Pheromones of three ambrosia beetles in the *Euwallacea fornicatus* species complex: ratios and preferences

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Three cryptic species in the *Euwallacea fornicatus* species complex were reared in laboratory colonies and investigated for the presence of pheromones. Collections of volatiles from combinations of diet, fungus, beetles, and galleries from polyphagous shot hole borer (*Euwallacea* sp. #1) revealed the presence of 2-heneicosanone and 2-tricosanone only in the presence of beetles, regardless of sex. Subsequent examination of volatiles from the other two species, tea shot hole borer (*Euwallacea* sp. #2) and Kuroshio shot hole borer (*Euwallacea* sp. #5), revealed these two ketones were present in all three species but in different ratios. In dual choice olfactometer behavioral bioassays, mature mated females were strongly attracted to the binary blend of ketones matching their own natural ratios. However, females in each species were repelled by the ketone blends in ratios corresponding to the other two species. Males of each species responded similarly to females when presented with ratios matching their own or the other two species. The presence of these compounds in the three beetle species, in ratios unique to each species, and their strong species-specific attraction and repellency, suggests they are pheromones. The ecological function of these pheromones is discussed. In addition to the pheromones, the previously known attractant (1*S*,4*R*)-*p*-menth-2-en-1-ol (also known as quercivorol) was discovered in the presence of the fungal symbionts, but not in association with the beetles. Quercivorol was tested in a dual-choice olfactometer and was strongly attractive to all three species. This evidence suggests quercivorol functions as a kairomone for members of the *E. fornicatus* species complex, likely produced by the symbiotic fungi.

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Abstract

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23   Three cryptic species in the *Euwallacea fornicatus* species complex were reared in laboratory

24   colonies and investigated for the presence of pheromones. Collections of volatiles from

25   combinations of diet, fungus, beetles, and galleries from polyphagous shot hole borer

26   (*Euwallacea* sp. #1) revealed the presence of 2-heneicosanone and 2-tricosanone only in the

27   presence of beetles, regardless of sex. Subsequent examination of volatiles from the other two

28   species, tea shot hole borer (*Euwallacea* sp. #2) and Kuroshio shot hole borer (*Euwallacea* sp.

29   #5), revealed these two ketones were present in all three species but in different ratios. In dual

30   choice olfactometer behavioral bioassays, mature mated females were strongly attracted to a

31   synthetic binary blend of ketones matching their own natural ratios. However, females in each

32   species were repelled by ketone blends in ratios corresponding to the other two species. Males

33   of each species responded similarly to females when presented with ratios matching their own or

34   the other two species. The presence of these compounds in the three beetle species, in ratios

35   unique to each species, and their strong species-specific attraction and repellency, suggests they

36   are pheromones. The ecological function of these pheromones is discussed. In addition to the

37   pheromones, the previously known attractant (1S,4R)-p-menth-2-en-1-ol (also known as

38   quercivorol) was discovered in the presence of the fungal symbionts, but not in association with

39   the beetles. Quercivorol was tested in a dual-choice olfactometer and was strongly attractive to

40   all three species. This evidence suggests quercivorol functions as a kairomone for members of

41   the *E. fornicatus* species complex, likely produced by the symbiotic fungi.

42

43   Key words: polyphagous shot hole borer, tea shot hole borer, Kuroshio shot hole borer,

44   attractant, repellent, pheromone, quercivorol, kairomone, chemical ecology

45

Introduction

46

47

48   Until several decades ago, ambrosia beetles were not considered economically or ecologically

49   important pests because the vast majority of them cultivate their ambrosia fungus within already

50   dead or dying trees and other woody host plants and function ecologically as decomposers (Batra

51   1963). However, recently it has been realized that some ambrosia beetles are capable of

52   attacking healthy trees where their ambrosia fungus functions as a plant pathogen, infecting trees

53   and causing branch dieback or tree mortality (Kühnholz et al. 2001; Hulcr et al. 2017). With the

54   sharp increase of global trade in recent years, we have also seen an increase of invasive ambrosia

55   beetles capable of causing major economic and ecological damage, and severely threatening

56   native forest ecosystems (Marini et al. 2011). Such is the case with members of the *Euwallacea*

57   *fornicatus* species complex (Coleoptera: Curculionidae: Scolytinae).

