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Evidence for Semiochemical Divergence Between Sibling Bark Beetle Species: *Dendroctonus brevicomis* and *Dendroctonus barberi*

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Abstract

We investigated geographic variation in the semiochemistry of major disturbance agents of western North American pine forests, *Dendroctonus brevicomis* Le Conte and *Dendroctonus barberi* Hopkins (Coleoptera: Curculionidae: Scolytinae), species separated by the Great Basin in the USA that until recently were synonymous. At 15 sites in the western USA and northern Mexico, beetle populations were examined to determine (1) pheromone production by solitary, mining females, (2) male electroantennogram amplitudes in response to known semiochemicals for the genus, or (3) relative attractiveness of two female-produced pheromone components (*endo*- and *exo*-brevicomin) and two host odors (*alpha*-pinene and myrcene) to beetles in the field. Compared to female beetles collected east of the Great Basin (*D. barberi*), western females (*D. brevicomis*) produced a consistently higher proportion of, and male antenna were correspondingly more sensitive to, the *exo*- than the *endo*-isomer of brevicomin. With the exception of one sampling location (where no preference was observed), beetles west of the Great Basin were more attracted to *exo*- than *endo*- brevicomin trap lures, whereas eastern beetles displayed the reverse preference. In contrast, there was not a consistent difference between these populations regarding relative attraction or olfactory response to myrcene or *alpha*-pinene, although some geographic variability was evident. These data show that the semiochemical systems of *D. brevicomis* and *D. barberi* have diverged and corroborate genetic and morphological evidence that they are distinct, allopatric species.

Keywords Western pine beetle · Southwestern pine beetle · Geographic variation · Pheromone · Host odor · Speciation

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Introduction

Bark beetles (Coleoptera: Curculionidae: Scolytinae) in the Holarctic genus *Dendroctonus* include some of the most economically and ecologically significant disturbance agents of the world's coniferous forests (Six and Bracewell 2015). Their capacity to kill trees relies on their use of semiochemicals to locate host trees and mates and to instigate mass attacks on individual trees for countering host defenses (Byers 1989; Raffa 2001). The central role of semiochemicals in the epidemiology of bark beetles spurred some of the earliest identifications of insect pheromones as well as attempts to use semiochemicals in insect control (Birch 1978; Vité 1970; Vité and Francke 1976). In *Dendroctonus*, females are the sex that arrives first on the host tree and begin releasing pheromone components attractive to both sexes (Renwick and Vité 1970). Upon arriving at the tree, males may release their own pheromone components that, among other effects, may be attractive or synergize attractiveness of the female-produced components (Borden et al. 1987; Hughes et al. 1976; Sullivan et al. 2007; Vité and Pitman 1969). The female-produced components initiate mass attack and attract males to female entrances for mating, whereas the compounds of both sexes may intensify and sustain mass attack (Niño-Domínguez et al. 2015, 2016; Pitman et al. 1969). Relatively few different compounds are utilized by species within the genus, with species often distinguished not by possession of unique compounds but rather by the particular combination produced and by the sex producing them (Skillen et al. 1997; Symonds and Elgar 2004a, b). Distinctive pheromone components or blends produced by females can influence male mate selection and thereby confer reproductive isolation between syntopic *Dendroctonus* species (Niño-Domínguez et al. 2015; Pureswaran et al. 2016a). Simultaneously, cross-attraction by different species to the same components or blends may mediate multi-species mass attacks of hosts (Økland et al. 2009; Pureswaran et al. 2016b).

Dendroctonus brevicomis Le Conte 1976 (Entomological Society of America-approved common name, "western pine beetle") and *Dendroctonus barberi* Hopkins 1909 (Hopkins' assigned common name, "southwestern pine beetle") are major destructive pests of North American pine forests (Fettig et al. 2019; Hopkins 1909; Valerio-Mendoza et al. 2019; Wood 1982). These were full species until synonymized by Wood (1963), but were recently separated again as full species (Valerio-Mendoza et al. 2019). According to this revised taxonomy, *D. brevicomis* ranges from British Columbia, Canada eastward to western Montana, USA and southward along the Pacific coast to Southern California; *D. barberi* occurs from Utah and Colorado, USA southward to Durango, Mexico (Hopkins 1909; Valerio-Mendoza et al. 2019). Their host ranges are restricted to species within the *Pinus* subsection *Ponderosae*. The two beetle species are separated by the largely host-free zones of the Great Basin and the Mojave and Sonoran deserts (hereafter, collectively called the "Great Basin"). Additionally, they are separated by a gap that includes Wyoming and eastern-central Montana, USA, although pines belonging to the *P. ponderosa* species group occur throughout this zone (Potter et al. 2015; Willyard et al. 2017).

A combination of evidence from morphological, genetic, and chemical ecology studies was used to justify the taxonomic revision (Valerio-Mendoza et al. 2019). Cytochrome oxidase I sequences from *D. brevicomis* sensu Wood (1963) specimens from eight sites west and northwest of the Great Basin (California, Oregon, and Idaho, USA; and British Columbia, Canada) and four sites to the east (Colorado, New Mexico, Utah, and Arizona, USA) were found to have a mean 6.9% sequence divergence (Kelley and Farrell 1998; Kelley et al. 1999). This level of divergence was similar to that between full species of *Dendroctonus*. Likewise, whole-genome sequencing of *D. brevicomis* sensu Wood (1963) indicated two widely-divergent, monophyletic populations separated by the Great Basin (Bracewell et al. 2018). A comprehensive examination of external morphological features and genitalia identified diagnostic characters that distinguish the two populations (Valerio-Mendoza et al. 2017, 2019). Fungal associates occupying the mycangia of these populations also differed taxonomically (Bracewell et al. 2018; Davis et al. 2010). Furthermore, populations of *D. brevicomis* sensu Wood (1963) in northern Arizona and northern California were shown to differ in the composition of their aggregation pheromone (Pureswaran et al. 2016b). The chemical ecology findings reported in the present paper provided further supporting evidence and were cited as unpublished data to justify the species separation (Valerio-Mendoza et al. 2019).

Prior to the 2000s, investigations of the chemical ecology of *D. brevicomis* sensu Wood (1963) were largely restricted to California and Oregon. The aggregation pheromone was shown to consist of female-produced (+)-*exo*-brevicomin and male-produced (-)-frontalin acting synergistically, and the host-produced monoterpene myrcene was found to be particularly synergistic with this attractive pheromone blend (Bedard et al. 1969; Libbey et al. 1974; Silverstein et al. 1968; Vité and Pitman 1969; Wood et al. 1976). This combination (employing the racemates of each respective pheromone component) has been the commercially-available lure for this taxon (Hofstetter et al. 2008; Skillen et al. 1997). Both sexes were shown to produce additional semiochemicals (particularly attraction disruptants) active with conspecific beetles (Bedard et al. 1980a; Bertram and Paine 1994; Byers 1982, 1983; Byers et al. 1984; Pitman et al. 1969), including *trans*-verbenol, verbenone, and ipsdienol. In subsequent years, research indicated the existence of geographic variation in the composition of the aggregation pheromone for *D. brevicomis* sensu Wood (1963). In Arizona, females produce

disproportionately the *endo*-isomer of brevicomin, in contrast to females of California and Oregon which produce predominantly the *exo*-isomer (Browne et al. 1979; Pureswaran et al. 2008, 2016a, b). Additionally, *D. brevicomis* sensu Wood (1963) in northern Arizona were more attracted to *endo*- than *exo*-brevicomin lures in baited traps whereas beetles in northern California display the reverse preference (Bedard et al. 1980b; Pureswaran et al. 2016b).

For many tree-killing *Dendroctonus*, the volatile constituents of the host's defensive resin – particularly monoterpenes – act as synergists for the aggregation pheromone despite often not being attractive alone (Raffa 2014; Seybold et al. 2006). The resin monoterpenes of pines commonly occur as complex blends (Bookwalter et al. 2019; Mirov 1961). However, a single monoterpene or a combination of a few often have superior attractant synergism, with some differences in preferences occurring among species (Borden et al. 2008; Fettig et al. 2004; Munro et al. 2020; Pureswaran and Borden 2005). The composition and concentration of these compounds associated with a tree may indicate host suitability and the identity of either a preferred or inappropriate host taxon (Byers 1995; Byers and Zhang 2011; Erbilgin et al. 2003; Liu et al. 2011; Pureswaran and Borden 2005). Trapping studies conducted in California indicated that myrcene was either a superior synergist for the aggregation pheromone of *D. brevicomis* sensu Wood (1963) (Pitman and Vité 1971) or similar in activity to other host monoterpenes (Bedard et al. 1980b). However, in Arizona, *alpha*-pinene was a superior pheromone synergist to myrcene (Hofstetter et al. 2008, 2012). The latter authors proposed that the difference was related to regional variation in resin composition of host species for *D. brevicomis* sensu Wood (1963). The resin composition of *P. ponderosa* sensu lato in California is low in *alpha*-pinene (typically less than 15%), whereas in northern Arizona it is typically the most abundant monoterpene present, comprising 35–50% of the monoterpene component (Smith 1977, 2000; Sturgeon 1979). It has been proposed that *D. brevicomis* sensu Wood (1963) has coevolved with its host, resulting in major genetic divergence and possibly speciation (Bracewell et al. 2018; Kelley et al. 1999). Monoterpene preferences by beetle populations may therefore represent adaptations to localized variation in the resin composition of their host trees.

