volume [e.g., (*R*)-(+)-limonene: $73.90 per 500 ml, (*S*)-(−)-limonene: $79.70 per 250 G, sigmaaldrich.com], (*R*)-(+)-limonene is probably the better choice. Based on the inconclusive nature of the Combination A assay, we considered including (*R*)-(+)-verbenone (SPLAT®) in the system for further testing as well. Verbenone is already well known for protecting trees from various bark beetle species and is likely to be readily recognized by landowners and natural resource managers. The ease of application and biodegradable nature of SPLAT® is also an attractive quality (ISCA Technologies Inc.).

The semiochemicals tested inhibited *P. juglandis* response to MBO aggregation pheromone in field-based, flight-intercept traps. The compounds proved effective enough to overcome the aggregation signal even when placed directly adjacent to the MBO lure on the same trap. Evaluating beetle responses to a potentially repellent semiochemical in this manner provided a conservative assessment. In an ideal situation, managers could utilize a repellent tool to protect trees prior to *P. juglandis* attacks in a manner consistent with semiochemical based tree protections reviewed in Seybold et al. (2018) and Seybold and Fettig (2020). Given the beetle's ubiquity across the western U.S.A., and particularly in California (Seybold et al. 2019), several walnut trees that are candidates for protection with semiochemical repellents may already be colonized by *P. juglandis*. Thus, evaluating a potential repellent tool's ability to overcome naturally occurring aggregation pheromone is valuable for successful tree protection.

An aspect not addressed with the flight intercept assays that is pertinent to a management strategy is the spatial limits of the repellency. In other words, at what distance from the release device(s) are *P. juglandis* actively pushed away? Knowing that the repellent can protect beetle colonization from the whole tree to stands of trees scale will be crucial to devising a successful management strategy against TCD. A major component of that will be knowing if a single tree will require a single device or multiple devices. Similarly, the density of repellent devices (i.e., on every tree, every other tree, etc.) in a stand or orchard needs to be tested empirically to inform a management strategy.

We believe the next logical step in developing a semiochemical-repellent management strategy for *P. juglandis* is to test the compounds discussed here on whole walnut trees. The repellent must be shown to overcome MBO aggregation (artificially and/or naturally produced) and host kairomones so as to protect walnut trees. The spatial extent (i.e., distance beyond the release device) of the repellent's influence on beetle landing behavior should also be assessed. Addressing both questions will allow for development of a more comprehensive management plan.

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Data Availability All data available upon request.

Compliance with Ethical Standards

Conflicts of Interest/Competing Interests None to report.

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Code Availability All code was generated using R statistical software with R Studio. Code can be made available upon request.

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