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Western Pine Beetle Populations in Arizona and California Differ in the Composition of Their Aggregation Pheromones

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Abstract We compared pheromone production and response for populations of western pine beetle, *Dendroctonus brevicomis* LeConte, from sites in northern Arizona and northern California. Volatiles were collected from individuals of both sexes that had mined as a pair in a *Pinus ponderosa* log for 1 d, and they were subsequently analyzed by gas chromatography coupled to mass-spectrometry. Principal component analysis of quantities of *Dendroctonus* pheromone components indicated strong site-associated clustering of blend composition for females but not males. Much of the clustering in females evidently was due to differences in the production of *endo*- and *exo*-brevicomin, which occurred in average ratios of 0.1:1 and 19:1 for populations in the California and Arizona sites, respectively. In the California site, *exo*- was better than *endo*-brevicomin in enhancing trap catches of both sexes to lures containing the host-tree odor α-pinene and the male-produced aggregation pheromone component frontalin. In an identical test in the Arizona site, *endo*- was a better adjuvant than *exo*-brevicomin for male attraction, whereas females did not show a significant preference. At neither location were the isomers antagonistic to one another in activity. Thus, one aggregation pheromone has apparently diverged between these populations, concurrent with published evidence that *D. brevicomis* on either side of the Great Basin are genetically distinct and are possibly different species. Furthermore, production of and response to the isomers of brevicomin by flying *Dendroctonus frontalis* Zimmermann in the Arizona site were similar to those of sympatric *D. brevicomis*. This interspecific signal overlap is likely sustainable since joint species mass-attacks may assist both species in overcoming host defenses, thereby increasing host availability.

Keywords Western pine beetle · Southern pine beetle · Interspecific attraction · Geographic variation · Pheromone · Character displacement · Scolytinae

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Introduction

The western pine beetle *Dendroctonus brevicomis* LeConte is a major agent of biotic mortality of pines within North America. Its range largely coincides with that of its principal host, *Pinus ponderosa* Laws., extending from southern British Columbia to southern California and from Idaho to Chihuahua State (Furniss and Carolin 1977; Wood 1982b). As is typical for "aggressive", tree-killing species of bark beetle, they can aggregate and attack *en masse* in order to deplete the primary defenses (i.e., resin) of the host tree and thereby permit colonization of healthy and high-quality hosts (Berryman 1972; Wood 1982a). Mass-aggregation is mediated by multi-component pheromone systems that have evolved in these species to both attract sufficient conspecifics for successful mass-attacks and simultaneously regulate attack densities to reduce deleterious effects of competition (Byers 1989; Raffa and Berryman 1987).

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In *Dendroctonus*, mass-attacks begin when females initiate mines (or "galleries") in the bark and emit pheromone components that attract conspecifics of both sexes, with males typically predominating (Six and Bracewell 2015). Male beetles that arrive and pair with females may release their own distinct and synergistic aggregation pheromone components (Wood 1982a). Production of attractants by the pair continues at least until the gallery is extended a short distance and oviposition commences, often coincident with the depletion of host resin defenses (Pureswaran and Sullivan 2012). Mass-attack of a host tree is terminated apparently by decline in both the host-generated synergists and aggregation pheromone production by the pair, and possibly by the presence of aggregation-inhibiting semiochemicals (Byers et al. 1984; Powell et al. 1998; Raffa et al. 1993; Wood 1982a). The aggregation pheromone blends of different species of *Dendroctonus* typically consist of differing combinations of the same few components (Borden 1982; Symonds and Elgar 2004), which are synergized by monoterpene odors from the host (e.g., α-pinene) which individually – as with the pheromone components themselves – confer limited specificity since they typically occur in many host and non-host tree species (Byers 1995; Seybold et al. 2006). Within *P. ponderosa*, the primary host, there is significant variation in the individual monoterpenes that dominate the chemical profile across its range, resulting in regionally specific chemical profile zones within the western United States (Smith 1977).

Published studies of the chemical ecology of *D. brevicomis* have been based predominantly on populations in California and Oregon. These studies indicate that attack-initiating females release the aggregation pheromone component *exo*-brevicomin that is biosynthesized *de novo*. The combination of this with myrcene from host resin attracts males that release the pheromone component frontalin (Bedard et al. 1969; Libbey et al. 1974; Silverstein et al. 1968; Vité and Pitman 1969a), which is also biosynthesized *de novo*. Frontalin strongly synergizes the attractiveness of *exo*-brevicomin and host-tree odors to both sexes of beetles (Bedard et al. 1980b; Renwick and Vité 1970; Vité and Pitman 1969b). Both sexes also have been found to release additional compounds that exhibit behavioral activity with conspecifics, most often as attraction inhibitors, e.g., verbenol, verbenone, ipsdienol (Bedard et al. 1980a; Bertram and Paine 1994; Byers 1982; Byers et al. 1984; Pitman et al. 1969; Renwick et al. 1976), but the summed evidence indicates that the key components of the aggregation pheromone are (+)-*exo*-brevicomin and (−)-frontalin (Bedard et al. 1985; Byers 1982; Wood and Bedard 1977; Wood et al. 1976). In studies of California and Oregon populations of *D. brevicomis*, the *endo*- isomer of brevicomin also has been detected (Browne et al. 1979; Libbey et al. 1974; Silverstein et al. 1968) but generally in similar or smaller quantities to the *exo*-isomer (Libbey et al. 1974), and laboratory and field tests did not indicate attractive or synergistic activity for *endo*-brevicomin (Bedard et al. 1980a; Silverstein et al. 1968). Relatively little research has been performed on the chemical ecology of *D. brevicomis* populations east of the Great Basin (Hofstetter et al. 2008, 2012; Pureswaran et al. 2008a), and the possibility of regional variation in pheromone composition has not been studied directly in *D. brevicomis*.