The objectives of this study initiated in 2012 were to assess geographic variation in *D. brevicomis* sensu Wood (1963) with respect to production of and response to female-produced pheromone components and preference for the host odor constituent of the aggregation attractant. We accomplished this by analyzing beetles at different sites for (1) the composition of volatiles released by host-attacking females, (2) the olfactory responses of males to *Dendroctonus* pheromone components and major host monoterpenes *alpha*-pinene and myrcene, and (3) behavioral responses of both sexes to traps baited with different combinations of these two monoterpenes and the two female-produced pheromone components *exo*- and *endo*-brevicomin. We hypothesized that composition of the aggregation pheromone would show substantially greater variation between than within populations on either side of the Great Basin and reflect the previously-reported pattern of genetic variation of these populations (Kelley et al. 1999). We also hypothesized that olfactory responses and attraction preferences to host monoterpenes would reflect regional variation in resin composition of the available host trees and genetic variation of the beetles. With regard to pheromone variation, we focused our investigation on the female-produced pheromone components *exo*- and *endo*-brevicomin since these had previously been found to distinguish populations in northern California and Arizona, USA (Pureswaran et al. 2016b) both in terms of production and response.

Methods and Materials

Experimental Insects Beetles used in pheromone production and electroantennogram studies were collected in multiple funnel traps baited with a five-component lure (frontalin, *endo*-brevicomin, *exo*-brevicomin, *alpha*-pinene, myrcene; with release devices identical to those used in attraction trials, see below) consisting of all components of the suspected aggregation attractant of populations both east and west of the Great Basin. Traps were typically suspended from non-host tree species in areas where activity by *D. brevicomis* sensu Wood (1963) was known. In order to maximize the longevity and health of the live-trapped beetles, trap collection cups had their lower interior packed with moistened paper towels to provide beetles a walking surface, elevated humidity, and refuges to minimize damage from mutual contact and predators. At 1–2 d intervals, the beetles were collected from trap cups and placed into ventilated, plastic, screw-cap specimen jars that were held under refrigeration (4° C for up to 10 d) prior to overnight shipment to the Pineville, Louisiana laboratory with ice packs. At Pineville, the original specimen jars were housed in a locked refrigerator (~ 4° C) within a USDA APHIS-PPQ-inspected containment facility. Beetles were trapped from 2012 to 2016 during the months of May through October and used in experiments as they became available. Quantitative pheromone production and/or electroantennogram analyses were performed on insects from 11 sites, with "site" normally defined as a single set of geographic coordinates (Table 1).

Pheromone Production We used methods previously described in Pureswaran et al. (2016b) and Sullivan et al. (2012) to determine the composition of known *Dendroctonus* pheromone components produced by gallery-

Table 1. Relevant data for study sites

Site #	Site	State	Country	Lat.	Long.	Trapping study	Pheromone & GC-EAD study	Region[a]	Available host taxon[b]
1	San Bernardino National Forest (Mountaintop District)	California	USA	N 34.-164	W 116.-912	X	X	West	*ponderosa*
2	Lassen National Forest (Hat Creek District)	California	USA	N 40.-688	W 121.-425		X	West	*ponderosa*
3	Lassen National Forest (Eagle Lake District)	California	USA	N 40.-455	W 120.-932	X		West	*ponderosa*
4	Mt. Hood National Forest (Barlow Mt. Hood District)	Oregon	USA	N 45.-397	W121. 424	X		West	*benthamaniana*
5	Wallowa-Whitman National Forest (La Grande District)	Oregon	USA	N 45.-365	W 118.-297	X	X	West	*ponderosa*
6	Boise National Forest (Mountain Home District)	Idaho	USA	N 43.-998	W 115.-862	X	X	West	*ponderosa*
7	Spring Mountains National Recreation Area	Nevada	USA	N 36.-257	W 115.-639	X	X	East	"canyonlands"
8	Fishlake National Forest (Fillmore District)	Utah	USA	N 39.-357	W 112.-221		X	East	"canyonlands"
9	Fishlake National Forest (Beaver District)	Utah	USA	N 38.-315	W 112.-405		X	East	"canyonlands"
10	Manti-LaSal National Forest (Moab District)	Utah	USA	N 38.-379	W 109.-206		X	East	*brachyptera*
11	Coconino National Forest (Flagstaff District)	Arizona	USA	N 35.-226	W 111.-792	X		East	*brachyptera*
12	Centennial Forest (Arizona State Trust Land)	Arizona	USA	N 35.-157	W 111.-738	X	X	East	*brachyptera*
13	Santa Fe National Forest (Jemez District)	New Mexic-o	USA	N 35.-819	W 106.-589	X		East	*brachyptera*
14	Davis Mountains Preserve, The Nature Conservancy	Texas	USA	N 30.-674	W 104.-146	X	X	East	*arizonica var. stormiae*
15	Galeana (adjacent)	Nuevo Leon	Mexico	N 24.-779	W 100.-045		X	East	*arizonica var. stormiae*

[a] "East" includes locations east and southeast, and "west" includes sites west and northwest, respectively, of the Great Basin. These designations coincide with those of Valerio-Mendoza et al. (2019)

[b] Species within the *Pinus ponderosa* group as indicated in Willyard et al. (2017).

establishing females. Females were forced to attack logs from mature *P. ponderosa* [*Pinus brachyptera* sensu Willyard et al. (2017)] cut within a single stand of the Centennial Forest of Northern Arizona University (N 35.1, W -111.4; ~2200 m elevation). As logs were needed, new trees were cut and logs immediately shipped to the Pineville laboratory. Logs were 25–35 cm long × 12–18 cm diam. and had their ends waxed to slow desiccation. Logs were stored under refrigeration at Pineville prior to use, and were generally used < 3 wk after the tree had been felled (an exception was a 60 d-old bolt used for 10 females when fresher material was not available). Females (9–25 per site) were confined within gelatin capsule halves over holes drilled into the bark (2 mm diam., spaced > 4 cm apart on the bark surface, phloem-depth) for 20–25 h at room temperature (19–31° C). Those that had entered the phloem and were expelling frass after this interval were dissected from

their gallery and sampled by placing them abdomen-first into a 100 µl-capacity, conical-interior glass vial containing a 2–3 mm depth of conditioned chemical adsorbent (HayeSep Q; polydivinylbenzene; 2–3 mg; 80–100 mesh; Hayes Separations Inc., Bandera, Texas, USA) in the vial tip. A PTFE rod confined the beetle to the space immediately above the adsorbent. The vial was capped with a septum closure with a 1 mm hole through the septum to allow limited ventilation through a layer of activated charcoal mesh pressed to the cap. The vial was then incubated for 20–25 h in darkness at 22° C, during which time the female released pheromone. Afterward, the female was removed, and the adsorbent was extracted sequentially with (1) 50 µl redistilled hexane spiked with 190 ng cycloheptanone (Sigma-Aldrich Inc., St. Louis, Missouri, USA) as an internal standard and (2) 50 µl redistilled pentane. The two extracts were combined and analyzed in splitless mode (inlet 200° C) on a coupled gas chromatograph-mass spectrometer (Hewlett-Packard 6890 − 5973, Palo Alto, California, USA) equipped with an HP-INNOWax column (60 m × 0.25 mm diam. × 0.25 µm film thickness; polyethylene glycol phase; Agilent, Santa Clara, California, USA). The flow rate of the carrier gas (helium) was 1.0 ml/min. The mass spectral detector was operated in electron ionization mode with ion source temperature 230° C, quadrupole temperature 150° C, and repeller voltage 29.5 V. The temperature program was 40° C for 1 min, then 16° C/min to 80° C, then 7° C per min to 230° C. Compounds were identified by matches of mass spectra and retention times to those of commercially-obtained standards (sources: Sigma-Aldrich; Synergy Semiochemicals Inc, Delta, British Columbia, Canada; Bedoukian Research Inc., Danbury, Connecticut, USA; Phero Tech Inc., Delta, British Columbia). Quantitation was performed relative to a response curve calculated from dilutions of these standards which had an identical concentration of cycloheptanone as the samples. Variation in site-associated, mean production of individual pheromone components and the ratio of *exo*- to *endo*-brevicomin were analyzed by a 1-way ANOVA on log-transformed values followed by a Ryan-Einot-Gabriel-Welsch multiple range test for all-pairwise comparisons. Additionally, to assess whether the difference between regions was greater than would be expected based on site-within-region differences, the eastern and western regions were contrasted with a mixed-model ANOVA, with region as the fixed effect and site nested within region as the random effect.