58

59   Independent studies have concluded that populations of beetles morphologically identified as *E.*

60   *fornicatus,* that stem from four separate invasions in the United States (Hawaii, Florida, and two

61   in southern California), are composed of three genetically distinct, cryptic species of ambrosia

62   beetles in what is now recognized as the *E. fornicatus* species complex (Eskalen and Stouthamer

63   2012; Eskalen et al. 2013; O'Donnell et al. 2015; Stouthamer et al. 2017). All three species

64   morphologically resemble *E. fornicatus*, but they are genetically different enough to be

65   considered different species, and carry different species of fungal symbionts in the genus

66   *Fusarium* (O'Donnell et al. 2015; Carrillo et al. 2016). They have yet to receive unique scientific

67   names, but they are commonly referred to as: the polyphagous shot hole borer (PSHB)

68   (*Euwallacea* sp. #1), which was first detected in Los Angeles County, CA in 2003 (Eskalen et al.

69   2012; Eskalen et al. 2013); the tea shot hole borer *sensu lato* (TSHB) (*Euwallacea* sp. #2), which

70   was first detected in Hawaii in 1910 (Schedl 1941) and more recently in Miami-Dade County,

71   FL in 2002 (Rabaglia et al. 2008); and the Kuroshio shot hole borer (KSHB) (*Euwallacea* sp.

72   #5), which was first detected in San Diego County, CA in November 2013 (Eskalen et al. 2013;

73   O'Donnell et al. 2015; Carrillo et al. 2016; Boland 2016; Stouthamer et al. 2017; Dodge et al.

74   2017). Each of these three beetle species carry different species of symbiotic *Fusarium* in their

75   mandibular mycangia (O'Donnell et al. 2015; Carrillo et al. 2016), and the inability of PSHB and

76   TSHB larvae to survive when fed *Fusarium* from the other species suggests that isolation

77   between species also takes place in their obligatory feeding requirements for their associated

78   *Fusarium* species as well (Freeman et al. 2013a). Differences were also found between the

79   cuticular hydrocarbon profiles of PSHB and TSHB which could potentially assist in species

80   diagnostics since they are morphometrically indistinguishable (Chen et al. 2016). These three

81   cryptic species are similar in their polyphagous nature, in that they can attack and spread their

82   *Fusarium* symbiont to hundreds of tree species in numerous families (Danthanarayana 1968;

83   Eskalen et al. 2013). According to Eskalen et al. (2013) and Eskalen (2016), there are now 49

84   known reproductive hosts of PSHB, and 15 known for KSHB (see also Boland 2016). These

85   lists continue to expand rapidly as research on these species continues to unveil their numerous

86   developmental hosts. They threaten numerous native tree species in California. For instance, in

87   riparian forests in San Diego county along the border with Mexico, KSHB has attacked and

88   severely damaged the majority of the three dominant native willow species, *Salix lasiolepis*, *S.*

89   *gooddingii,* and *S. laevigata*, which profoundly affects the entire ecosystem (Boland 2016).

90   California sycamore, *Platanus racemosa,* is another dominant native tree species that is

91   susceptible to mass attack and killed by PSHB and KSHB (Coleman et al. 2013; Boland 2016).

92 Avocado is now threatened in California and Florida, and more than one quarter of all street trees

93 in southern California are reproductive hosts susceptible to attack (Lesser 1996; Eskalen and

94 Stouthamer 2012; Mendel et al. 2012; Freeman et al. 2013b; Eskalen et al. 2013; Carrillo et al.

95 2016; Cooperband et al. 2016; Kendra et al. 2017; Stouthamer et al. 2017).

96

97 These three cryptic species of beetles collectively bring with them at least five species of

98 phytopathogenic *Fusarium* ambrosia which they cultivate, and upon which they feed and

99 develop inside galleries in trees and woody plants (O'Donnell et al. 2015; Carrillo et al. 2016).

100 Infection of trees with these fungi cause the disease known as Fusarium dieback. Additional

101 fungi, *Graphium euwallaceae*, *Paracremonium pebium*, and *Acremonium* sp. were found in the

102 heads of beetles from California and Florida (Lynch et al. 2016; Carrillo et al. 2016). The fungal

103 symbionts help the beetles overcome defenses of a seemingly healthy tree by blocking the

104 vascular tissues of the tree, subsequently lead to staining, branch dieback, and large scale tree

105 mortality (Eskalen et al. 2013; Lynch et al. 2016). Interestingly, a positive association has been

106 seen between water abundance and beetle infestation rate (Boland 2016).