Hindguts of female *D. brevicomis* live-trapped at a site in northern Arizona, however, contained *endo*-brevicomin in quantities that greatly exceeded the *exo*-isomer. This suggested a geographic variation in pheromone blends between Arizona and coastal populations (Pureswaran et al. 2008a). Additionally, cytochrome oxidase I sequences have indicated that populations of *D. brevicomis* on either side of the Great Basin desert, although apparently monophyletic (Kelley and Farrell 1998), are genetically distinct and possess a degree of genetic distance comparable to that observed for recognized sibling species of *Dendroctonus* (Kelley et al. 1999). It is possible that a divergence in pheromone composition may have accompanied the genetic split, and that host chemical profiles may have contributed to that divergence.

Dendroctonus brevicomis is sympatric with sibling species *Dendroctonus frontalis* Zimmermann (the southern pine beetle) within a small zone that includes Arizona and New Mexico (Pureswaran et al. 2016; Wood 1982b). As with *D. brevicomis*, *D. frontalis* utilizes an aggregation pheromone to organize mass attacks. However, in the latter species the pheromone is composed of female-produced frontalin and male-produced *endo*-brevicomin (Sullivan 2011). The two species are syntopic on *P. ponderosa* within the sympatric zone: they infest the same portions of the bole of individual trees simultaneously, and their galleries often occur adjacent to one another (Davis and Hofstetter 2009; Sanchez-Martinez and Wagner 2002). Since this interaction between species likely generates selective pressures on pheromone composition and behavioral responses, we hypothesize that shifts in pheromone composition of the eastern populations of *D. brevicomis* might be shaped by its coexistence with *D. frontalis*.

We tested the hypotheses that (1) the composition of the aggregation pheromone differs between populations of *D. brevicomis* in sites in northern California and Arizona, i.e., on either side of the Great Basin, and (2) the compositions of the aggregation pheromones of sympatric *D. brevicomis* and *D. frontalis* resemble each other, as cross attraction might enhance mutual capacity of the species to mass-attack host trees successfully. We tested this by comparing pheromone production of the allopatric *D. brevicomis* populations and performing trapping experiments at sites in both California and Arizona with synthetic lures that reflected identified differences in production. Additionally we assayed *D. frontalis* responses to the same lures, and compared the pheromone

blend of *D. brevicomis* in the Arizona site to that previously published for *D. frontalis*.

Methods and Materials

Collection and Treatment of Beetles Bark containing late larvae through callow adult life stages of the beetles was excised from naturally-infested *P. ponderosa*, transported to the laboratory, and placed inside polyester-cotton, zippered pillow covers (51 × 91 cm; Target Corp.) held inside a rearing room maintained at room temperature. Beetles that emerged from the bark were collected 1–2 times daily from the inside surface of the pillow covers and housed at 6 °C in plastic petri plates (9 cm diam) lined with moistened paper wipers for a maximum of 32 d (typically <12 d) prior to use. Beetles were separated by sex by the presence of the pronotal callus (mycangium) on females and frontal protuberances on males (Osgood and Clark 1963; Wood 1982b).

Bark infested with *D. brevicomis* from multiple trees in Arizona was collected between September and November 2011 from two sites, one in the Prescott National Forest (1 tree; 35°10' N 111°45' W elev. 1830 m) and the second in the Coconino National Forest (2 trees; 35°14' N 111°49' W; elev. 2220 m), both in northern Arizona. Bark infested with *D. brevicomis* from two trees in northern California was collected in September 2011 from a single site on the Plumas National Forest (40°09' N, 120° 47'0 W; elev. 2012 m).

Healthy, uninfested *P. ponderosa* logs (14–15 cm diam., 22–24 cm long) from two trees cut in the same area in Arizona where infested bark was obtained were inoculated with up to 20 beetle pairs per bolt. The ends of logs were sealed with wax immediately after cutting to minimize desiccation, and after 5–12 d stored at 2 °C for a maximum of 32 d prior to use. All beetles used to infest logs were active and apparently healthy. Logs first were allowed to equilibrate to room temperature, and then females were inoculated into the bolt by enclosing them individually within half of a #0 gelatin capsule secured over a 2 mm-diam. hole drilled through the outer bark. Attacks on bolts were spaced >4 cm apart, and, when beetles from the two populations were inoculated into the same log, they were confined to opposite sides. After 22–26 h, a male from the same region was introduced into the gel caps of females that had entered the bark and were producing frass. Both sexes were allowed to tunnel together for another 14–22 h after which they were carefully excised with a knife and soft forceps and immediately transferred to volatile sampling vials. The artificially-infested logs were maintained at 23–27 °C (room temperature).