Olfactory Responses Electrophysiological studies of olfaction were performed only with males to conserve females for pheromone production experiments (often insect availability was limiting) and because males are the responding sex with regard to pheromone-mediated mate location in this genus. We used coupled gas chromatography-electroantennographic detection (GC-EAD) to quantify the responsiveness of the antenna of male beetles to seven different semiochemicals known to influence the behavior of pine-infesting *Dendroctonus* (Skillen et al. 1997). The GC-EAD apparatus was described in detail previously (Asaro et al. 2004) and was operated with the same column and temperature program used for the pheromone production analyses above. Briefly, it consisted of a Hewlett-Packard 5890 GC with half of the column effluent delivered to a flame ionization detector and half into a stream of humidified air (400 ml/min) in which the antennal preparation was centered. Antenna electrodes were attached to a Syntech (Buchenbach, Germany; Universal AC/DC Probe) preamplifier, and the resulting signal was conditioned with a Syntech Auto-Spike 2/3 IDAC. Data were recorded with an SRI Instruments (Torrance, California, USA) model 202 computer interface operated with Peak Simple software (SRI Instruments). Electrodes consisted of tapered glass capillaries filled with Beadle-Ephrussi Ringer saline solution (with 0.5% polyvinylpyrrolidone) and an $Ag/AgCl_2$ wire that maintained electrical contact with the saline. The reference electrode was inserted into the base of the beetle's ablated head, and the opening of the signal electrode tip (and meniscus of saline) was placed into contact with one entire side of the unaltered antennal club. Test compounds were diluted in hexane to produce a single mixture with approximately 2.0 µg/µl of the host monoterpenes (±)-*alpha*-pinene and myrcene and 0.20 µg/µl of pheromone components (±)-frontalin, (±)-*exo*-brevicomin, (±)-*endo*-brevicomin, (±)-ipsdienol and 33%(+):67%(-) verbenone. With the exception of myrcene (80%), purities were > 96% for all compounds (sources same as GC-MS standards). With a 1:19 split ratio in the GC inlet, an approximate 1:1 split between the GC's flame ionization detector and the EAD, and an injection volume of 2 µl, we roughly approximate that 0.1 µg of the monoterpenes and 0.01 µg of the pheromone components were delivered to the antenna. A higher concentration of the monoterpenes relative to pheromone components was used in the GC-EAD test stimulus because of the relatively low olfactory voltages produced in *D. brevicomis* sensu Wood (1963) by the former compounds. This disparity did not impact data interpretation since we wished to compare different populations for responses to identical stimuli rather than establish the beetles' relative sensitivities to the different compounds.

Before and after elution of all test compounds during each GC-EAD run, the antennal preparation was exposed to a 30 ml/min, 2 sec duration puff of air through a Pasteur pipette containing a piece of filter paper treated with 10 µl of a mineral oil standard solution with 0.2-2 µg/µl *alpha*-pinene, myrcene, frontalin, and *exo*- and *endo*-brevicomin. The linear equation $y = bx + c$ was calculated for response amplitude (y) and retention time (x) from the response amplitudes and retention times of the first and second puffs, and response amplitude to each compound in the EAD standard mixture was normalized by dividing it by the y value of this equation with x

equal to the retention time of the compound. This normalization was intended to correct for loss of antennal vigor during each run. The normalized response amplitude for each compound was divided by the sum of responses of all seven compounds to give a relative amplitude value for each compound. These values were adjusted with the arcsine square root transformation and analyzed with a principal component analysis (derived from the covariance matrix) to identify overall patterns of variation in olfactory responsiveness. Additionally, for each compound separately, we (1) contrasted responses by the eastern and western regions with a mixed-model ANOVA using region as the fixed effect and site as a random effect, and (2) tested for significant differences among all sites with $N > 1$ (eight sites) by a one-way ANOVA followed by Ryan-Einot-Gabriel-Welsch multiple range tests for all-pairwise comparisons.

Behavioral Responses Trapping studies were limited to combinations of just four semiochemicals (*endo*-brevicomin, *exo*-brevicomin, *alpha*-pinene, and myrcene) since previous studies had indicated geographic variation in behavioral responses by *D. brevicomis* sensu Wood (1963) to them. Trapping trials were conducted within the months of July through October in 2012 to 2015 at each of ten sites within forests with evident *D. brevicomis* sensu Wood (1963) activity (Table 1). At nine of the sites, two lines (factor "trapline") of four 12-unit multiple-funnel traps were established, with 8–10 km between lines and 50–100 m spacing between adjacent traps in each line. Each trap was suspended from a pole, a non-host tree, or rope between trees, with its center 1.5–2 m above the ground. All traps had a lure releasing frontalin (racemic, 94–98% purity; consisting of a closed polyethylene microcentrifuge tube releasing approximately 6 mg/d). Treatments (factor "lure") were the addition of either (a) *exo*-brevicomin + myrcene, (b) *exo*-brevicomin + *alpha*-pinene, (c) *endo*-brevicomin + myrcene, or (d) *endo*-brevicomin + *alpha*-pinene. The *endo*-brevicomin (racemic, 84–95% approximate purity; 1.4–2.4% *exo*-isomer present; 4 mg/d approximate release) and *exo*-brevicomin (racemic, 96–97% purity; 0.5–0.7% *endo*-isomer present; 8 mg/d approximate release) were each released from single, closed polyethylene microcentrifuge tubes. *alpha*-Pinene [66–95% purity; ~75% (+)-enantiomer; 0.18 g/d] and myrcene (65–91% purity; 0.27 g/d) were each released from a single, capped polyethylene bottle[1]. Release rate measurements were taken gravimetrically in a fume hood at 24 ± 2° C over 1 wk. Approximate purities of the volatile composition of lure contents were derived from total ion chromatogram integration areas of GCMS analyses, and these generally corroborated purities provided by the manufacturer (Synergy Semiochemicals). Treatments were assigned randomly to traps within each trapline. Catches were collected at intervals of 2–11 days (typically 1 wk), and treatment positions were rerandomized without replacement until every treatment had been at every trap position once (four collections per trap; factor "collection"). This arrangement at each site comprised a multiple Latin squares design consisting of two squares with traps as columns and collection dates as rows within each square. A different design was used at the tenth site (#14 Davis Mountains, Texas) due to time and space constraints. A randomized complete block design was executed with blocks consisting of lines of four traps (as described above) and the four treatments assigned randomly to each trap without subsequent rotation (factor "block"). Five blocks were completed on 1–2 October 2015 and six on 6–7 October 2015 (factor "date", two levels). Blocks on the two dates did not overlap spatially.

Two different sets of analyses were performed: (1) To identify any influence of region (eastern or western) and site within each region on response to the four lure treatments, we performed a mixed-model ANOVA with fixed effects lure, region, region × lure, site nested in region, and lure × site nested within region. Since the experimental design at the Texas site was different from the other sites, indicator variables (i.e., variables taking on the values 0 or 1) were used in the SAS code to include different random effects appropriate for the sites with different experimental designs. Random effects applied to Texas data were site × region, date nested in site × region, and block nested in date × site × region; for all other data, the random effects were trapline nested in site × region and collection nested in site × region. (2) To identify any influence of region on response to the two lure factors (i.e., brevicomin component, "brevicomin," and terpene component, "terpene"), a mixed-model ANOVA was performed with fixed factors region, brevicomin, terpene and all possible interactions. Random effects common to all sites were site nested in region and lure × site nested in region. Additional random effects were, for the Texas site, date nested in site × region, and block nested in date × site × region; for all other sites the additional random factors were trapline nested in site × region and collection nested in site × region.

Treatment effects were additionally analyzed for all sites individually. Texas catch data were analyzed with a mixed-model ANOVA with lure as the fixed factor and random factors date, block nested in date, and lure × date. For all sites other than Texas, fixed factors were lure, trapline, and collection; random factors were trap nested in trapline and lure × trapline. A Tukey test ($\alpha = 0.05$) was used for all-pairwise contrasts among treatments, and tests for the main and interaction effects of lure factors brevicomin and terpene were executed with ESTIMATE statements.

[1] We do not believe that the low purity discovered for the contents of a portion of the lures would have significantly influenced the results. The contamination was due to compounds that would have had substantially lower permeability through polyethylene (i.e., oxygenated and higher molecular weight compounds) than hydrocarbon monoterpenes *alpha*-pinene and myrcene and thus would have been released from the lures at a considerably lower rate than the primary monoterpene component. No more than trace levels of the two monoterpenes *alpha*-pinene and myrcene were present in the reciprocal lure.

Spearman rank-order correlation was used to test how strongly regional variability in beetle olfactory and behavioral responsiveness to host monoterpenes (*alpha*-pinene and myrcene) and pheromone components (*exo*- and *endo*-brevicomin) was associated with regional variation in host resin composition. The resin composition variable was the proportion of *alpha*-pinene/myrcene present in *P. ponderosa* resin recorded at the closest sampling site in Smith (1977) to where the analyzed beetles were derived or trapping experiments were performed (Online Resource 1). The distances between the Smith (1977) sites and our closest data collection sites ranged from 10 to 296 km (mean 91 km). Responsiveness (olfactory or behavioral) was calculated as a single value for each site as the ratio of EAD response amplitudes or trap catches between the two monoterpenes or pheromone components. The Spearman's non-parametric correlation was chosen because there was no *a priori* basis for selecting a particular function for describing the association of our variables.

For all statistical analyses, data were transformed to optimize homoscedasticity and normality if necessary, and transformation choice was based on examination of residuals plots. However, means and standard errors presented in figures and tables represent the untransformed data. All analyses were performed with SAS 9.4 (SAS Institute, Cary, North Carolina, USA).

Results

Pheromone Production Eight ostensibly beetle-produced compounds previously demonstrated to have behavioral activity with *Dendroctonus* (Skillen et al. 1997; Sullivan 2005) were identified in the volatiles isolations from individual females (Table 2). These were the bicyclic ketals *exo*- and *endo*-brevicomin, the aromatic ketone acetophenone, and the oxygenated monoterpenes *cis*- and *trans*-verbenol, *trans*-pinocarveol, borneol and myrtenol.