107

108 Mating typically occurs between haploid brothers and diploid sisters in their natal galleries prior

109 to female dispersal (Cooperband et al. 2016). A female that has not found a mate may initiate a

110 new colony by producing haploid male offspring through parthenogenesis, mating with a son,

111 then producing female offspring (Cooperband et al. 2016). Therefore, inbreeding is the rule, and

112 outbreeding depression is likely (Peer and Taborsky 2005). A crossing study conducted between

113 PSHB and TSHB revealed that when forced to interbreed, most crosses failed, but a small

114 amount of hybridization resulted in low fitness or reproductive compatibility between the two

115   species (Cooperband et al. 2015). Results were similar when attempting to cross PSHB and

116   KSHB, demonstrating that there is reproductive isolation between the species (Cooperband et al.

117   2017).

118

119   The three beetle taxa in the *E. fornicatus* species complex originate in southeast Asia, and there

120   are regions where they occur in sympatry (Stouthamer et al. 2017). The most genetically diverse

121   populations of TSHB were in Thailand, PSHB in Vietnam and Taiwan, and KSHB in Taiwan,

122   suggesting their possible evolutionary origins. However, all three species were found in Taiwan,

123   PSHB and KSHB were both found in Okinawa, and PSHB and TSHB were both found in

124   Thailand (Stouthamer et al. 2017). Although geographical barriers play a role in genetic

125   isolation between species, with overlapping host tree and geographical ranges, other character

126   displacements may also play a role in the genetic isolation between the three species.

127

128   With the need for improved detection tools soon after the invasion of PSHB in southern

129   California, the initial goal of this study was to investigate the possible presence of a pheromone.

130   As studies began to emerge establishing that three distinct cryptic species occur in the US, the

131   scope of this study expanded to encompass all three species. The goal, if pheromones were

132   found, was to identify and quantify them, and demonstrate their behavioral function. Because of

133   the potentially confounding presence of behaviorally active volatiles from the host plant and the

134   symbionts, experiments were designed to isolate volatiles originating from beetles while

135   controlling for those that originated from their fungal symbionts or host plant.

136

137   **Materials and methods**

138

139 *Insects*

140

141 Initial exploratory volatile collections focused only on beetles from the population of PSHB (*E.*

142 sp. #1) collected in Altadena, in Los Angeles County in southern California, which has been

143 maintained in colony in the insect containment facility of the Otis Laboratory since August, 2013

144 (USDA permit P526P-13-01673) (Cooperband et al. 2016).

145

146 Subsequent volatile collections and extracts to compare the three members of the species

147 complex involved PSHB as well as TSHB (*E.* sp. #2) isolated from Miami-Dade County in

148 Florida and reared in a laboratory colony since early 2014, and KSHB (*E.* sp. #5) which was

149 isolated from San Diego County, CA and kept in a laboratory colony since the end of 2014.

150 Rearing took place under LD 16 : 8 h photocycle at 24 ∘C, using protocols described in detail in

151 Cooperband et al. (2016).   Briefly, sib-mated females were placed individually into 50 ml

152 polyethylene centrifuge tubes (Fisher Scientific, Waltham, MA) containing 15 ml of artificial

153 diet. Diet was based on sawdust from either boxelder (for PSHB) or avocado (for TSHB and

154 KSHB), corresponding to host tree from which they were originally collected. Initially each

155 foundress excavated into the diet, seeding it with *Fusarium* fungus from her mycangia (Freeman

156 et al. 2013a; O'Donnell et al. 2015), and forming galleries lined with *Fusarium* which would be

157 fed upon by her and her offspring over the next 5-8 weeks, during that time the 15 ml diet plug

158 became completely permeated with the fungus. On average, a typical foundress produced

159 between 25 to 35 females and one to three male offspring in 5-8 weeks (Cooperband et al. 2016).

160 The three species were reared separately, and to avoid contamination between colonies they were

161   kept in separate triple-nested containers which were never opened at the same time. Containers

162   and work areas were wiped with a 10% solution of bleach before and after use. Beetles and

163   *Fusarium* species were confirmed by DNA to match those described by O'Donnell et al. (2015)

164   (Cooperband et al. 2016).