On 30 September 2011, four Arizona pairs and 19 California pairs were sampled from a single log; on 3 November 2011 seven Arizona pairs and six California pairs were sampled from a single log (different log derived from same tree as the 30 September log), and on 29 November 2011, 17 Arizona pairs were sampled from a single log derived from a different tree. These data were pooled and the emitted pheromones were analyzed from individuals in four categories of *D. brevicomis* derived from pairings: females ($N = 30$) and males ($N = 28$; two were destroyed during excision) from the Arizona sites as well as females ($N = 25$) and males ($N = 25$) from the California site.

Analyses of Emitted Pheromones Volatiles released from the beetles were sampled by confining them individually in vertically-maintained 100 µl conical-interior glass vials containing a 2–3 mm-depth (approximately 0.3 mg) of the adsorbent HayeSep-Q® (80–100 mesh; Hayes Separations, Bandera, TX, USA) in their tip (Pureswaran et al. 2008b; Sullivan 2005). A 2 mm diam. Polytetrafluoroethylene (PTFE) rod was used to secure the live beetle in the vial tip such that the apex of its abdomen was <2 mm above the adsorbent. The vial was closed with a septum cap possessing a ~ 1 mm hole in its center to permit ventilation for beetle respiration, and the vials were incubated for 23–25 h at 21–22 °C with layers of activated charcoal mesh (Universal Replacement Pre-filter, Honeywell, Southborough, MA, USA) pressed against the vial caps to reduce incursion of outside volatiles. Afterward, the beetle and PTFE rod were removed, and adsorbent and beetle excrement in the vial were extracted with 50 µl of hexane spiked with 3.8 ng/µl cycloheptanone as an internal standard. This extract and a further 50 µl wash of the conical vial made with redistilled pentane were combined in an autosampler vial with 200 µl insert and stored at −80 °C prior to chemical analyses.

Each extract sample (1 µl) was analyzed in splitless mode on a coupled GC-MS (Hewlett-Packard GCD 1800C) equipped with an HP-INNOWax (Agilent Technologies, Santa Clara, CA, USA; 60 m long × 0.25 mm diam. × 0.25 µm film thickness) polyethylene glycol-phase microcapillary column. The oven temperature program was held at 40 °C for 1 min, then increased at 16 °C/min to 80 °C, then 7 °C per min to 230 °C and held 12 min. Ten previously identified pheromone components for pine-infesting *Dendroctonus* were quantified (in ng/beetle) based on standard curves generated by analyzing serial dilutions of known quantities of commercially-obtained synthetic versions of the compounds. Control aeration vials without beetles were prepared at the same time as the sample vials, and these were found to be free of detectable levels of pheromone components. Four Arizona males produced no excrement and merely trace amounts of pheromone components were detected from these as, in feeding bark beetles, pheromone is released during defecation. These four strong outliers were removed from the statistical analyses, as recommended for data sets analyzed by multivariate ordination methods (McGarigal et al. 2000).

Quantities (ng) were transformed by $\log_{10}$ (x + 1) to improve homoscedasticity, and 1 ng was the approximate threshold of detection of the mass spectral detector. Pheromone component quantities were analyzed within sex using multivariate analysis of variance (MANOVA, proc GLM) with factors being geographical source (California or one of the two sites in Arizona) and the specific log on which the beetles were infested, since log age and condition of source tree could potentially influence pheromone production. Compounds included in the model for either sex were limited to those that were present in more than trace quantities. A principal components analysis (PCA) using a covariance matrix was calculated for each sex separately (on the log transformed quantities of the same pheromone compounds included in the MANOVAs) to compare variation in pheromone emission profiles between populations of *D. brevicomis* in the California and Arizona sites. Biplots for principal components 1 and 2 were generated with symmetric scaling (Yan and Hunt 2002) to provide a means of visually interpreting the relative contribution of each compound to the total variance within the principal components. Univariate contrasts of quantities produced by individual beetles from the two sites were performed within sex by means of a SAS estimate statement within the MANOVA. The ratios of *endo*/*exo*-production by individual females were plotted in a histogram for *D. brevicomis* samples in addition to pheromone production data for five Arizona-derived female *D. frontalis* reported by Pureswaran et al. (2016). For these five beetles the pheromone sampling techniques were essentially identical to those used in the present paper.

Evaluation of Responses to Pheromones in the Field Since the proportions of *endo*- to *exo*-brevicomin appeared to be a major variable responsible for PCA clustering of female *D. brevicomis* from California and Arizona, trapping trials were performed in sites in Arizona and California to look for corresponding regional differences in responses to these compounds. In Arizona (35° 10' N, 111° 45' W, elev. 2080 m), the experiment was conducted between 6- Jun-2010 and 1-Jul-2010. In California (40° 34' N, 120° 47' W, elev. 2020 m), the experiment was conducted from 16-Aug-2010 to 9-Sep-2010. Twelve-unit multiple funnel traps (Lindgren 1983) were baited uniformly with frontalin, a male-produced pheromone component that did not vary between populations, and α-pinene, a host pine monoterpene that can enhance attraction of *D. brevicomis* to components of its aggregation pheromone (Hofstetter et al. 2008). To these were added either: 1) nothing (control), 2) *exo*-brevicomin, 3) *endo*-brevicomin, or 4) both *exo*- and *endo*-brevicomin. Compounds were released from commercially-obtained lures (Synergy Semiochemicals Inc., Vancouver, BC, CA) with the following purities and release rates measured gravimetrically at 23 °C: α-pinene (> 93 %; ~150 mg/d), racemic frontalin (99 %; ~5.2 mg/d), racemic *exo*-brevicomin (97 % *exo*-brevicomin, 2 % *endo*-brevicomin; ~1.7 mg/d) and racemic *endo*-brevicomin (95 % *endo*-brevicomin, 4 % *exo*-brevicomin; ~2.8 mg/d). The four lure treatments were deployed in 10 blocks of four traps in a randomized complete block design. Blocks were 0.5 km apart and traps within each block were 10–20 m apart. Traps were checked and beetles collected every 3 to 7 d, at which time the positions of lures were moved forward one position, and the experiment was continued until each lure had occupied every trap position. The species of captured beetles were identified (using Wood 1982b), their sex was determined (Osgood and Clark 1963), and their total numbers were counted. Data from each site and species were cube-root transformed and analyzed using a mixed model analysis of variance with treatment, sex, and treatment-by-sex as fixed factors, and block as a random factor. Pairwise comparisons were produced by Fisher LSD with a Bonferroni adjustment for six comparisons (within state, sex, and species; $\alpha = 0.05$).

