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Research

Trapping Failure Leads to Discovery of Potent Semiochemical Repellent for the Walnut Twig Beetle

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Abstract

The walnut twig beetle, *Pityophthorus juglandis* Blackman, and its associated fungal pathogen that causes thousand cankers disease, currently threaten the viability of walnut trees across much of North America. During a 2011 assessment of seasonal flight patterns of *P. juglandis* with yellow sticky traps baited with the male-produced aggregation pheromone component, 3-methyl-2-buten-1-ol, dramatically reduced catches were recorded when Tree Tanglefoot adhesive was used to coat the traps. In summer 2011, two trap adhesives were tested for potential repellency against *P. juglandis* in a field trapping bioassay. SuperQ extracts of volatiles from the most repellent adhesive were analyzed by gas chromatography–mass spectrometry, and limonene and α-pinene were identified as predominant components. In field-based, trapping experiments both enantiomers of limonene at a release rate of ~700 mg/d conferred 91–99% reduction in trap catches of *P. juglandis* to pheromone-baited traps. (+)- and (−)-α-Pinene reduced trap catch by 40 and 53%, respectively, at the highest release rate tested. While a combination of *R*-(+)-limonene and (+)-α-pinene resulted in a 97% reduction in the number of *P. juglandis* caught, the combination did not consistently result in greater flight trap catch reduction than individual limonene enantiomers. The repellent effect of limonene may be valuable in the development of a semiochemical-based tool for management of *P. juglandis* and thousand cankers disease.

Key words: limonene, α-pinene, trap adhesive, thousand cankers disease, repellent

Walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), is an invasive bark beetle that vectors the fungal pathogen *Geosmithia morbida* M. Kolařík, E. Freeland, C. Utley, N. Tisserat (Ascomycota: Hypocreales) (Tisserat et al. 2009, Kolařík et al. 2011, Seybold et al. 2016). Together, these organisms cause thousand cankers disease (TCD), a serious necrosis of the branch and trunk phloem of walnut trees (*Juglans* spp.) (Tisserat et al. 2009, Seybold et al. 2016) and wingnut trees (*Pterocarya* spp.) (Hishinuma et al. 2016). Both organisms have been introduced and established in areas well beyond their putative historic ranges of Arizona, New Mexico, and northern Mexico (Zerillo et al. 2014, Rugman-Jones et al. 2015), and threaten the viability of valuable agricultural and timber resources across the western and eastern United States, and in Italy (European Plant Protection Organization [EPPO] 2015; Faccoli et al. 2016; Montecchio et al. 2016; Seybold et al. 2016, 2019; Moricca et al. 2019). Continued anthropogenic spread may compromise walnut resources on a global scale (e.g., Asia, Australia, and South America) (Hefty et al. 2018).

Nowhere has the impact of *P. juglandis* and TCD been more visible than in California, United States, the dominant edible walnut-growing region in North America. TCD has significantly impacted English (Persian) walnut, *Juglans regia* L., the staple of the >$1 billion (USD) per year edible walnut industry (California Walnut Board 2019), and is widespread within the limited native range of Northern California black walnut, *Juglans hindsii* (Jeps.) Rehder, and Southern California black walnut, *Juglans californica* S. Wats., two important riparian tree species in California and Oregon, United States (Griffin and Critchfield 1976, Keeley 1990, Beede and Hasey 1998, Callahan 2008, Leslie et al. 2009, Utley et al. 2013, Yaghmour et al. 2014, Seybold et al. 2016, Hefty et al. 2018). In California, as elsewhere, TCD causes progressive crown decline and tree mortality (Seybold et al. 2019).

In 2008, when *P. juglandis* and *G. morbida* were first observed together and recognized as TCD in California (Flint et al. 2010, Seybold et al. 2012a), a cooperative research team composed of members of the scientific staffs of the USDA Forest Service Pacific Southwest Research Station and University of California Davis Departments of Plant Pathology and of Entomology and Nematology initiated flight monitoring of *P. juglandis*. This monitoring effort and the associated analysis of volatile organic compounds from feeding beetles culminated in isolation, identification, and patenting of a male-produced aggregation pheromone component, 3-methyl-2-buten-1-ol (MBO) (Seybold et al. 2015) that became the centerpiece of a trapping technique and guidelines for detection of this invasive bark beetle in the western and eastern United States (Seybold et al. 2013). This approach was implemented on a national scale as part of an annual detection effort by cooperating states and led by the USDA Forest Service and the USDA Animal Plant Health Inspection Service (USDA 2019).

Initial trapping methods employed a yellow 'sticky card' trap used in California walnut orchards to monitor for walnut husk fly, *Rhagoletis completa* Cresson (Diptera: Tephritidae). The yellow, waxed cardboard traps were coated with a trapping adhesive and baited with small *J. hindsii* branch sections with and without live *P. juglandis* adults present in the phloem (Graves et al. 2008a). When aggregation pheromone became available, branch lures were replaced by bubble capsule-style release devices (~1 mg/d) or 15-ml polyethylene bottles (~5 mg/d) (Seybold et al. 2013, 2015). In 2010, continuous monitoring of the flight of *P. juglandis* began in northern California, utilizing sticky card traps in Davis, California. In 2011, following the implementation of an alternate trapping adhesive, an unexpected trapping failure occurred. Not a single *P. juglandis* was caught during the 4-mo period, despite the fact that baited funnel traps at other northern California sites caught numerous beetles during the same period (Dallara and Seybold, unpublished data). We hypothesized that volatiles emanating from the secondary trapping adhesive may have interrupted (repelled) flight responses of *P. juglandis* to the baited traps.

This paper describes a series of laboratory chemical analyses and field trapping experiments that elucidated the repellent effect of certain volatile compounds from an insect trapping adhesive (Tree Tanglefoot) on the flight behavior of *P. juglandis*. These studies establish a timeline and research primacy for the discovery of limonene as a repellent for *P. juglandis* and serve as a useful lesson about the care necessary when altering materials used in scientific research.

Materials and Methods

Field bioassays were conducted in three locations: 1) a riparian stand of *J. hindsii* along the north fork of Putah Creek in Davis, CA (38°32′20.66″N, 121°44′21.42″W); 2) a multispecies tree planting at the United States Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository (NCGR) *Juglans* collection at Wolfskill Experimental Orchards in Winters, CA (38°30′00.4″N, 121°58′37.1″W); and 3) an abandoned *J. regia* orchard at 3020 Fostoria Way in Danville, CA (N 37.781684°, W 121.975364°). The Putah Creek site contained ~15 stems of *J. hindsii*, as well as fewer than five stems each of boxelder, *Acer negundo* L., Fremont cottonwood, *Populus fremontii* S. Watson, valley oak, *Quercus lobata* Neé, and tree-of-heaven, *Ailanthus altissima* (Mill.) Swingle, all non-hosts for *P. juglandis*. The NCGR site contained ~1,000 *Juglans* trees representing 15 species planted in 25 rows of 40 trees each. The Danville site contained >200 *J. regia* stems.

Seasonal Flight Trapping

Seasonal flight patterns of male and female *P. juglandis* were evaluated from 6 May 2010 to 2 June 2014 using catches from two yellow sticky traps (PHEROCON AM, AM/NB Trap Trécé, Adair, OK or ACP Trap Alpha Scents, Inc., West Linn, OR) situated at the Putah Creek site. Traps were coated with Stikem Special (Formulation Regular, Seabright Laboratories, Emeryville, CA) (hereafter Stikem) throughout the trapping period except from 25 April to 25 August 2011, when a second source of trap adhesive, Tree Tanglefoot Insect Barrier (Product No. 99080, The Tanglefoot Company, Grand Rapids, MI) (hereafter Tanglefoot), was used as the supply of Stikem had been depleted. Each trap was baited with a 15-ml polyethylene screw-cap bottle (Products A1-1050/002, A1-1055/002, Contech Enterprises Inc., Delta, BC, Canada) charged with 15 ml of MBO (Product W364703, Sigma-Aldrich, St. Louis, MO) released at ~5 mg/d. Insects were collected from the traps weekly.