Quantities of *exo*-brevicomin, *endo*-brevicomin, and acetophenone varied significantly depending on site of female origin ($F = 63.1$; $df = 9, 150$; $P < 0.001$; $F = 8.14$, $df = 9, 150$; $P < 0.001$; $F = 10.6$; $df = 9, 150$; $P < 0.001$; respectively) (Table 2). Females in the western region produced significantly more *exo*-brevicomin (typically 10^4 ng/beetle) than those in the eastern region ($10^2 - 10^3$ ng/beetle) ($F = 66.0$; $df = 1,8$; $P < 0.001$). *endo*-Brevicomin quantities were marginally significantly higher in the eastern than the western regions ($F = 5.57$; $df = 1, 8$; $P = 0.046$) which differed by less than an order of magnitude. Sites varied strongly in the ratio of *exo*- to *endo*-brevicomin produced by individual females ($F = 684.26$; $df = 1, 8$; $P < 0.001$, Fig. 1). There was no overlap in *exo*/*endo*-brevicomin ratios for individual beetles from different regions [a ratio of 1.3–17 to 1 in the western region (with a single outlier at 0.21 to 1; $N = 70$), and 0.011–0.11 to 1 in the eastern region ($N = 89$)] (Fig. 1, Online Resource 2). Mean *exo*/*endo*-brevicomin ratios were significantly different among the four western sites but not among the six eastern sites (Fig. 1); however, the western sites displayed extensive overlap in the distributions of ratios for individual beetles (Online Resource 2). Female acetophenone production varied significantly among sites ($F = 10.6$; $df = 9,150$; $P < 0.001$; Table 2); however, the eastern and western regions did not differ significantly ($F = 2.93$; $df = 1, 8$; $P = 0.13$). Full statistical analyses were not performed for the other five semiochemicals because of evidence of strong correlation of their concentrations with those of host-produced hydrocarbon monoterpenes in the samples (see discussion).

Olfactory Responses Principal component analysis of male EAD response amplitudes to seven semiochemicals indicated two widely-separated clusters divided almost solely along PC1 (69.5% of total variation). These clusters were perfectly associated with the insects' region of origin (i.e., eastern or western) (Fig. 2a). According to the PC eigenvectors (Fig. 2b), PC1 was explained predominantly by variation in antennal response amplitudes to *endo*- and *exo*-brevicomin (which had opposite influences) and to a much smaller extent by variation in response to ipsdienol. Within clusters associated with either region, there was a high degree of overlap along PC1 among all sites, but there was some evidence of segregation along PC2 (10.3% of total variation) within the eastern region [e.g., there was no overlap between Davis Mountains, Texas beetles ($N = 8$) and Centennial Forest, Arizona beetles ($N = 10$)]. PC2 was explained largely by trends in antennal response amplitudes to both brevicomin isomers relative to the other tested semiochemicals. Eastern and western regions were distinguished by a wide separation in *exo*/*endo*-brevicomin response amplitude ratios for individual beetles (western region, 1.19–2.98; eastern region, 0.14–0.57), however there was substantial overlap in response amplitude ratios for *alpha*-pinene/myrcene (Online Resource 3). Mean site-associated antennogram response amplitude ratios for *exo*/*endo*-brevicomin and *alpha*-pinene/myrcene were not significantly correlated (Spearman correlation, $R = -0.418$, $P = 0.213$, $N = 10$).

When compounds were examined individually, eastern and western regions differed significantly in olfactory responses to *endo*-brevicomin, *exo*-brevicomin, and ipsdienol; but not to *alpha*-pinene, myrcene, frontalin, or verbenone (Table 3). Significant variation in olfactory sensitivity occurred among sites in the western region for *alpha*-pinene, frontalin, and *endo*-brevicomin; and in the eastern region for *alpha*-pinene, myrcene, frontalin, *exo*-brevicomin and ipsdienol (Table 3).

Table 2 Quantities (± SE per beetle) of *Dendroctonus* semiochemicals isolated in static headspace samples from individual females excised after mining 1 d in a host log [*Pinus brachyptera* sensu Willyard et al. (2017)]

Site		State	Region	*N*	*alpha*-Pinene (μg)[b]	*exo*-Brevicomin (μg)[b]	*endo*-Brevicomin (μg)[b]	Aceto phenone (ng)[b]	*trans*-Pinocarveol (ng)	*cis*-Verbenol (μg)	*trans*-Verbenol (μg)	Borneol (ng)	Myrtenol (μg)
1[a]	San Bernardino NF	CA	W	13	8.4 ± 2.4ef	2.5 ± 0.7d	0.32 ± 0.09a	4.1 ± 0.8 cd	21.6 ± 5.1	0.26 ± 0.07	2.5 ± 0.6	19.5 ± 4.1	0.80 ± 0.20
2	Lassen NF	CA	W	12	0.47 ± 0.10bc	4.1 ± 0. 9d	0.67 ± 0.26ab	15.8 ± 5.7d	13.8 ± 3.8	0.092 ± 0.019	0.84 ± 0.17	7.5 ± 1.6	0.34 ± 0.07
5	Wallowa-Whitman NF	OR	W	22	0.83 ± 0.20bc	2.0 ± 0.4d	0.55 ± 0.11ab	0.59 ± 0.18ab	5.4 ± 1.0	0.038 ± 0.011	0.40 ± 0.11	5.2 ± 0.6	0.131 ± 0.044
6	Boise NF	ID	W	24	0.28 ± 0.07b	2.6 ± 0.3d	0.58 ± 0.07ab	2.7 ± 0.8bc	4.3 ± 0.7	0.038 ± 0.005	0.40 ± 0.06	5.0 ± 0.4	0.140 ± 0.018
7	Spring Mountains NRA	NV	E	9	2.5 ± 0.7cde	0.023 ± 0.009a	0.85 ± 0.40ab	1.78 ± 0.83abc	10.2 ± 2.3	0.075 ± 0.022	0.97 ± 0.30	9.2 ± 1.4	0.116 ± 0.039
8/9	Fishlake NF	UT	E	9	0.09 ± 0.06a	0.025 ± 0.011a	1.04 ± 0.42ab	1.50 ± 0.42abc	0.78 ± 0.35	0.0086 ± 0.0039	0.10 ± 0.05	2.1 ± 0.5	0.0116 ± 0.0064
10	Manti-LaSal NF	UT	E	25	13.7 ± 1.4f	0.053 ± 0.008ab	1.8 ± 0.3bc	0.40 ± 0.10a	11.9 ± 0.9	0.31 ± 0.03	3.4 ± 0.3	28 ± 2	0.38 ± 0.04
12	Centennial Forest	AZ	E	14	2.3 ± 0.5cde	0.056 ± 0.008bc	2.4 ± 0.4c	1.79 ± 0.69abc	12.4 ± 2.0	0.24 ± 0.03	2.2 ± 0.3	9.8 ± 1.3	0.26 ± 0.03
14	Davis Mts. Preserve	TX	E	21	1.42 ± 0.43bcd	0.027 ± 0.005ab	0.91 ± 0.25ab	2.17 ± 0.56abc	9.9 ± 1.5	0.067 ± 0.012	0.71 ± 0.11	7.2 ± 1.0	0.091 ± 0.015
15	Galeana	NL	E	10	7.5 ± 1.6def	0.162 ± 0.027c	3.7 ± 0.5c	1.00 ± 0.39ab	9.9 ± 1.3	0.20 ± 0.03	2.8 ± 0.5	34 ± 4	0.23 ± 0.04
-	$F_{9,150}$ (P)[c]				23.4 (< 0.001)	63.1 (< 0.001)	8.14 (< 0.001)	10.55 (< 0.001)					

[a] Numbered headings correspond to site designations of Table 1

[b] Means associated with the same letter did not differ significantly [Ryan-Einot-Gabriel-Welsch multiple range test, $\alpha = 0.05$, following 1-way ANOVA on log-transformed compound quantities] within compound. Statistical analyses were not performed for the oxygenated monoterpenes (i.e., *trans*-pinocarveol, *cis*-verbenol, *trans*-verbenol, borneol, and myrtenol) since their quantities were likely influenced by variability in the resinousness of the artificially-infested logs and quantities of hydrocarbon monoterpenes to which the beetles were exposed (see text)