Results

Analysis of Pheromone Emission Profiles For *D. brevicomis*, due to imbalances in the collection of data regarding the tree and specific log on which beetle attacks were produced, it was not possible to completely separate effects of beetle source population from tree and log in the MANOVAs. The MANOVA analysis (Wilks' Lambda statistic) indicated a significant effect on blend composition for the site from which the beetles were derived (for males: $F = 29.24$, $df = 8, 39$, $P < 0.001$; for females: $F = 686.5$, $df = 7, 42$, $P < 0.001$), but also a significant interaction between the location sampled and the specific log onto which the beetles were infested (for males: $F = 11.17$, $df = 32, 138.04$, $P < 0.001$; for females: $F = 27.9$, $df = 28, 145.6$, $P < 0.001$). The interaction may have arisen from variation in the resin concentrations and composition in the different logs that were infested for sampling. The quantity and quality of host resin monoterpenes to which a beetle is exposed can substantially alter the quantities of certain pheromone components produced, particularly the oxygenated monoterpenes that can be produced by direct oxidation of resin monoterpenes (Hughes 1973; Seybold et al. 2006). However, the PCA of the pheromone compositions of female beetles indicated two distinct, widely-spaced clusters representing either the single California site or both Arizona sites (Fig. 1). Since some samples from the same and different logs are included in each cluster, the implication is that grouping of pheromone blends associated with the state of beetle origin far exceeded any segregation due to other potentially confounding sampling variables. Biplot vectors indicated that the variables most strongly associated with the clustering of female samples were relatively higher levels of *exo*-brevicomin produced by California females and higher

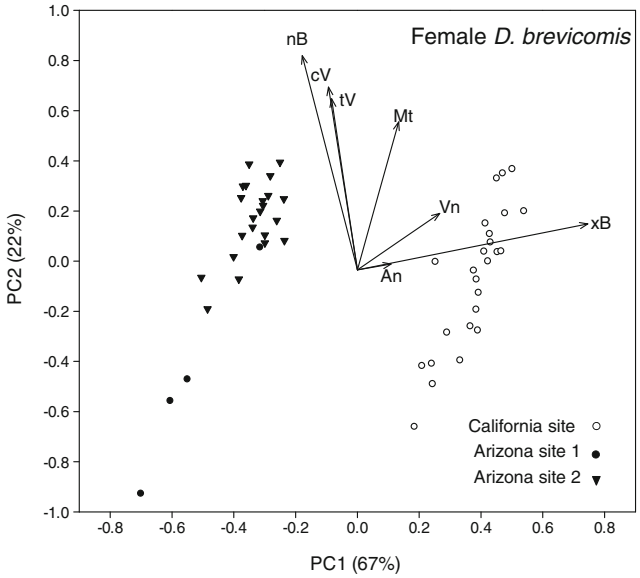

Fig. 1 Biplot of principal component analysis (Yan and Hunt 2002) of quantities of *Dendroctonus* pheromone components isolated from paired, mining *D. brevicomis* females originating from one site in northern California (40°09' N, 120° 47'0 W) and two sites in northern Arizona (35°10' N 111°45' W and 35°14' N 111° 49' W). Biplot vectors (indicating the contribution of individual compounds to total variation within the first two principal components) are labelled as *endo*-brevicomin (nB), *cis*-verbenol (cV), *trans*-verbenol (tV), myrtenol (Mt), verbenone (Vn), acetophenone (An), and *exo*-brevicomin (xB)

levels of *endo*-brevicomin and *cis*- and *trans*-verbenol associated with Arizona females. These associations were corroborated by univariate statistics within the MANOVA (Fig. 2): significantly higher quantities of *exo*-brevicomin, acetophenone, verbenone, and myrtenol were produced by California females, and significantly higher levels of *endo*-brevicomin, *cis*-verbenol, and *trans*-verbenol were produced by Arizona females. Within the biplots of females, vectors for *endo*- and *exo*- brevicomin were the two most strongly opposed, and the histogram of the *endo*/*exo*-brevicomin ratios for females indicated two completely non-overlapping clusters for California and Arizona *D. brevicomis* (greater than an order of magnitude separation), with a median ratio of 0.090 for California and 29.0 for Arizona *D. brevicomis* (Fig. 3).