Comparative Effect of Tanglefoot Versus Stikem Adhesives on Flight Response of *P. juglandis* to Its Aggregation Pheromone

We compared trap catches on acrylic pane traps (two clear 23-cm × 14-cm acrylic panes hinged together along the top edge with string loops) coated with either Stikem or Tanglefoot and baited with the same MBO lure used in the seasonal flight study. Five blocks of two treatments each were tested at the NCGR site from 18 to 28 July 2011 (Table 1). Traps were hung from a vertical 1.27-cm × 304.8-cm thin-wall conduit placed over a rebar stake (Seybold et al. 2013) and positioned with sticky surfaces vertical and facing outward with panes straddling the conduit. The MBO lure was inserted between trap panes and secured with thin wire or string to a wire hanger used to connect each trap to the top of the conduit. Traps within a block were spaced 15 m apart, and the distance between blocks was ≥15 m.

Traps were checked daily, and if at least six *P. juglandis* were caught on one of the traps in four of the five blocks, all traps were collected. When traps were collected, two cardboard spacers were placed on the adhesive surfaces before hinging the trap closed. Closed traps were sealed individually in a ziplock bag and taken to the laboratory where all insects were removed, sorted, identified, and tallied by sex. New traps were labeled and then positioned according to the randomization schedule. Traps were collected every 1–3 d and fresh traps were deployed in re-randomized positions for a total of five time periods (25 replicates). Only traps (i.e., adhesive treatments) were re-randomized; lures, hangers, and poles remained in place. Traps were collected before 1400 PDT to minimize chances of disturbing beetle flights as flight activity of *P. juglandis* is primarily crepuscular and lowest during midday (Seybold et al. 2012b, Chen and Seybold 2014).

Volatile Collection and Analysis

To identify compounds potentially repellent to *P. juglandis*, we collected volatiles from Tanglefoot and Stikem adhesives and analyzed them by gas chromatography–mass spectrometry (GC–MS). The adhesives were applied to clean petri dishes and placed in separate, pre-cut 1-liter glass storage bottles fitted with a ground-glass side arm. For Tanglefoot aerations in 2012, we placed one empty control dish and three dishes containing adhesive, one with ~7 g and two with ~14 g, into separate bottles. For follow-up aerations in 2018, two dishes (no control) containing ~54 g of adhesive were placed into separate bottles. Compressed air was filtered through a column containing 1 g of Poropak Q (50/80 Mesh Size, Product #20333, Supelco, Bellefonte, PA) before flowing through the bottles at 30 ml/min. On the side arm, a small collection column of SuperQ (80/100

Table 1. Summary of field-based, flight trapping experiments measuring response of the walnut twig beetle, *Pityophthorus juglandis*, to potential repellent semiochemicals, California, United States

Experiment number	Date range	Experimental description	Location
Experiment 1	18–28 July 2011	Flight response of *P. juglandis* to its aggregation pheromone and Tree Tanglefoot vs Stikem Special trap adhesives	National Clonal Germplasm Repository (NCGR) Site Solano Co.
Experiment 2	5–22 September and 22 September–2 October 2012	Flight response of *P. juglandis* to its aggregation pheromone and limonene enantiomers at one release rate	Danville Site Contra Costa Co.
Experiment 3	21 April–18 May and 10 June–1 July 2016	Flight response of *P. juglandis* to its aggregation pheromone and limonene enantiomers at three release rates	NCGR Site Solano Co.
Experiment 4	17 July–10 August 2017	Flight response of *P. juglandis* to its aggregation pheromone and α-pinene enantiomers at three release rates	NCGR Site Solano Co.
Experiment 5	4–27 October 2017	Flight response of *P. juglandis* to its aggregation pheromone and (+)-α-pinene and *R*-(+)-limonene	NCGR Site Solano Co.

Mesh Size, Product #2735, Alltech Associates, Inc., Deerfield, IL) (~115 mg in a short glass column, plugged on each end with glass wool) was used to trap the effluent (Bartlet et al. 2004). The SuperQ columns were collected after 48 h of aeration. For Stikem aerations, one control dish and three dishes containing ~24 g each were placed into separate bottles. The aeration apparatus was configured in the same manner, but the air flow rate was 100 ml/min and columns were collected after 24 h. All columns were extracted with pentane (Product #V557-10, Mallinckrodt Chemicals, Phillipsburg, NJ) to yield 100 µl samples.

GC–MS analyses were performed on an Agilent 6890 GC coupled with the 5973 MSD, with Agilent Chemstation data analysis software G1701CA version C.00.00 (Agilent, Santa Clara, CA). The GC–MS was equipped with a DB-WAX-fused silica capillary column (60 m × 0.250 mm ID, 0.25 µm film thickness) (Agilent) and operated in splitless mode. A 1 µl aliquot of each extract was injected into the GC inlet and analyzed by using a temperature program from 37 to 195°C at 3°C/min with a final hold of 5 min. Helium was the carrier gas and the flow rate was 1 ml/min. Electron impact (EI) mass spectra were obtained at 70 eV.

To quantify enantiomeric composition of Tanglefoot volatiles, extracts from the 2018 aeration were analyzed using the same GC–MS equipment and software, but with a CYDEX-B chiral column (50 m × 0.220 mm ID × 0.25 µm film thickness) (SGE, Melbourne, Australia). The injection was made with a split ratio of 20:1, and run isothermally at 110°C for 35 min. Other analysis parameters were the same as above. Compounds were identified by comparison with the NIST Mass Spectral Library (version 2.2, 2014) and authentic standards (Aldrich Chemical Co., Milwaukee, WI).

Gravimetric Analyses of *R*-(+)-Limonene and *S*-(−)-Limonene Release Devices

Gravimetric analyses were conducted to determine the daily release of *R*-(+)- and *S*-(−)-limonene (Products 183164 and 218367-250G, respectively, Sigma-Aldrich Chemical Co., St. Louis, MO) from the three release devices used in the field trapping experiments. Both enantiomers were dispensed among 10 replicates for each of three release devices for a total of 60 devices. In the first device, 15 ml of limonene was dispensed into 15-ml screw-cap polyethylene bottles. Next, 400 µl was dispensed into 400-µl polyethylene microcentrifuge tubes. Finally, 1.5 ml of limonene was dispensed into 1.5-ml polyethylene tubes. The tubes were heat-sealed by melting a small tab on each cap onto the lower lip of the tube opening using a soldering iron. All devices were held in a freezer for 7 d prior to the experiment. Glassware and utensils used in the transfer of limonene were washed between enantiomers by sonicating each piece for ~5 min each with hexane, acetone, and methylene chloride.

All devices were removed from the freezer, weighed, and hung by wire from holding racks inside a gravimetric chamber held at a constant 30°C. Devices were removed from the chamber at 24-h intervals for 41 d, weighed, and replaced.

The 400-µl tubes were of insufficient volume to hold limonene throughout the experiment. New tubes were filled and original tubes were replaced (every 8–12 d) in the chamber. Three replacements were made during the experiment. One replacement of the 15-ml screw-cap bottles was also made after 26 d.