[c] Results of 1-way ANOVA on log-transformed compound quantities

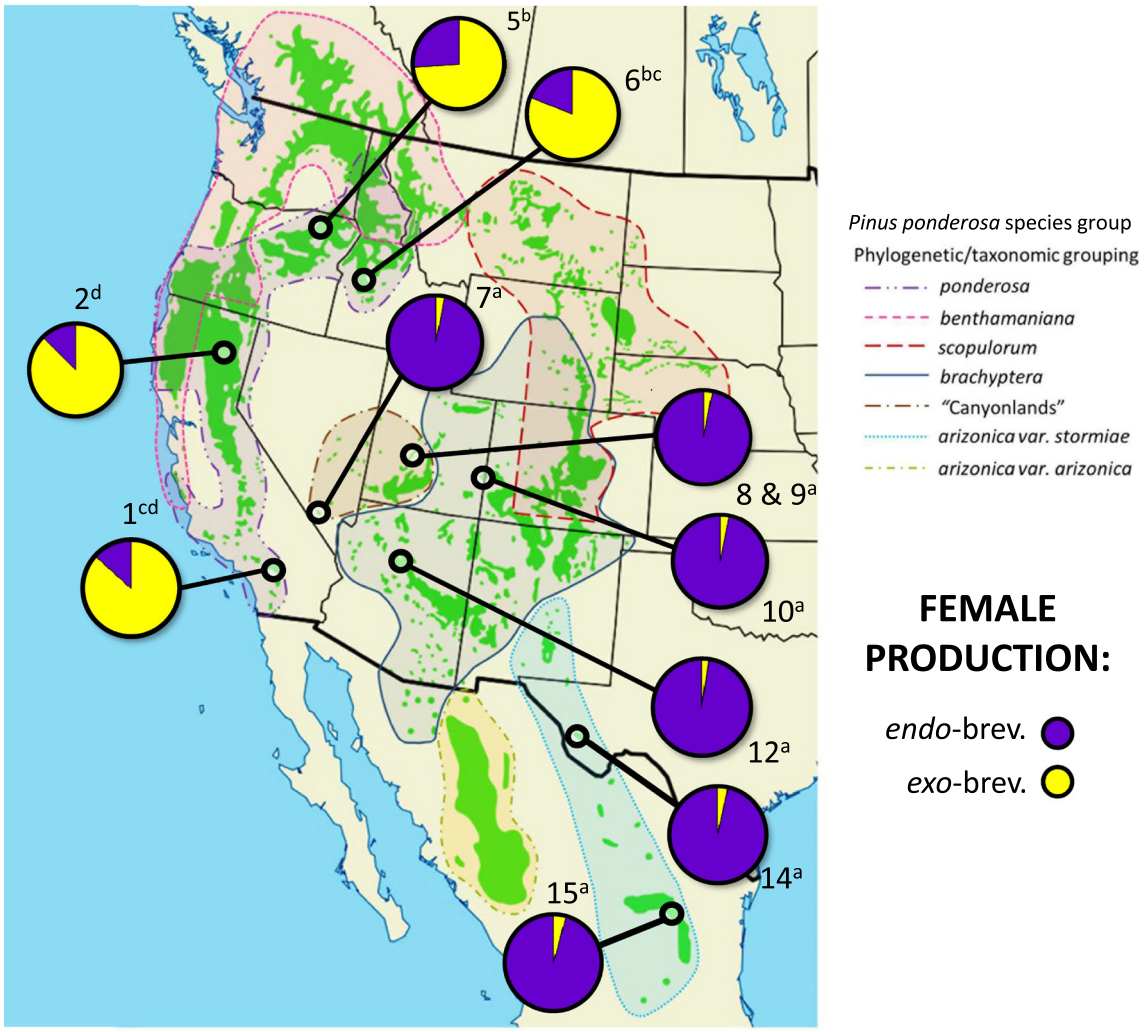

Fig. 1 Proportions of two isomers (*endo-* and *exo-*) of the pheromone component brevicomin released by female *D. brevicomis* sensu Wood (1963) during ~24 h following their removal from a host log [*Pinus brachyptera* sensu Willyard (2017)] where they had been mining singly for 1 d. Figure background represents the taxonomic map of the *P. ponderosa* species group according to Willyard et al. 2017. Numbers adjacent to pies correspond to sites indicated in Table 1. Pies associated with the same lower-case letter did not differ in the ratio of *endo-* to *exo-*brevicomin (1-way ANOVA on log-transformed ratios, $F_{1,8}$ = 684.3, $P < 0.001$; and Ryan-Einot-Gabriel-Welsch multiple range test, $\alpha = 0.05$)

Results of all-pairwise comparisons among all sites with more than one sampled individual are displayed in Table 4. There was not a significant Spearman rank-order correlation between reported ratios of *alpha*-pinene to myrcene in hosts located in proximity to beetle collection sites (Smith 1977; Online Resource 1) and ratio of EAD response amplitudes to these monoterpenes (R = -0.300, $P = 0.407$, $N = 9$).

Behavioral Responses One of the ten trapping sites (#8, Filmore District, Fishlake National Forest, Utah) was excluded from analyses because of insufficient catches. ANOVA on catches from the other nine sites yielded a significant effect (ANOVA model 1) due to lure composition ($F = 6.23$; $df = 3$, 217; $P < 0.001$), an interaction between lure composition and region (eastern or western; $F = 122.3$; $df = 3$, 217; $P < 0.001$), and an interaction between lure (nested within region) and site ($F = 5.08$; $df = 21$, 217; $P < 0.001$). There was also a significant interaction (ANOVA model 2) between region and brevicomin isomer (*exo-* or *endo-*; $F = 65.6$; $df = 1$, 217; $P < 0.001$) and between region and monoterpene component (*alpha*-pinene or myrcene; $F = 4.99$; $df = 1$, 217; $P = 0.027$) present in the lure.

The brevicomin isomer in the lure significantly influenced trap catches at all sites except the Lassen National Forest, California (t = -2.42, $df = 3$, $P = 0.094$; Table 5; Fig. 3). At the other four western sites, traps with *exo*-brevicomin were more attractive than those with *endo*-brevicomin, whereas at all four eastern sites *endo*-brevicomin was more attractive than *exo*-brevicomin. A lower level of discrimination was observed to the monoterpene component (*alpha*-pinene or myrcene) of

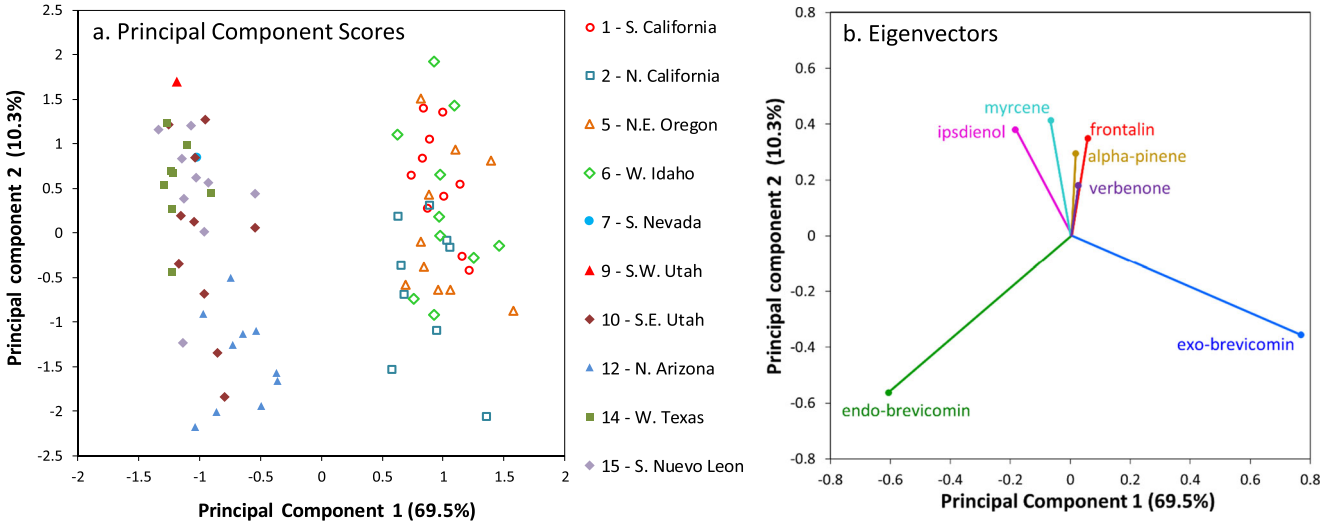

Fig. 2 Principal components scores (**a**) and eigenvectors (**b**) for GC-EAD responses by antenna of males of eastern and western populations of *D. brevicomis* sensu Wood (1963) to seven semiochemicals associated with attacks of pine-infesting *Dendroctonus* species (two host-associated compounds: *alpha*-pinene and myrcene; five pheromone components: *endo*-brevicomin, *exo*-brevicomin, frontalin, ipsdienol, and verbenone). Numbers for figure legend correspond to those in Table 1

the lure (Table 5; Fig. 3). Statistically significant levels of discrimination occurred in only four of the nine sites, with beetles at three sites showing a preference for myrcene (two in the western region: San Bernardino National Forest, California, and Boise National Forest, Idaho; one in the eastern region: Spring Mountains, Nevada) and with only northern Arizona showing a significant preference for *alpha*-pinene. At no site was there a significant 2-way interaction detected between the type of brevicomin lure and monoterpene lure component present ($P \geq 0.12$). Although catches were too low for statistical analyses, results at the Utah site were consistent with a preference for *endo*- over *exo*-brevicomin as was observed at the other eastern sites (11 beetles trapped with *endo*- and one with *exo*-brevicomin).

In correlation analyses of site means, we detected a marginally-significant correlation in the ratio of catches between *alpha*-pinene and myrcene lures and the ratio of catches between *endo*-brevicomin and *exo*-brevicomin lures (Spearman correlation, $R = 0.583$, $N = 9$, $P = 0.087$); also between these *alpha*-pinene/myrcene catch ratios and the ratios of *alpha*-pinene to myrcene reported in the composition of resin of *P. ponderosa* sampled by Smith (1977) in closest proximity to the trapping sites ($R = 0.603$, $N = 9$, $P = 0.077$; Online Resource 1). In contrast, there was a highly significant correlation between these *alpha*-pinene/myrcene resin content ratios and *endo*-brevicomin/*exo*-brevicomin catch ratios (Spearman correlation, $R = 0.854$, $N = 9$, $P < 0.001$).