The *endo*/*exo*-brevicomin ratios for five sampled *D. frontalis* females from Arizona (Pureswaran et al. 2016) ranged from 8.00 to 26.5, and grouped with the lower half of the Arizona cluster.

PCA of the pheromone productions of paired male *D. brevicomis* beetles generated merely a single cluster, although with apparent concentration of data points for each site into opposite halves of this cluster (Fig. 4). Males from the California and Arizona sites produced similar levels of frontalin. Differences between the California and Arizona sites were seen in the amounts of *cis*-verbenol, ipsdienol, and 2-phenylethanol (Fig. 5).

Evaluation of Responses to Pheromones in the Field At the California site, there was a significant effect for treatment ($F = 63.8$, $df = 3, 27$, $P < 0.001$), treatment by sex ($F = 3.91$, $df = 3, 27$, $P = 0.019$), but not sex ($F = 4.06$, $df = 1, 9$, $P = 0.075$) on the number of *D. brevicomis* trapped. Attraction of both sexes was much greater when *exo*- rather than *endo*-brevicomin lures were added to the control blend of α-pinene and frontalin, although *endo*-brevicomin lures increased catches substantially over the control blend alone (Fig. 6a). Addition of *endo*-brevicomin lures to lures with the *exo*-isomer did not significantly alter catches. For *D. brevicomis* trapped in the Arizona site, there was a significant effect for treatment ($F = 23.8$, $df = 3, 27$, $P < 0.001$), treatment by sex ($F = 6.42$, $df = 3, 27$, $P = 0.002$), but not sex ($F = 1.68$, $df = 1, 9$, $P = 0.23$). As in the California site, catches of both sexes were

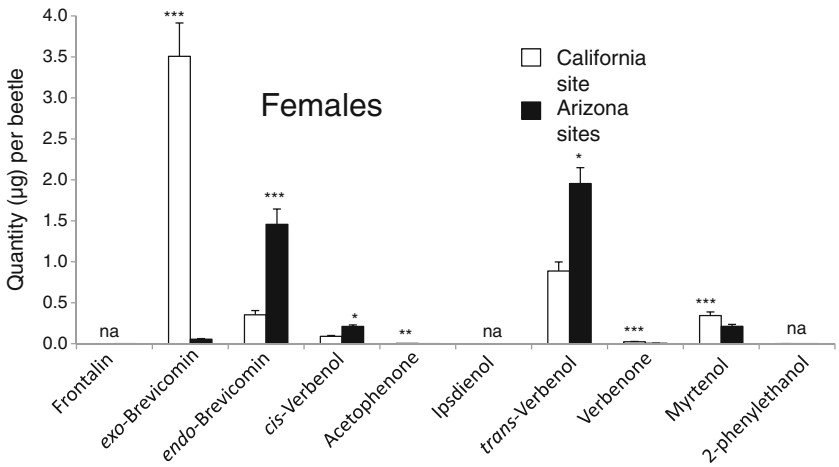

Fig. 2 Quantities of *Dendroctonus* pheromone components isolated from paired, mining *D. brevicomis* females originating in sites from northern California or northern Arizona (sites given in Fig. 1 legend; Arizona data pooled). Statistical significance in univariate contrasts between locations was $P < 0.05$*, $P < 0.01$**, and $P < 0.001$*** (MANOVA with estimate statements for all-pairwise contrasts and a Bonferroni correction). The notation "*na*" indicates that the compound was not analyzed due to insufficient quantities

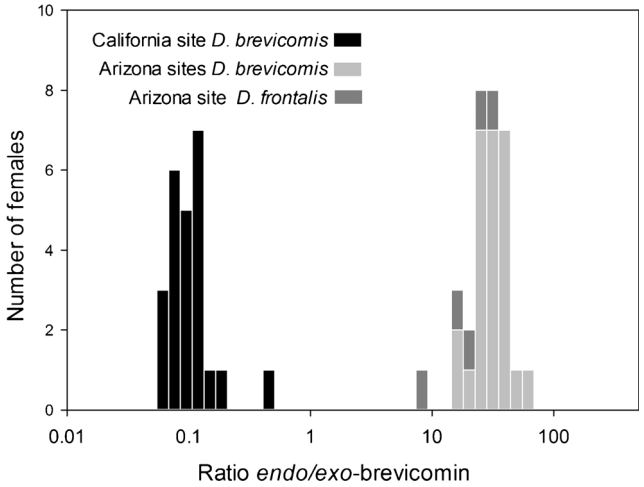

Fig. 3 Histogram illustrating the distribution of raw ratios of *endo*/*exo*-brevicomin in volatiles isolated from paired female *Dendroctonus brevicomis* from a site in northern California and two sites in northern Arizona (both pooled). Data for five male *Dendroctonus frontalis* Zimmermann collected in northern Arizona and sampled by identical methods as *D. brevicomis* are also shown

significantly increased by the addition of either or both isomers of brevicomin to traps (Fig. 6b). However, males were more attracted to *endo*- than *exo*-brevicomin, and addition of *exo*- to the *endo*-brevicomin treatment did not alter responses. Females did not differ significantly in their attraction to either isomer alone. However, traps with the combination of *endo*- and *exo*-brevicomin trapped significantly more than traps amended only with *exo*-brevicomin.