Field Trapping Experiments of *P. juglandis* Flight Response to Limonene Enantiomers

Two field trapping bioassays of *P. juglandis* in response to its aggregation pheromone and limonene at a single release rate were

conducted in fall 2012 at the Danville site (Table 1) using a randomized complete block design with 4-unit funnel traps (Product #4050, Synergy Semiochemicals Corp., Delta, BC, Canada). Five blocks each with four trap stations (20 traps) were established in the orchard. In the first bioassay, run from 5 to 22 September 2012, treatments included: 1) MBO alone, 2) MBO plus *R*-(+)-limonene, 3) *R*- (+)-limonene, and 4) an unbaited trap. Treatments in the second bioassay, run from 22 September to 2 October 2012, included: 1) MBO alone, 2) MBO plus *S*-(−)-limonene, 3) *S*-(−)-limonene, and 4) an unbaited trap. MBO was dispensed at ~1.2 mg/d from a sponge-stabilized bubble cap (Product RD-1058, Scotts Canada, Delta, BC, Canada). Each enantiomer of limonene was dispensed at ~700 mg/d from 15-ml screw-cap polyethylene bottles. The pouch and/or bottle was suspended in the middle of the funnel trap that was fastened to the top of a 3-m tall metal conduit pole slid over an ~1 m long piece of rebar hammered into the ground (Seybold et al. 2013). All collection cups were filled with non-toxic, ethanol-free, propylene glycol (Winter Safe −50°F/−46°C Anti-freeze, Kinpak Inc., East Montgomery, AL) to a depth of ~2.5–3 cm. Traps in the first bioassay were emptied every 2–5 d with positions re-randomized (each trap and associated lures were moved to a new trapping station) within each block at varying time periods (N = 76). Traps in the second bioassay were emptied every 2 d with positions re-randomized within each block for a total of 5 time periods (N = 100).

Two field trapping bioassays of *P. juglandis* response to its aggregation pheromone and limonene at variable release rates were conducted in spring 2016 at the NCGR site (Table 1). Four randomized complete blocks were established, each with five trap stations (20

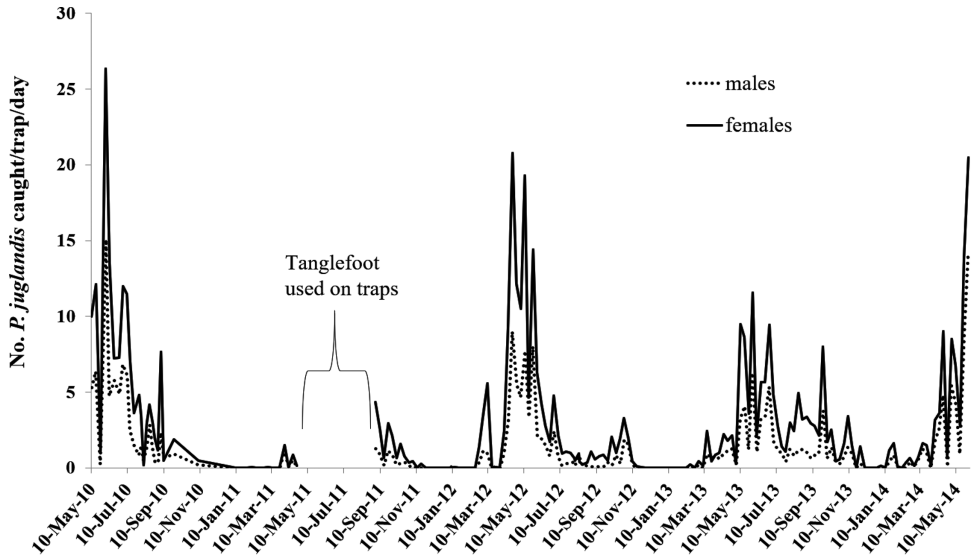

Fig. 1. Seasonal flight response of *Pityophthorus juglandis* to the aggregation pheromone component 3-methyl-2-buten-1-ol, Davis, California, United States, 6 May 2010–2 June 2014.

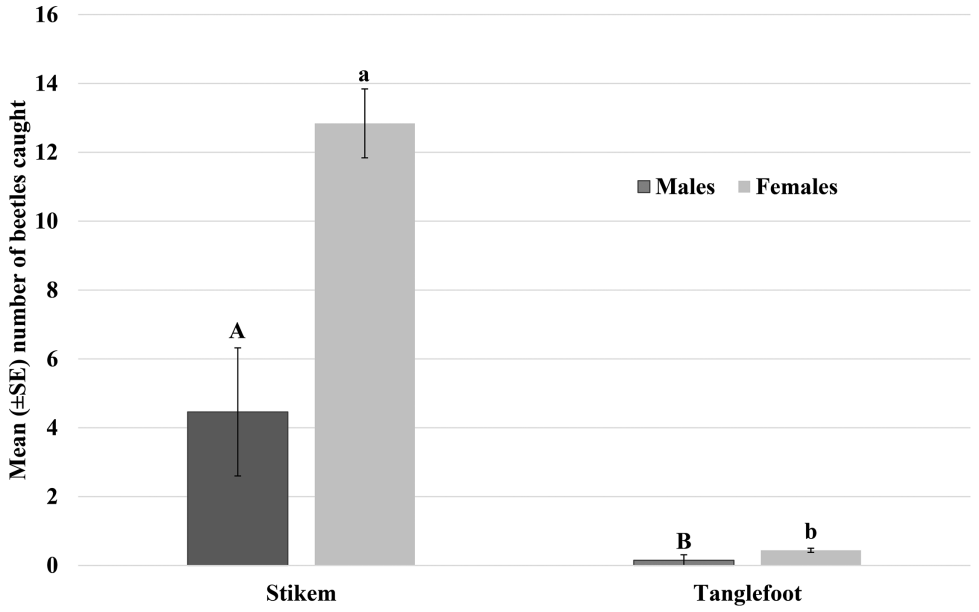

Fig. 2. Mean number of male and female *Pityophthorus juglandis* caught on traps coated with Tree Tanglefoot or Stikem Special. Treatments are significantly different within each sex (P < 0.001), two-sided sign test (test performed on the medians and applied to display of the means). Means with the same letter are not significantly different.

traps within 5 m of the stem of adjacent trees), and consisted of traps set-up as previously described.

R-(+)-limonene was tested from 21 April to 18 May 2016 and *S*-(−)-limonene was tested from 10 June to 1 July 2016. In each bioassay, treatments were: 1) MBO alone (15-ml screw-cap polyethylene bottle, ~5 mg/d at 30°C), 2) MBO plus low limonene (1.5-ml polyethylene tube, ~20 µg/d at 30°C), 3) MBO plus medium limonene (400-µl polyethylene tube, ~45 mg/d at 30°C), 4) MBO plus high limonene (15-ml screw-cap polyethylene bottle, ~700 mg/d at 30°C), and 5) unbaited trap. All traps were emptied every 1–3