Discussion

Our results indicated that the composition of the aggregation pheromone differed between beetle populations separated geographically by the Great Basin. The predominant isomer

Table 3 Univariate ANOVA results for tests of variation in male electroantennographic detector (EAD) responses to individual compounds between the western (W) and eastern (E) regions[a] and among sites within each region

	W versus E		Within W[b]		Within E[b]	
alpha-Pinene	$F_{1,8} = 0.01$	$P = 0.94$	$F_{3,35} = 4.24$	$P = 0.012$*	$F_{3,33} = 6.34$	$P = 0.002$*
Myrcene	$F_{1,8} = 2.10$	$P = 0.19$	$F_{3,35} = 2.58$	$P = 0.069$	$F_{3,33} = 3.97$	$P = 0.016$*
Frontalin	$F_{1,8} = 0.60$	$P = 0.46$	$F_{3,35} = 6.91$	$P < 0.001$*	$F_{3,33} = 10.3$	$P < 0.001$*
exo-Brevicomin	$F_{1,8} = 112$	$P < 0.001$*[c]	$F_{3,35} = 0.82$	$P = 0.49$	$F_{3,33} = 19.1$	$P < 0.001$*
endo-Brevicomin	$F_{1,8} = 240$	$P < 0.001$*	$F_{3,35} = 4.18$	$P = 0.013$*	$F_{3,33} = 2.06$	$P = 0.13$
Ipsdienol	$F_{1,8} = 6.52$	$P = 0.034$*	$F_{3,35} = 2.57$	$P = 0.069$	$F_{3,33} = 5.45$	$P = 0.004$*
Verbenone	$F_{1,8} = 0.97$	$P = 0.35$	$F_{3,35} = 0.40$	$P = 0.76$	$F_{3,33} = 0.12$	$P = 0.95$

[a] Indicated in Table 1

[b] Included only sites from which more than one beetle was assayed

[c] Asterisk denotes a significant test result ($\alpha = 0.05$)

Table 4 Electroantennographic detector (EAD) responses[a] of males to components in a synthetic mixture of known semiochemicals for pine-infesting *Dendroctonus* species

	Site	State	*N*	Region	*alpha*-Pinene	Myrcene	Frontalin	*exo*-Brevicomin	*endo*-Brevicomin	Ipsdienol	Verbenone
1[b]	San Bernardino NF	CA	10	W	4.0 ± 0.7ab	5.5 ± 0.5a	14.7 ± 1.2a	40.2 ± 1.3a	21.9 ± 0.6c	8.1 ± 1.0b	5.6 ± 0.5a
2	Lassen NF	CA	9	W	1.8 ± 0.3c	4.0 ± 0.5a	10.5 ± 0.9bc	42.8 ± 2.0a	27.1 ± 1.2b	8.6 ± 0.7b	5.4 ± 1.1a
5	Wallowa-Whitman NF	OR	10	W	2.2 ± 0.4abc	4.0 ± 0.8a	9.6 ± 1.0bc	43.6 ± 1.8a	22.0 ± 1.3c	12.1 ± 1.6ab	6.4 ± 1.6a
6	Boise NF	ID	10	W	4.0 ± 0.6a	6.0 ± 0.7a	9.6 ± 0.6bc	43.0 ± 1.6a	22.2 ± 1.4c	10.4 ± 0.9b	4.7 ± 0.3a
7	Spring Mts. NRA	NV	1	E	4.7	5.3	16.6	11.4	45.2	12.5	4.2
8/9	Fishlake NF	UT	1	E	4.2	15.8	9.4	9.2	42.6	15.1	3.6
10	Manti-LaSal NF	UT	10	E	3.1 ± 0.5abc	6.3 ± 0.9a	10.1 ± 0.9bc	14.4 ± 1.2c	48.0 ± 1.3a	12.6 ± 1.8ab	5.5 ± 1.7a
12	Centennial Forest	AZ	10	E	1.9 ± 0.3bc	3.9 ± 0.6a	6.4 ± 0.3d	21.9 ± 1.1b	50.5 ± 1.2a	10.6 ± 0.6b	4.8 ± 0.6a
14	Davis Mts. Preserve	TX	8	E	1.8 ± 0.4c	6.5 ± 0.7a	11.7 ± 0.8ab	11.0 ± 0.6c	47.0 ± 0.9a	17.5 ± 1.3a	4.4 ± 0.9a
15	Galeana	NL	9	E	3.9 ± 0.3a	6.6 ± 0.4a	7.4 ± 0.7dc	13.7 ± 1.1c	45.9 ± 1.6a	17.7 ± 1.6a	4.8 ± 0.5a
	$F_{7,68}$ (P)[d]				4.34 (< 0.001)	3.48 (0.003)	9.35 (< 0.001)	114.2 (< 0.001)	112.2 (< 0.001)	6.88 (< 0.001)	0.34 (0.935)

[a] Expressed as a percentage of the sum of response amplitudes to all seven compounds. Within compound, means followed by the same letter were not significantly different (1-way ANOVA followed by Tukey-Kramer test on arcsine square root transformed data; $\alpha = 0.05$). Antenna were exposed to monoterpenes *alpha*-pinene and myrcene at approximately 10-fold the concentration of the other compounds, so response amplitudes should not be directly compared among compounds

[b] As given in Table 1

of the brevicomin component of the pheromone was *endo*- to the east of the Great Basin and *exo*- to the west (Table 1). This pheromone difference was indicated by significantly greater female production of, male olfactory responses to, and attractiveness of, these respective isomers for populations in each region. The average *exo*:*endo* ratios of brevicomin isomers produced by females in the western and eastern regions were 6:1 and 1:35, respectively. Although pheromone production in males was not examined in this study, brevicomin has not been detected in male *D. brevicomis* sensu Wood (1963) in significant quantities in past studies conducted with either eastern or western populations (Byers et al. 1984; Libbey et al. 1974; Pureswaran et al. 2016a, b). Thus, it is probable that only females contribute the brevicomin component of the aggregation pheromone and the isomer ratios detected in our study are those of the aggregation pheromone of the respective populations.

Behavioral discrimination of the brevicomin isomers was somewhat less consistent and typically less pronounced than the differences in production of, and olfactory responses to, the isomers. Proportions of beetles responding to either *exo*- or *endo*-brevicomin baited traps was 3:1 and 1:6 for western and eastern regions, respectively. No behavioral discrimination between brevicomin isomers was observed in the trapping tests at Lassen National Forest, in northern California (Table 5; Fig. 3), despite a seven-fold greater production of the *exo*- than the *endo*- isomer by females from this site (Table 2; Fig. 1). However, this trapping result contrasts with a study conducted in the same forest in 2010 in which *exo*-brevicomin lures were significantly (more than five-fold on average) more attractive to *D. brevicomis* sensu Wood (1963) than *endo*- lures (Pureswaran et al. 2016b), an outcome that was consistent with the other western sites in the present study. In twin tests performed in northern Arizona and northern California, both *exo*-brevicomin and *endo*-brevicomin lures significantly enhanced attraction to frontalin and host odors at both locations, although with the same region-associated preference for one isomer as observed in the present study (Pureswaran et al. 2016b). Additionally, at both locations, the preferred brevicomin isomer was not reduced in attractiveness when released simultaneously and at roughly similar rates as the less preferred isomer (Pureswaran et al. 2016b). Assuming this result occurs generally across both the eastern and western regions, it suggests the existence of a degree of cross-attractiveness to both isomers and the absence of anti-attractant effects from the less abundant isomer for each respective population. It should be noted that the *exo*- and *endo*-brevicomin release devices used in both the aforementioned and present studies were contaminated with some (up to 4%) of the other isomer, and this may have obscured the degree of isomer discrimination that might have been observed with lures of greater isomeric purity.

We saw limited evidence that populations east and west of the Great Basin differed in their pheromone composition in additional respects; however, our study intentionally focused on a small number of compounds and was not a comprehensive investigation of pheromone composition. There was considerable variation among sites in female production of oxygenated monoterpene pheromone components of *Dendroctonus* (*cis*- and *trans*-verbenol, *trans*-pinocarveol, borneol and myrtenol) (Table 2). However, exposure of *Dendroctonus* spp. to the host's hydrocarbon monoterpenes can result in production of these oxygenated monoterpenes in a concentration-dependent manner, as the former compounds may be converted into the latter (Gries et al. 1990; Renwick and Hughes 1975). Since the level of insect exposure to host

Table 5 Beetle captures and relevant statistical tests for trapping experiments at nine sites in the western USA

					Pairwise contrasts of lure combinations[a] (Tukey test; $\alpha = 0.05$)	
Site #[b]	Location	State	Dates	ANOVA (lure effect)[c]	*exo*-brevicomin + myrcene	*exo*-brevicomin + *alpha*-pinene
1	San Bernardino NF	CA	7 Aug − 4 Sep 2012	$F_{3,3} = 15.75$, $P = 0.024$	9.1 ± 3.0a	1.9 ± 0.6ab
3	Lassen NF	CA	3 Jul − 3 Aug 2012	$F_{3,3} = 2.78$, $P = 0.211$	20.8 ± 5.2a	25.3 ± 5.0a
4	Mt. Hood NF	OR	10 Aug − 7 Sep 2012	$F_{3,3} = 20.50$, $P = 0.017$	176.1 ± 57.5a	116.5 ± 37.1ab
5	Wallowa-Whitman NF	OR	5 Sep − 7 Oct 2013	$F_{3,3} = 9.44$, $P = 0.049$	30.2 ± 10.8a	10.4 ± 3.2ab
6	Boise NF	ID	10 Jul − 9 Aug 2014	$F_{3,3} = 27.35$, $P = 0.011$	295.4 ± 45.6a	194.0 ± 36.2a
7	Spring Mts. NRA	NV	21–30 Sept 2015	$F_{3,3} = 8.71$, $P = 0.054$	7.8 ± 1.0ab	5.6 ± 1.9b
11, 12	Coconino NF & Centennial For.	AZ	10 Aug − 6 Sep 2012	$F_{3,3} = 30.89$, $P = 0.009$	0.8 ± 0.2c	4.2 ± 1.1bc
13	Santa Fe NF	NM	18 Jul − 19 Aug 2012	$F_{3,3} = 44.25$, $P = 0.006$	2.3 ± 0.8b	2.6 ± 1.1b
14	Davis Mts. Pres.	TX	1–7 Oct 2015	$F_{3,3} = 11.00$, $P = 0.040$	15.5 ± 7.2b	19.0 ± 4.8ab