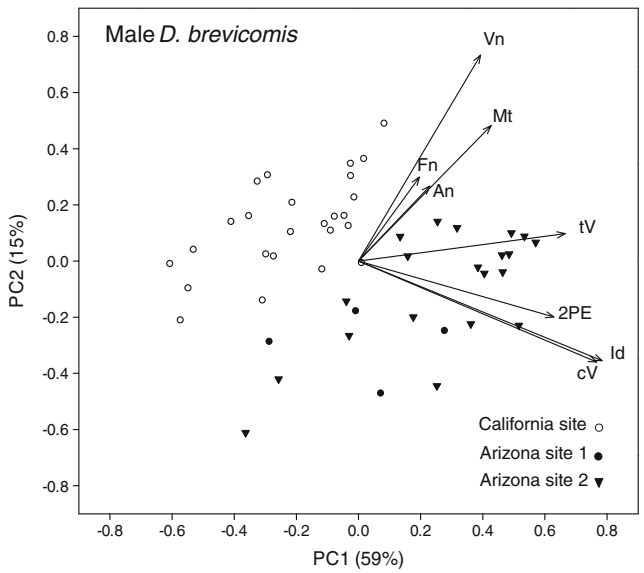

Fig. 4 Biplot of principal component analysis (Yan and Hunt 2002) of quantities of *Dendroctonus* pheromone components isolated from paired, mining *D. brevicomis* males originating from a site in northern California and two sites in northern Arizona (i.e., mates of females in Fig. 1). Biplot vectors are labelled as verbenone (Vn), frontalin (Fn), acetophenone (An), myrtenol (Mt), *trans*-verbenol (tV), 2-phenylethanol (2PE), ipsdienol (Id), and *cis*-verbenol (cV)

For *D. frontalis* trapped in the Arizona site, there was a significant effect for treatment ($F = 5.32$, $df = 3, 27$, $P = 0.005$), sex ($F = 21.7$, $df = 1, 9$, $P = 0.001$) and a treatment-by-sex interaction ($F = 11.06$, $df = 3, 27$, $P < 0.001$). For both sexes of *D. frontalis* in the Arizona site, attraction did not differ between *exo*- and *endo*-brevicomin-amended lures (Fig. 6c). For males, amendment of the control blend with either isomer alone did not significantly increase catches, whereas the combination of the two isomers together increased catches over the control blend as well as over the *endo*-isomer alone. Catches were not greater to the two-isomer combination than the *exo*-isomer alone. For females, both the *exo*- and the *endo*- isomers alone as well as their combination significantly increased attraction to the control blend, and there were no significant differences in responses among these three treatments. The poor discrimination of lures by *D. frontalis* might be attributable to the close spacing of traps within blocks (10–20 m), which has been shown to cause anomalous lure comparison results (Sullivan and Mori 2009).

Discussion

Published studies on the pheromone biology of *D. brevicomis* indicate that females produce both the *exo*- and *endo*-isomers of brevicomin (Libbey et al. 1974; Silverstein et al. 1968). However, no behavioral activity or ecological function has been attributed to *endo*-brevicomin. In contrast, *exo*-brevicomin has been shown in numerous studies to be attractive both alone and as a synergist with frontalin when released with host odors to populations in the coastal United States (Bedard et al. 1980a; Vité and Pitman 1969a; Wood et al. 1976), and it has been concluded to be an essential part of the aggregation pheromone. Furthermore numerous studies indicate the attractiveness of the myrcene/*exo*-brevicomin/frontalin combination to *D. brevicomis* (Skillen et al. 1997). We provide compelling evidence that *endo*-brevicomin is an aggregation pheromone component for *D. brevicomis* in central Arizona and may replace or duplicate *exo*-brevicomin as the major female-produced bicyclic acetal pheromone component in this region. We also observed significant attraction enhancement by *endo*-brevicomin lures in the California site, suggesting behavioral activity with this population as well, although this response was much lower than to the *exo*-isomer and could be attributable to the 4 % *exo*- contamination of the *endo*-brevicomin lures.

In both the California and Arizona sites, *D. brevicomis* lures including both brevicomin isomers in roughly equal quantities did not differ in attractiveness from lures with the apparently most attractive isomer presented alone, indicating that the isomers were neither additive, synergistic, nor antagonistic, in activity. Thus, the narrow range of isomer ratios produced by beetles at either site (Fig. 3), contrasts with their

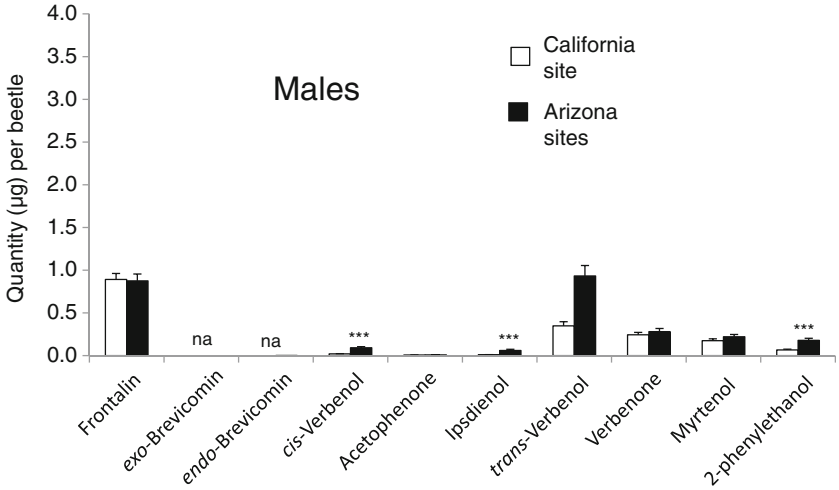

Fig. 5 Quantities of *Dendroctonus* pheromone components isolated from paired, mining *D. brevicomis* males originating from sites in northern California and northern Arizona. (Other details as Fig. 2)