165

166   *Volatile collections for qualitative comparisons with PSHB*

167

168   The first phase involved exploration for a pheromone by collecting volatiles from sources with

169   and without PSHB beetles and comparing volatile profiles for qualitative differences. To

170   maximize this phase, we employed several approaches to collect volatiles: solid phase micro-

171   extraction (SPME) fibers, volatile collections, and solvent extracts or rinses on subjects with

172   setups described below.

173

174   All SPME sampling utilized 100-μm polydimethylsiloxane coated fibers (Supelco, Bellefonte,

175   PA). SPME fibers were exposed either: 1) in the headspace of a closed rearing tube or jar

176   containing the volatile source, 2) inside a Pasteur pipette containing the volatile source, 3) inside

177   the galleries of beetle colonies established in artificial diet, or 4) swiping or briefly touching the

178   volatile source with the SPME fiber. Colonies were on average 47 d old when used and SPME

179   fibers were exposed inside Pasteur pipettes for an average of 12 h. To sample the volatiles inside

180   a gallery, the diet plug was tapped out of the rearing tube containing a mature beetle colony, and

181   the bottom of the plug was chipped away incrementally until a gallery was revealed. A SPME

182   fiber was inserted directly into the gallery and held in place for 2 min on average. After

183   exposure, the diet plug was dissected, and the number and sex of beetles within that colony was

184   quantified. In some cases, the foundress had died and no beetles were in the galleries, and these

185   were re-categorized as part of the "diet + fungus" treatment (described below). To sample

186   volatiles using a Pasteur pipette, approximately 150 mg of the source material or a known

187   number of beetles was placed inside a glass Pasteur pipette, with the larger opening covered with

188   aluminum foil, and the SPME fiber inserted and exposed through the smaller opening for on

189   average 105 min. Alternatively, SPME fiber exposures in other containers such as the headspace

190   inside a rearing tube lasted on average 272 min, and exposure inside galleries was on average 1

191   min.

192

193   Volatile collections in this phase were conducted by passing odor-laden air through volatile traps

194   containing approximately 20 mg of either activated charcoal (50-200 mesh, Fisher Scientific,

195   Waltham, MA) or Hayesep Q (80-100 mesh, Hayes Separations, Inc., Bandera, TX) packed

196   between two small plugs of glass wool inside a Pasteur pipette. Air passed through an activated

197   charcoal in-line air filter (Analytical Research Systems, Inc.) at 0.2 L/min, then into a 50 ml

198   rearing tube, 20 ml vial, or 0.24 L jar containing the odor source, and then exited the container

199   through the volatile collection trap. Volatile samples were eluted with approximately 1 ml of

200   hexane through the trap into a collection vial.

201

202   Extracts in this phase were made by placing the beetles into a 2 ml autosampler vial containing

203   just enough solvent to cover them, and allowing them to soak for a period of time, from 30 min

204   to several days. To make a rinse, live beetles were removed from their galleries, placed into a

205   Pasteur pipette. The pipette was placed in a stand over an empty 2 ml autosampler vial and

206   approximately 1 ml of hexane was dispensed into the pipette rinsing over the beetles and

207   collecting in the autosampler vial. One rinse was made by dispensing the hexane directly into a

208   gallery of a live colony of beetles, and immediately recovering the hexane with a Pasteur pipette.

209

210   For qualitative comparisons, odor sources were categorized into six treatments as follows:

211   (1) "Control" consisted of a clean container such as an empty Pasteur pipette or rearing tube.

212   (2) "Diet" consisted of sterile diet that had never been in contact with beetles or their fungal

213   symbionts.

214   (3) "Diet + Fungus" consisted of the *Fusarium*-infested diet from the middle of a diet plug

215   from a rearing tube, taken from an area that did not contain any galleries or beetles. One

216   exception in which the gallery was included in this category occurred when a gallery was

217   sampled from a rearing tube, but after dissection it was found that there were no living

218   beetles in that tube.

219   (4) "Diet + Fungus + Beetles" consisted of non-gallery *Fusarium*-infested diet from rearing

220   tubes as in "Diet + Fungus" above, but with beetles added (either male or female or both).

221   This category mostly consisted of SPME samples taken from material placed inside of a

222   pipette. However, this category also included head space volatile collections of rearing

223   tubes containing complete colonies.