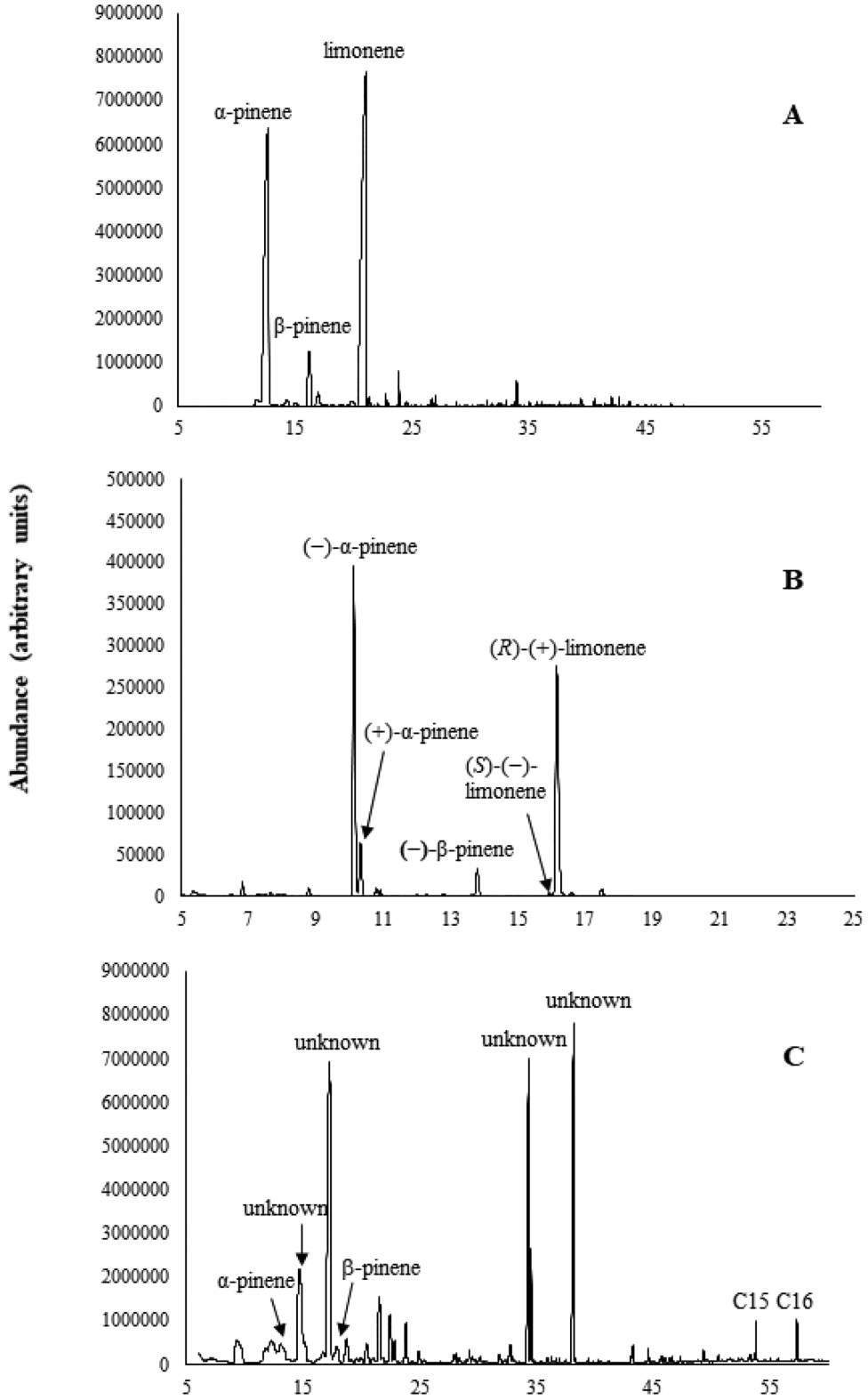

Fig. 3. Representative total ion chromatograms from GC–MS analyses of volatiles trapped from (A) Tree Tanglefoot run on a DB-WAX column; (B) Tree Tanglefoot run on a CYDEX-B chiral column, and (C) Stikem Special run on a DB-WAX capillary column.

d, and treatments were re-randomized 14 times within each block (N = 280). Daily temperature data were obtained from University of California weather station 139 located on the NCGR site. During the *R*-(+)-limonene trial, average daily temperatures (high/low) were 24.6°C (range 16.1–33.9°C) and 12.5°C (range 5.6–20.6°C), respectively. During the *S*-(−)-limonene trial, average daily temperatures (high/low) were 32.1°C (range 22.2–39.4°C) and 14.9°C (range 9.4–18.9°C), respectively.

Field Trapping Experiment of *P. juglandis* Flight Response to α-Pinene Enantiomers

Two behavioral bioassays were run concurrently from 17 July to 10 August 2017, for (−)- and (+)-α-pinene (Sigma-Aldrich Chemical Co.) at the NCGR site (Table 1). Each enantiomer was tested at low, medium, and high release rates using the same release devices that were used for limonene. The release rates for α-pinene were assumed to be similar to those for limonene. Three trapping blocks were established for each enantiomer, following the same establishment criteria previously described. Traps were emptied every 1–3 d and the positions of each treatment were re-randomized 20 times within each block (N = 300). Average temperatures (high/low) were 35.2°C (range 31.7–39.4°C) and 15.2°C (range 12.2–22.2°C), respectively.

Field Trapping Experiment of *P. juglandis* Flight Response to (+)-α-Pinene and *R*-(+)-Limonene Combined

A combination of (+)-α-pinene and *R*-(+)-limonene was tested at the highest release rates (most effective in previous bioassays) for additive or synergistic repellency. Although (−)-α-pinene performed better than (+)-α-pinene, we tested (+)-α-pinene because of a low supply of the antipode. This bioassay was conducted from 4 to 27 October 2017 utilizing the original four trapping blocks at NCGR (Table 1) following the same procedures as previously described. Treatments included: 1) MBO alone, 2) MBO plus high release rate (+)-α-pinene, 3) MBO plus high release rate *R*-(+)-limonene, 4) MBO plus high release rate of both (+)-α-pinene and *R*-(+)-limonene, and 5) unbaited trap. Treatments were repeated across the four trapping blocks (20 traps) with trap placements re-randomized 14 times (N = 280). Average temperatures (high/low) were 26.7°C (range 17.8–32.2°C) and 7.8°C (range 3.9–11.7°C), respectively.

Data Handling and Statistical Analyses

All *P. juglandis* collected from traps were tallied by sex, based on the presence of dense setae on the frons (females) and the presence of asperities on the declivity (males), using a stereo dissecting scope (Seybold et al. 2013). Catches were normalized by number of beetles caught per day (rounded to the nearest whole number) and analyzed separately for each sex. Counts of beetles captured on each of the two adhesives (Experiment 1) were compared by determining exact P values by using a two-sided sign test (Zar 2010).

Linear regressions were used to analyze the release rates for limonene enantiomers from each release device. Because almost all contents were depleted after 6 d and 17 d of exposure for 400-µl tubes and 15-ml bottles, respectively, observations beyond those durations were removed from the analyses. One replicate for *R*-(+)-limonene in 400-µl tubes was omitted from analysis based on an anomalous, sharp drop in weight after 1 d of exposure, likely because of a spill. Changes in weights were modeled using the linear regression function (lm). A 95% CI was generated for the average daily change for each of the release devices. Analyses were conducted using the Stats package with R Statistical Software (version 3.4.4) via RStudio (version 1.2.1335) statistical software (R Core Team 2019).

The flight responses to limonene enantiomers at a single release rate (Experiment 2) were analyzed using Friedman's non-parametric analysis of variance (Zar 2010). Treatments within experiments yielding a significant treatment effect were compared using an a posteriori Nemenyi test, experimentwise α = 0.05 (Zar 2010). Analyses for Experiment 2 were performed using Excel software.