behavioral responsiveness to an apparently broad range of isomer ratios, particularly, in the case of the Arizona population. Byers (1988) demonstrated that California *D. brevicomis* were not particularly sensitive to the precise ratios of the two major components of their aggregation pheromone (*exo*-brevicomin and frontalin), and it is possible that *D. brevicomis* may be similarly insensitive to the precise ratios between the brevicomin isomers. Nonetheless, the dominant isomer produced by each *D. brevicomis* population was the most attractive to it, indicating a parallel shift in production and response to the isomers between the populations. The rather unselective behavioral responses to isomer ratios in the test lures indicate that these pheromone composition differences would have a limited capacity to act as an effective mechanism of reproductive isolation between populations in a zone of sympatry (at least for flying insects). The observed response specificity, however, of either population to the brevicomin isomers should be interpreted cautiously because of the aforementioned contamination of lures with the reciprocal isomer.

Reproductive compatibility (and thus species validity and integrity) of the sampled *D. brevicomis* populations in sites in California and Arizona has not been adequately investigated. Lanier et al. (1988) succeeded in producing F1 brood from crosses of Arizona females and California males, but two attempted reciprocal crosses failed to produce larvae, and no F1 backcrosses were performed (Lanier et al. 1988). However, cytochrome oxidase I sequences indicate that populations of *D. brevicomis* on either side of the Great Basin desert, although apparently monophyletic (Kelley and Farrell 1998), are genetically isolated and possess a degree of genetic distance comparable to that observed for recognized sibling species of *Dendroctonus* (Kelley et al. 1999). Thus, divergence in pheromone composition of *D. brevicomis* in Arizona and California could be explained potentially by genetic drift of isolated populations and/or character displacement within existing or past zones of sympatry between the two genetically-distinct and possibly reproductively incompatible populations (Kelley et al. 1999). Interestingly, Bracewell and Six (2014) also found that *D. brevicomis* beetles from Montana are unable to acquire the mutualistic fungus of *D. brevicomis* from New Mexico, and the resulting brood females were aposymbiotic. This implies that beetles may have diverged along with their fungi and that they are adapted to particular genotypes of symbionts enforcing a high degree of specificity and fidelity.

Regional variation in the chemical profile of *P. ponderosa* also has contributed to differences in kairomones used and pheromone composition within Arizona (Hofstetter et al. 2008). Enhanced host tree availability conferred to *D. brevicomis* through convergence of its aggregation pheromone composition with that of *D. frontalis* (see below) in their sympatric zone in the southwestern United States and northern Mexico also could have promoted or generated a pheromone divergence between southwestern and coastal *D. brevicomis* populations. Recent evidence, however, indicates that *D. brevicomis* of the pheromone type from the Arizona site occur in zones of allopatry with *D. frontalis* (e.g., east-central Utah; authors' unpublished data), suggesting that sympatry with *D. frontalis* may not have been the major factor in the evolution of the *D. brevicomis* pheromone blend in Arizona.

The California/Arizona divergence in pheromone composition is perhaps the first recognized, ecologically-relevant phenotypic difference that distinguishes the eastern and western *D. brevicomis* genotypes identified by Kelley et al. (1999) and supports the hypothesis that these populations are cryptic species within *D. brevicomis*. The divergence in pheromone composition was most conspicuous within females (Figs. 1, 4), the pioneer sex. Since the female pheromone blend is presumably responsible for mediating mate recognition by males seeking female-initiated gallery entrances, this suggests that the shift may have been governed by sexual selection. The pheromone blend divergence in males, by