	Pairwise contrasts of lure combinations[a] (Tukey test; $\alpha = 0.05$)		Factorial effects		
Site #[b]	*endo*-brevicomin + myrcene	*endo*-brevicomin + *alpha*-pinene	Factor brevicomin isomer	Factor monoterpene	Interaction between factors
1	3.3 ± 2.0ab	1.7 ± 1.3b	$t_3 = -4.30$, $P = 0.023$	$t_3 = 5.12$, $P = 0.014$	$t_3 = -1.60$, $P = 0.208$
3	15.0 ± 3.5a	14.8 ± 6.1a	$t_3 = -2.42$, $P = 0.094$	$t_3 = -0.17$, $P = 0.877$	$t_3 = 1.56$, $P = 0.216$
4	45.1 ± 18.9bc	26.2 ± 9.4c	$t_3 = -7.49$, $P = 0.005$	$t_3 = 2.31$, $P = 0.104$	$t_3 = -0.23$, $P = 0.835$
5	10.2 ± 6.4ab	3.6 ± 1.5b	$t_3 = -4.58$, $P = 0.020$	$t_3 = 2.67$, $P = 0.076$	$t_3 = -0.42$, $P = 0.700$
6	88.8 ± 19.9ab	35.9 ± 11.9b	$t_3 = -8.15$, $P = 0.004$	$t_3 = 3.71$, $P = 0.034$	$t_3 = 1.39$, $P = 0.257$
7	39.4 ± 8.0a	15.2 ± 5.2ab	$t_3 = 3.61$, $P = 0.036$	$t_3 = 3.25$, $P = 0.047$	$t_3 = 1.57$, $P = 0.214$
11, 12	19.1 ± 4.4ab	65.0 ± 14.7a	$t_3 = 8.58$, $P = 0.003$	$t_3 = -4.13$, $P = 0.026$	$t_3 = -0.60$, $P = 0.590$
13	20.2 ± 4.2a	53.6 ± 12.8a	$t_3 = 11.11$, $P = 0.002$	$t_3 = -2.18$, $P = 0.117$	$t_3 = -2.15$, $P = 0.121$
14	62.9 ± 21.1ab	58.2 ± 14.4a	$t_3 = 5.39$, $P = 0.013$	$t_3 = -1.81$, $P = 0.168$	$t_3 = 0.82$, $P = 0.472$

[a] Lure combinations uniformly included a device releasing frontalin in addition to the variable bait components indicated. Pairwise contrasts are within rows . Treatments with the sa me lower-case letter did not differ significantly (Tukey test on transformed data; $\alpha = 0.05$).

[b] Site number indexed in Table 1

[c] Data were log transformed

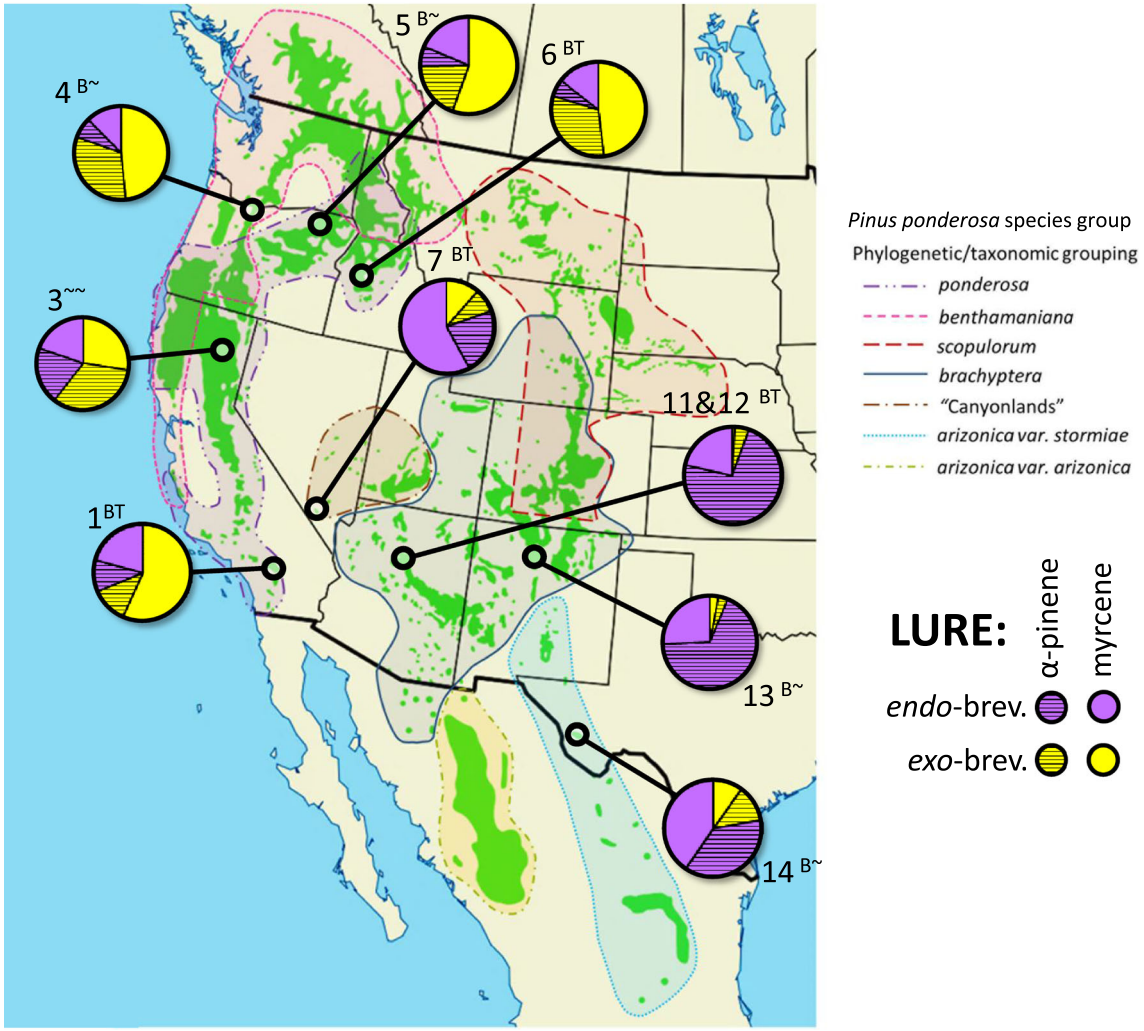

Fig. 3 Catches of *D. brevicomis* sensu Wood (1963) at nine sites in traps baited with the pheromone component frontalin and one of four possible combinations of pheromone components *endo-* or *exo*-brevicomin with host terpenes *alpha*-pinene or myrcene. Numbers adjacent to pies correspond to sites indicated in Table 1. Presence of a superscript capital letter indicates that there was a significant preference by beetles at that site for a terpene ("T") or brevicomin isomer ("B") in the lure (mixed-model factorial ANOVA on log-transformed catches, $\alpha = 0.05$). Figure background represents the taxonomic map of the *P. ponderosa* species group according to Willyard et al. (2017)

odors was not controlled in our pheromone production study, oxygenated monoterpenes were excluded from site-level statistical analyses. Evidence that females differed in their exposure to host odors when infested onto logs included large, site-level variation in levels of hydrocarbon monoterpenes in the headspace samples of the beetles (e.g., *alpha*-pinene amounts fluctuated over three orders of magnitude among sites; $F = 23.4$; $df = 9,150$; $P < 0.001$; Table 2). Resin was visible on the cuticles of a portion of females placed into headspace sampling vials. This resin was undoubtedly a source for host monoterpenes in samples, and variability in its presence no doubt signified variation in female exposure to host monoterpenes. Site-level variation likely occurred because only 1–2 logs were used for sampling females from each site, and logs typically were derived from different trees cut in different years and times of year, and they were stored for different durations. Consistently strong positive correlations (Spearman rank-order correlation coefficients > 0.5) between levels of the five oxygenated monoterpene semiochemicals and host monoterpenes *alpha-* and *beta-* pinene in individual samples support our conclusion that site-associated variation in detected oxygenated monoterpenes was to some extent an artifact of log condition.

However, if results are viewed with caution, regions can be contrasted regarding female production of oxygenated monoterpene semiochemicals. Logs were used indiscriminately (albeit not randomly) among sites, and there was not a significant difference between the two regions in mean concentrations of major host monoterpene *alpha*-pinene in headspace samples (with site used as the unit of replication; $F = 0.20$; $df = 1, 8$; $P = 0.67$). Therefore, we believe that variation in log qualities did not increase type 1 error risk sufficiently to invalidate

region-level contrasts. That being said, production of *Dendroctonus*-associated oxygenated monoterpene pheromone components by females did not differ significantly between eastern and western regions: *cis*-verbenol ($F = 0.20$; $df = 1, 8$; $P = 0.66$), *trans*-verbenol ($F = 0.26$; $df = 1, 8$; $P = 0.62$), *trans*-pinocarveol ($F = 0.01$; $df = 1, 8$; $P = 0.91$), borneol ($F = 0.52$; $df = 1, 8$; $P = 0.49$), and myrtenol ($F = 0.72$; $df = 1, 8$; $P = 0.42$). However, the apparently high level of variability in quantities of precursor monoterpenes in different logs likely obscured differences in oxygenated monoterpene production that may have been apparent under more uniform host conditions.