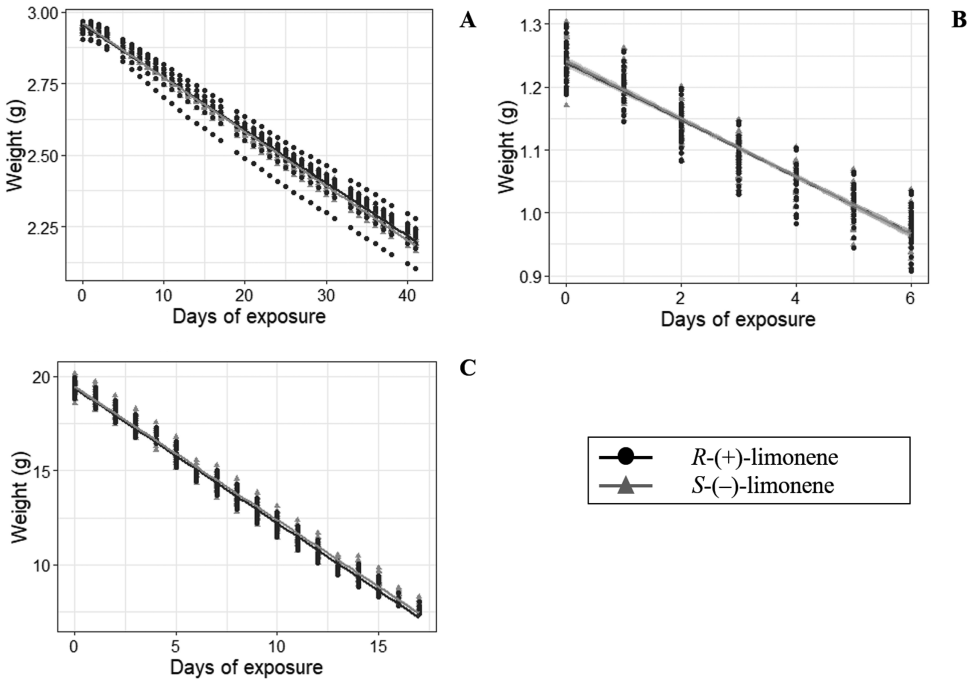

Fig. 4. Daily weight loss of three polyethylene release devices, 1.5-ml tubes (A), 400 µl-tubes (B), and 15-ml bottles (C), each filled with one of two compounds, *R*-(+)-limonene (black) or *S*-(−)-limonene (gray), when exposed to 30°C in a gravimetric chamber. Daily weight change was used to calculate the release rate for both compounds from each of the three release devices.

All other analyses (Experiments 3–5) employed 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 combination experiment (Experiment 5) models, in which trap position was treated as a fixed effect or dropped. Model selections were informed by Akaike information criterion (AIC) values and likelihood ratio tests (Zuur et al. 2009). Multiple means comparisons were made with post hoc pairwise, least squares means (lsmeans) tests with the Bonferroni correction ($\alpha = 0.05$) using the emmeans package. Repellent efficacy for male, female, and total *P. juglandis* trap catches across Experiments 2–5 was determined by comparing percent trap catch reductions. Percent trap catch reductions were calculated by comparing the proportion of beetles caught to the MBO alone. Finally, a two-sample test for equality of proportions with a continuity correction (prop.test) was used to compare percent trap catch reductions by sex (within trials) and by enantiomers (between trials). Analyses for Experiments 3–5 utilized the lme4, emmeans, MASS, and FSA packages with R Statistical Software (version 3.4.4) via RStudio (version 1.2.1335) statistical software (R Core Team 2019).

Results

Seasonal Flight Trapping

Flight of *P. juglandis* peaked between April and September (Fig. 1). Females were trapped as late as late-November, and both sexes were caught as early as January. During 25 April–25 August 2011, no *P. juglandis* were caught on sticky traps (Fig. 1) when Tanglefoot replaced Stikem as an adhesive, suggesting a repellent effect.

Comparative Effect of Tanglefoot Versus Stikem

Mean daily catches of *P. juglandis* on traps coated with Tanglefoot and Stikem were significantly different for each sex ($P < 0.001$,

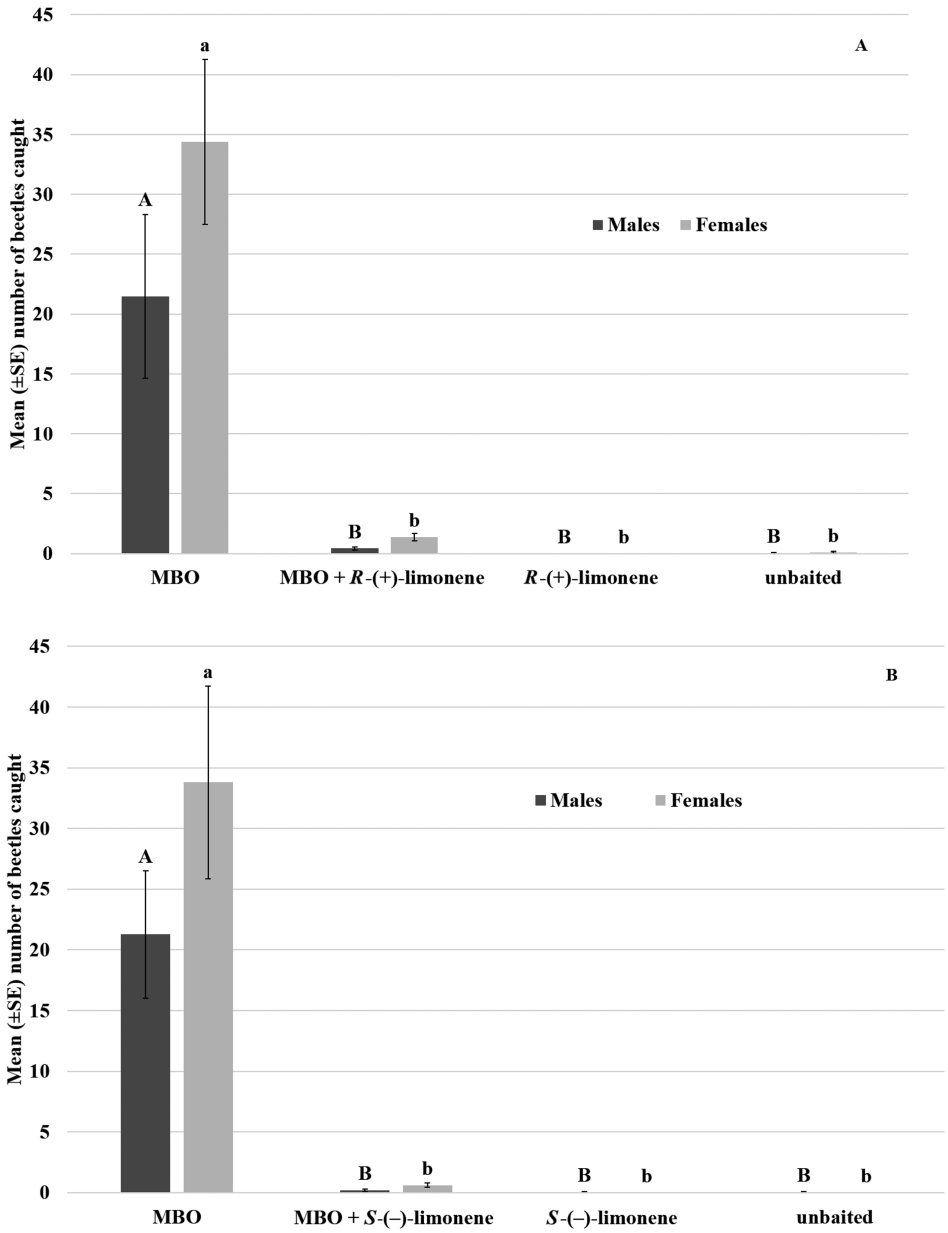

Fig. 5. Response of *Pityophthorus juglandis* to 4-unit funnel traps baited with the aggregation pheromone component 3-methyl-2-buten-1-ol (MBO) and the individual enantiomers of the potential interruptant limonene, *R*-(+)- (A) and *S*-(-)- (B), at a single release rate (Experiment 2, Table 1). Means labeled with the same letter are not significantly different (a posteriori Nemenyi test, experimentwise $\alpha = 0.05$).