224   (5) "Gallery" refers to volatile samples that were taken from the gallery itself, in which live

225   beetles were present. These were accomplished either by inserting a SPME fiber directly

226   inside an inner gallery near the bottom of the diet tube, or by removing a section of inner

227   gallery and placing it inside a Pasteur pipette, and then inserting the SPME fiber into the

228   pipette. Also included in this treatment was the single hexane rinse of a gallery,

229   described above. Each rearing tube from which a gallery was sampled was dissected and

230   the number of males and females living in that tube was recorded and attributed to that

231   gallery sample. Therefore, diet and fungus and beetles were all components of galleries.

232   (6) "Beetles" consisted of only beetles. They were removed from their galleries in a rearing

233   tube and immediately sampled for volatiles in the absence of their diet and fungus rearing

234   media. The beetle category was later broken down into three subcategories, male,

235   female, or male + female, and compared to each other and to non-beetle samples.

236

237   *Volatile collections for qualitative comparisons with TSHB*

238

239   While conducting the above sampling with PSHB, the first TSHB colony in a diet tube arrived

240   from Florida. The TSHB colony had been initiated by a single field-collected foundress, surface

241   sterilized in 70% ethanol for 10 s prior to introduction onto the diet. After developing for nine

242   weeks it was used to test for volatiles. The colony was dissected and found to be densely

243   populated with 69 adult females and 5 adult males. At this advanced colony age, all 15 ml of

244   diet in the tube contained the *Fusarium*. Four volatile sources were selected from within the

245   rearing tube and sampled with SPME fibers: (1) approximately 150 mg of diet and fungus from a

246   solid area without beetles or galleries was placed inside a Pasteur pipette, (2) approximately 150

247   mg of the same diet and fungus from an area without beetles or galleries was placed in a second

248   Pasteur pipette, and five adult male beetles were added, (3) 46 female beetles were placed in a

249   sterile 120 ml specimen jar, and (4) the space inside beetle galleries. SPME fibers were exposed

250   for 10, 10, 1, and 2 min to these four treatments, respectively.

251

252   *Exploratory sample analysis*

253   Samples were analyzed by injection into an Agilent 7890B gas chromatograph coupled with a

254   5977A mass-selective detector (GC-MS) (Agilent Technologies, Inc., Santa Clara, CA, USA).

255   The GC was equipped with an HP-5MS column (30 m x 0.25 mm I.D. x 0.25 micron film

256   thickness; Agilent Technologies, Inc., Santa Clara, CA, USA). The column effluent was split in

257   half by a Gerstel uFlow Manager (Gerstel Inc., Linthicum, MD, USA), such that half the effluent

258   was directed into the MS and half to another detector that was not used in this study. Helium

259   was used as the carrier gas (constant pressure 13.8 psi) and samples were injected in splitless

260   mode. The GC injector was held at 250 ˚C, and the column starting temperature was 50 ˚C, held

261   for 0.75 min, then ramped at 10 ˚C/min to 250 ˚C and held for 25 min. Initial GC-MS

262   identifications were made by using libraries (Wiley and NIST), and subsequent verification of

263   compounds compared Kovat's indices, mass spectra, and retention times with those of synthetic

264   standards (see Chemical synthesis section below). GC-MS results from different treatments

265   were compared to look for compounds unique to beetles.

266

267   *Whole beetle extracts to compare pheromone component ratios between species*

268

269   Beetles from each of the three species were gathered from galleries and groups of 9 to 31 mature

270   females (each group harvested from a different diet tube), and 3 to 10 males (combined from

271   multiple tubes) were extracted in pentane for 30 min, after which a known amount of 2-

272   tridecanone was added as an internal standard to allow for accurate quantification. Samples were

273   analyzed on an Agilent 7890 GC equipped with a flame ionization detector (FID), using the

274   above mentioned GC column and GC run settings.

275

276   *Bioassay design*

277

278   Rearing tubes that were 5-11 weeks old were harvested and the mature adult females were placed

279   in a holding jar with a piece of filter paper and allowed to acclimate for at least 1 h prior to use in

280   behavioral bioassays.