Studies of bark beetle pheromone production that use host substrate derived from a single species and location [in this study, *P. brachyptera* sensu Willyard et al. (2017) from northern Arizona] may fail to detect some geographic variation, since host chemistry varies geographically and can affect pheromone production (Cale et al. 2016; Clark et al. 2014; Taft et al. 2015). The taxonomic revision of Willyard et al. (2017) indicates that the native hosts for many sampled females differed taxonomically from the logs used in our tests, hence such females were being forced to attack an "unnatural" host. [However, host log acceptance and mining appeared to be similar regardless of a female's site of origin.] The resin composition of hosts on either side of the Great Basin differs substantially (Smith 1977), and this difference is likely to cause some differences in pheromone production in nature that are unrelated to beetle genotype (Blomquist et al. 2010; Hughes 1973; Renwick 1976; Taft et al. 2015).

Male pheromone production was not examined in our study, although this sex contributes components to the aggregation pheromone of *D. brevicomis* sensu Wood (1963) [importantly, the synergist frontalin (Libbey et al. 1974; Pureswaran et al. 2016a; Renwick and Vité 1970)], and it is possible that the male pheromone contribution shows regional differences. In the present study, beetles from the two regions differed significantly in their olfactory responses to ipsdienol [a pheromone component produced by male *D. brevicomis* sensu Wood (1963) as well as numerous sympatric bark beetle species (Skillen et al. 1997; Sullivan et al. 2012)], with eastern populations displaying greater average electrophysiological response amplitudes (Tables 3 and 4). Simultaneously, males from either region did not differ in their average antennal responses to frontalin, although considerable within-region variation was apparent (Tables 3 and 4). A previous study which compared production of pheromone components by newly-paired, mining *D. brevicomis* sensu Wood (1963) males from either northern California or northern Arizona found that the latter produced significantly higher quantities of ipsdienol as well as *cis*-verbenol and 2-phenylethanol (Pureswaran et al. 2016b). In their totality, these data suggest that, in particular, ipsdienol should receive additional investigation as a possible pheromone component whose activity or role may differ between *D. brevicomis* and *D. barberi*.

Our results are aligned with evidence indicating genetic isolation of populations on either side of the Great Basin (Bracewell et al. 2018; Kelley et al. 1999; Lanier et al. 1988), and they contributed to the totality of evidence used to justify the separation of *D. brevicomis* (western region) and *D. barberi* (eastern region) into distinct species (Valerio-Mendoza et al. 2019). These species can be distinguished morphologically by (1) a difference in the uniformity in length of setae on the elytral declivity (i.e., uniformly short in *D. barberi* but with significant variation in *D. brevicomis*) and (2) inconspicuous but quantifiable differences in shape (measured by geometric morphometrics) of the antennal club and genitalia of both sexes (Valerio-Mendoza et al. 2019). The authors were successful in separating western and eastern specimens in the present study using character (1) above. *Dendroctonus brevicomis* and *D. barberi* additionally differ in the geometry of their galleries, being either subtransversely or transversely winding, respectively (Hopkins 1909; Valerio-Mendoza et al. 2019). The species also differ in composition of their mycangial fungi (Davis et al. 2010; Bracewell et al. 2018). No additional biological differences have been reported for these species, but they likely exist and are an important topic for future investigations, particularly as they relate to management.

Geographic variation in pheromone production or responses within individual bark beetle species is not uncommon (Berisford et al. 1990; Borden et al. 1996; Lanier et al. 1972; Miller et al. 1996), and the existence of non-overlapping differences in pheromone production and response between *D. brevicomis* and *D. barberi* is not alone a sufficient argument for their taxonomic separation. No geographical zones of cooccurrence appear to exist between *D. brevicomis* and *D. barberi*, hence pheromone differences ostensibly play no current role in maintaining the existing reproductive isolation indicated by genetic evidence (Bracewell et al. 2018; Kelley et al. 1999). Nevertheless, our observation of greater attraction by flying individuals of each species to their own pheromone blend indicates that the pheromone difference would contribute in some degree to reproductive isolation if the two species were to come into contact. Additionally, pheromone differences in *Dendroctonus* species may have more potent isolating effects at close range [e.g., more for beetles interacting on and within the bark than for beetles in flight (Niño-Domínguez et al. 2015, 2016; Pureswaran et al. 2016a; authors' unpublished data)], hence the potential of our identified pheromone differences to mediate reproductive isolation may not have been fully revealed by the trapping experiments. A limited study of crosses between Arizona and California populations of, respectively, *D. barberi* and *D. brevicomis* reported evidence of

nonreciprocal fertility (Lanier et al. 1988), suggesting that premating reproductive isolation mechanisms such as pheromone differences would be adaptive in a zone of cooccurrence between the two species.

Our analyses also detected significant intraspecific, geographic variation in pheromone production and response, although it is unknown whether such differences would be sufficient to affect population connectivity and hence promote the local isolation of populations. There was no evidence of a north-south gradient in pheromone production or response along the west coast of the USA in parallel with a strong genetic gradation that exists there (Bracewell et al. 2018). Based on morphometric analyses, Valerio-Mendoza et al. (2019) proposed that *D. barberi* may be composed of two allopatric, cryptic species with one restricted to the states of Coahuila, Nuevo León, and Tamaulipas in northeastern Mexico ("SMOR" morphotype), and the other occurring in the central-northern Mexican states of Chihuahua and Durango as well as the entire USA range of *D. barberi* ("East-SMOC" morphotype). The present paper examined beetles from just one site within the range of the SMOR morphotype (#15, Galeana, Nuevo Leon) from which data on female pheromone production and male antennal response were collected. Beetles from this site could not be distinguished semiochemically in any examined trait from all other sites (Tables 2 and 4; Figs. 1 and 2; Online Resource 2,3), although sometimes characteristics of the Geleana beetles had a site-associated mean value that represented the extreme among *D. barberi* sample sites (e.g., greatest mean quantities of both brevicomin isomers). Although these data provide minimal support for distinguishing the SMOR population as a distinct species, the present study was incomplete both with regard to numbers of sites sampled, the kinds of semiochemistry data investigated, and which sex was examined.

The aggregation attractant of many bark beetle species incorporates odors from the host tree, with monoterpenes of the host resin commonly dominating this blend (Miller and Borden 2000; Seybold et al. 2006). The composition of the odor blend of a potential host tree may allow foraging bark beetles to discriminate suitable host taxa (Chapman 1963; Gitau et al. 2013; Pureswaran and Borden 2003). For example, odors of non-hosts (in particular, those from hardwoods) can deter response by conifer-infesting bark beetles to attractants (Byers 2007; Zhang and Schlyter 2004). However, in conifer-infesting bark beetles, minimal association has been observed between attractive preferences for monoterpenes and the constituency of these compounds in the resin of preferred conifer taxa (Borden et al. 2008; Pureswaran and Borden 2005; Pureswaran et al. 2004). Similarly, data from the current study found merely a weak association between the resin composition of locally available hosts and behavioral and electrophysiological responses to host resin constituents myrcene and *alpha*-pinene. Available hosts within the range of *D. barberi* in the USA contain greater amounts of *alpha*-pinene than myrcene (Texas data were unavailable), whereas hosts in the range of *D. brevicomis* display the reverse relationship (Davis and Hofstetter 2012; Smith 1977; Online Resource 1). However, we did not observe consistent preferences for either myrcene or *alpha*-pinene lures corresponding to geographical distribution of the beetle species. We likewise observed only a weak correlation overall between proportions of beetles trapped by either *alpha*-pinene or myrcene lures and the proportions of these two monoterpenes represented in the resin of the closest host population for which data was available. Simultaneously, antennal responses to myrcene and *alpha*-pinene similarly failed to display any correspondence to local host resin composition or association with the taxonomic division of *D. brevicomis* and *D. barberi*. Thus, our data do not support the hypothesis that adaptation to local host biochemistry has caused the regional variation in behavioral responses to myrcene and *alpha*-pinene observed in *D. brevicomis* and *D. barberi*. Some caution should be taken in interpreting our results since our antennogram and behavioral experiments utilized a specific enantiomeric ratio of *alpha*-pinene, whereas chirality has been demonstrated to influence attractiveness and antennal responses of *alpha*-pinene with species of *Dendroctonus* (Hobson et al. 1993; Staeben et al. 2015; White and Hobson 1993).

Our research indicates that the operational trap lures for *D. brevicomis* and *D. barberi* should be formulated differently. For *D. barberi*, replacing the *exo*-isomer of brevicomin [as is currently used for the *D. brevicomis* sensu Wood (1963) lure] with *endo*-brevicomin should cause catches to increase by an order of magnitude. Our research agreed with previous findings that *alpha*-pinene is more attractive than myrcene for *D. barberi* in Arizona (Hofstetter et al. 2008); however, this preference appears to be locally variable (e.g., myrcene was preferred in southern Nevada), and thus there was no indication that either was a superior monoterpene synergist for the entire species. For *D. brevicomis*, the general trend among trapping sites indicates myrcene is the superior attractant synergist, and thus no change is recommended for the *D. brevicomis* lure (i.e., *exo*-brevicomin, frontalin, and myrcene).

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