Table 2. Comparison of percent *Pityophthorus juglandis* trap catch reductions at the highest doses of limonene and α-pinene added to MBO in Experiments 2–5 for males, females, and both sexes combined to catches in traps baited with MBO alone

Compound	Males	% Reduction		Females	% Reduction		Total	% Reduction	
R-(+)-Limonene[a]	8/408	96.0	AB	26/653	98.0	B	34/1,061	96.8	B
S-(−)-Limonene[a]	5/532	98.2	A	15/845	99.1	A	20/1,377	98.5	A
R-(+)-Limonene[b]	24/624	96.1	B	95/1,622	94.1	B	119/2,246	94.7	C
S-(−)-Limonene[b]	23/1,034	97.8	AB	136/2,561	94.7	B	159/3,595	95.6	BC
(+)-α-Pinene	82/232	64.7	F	338/465	27.3	F	420/697	39.7	G
(−)-α-Pinene	60/350	82.9	D	396/846	53.2	D	456/1,196	53.5	E
R-(+)-Limonene[c]	25/360	93.1	C	63/660	90.5	C	88/1,020	91.4	D
(+)-α-Pinene[c]	120/360	66.7	E	379/660	42.6	E	499/1,020	51.1	F
Combination[c]	2/360	99.4	A	29/660	95.6	B	31/1,020	97	B

Pairwise comparisons were made by using a two-sample test for equality of proportions with a continuity correction (prop.test), using the R base package in RStudio (R Core Team 2019). Percent catch reductions with the same letter within a category indicate no significant difference (α = 0.05).

[a]Experiment 2.

[b]Experiment 3.

[c]Compounds tested individually and in combination simultaneously in Experiment 5.

two-sided sign test), 4.6 ± 1.0 versus 0.2 ± 0.1 for males and 12.8 ± 1.9 versus 0.4 ± 0.2 for females (Fig. 2). Tanglefoot traps caught 94.8% fewer males and 95.9% fewer females than did Stikem traps (Fig. 2).

Volatile Collection and Analysis

The most abundant volatiles collected from Tanglefoot were limonene, α-pinene, and β-pinene (Fig. 3A). Minor compounds included other monoterpenes, sesquiterpenes, and aldehydes. The amounts of limonene, α-pinene, and β-pinene relative to the total volatiles detected ranged from 47.8 to 59.2%, 35.8 to 43.0%, and 3.9 to 5.1%, respectively. The control sample presented trace quantities of monoterpenes, including α-pinene, β-pinene, and limonene, and several unidentified compounds. The enantiomeric composition of the major volatile components collected from Tanglefoot expressed as percent of total was (+)-α-pinene 41.20%, (−)-α-pinene 7.0%, (−)-β-pinene 4.1%, *S*-(−)-limonene 0.4%, and *R*-(+)-limonene 39.8% (Fig. 3B).

Little or no limonene was detected in volatiles from Stikem. Respective proportions of monoterpenes in Samples 1–3 were 10.4, 2.5, and 1.5% for α-pinene, 6.5, 1.8, and 1.9% for β-pinene, and 1.6, 0.0, and 0.0% for limonene. Samples 2 and 3 had large amounts of four unidentified compounds (Fig. 3C).

Emission of *R*-(+)-Limonene and *S*-(−)-Limonene From Release Devices

Mean release (at 30°C) of *R*-(+)-limonene and *S*-(−)-limonene in 15-ml bottles was 709.2 mg/d (95% CI: 704.4–714.0 mg/d) and 704.1 mg/d (95% CI: 699.5–708.8 mg/d), respectively, and 45.1 mg/d (95% CI: 44.4–45.7 mg/d) and 45.5 mg/d (95% CI: 44.8–46.1 mg/d) in 400-µl tubes. Mean release of *R*-(+)-limonene and *S*-(−)-limonene in 1.5-ml tubes was 18.5 mg/d (95% CI: 18.4–18.6 mg/d) and 19.0 mg/d (95% CI: 18.9–19.1 mg/d), respectively. The adjusted R^2 value was >0.98 for each of the six models, indicating a strong model fit for each data set (Fig. 4).

Reduction of Catches in Traps Baited With Limonene Enantiomers

Addition of *R*-(+)-limonene to MBO reduced trap catches compared to MBO alone by 98% for males and 96% for females (Friedman test, males $\chi^2 = 47.0$, $P < 0.001$; females $\chi^2 = 52.5$, $P < 0.001$, Nemenyi test, $P = 0.05$) (Fig. 5A). Similarly, addition of *S*-(−)-limonene to MBO reduced trap catches compared to MBO alone by 99.1% for males and 98.2% for females (Friedman test, males $\chi^2 = 59.7$, $P < 0.001$, females $\chi^2 = 65.9$, $P < 0.001$; Nemenyi test, $P = 0.05$) (Fig. 5B). Both enantiomers reduced the number of male beetles caught equally. Conversely, *S*-(−)-limonene reduced female trap catches more than the antipode did (two-sample proportion test, $\chi^2 = 59.7$, $P = 0.015$) (Table 2).

Addition of *R*-(+)- and *S*-(−)-limonene to MBO at low, medium, and high release rates significantly reduced catches of male and female *P. juglandis* in a dose-dependent manner (Fig. 6A and B). For *R*-(+)-limonene, post hoc least squares means comparisons with a Bonferroni correction indicated highly significant differences ($P < 0.001$) in mean trap catches between MBO and MBO + limonene at all doses, however, even at the high release rate (96.1% reduction compared to MBO for males and 94.1% for females) catches were not reduced to the same level as the unbaited control traps (Fig. 6A). Trap catch reduction was not statistically significant between males and females (two-sample proportion test, $\chi^2 = 3.242$, $P = 0.072$).