281

282   Custom bioassay "Y-plates" designed by M. Cooperband and manufactured by Applied Plastics

283   Technology, Inc. (Bristol, RI) were used to conduct dual choice behavioral bioassays within the

284   insect containment facility at the Otis Laboratory. Each Y-plate consisted of a block of solid

285   Teflon (16.5 long x 12.7 wide x 1.3 cm high) from which a channel was cut in the shape of a Y

286   (**Fig. 1**). The single stem of the Y was 7.6 cm long, and two arms diverged at 90 degrees from

287   each other. The two arms each had a 5.7 cm long section extending from the split, then a 45

288   degree bend which brought the final 1.8 cm sections parallel to each other. Each arm was 1.9 cm

289   across. At each end of the two upper arms, a 0.635 cm hole was bored for the insertion of Teflon

290   tubing (0.635 cm OD) for airflow into the bioassay. A transparent sheet of acetate was placed

291   against the top and bottom of the bioassay plate and sealed in place with a thin film of electrode

292   gel, so air entering the two upper arms could only exit through a 1.905 cm diam. hole at the end

293   of the stem. An oiless air compressor provided air flow through the apparatus via a regulator,

294   activated charcoal filtration, Teflon tubing, humidifier, and a flow meter set to 0.6-0.7 L/min.

295   Air was directed through a Y-splitter which delivered even flow to both upwind arms of the Y-

296   plate. Visualization of the plume using smoke revealed that the plumes entering the two arms of

297   the Y remained separate until they were practically to the end of the stem. A hotwire

298 anemometer placed at the downwind end of the Y was used to measure the wind speed, which

299 was in the range of 30-35 cm/s.

300

301 Beetles were inserted into the bottom of the Y using a paint brush. A preliminary attempt at odor

302 delivery into the bioassay consisted of passing air through two flasks, one containing a lure and

303 the other a control, and air from the two flasks was directed into the two arms of the Y. When

304 visualized using smoke, it was found this produced a homogeneous odor plume on one half of

305 the Y. However, this approach did not produce clear results as beetles responding to known

306 attractants chose the control arm. It was suspected that the plume was too homogenous for

307 beetles to navigate upwind in its center, and by navigating along the edge of clean air, they ended

308 up in the wrong arm. In order to produce a heterogeneous plume composed of clean air

309 interspersed with bursts of odors to allow optomotor anemotaxis to take place, custom nozzles

310 were constructed which produced the desired effect and greatly improved the bioassay

311 performance (**Fig. 2**).

312

313 Air entered the two arms of the Y through a pair of custom nozzles crafted out of disposable

314 pipette tips of two sizes, 1000 µl and 100 µl (Finntip, Thermo Scientific, Waltham, MA). Both

315 tips were cut as shown in **Fig. 2a-b.** One eighth of a rubber septum was inserted into the smaller

316 pipette tip, which was then inserted into the larger pipette tip (**Fig. 2c**). Ridges around the base

317 of the smaller tip functioned as channels, allowing clean air to flow between the smaller tip and

318 the larger tip (**Fig. 2d**). Air flowing through the inner tip flowed past the loaded septum and

319 carried volatile compounds into the clean air stream which surrounded it. Solvent only rubber

320 septa were used for controls. The tips of the nozzles were cut at an angle, the open side of which

321   was directed toward the middle of the Y-plate. Nozzles were newly crafted for every set of tests

322   and discarded afterwards.

323

324   *Chemical synthesis*

325

326   The compounds 2-heneicosanone (2-21:Kt) and 2-tricosanone (2-23:Kt) were prepared from 1-

327   bromooctadecane and 1-bromoeicosane respectively using previously described methodology

328   (Mason et al. 1990). The resulting ketoesters were saponified, and after hydrolysis, provided the

329   appropriate ketones. These ketones were recrystallized from heptane to provide more than one

330   gram of each as a crystalline solid. The resulting material was 96.5 and 95.8% pure,

331   respectively. Quercivorol, (1*S*,4*R*)-*p*-menth-2-en-1-ol, was prepared by following Mori (2006)

332   and was 98% pure.

333

334   *Lures for behavioral bioassays*

335

336   Red rubber septa were extracted and loaded according to Zilkowski et al. (2006). The two

337   synthetic ketones, 2-21:Kt and 2-23:Kt, were weighed and combined in hexane to produce stock

338   solutions of each of the three ratios 45:55, 68:32, and 87:13. They were then serially diluted

339   such that 100 ul contained either 0.25, 2.5, or 25 ug of the total combined ketones at the three

340   different ratios. Septa loaded with the different doses and ratios of the two ketones were sliced

341   into eighths, which were used in behavioral bioassays. Thus septum eighths used in the dose

342   response assays contained approximately 31, 313, or 3125 ng of the two ketones combined, at a

343   ratio of 45:55. Septum eighths used to compare attraction for all three species contained 313 ng

344   of the two ketones combined, at either 45:55, 68:32, or 87:13. Septa were stored inside glass

345   vials at -20 ˚C when not in use.