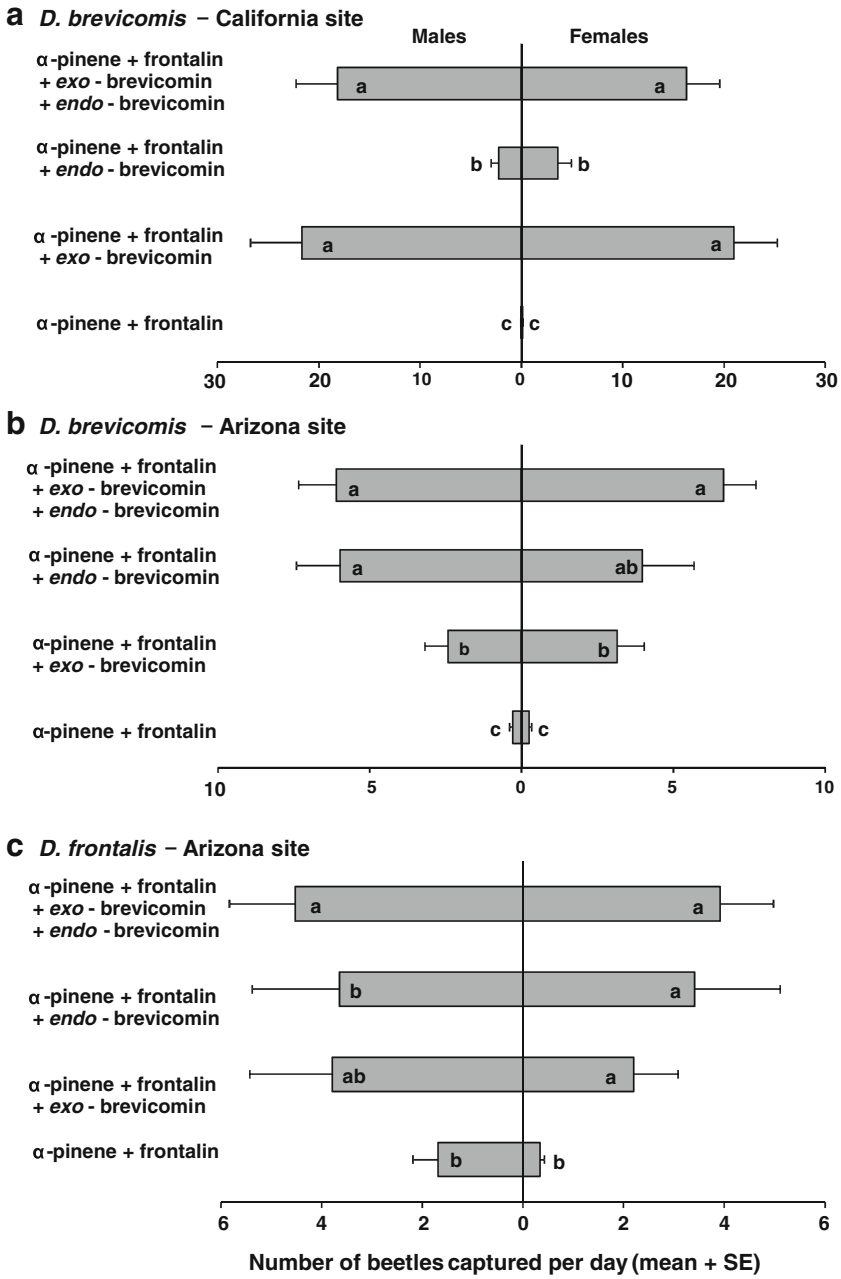

Fig. 6 Behavioral response of sympatric *Dendroctonus frontalis* and *Dendroctonus brevicomis* at a site in northern Arizona and *D. brevicomis* at a site in northern California to funnel traps baited with combinations of semiochemicals produced by these species. Within species, site, and sex, bars associated with the same lower case letter were not significantly different (ANOVA with Fisher LSD and Bonferroni correction for six contrasts; $\alpha = 0.05$)

contrast, was relatively small (Fig. 5). However, males from the Arizona sites, despite producing similar quantities of the attractive synergist frontalin as their California site counterparts, produced significantly more ipsdienol, *cis*-verbenol, and 2-phenylethanol, and these may represent population-specific differences in the male contribution to the aggregation pheromone blends. The potential behavioral roles of these three compounds for Arizona populations of *Dendroctonus* merit further investigation.

Our data corroborate other evidence that the aggregation pheromone blend of the sympatric populations of *D. frontalis* and *D. brevicomis* in Arizona sites are similar and that cross-attraction likely occurs in nature. *Dendroctonus frontalis*, like the sympatric *D. brevicomis* of Arizona of the present study, utilize both frontalin and *endo*-brevicomin as the major components of their aggregation pheromone (Moreno et al. 2008; Sullivan et al. 2007; Vité et al. 1985). In their sympatric zone in Arizona, both *D. brevicomis* and *D. frontalis* exhibit similarly strong attraction and discrimination of field trapping baits that incorporate frontalin and brevicomin (Hofstetter et al. 2008, 2012). In a field study in which traps were baited with screened logs infested manually with either *D. frontalis* or *D. brevicomis* females (and thus releasing half the aggregation pheromone), beetles of both species were trapped in small numbers by the *D. brevicomis* logs but not the *D. frontalis* logs (Davis and Hofstetter 2009). In reverse of

D. brevicomis, *D. frontalis* males are the sex that produces brevicomin (and predominantly the *endo*-isomer) whereas females release frontalin (Pureswaran et al. 2006, 2008a). The difference in the pheromone components produced by the females could confer some degree of reproductive isolation between species despite similarities of the aggregation pheromone that might cause mutual aggregation (Niño-Domínguez et al. 2015). Species with overlapping ranges actually have greater pheromone similarity than allopatric species, and there is no evidence for reproductive character displacement in the pheromone blends of sympatric species that share host species (Symonds and Elgar 2004). Pheromone overlap likely improves the capacity of aggressive beetles to locate and overcome the defenses of live and vigorous trees, and this may outweigh the cost of potentially more frequent interspecific reproductive encounters and competition (Davis and Hofstetter 2009).

The comparison of trap lures involved alterations of merely a single compound (brevicomin), and additional compounds may be involved in cross-species responses, as suggested by the significant differences in quantities of multiple compounds produced by either California or Arizona *D. brevicomis*. Additionally, cross-attraction between the naturally-produced odors of attacking *D. frontalis* and *D. brevicomis* pairs has yet to be confirmed directly in the field.

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