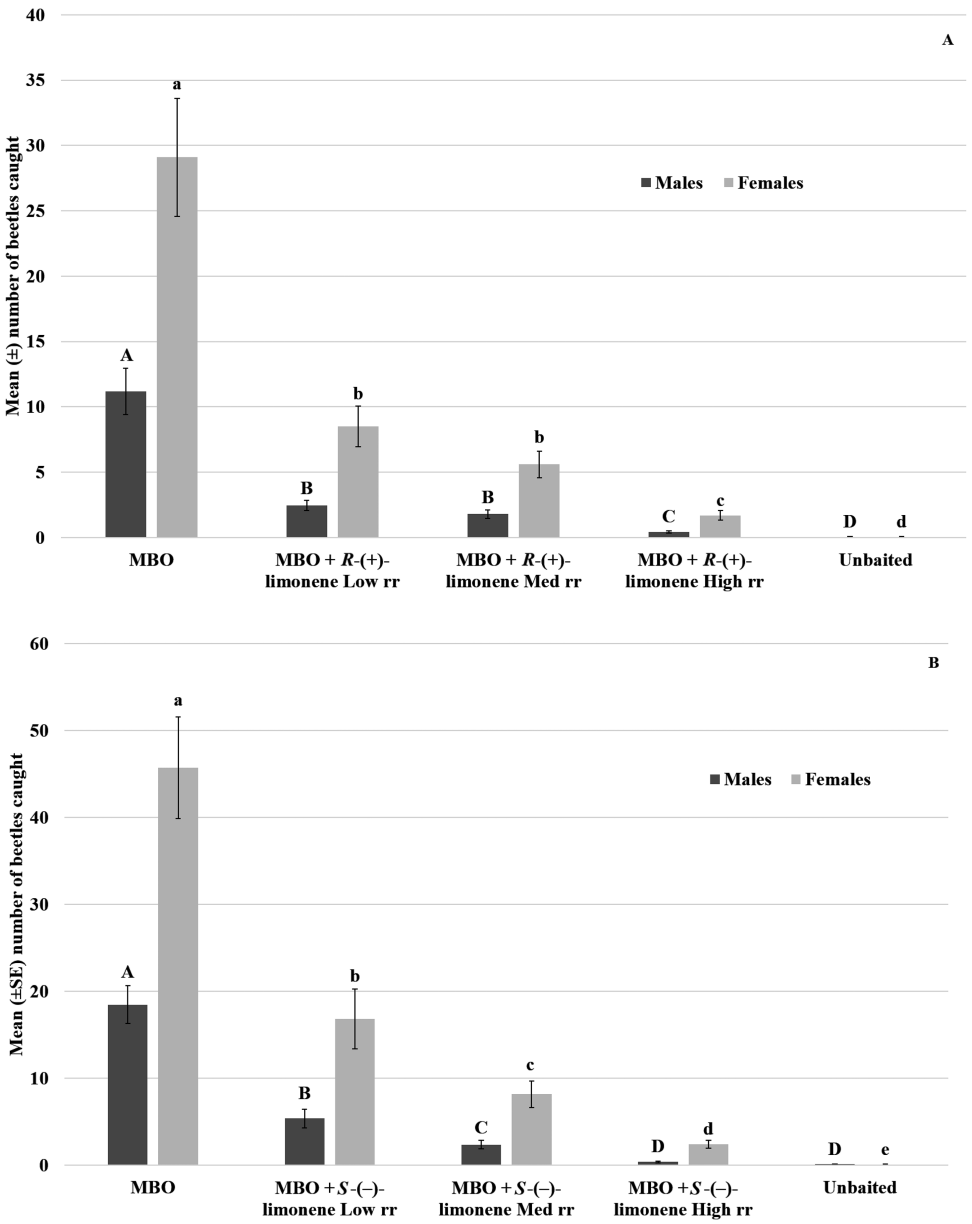

Fig. 6. Response of *Pityophthorus juglandis* to 4-unit funnel traps baited with the aggregation pheromone component 3-methyl-2-buten-1-ol (MBO) and the individual enantiomers of the potential interruptant limonene, *R*-(+)- (A) and *S*-(-)- (B), at multiple release rates (Experiment 3, Table 1). Means with the same letter are not significantly different (post hoc pairwise, least squares means tests with the Bonferroni correction, $\alpha = 0.05$).

Negative binomial GLMM analyses of male and female trap catches indicated there was also a dose-dependent reduction in trap catches caused by adding *S*-(−)-limonene to MBO (Fig. 6B), similar to that found for *R*-(+)-limonene (Fig 6A). However, at the highest release rate the 97.8% reduction in trap catch for males lowered trap catches to a level not significantly different from those in unbaited control traps (Fig. 6B). Trap catch reduction compared to the MBO alone traps was significantly greater for males (97.8%) than for females (94.7%) (two-sample proportion test, $\chi^2 = 15.873$, $P < 0.001$). Both enantiomers reduced trap catches compared to the MBO alone traps equally for male and female *P. juglandis* (Table 2).

Reduction of Catches in Traps Baited With α-Pinene Enantiomers

Negative binomial GLMM analysis indicated that adding (+)- or (–)-α-pinene to MBO reduced the mean trap catches of *P. juglandis* in a dose-dependent manner, but only at the medium and high doses for males and only the high dose for females (Fig. 7A and B). Even at the highest dose, the maximum reduction in trap catch (82.9% reduction in the number of males caught in traps baited with (–)-α-pinene) left catches of both sexes at much higher levels than those in unbaited control traps, indicating weaker repellency than for limonene (Table 2).

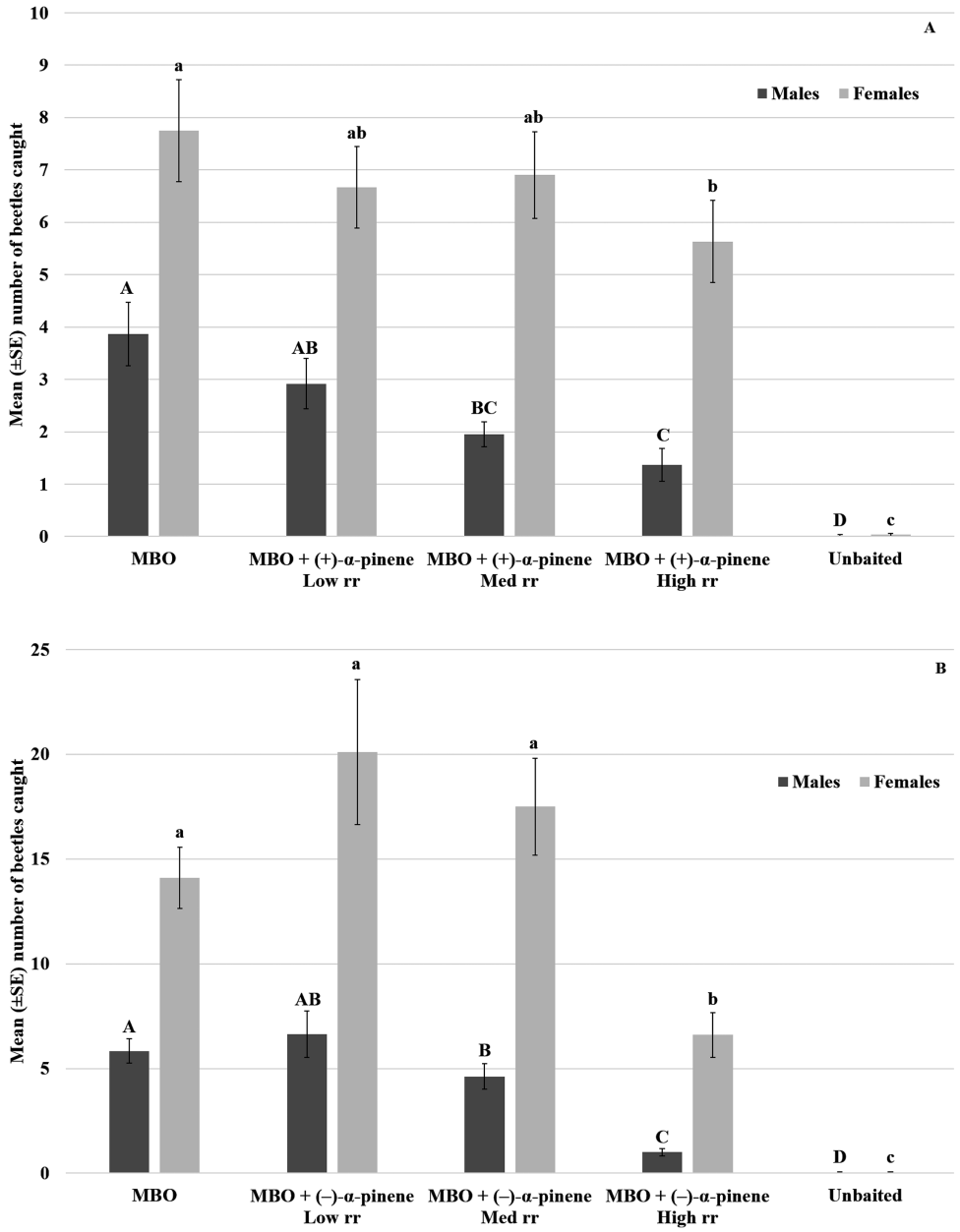

Fig. 7. Response of *Pityophthorus juglandis* to 4-unit funnel traps baited with the aggregation pheromone component 3-methyl-2-buten-1-ol (MBO) and the individual stereoisomers of the potential interruptant α-pinene, *R*-(+)- (A) and *S*-(-)- (B), at multiple release rates (Experiment 4, Table 1). Means with the same letter are not significantly different (post hoc pairwise, least squares means tests with the Bonferroni correction, α = 0.05).