346

347   *Behavioral bioassays*

348

349   To avoid issues of contamination a clean Y-plate was used for every set of 15 or fewer replicates.

350   Clean filter paper cut into the shape of the Y was inserted into the Y-plate to provide beetles with

351   traction, and was discarded after each set. At the onset of each session, tests commenced by first

352   offering beetles a choice in the Y-plate containing no odors (controls on both sides) in order to

353   ensure that there was no bias in the apparatus due to lighting, airflow, contamination, or other

354   factors. Once control beetles showed no bias, the lures were placed into the nozzles as described

355   above and beetles were given a choice between a clean septum and an odor-laden septum.

356   Beetles were individually placed into the Y through the hole at the bottom using a paint brush

357   and allowed three minutes to make a choice. Beetles that entered one of the two arms and

358   traveled at least half of the remaining distance from the junction to either side were scored as

359   having made a choice. All other beetles were scored as non-responders. Once a beetle made a

360   choice, that trial ended. The side of the Y-plate used to test the volatiles was alternated, and

361   plates were cleaned thoroughly between changing sides or compounds. Behavioral testing was

362   conducted between 1030-1330 hrs, under ambient fluorescent lighting, at 17-25 ˚C.

363

364   Using the Y-plate bioassays, a dose response test of the synthetic PSHB blend was conducted

365   with mature female PSHB to evaluate the behavioral function of the two synthetic ketones, as

366   well as to determine their optimal dose. Subsequently, the optimal dose was used to test mature

367   females of each species for attraction to the two synthetic ketones at the three different ratios.

368   Males of all three species were tested in Y-plate bioassays to determine their response to the

369   blends as well. Finally, quercivorol at a dose of approximately 363 ng was tested for attraction

370   with all three species as a positive control to confirm that the assays were working properly.

371

372   *Statistical analysis*

373

374   Pheromone component ratios for the three species were compared by dividing the amount of 2-

375   21:Kt by the amount of 2-23:Kt in each extract of groups of beetles. After verifying equal

376   variances, ratios were analyzed using ANOVA and Tukey means separations ($\alpha=0.05$) (JMP

377   10.0.0, SAS Institute, Inc.).

378

379   Dual choice bioassays conducted in the Y-plate olfactometer were used to test the null

380   hypothesis that both stimuli were chosen at the same frequency of 0.5. Because the requirements

381   for using a Chi Square Goodness-of-fit test were frequently violated when fewer than 5 beetles

382   selected one side (Sokal and Rohlf 1995), the non-parametric two-tailed sign test was used to test

383   the null hypothesis and significance level was determined using Statistical Table Q (Rohlf and

384   Sokal 1995).

385

386   **Results**

387

388   *Qualitative analysis of PSHB samples*

389

390 Preliminary studies explored volatiles from combinations of diet, fungus, and beetles. Using

391 PSHB colonies, this exploratory phase revealed two hydrocarbon ketones found only in the

392 presence of beetles: 2-heneicosanone (2-21:Kt) and 2-tricosanone (2-23:Kt) (**Fig. 3**). Of 17

393 PSHB galleries sampled with SPME fibers, the ketones were detected in all except one. Upon

394 dissection of those rearing tubes, the gallery that did not contain either ketone was from a tube

395 that had no live beetles present, and was thus reclassified into the "diet + fungus" treatment. The

396 two ketones were also not found in any samples containing diet + fungus taken from the non-

397 gallery parts of rearing tubes that contained active colonies, or diet + fungus from rearing tubes

398 without live beetles. These two ketones were, however, isolated from both the headspace and

399 extracts of beetles alone. The ketones were found in samples from mature adult females as well

400 as virgin teneral females (**Fig. 4**). These two ketones were also discovered in small amounts in

401 volatiles from males (**Fig. 4g**). Whole body extracts contained compounds also found in diet +

402 fungus, as well as large hydrocarbon peaks possibly originating from the cuticle (**Fig. 4g**).

403

404 Quercivorol, also known as (1*S*,4*R*)-*p*-menth-2-en-1-ol, was found to be associated with volatile

405 samples containing fungus, but was not detected in samples from diet or beetles in the absence of

406 fungal growth. Quercivorol was found in both galleries and non-gallery samples of diet +

407 fungus, both in the presence or absence of beetles (**Fig. 3**).