Reduction in Trap Catches in Traps Baited With a Combination of (+)-α-Pinene and *R*-(+)-Limonene

Adding both *R*-(+)-limonene and (+)-α-pinene to MBO significantly reduced catches of both sexes (Fig. 8). However, the combination did not reduce the catches of either sex to the levels of catches in the unbaited control traps. The addition of *R*-(+)-limonene caused a significantly more pronounced reduction in catches of both sexes than caused by the addition of (+)-α-pinene (Fig. 8), confirming the pronounced repellency imparted by *R*-(+)-limonene in Experiments 2 and 3 (Figs. 5A and 6A). This result affirms that of the comparisons across bioassays (Table 2). The combination treatment of (+)-α-pinene and *R*-(+)-limonene (both at the highest release rate) in Experiment 5 induced the greatest overall trap catch reduction of 99.4% for male *P. juglandis*; however, this result was not significantly different from the reductions observed from the reductions observed from either enantiomer of limonene in Experiment 2 or from *S*-(−)-limonene in Experiment 3 (Table 2). Comparisons across bioassays reveal that the addition of (+)-α-pinene did not consistently improve trap catch reduction compared with limonene alone.

Discussion

An unexpected trapping failure of both sexes of *P. juglandis* when Tanglefoot was used as a trap adhesive influenced the discovery of a repellent effect of *R*-(+)- and *S*-(−)-limonene and (+)- and (−)-α-pinene on *P. juglandis*. The unexpected repellency of Tanglefoot may serve as a useful lesson about the care necessary when altering materials used in scientific research. It also suggests that results of other

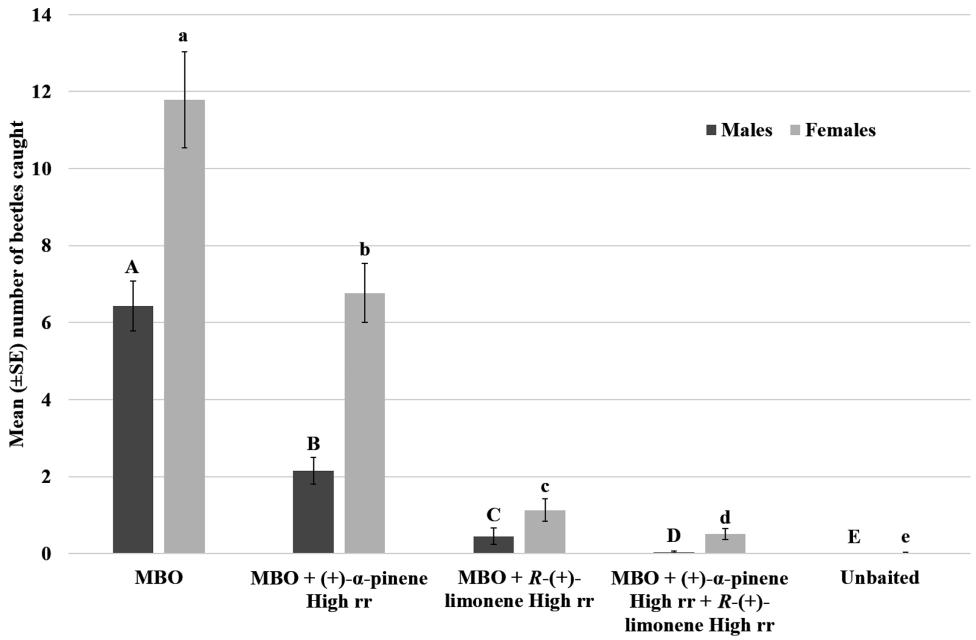

Fig. 8. Response of *Pityophthorus juglandis* to 4-unit funnel traps baited with the aggregation pheromone component 3-methyl-2-buten-1-ol (MBO) and two interruptant semiochemicals, limonene and α-pinene (Experiment 5, Table 1). Means with the same letter are not significantly different (post hoc pairwise, least squares means tests with the Bonferroni correction, α = 0.05).

studies in which Tanglefoot was employed as a trap adhesive, for *P. juglandis* as well as other taxa, should be re-examined. Both limonene and α-pinene were major constituents of the volatile profile of Tanglefoot (Fig. 3) and the influence of both compounds (as with many other monoterpenes) on the behavior of numerous insects are well documented (reviewed in Schiestl 2010). Based on our analyses, it appears likely that the two terpenes, especially limonene, were the driving factors explaining the observed repellency of Tanglefoot on *P. juglandis*. However, not all volatile components of the adhesive were tested (Fig. 3A and B) and other compounds could also have influenced the beetle's response. Similarly, the two trapping adhesives may have presented different visual cues to *P. juglandis* thus influencing the observed trapping differences. Another possible, although less likely explanation, could be a difference in trapping efficacy specific to beetles of the two adhesives. Given the well-documented influence of volatile compounds on bark beetle behaviors, a semiochemical-based explanation seems the most plausible.

The terpene limonene was demonstrated to have a particularly strong repellent effect. We consistently found that a dose of ~700 mg/d of both enantiomers reduced the numbers of both sexes caught by greater than 90% compared with the number of beetles caught in MBO alone baited traps (Figs. 5–8; Table 2). Our findings corroborate Blood et al. (2018) who reported that *R*-(+)-limonene inhibited the response of *P. juglandis* to MBO-baited traps in Tennessee. Limonene also interrupted responses to ethanol-baited traps for three ambrosia beetle species: *Xylosandrus compactus* (Eichhoff), *Xylosandrus crassiusculus* (Motschulsky), and *Xyleborinus saxsesni* (Ratzeburg) in Hawaii, United States (Burbano et al. 2012). Conversely, Miller (2007) reported that limonene was attractive to the cone-feeding beetle *Conophthorus coniperda* (Schwarz) in North Carolina, United States.

The strong repellency to both sexes caused by both enantiomers of limonene and the combination of *R*-(+)-limonene and *R*-(+)-α-pinene suggests that these terpenes could be used in a semiochemical-based management strategy for *P. juglandis*. Male *P. juglandis* initiate host colonization (Seybold et al. 2016). Once a male enters a suitable host it begins producing the aggregation pheromone and is subsequently joined by female and other male *P. juglandis*. Given this aspect of *P. juglandis* biology, it follows that interruption of male host searching behavior is perhaps more critical than interruption of female beetles. Conveniently, both enantiomers of limonene appear to confer strong repellency in baited funnel trap assessments. We conclude that the differences in both male and female *P. juglandis* responses to either *R*-(+)- and *S*-(−)-limonene are likely neither strong enough nor consistent enough to justify favoring one compound over the other. Employing a racemic blend would likely confer operationally effective bioactivity.

Both enantiomers of α-pinene were repellent to *P. juglandis*, but (−)-α-pinene was repellent only at the highest release rate tested. In contrast, Blood et al. (2018) reported racemic α- and β-pinene was attractive to *P. juglandis* in a laboratory olfactometer. Our field-based results showing increased, however not significantly so, trap catches in response to low and medium doses of (−)-α-pinene may indicate that attraction at low doses is real, and that there is a threshold beyond which the compound becomes repellent. Given the presence of both α- and β-pinene in the volatile headspace of *Juglans nigra* foliage (Blood et al. 2018), it may be that low concentrations of these terpenes help inform host selection for *P. juglandis*. Our results, however, suggest that α-pinene is not a strong candidate for further pursuit as a repellent for *P. juglandis* management.

The results of our field-based, trapping experiments combined with evidence of in-flight host discrimination (Hishinuma 2017, Audley et al. 2020, Lona et al. 2020) suggest that limonene could be useful in tree protection, similar to the use of verbenone to disrupt host colonization by mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Progar et al. 2014, Seybold et al. 2018, Seybold and Fettig 2020). While reduction of trap catches in field-based bioassays provides evidence of behavioral response of

dispersing beetles, further study of limonene is necessary before determining efficacy and utility as a management tool. Specifically, the ability to protect individual walnut trees from *P. juglandis* colonization should be evaluated following an approach like that of Graves et al. (2008b). We believe limonene may be used as a component in a semiochemical interruptant to protect both individual and stands of walnut trees, with the ultimate goal of providing protection for important economic resources in walnut orchards and plantations.

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