408

409 *Qualitative analysis of TSHB samples*

410

411 Analysis of non-gallery diet and fungus, gallery, and males of the single TSHB colony revealed a

412 similar pattern to that seen in PSHB. Headspace was sampled (SPME) from four treatments of a

413   single 9-wk old TSHB rearing tube. The treatments were: diet + fungus, diet + fungus + males,

414   females, and gallery. The two ketones, 2-21:Kt and 2-23:Kt, were found in all of these

415   treatments except for the diet + fungus. It was noted that the ratio of the two ketones appeared to

416   be different from that of PSHB based on peak area, which led to a quantitative examination of

417   ratios for all three species.

418

419   *Quantitative analysis of beetle-associated volatiles*

420

421   Extracts of male or female groups of PSHB, TSHB, and KSHB conducted with an internal

422   standard revealed that the two ketones, 2-21:Kt and 2-23:Kt, were found at three different ratios

423   among the three species, regardless of sex (**Table 1, Fig. 5**). Mean ratios of 2-21:Kt and 2-23:Kt

424   in mature females were 45:55, 68:32, and 87:13 in PSHB, TSHB, and KSHB, respectively. They

425   were also present at similar ratios in teneral adult females. When 2-21:Kt and 2-23:Kt were

426   combined, mature female PSHB, TSHB, and KSHB produced about 91, 48, and 75 ng/beetle,

427   respectively. Females produced more than twice as much of these two compounds than males

428   (**Table 1**). Ratios of the two components differed significantly between the three species (**Fig. 5**)

429   (ANOVA and Tukey means separation, df=2, F=179.93, P<0.0001, α=0.05).

430

431   *Behavioral bioassays*

432

433   Behavioral bioassays were conducted with female PSHB in a dose-response test to the synthetic

434   blend of 2-21:Kt and 2-23:Kt at a ratio of 45:55, and doses of 0, 31, 313, and 3125 ng. PSHB

435   females responded dose-dependently with significant attraction to the 313 ng dose (**Fig. 6**).

436

437   Lures with the 313 ng dose were tested subsequently with females of the three species to

438   compare walking responses to blends with ratios corresponding to their natural ratios and to

439   assess cross-attraction. Each of the three species were significantly attracted to synthetic

440   versions of their own blend ratio, and significantly repelled by the blend ratios of the other two

441   species (**Fig. 7**). Males were similarly found to be attracted to their own synthetic blends but not

442   to blends matching the other two species (**Fig. 8**). The limited availability of males due to the

443   extremely female-biased sex ratio resulted in fewer replicates. However, significant conspecific

444   attraction by males was observed in all three species, and the same pattern of repellency trends

445   were observed.

446

447   Quercivorol was found to be significantly attractive to mature females of all three species, and

448   was used as a positive control to validate the walking assays (**Fig. 9**).

449

450   **Discussion**

451

452   A variety of exploratory techniques revealed the presence of two ketones, 2-heneicosanone and

453   2-tricosanone associated with PSHB beetles. The most successful technique for demonstrating

454   the presence or absence of these ketones in different treatments was by collecting head space

455   volatiles with a SPME fiber inside a glass pipette which contained the volatile source. The

456   systematic exploratory sampling of volatiles demonstrated that these two ketones were of beetle

457   origin and not produced by the host plant material, diet, or symbiotic fungi. These ketones were

458   found in both males and females (both teneral and mature females), and each of the three

459   *Euwallacea* species were found to have the same two ketones, but at different ratios. Although

460   ratios within a species were consistent for both males and females, females consistently produced

461   more than two times the quantity of the two ketones than males. A dose-response test on the

462   synthetic two-ketone blend, using the appropriate ratio for PSHB, demonstrated peak attraction

463   by female PSHB beetles to the lure containing 313 ng of the blend. When the two ketones were

464   tested in three ratios corresponding to the three *Euwallacea* species (PSHB, TSHB, and KSHB),

465   females of each species were found to be significantly attracted to their own ratio, and

466   significantly repelled by the other two ratios. Similarly, males were primarily attracted to their

467   own ratio and not by ratios of the other species.

468

469   Although attraction to the pheromone blends was species-specific, quercivorol was found to be

470   highly attractive to all three species. In volatile collections, quercivorol was not associated with

471   beetles and was only detected in samples containing *Fusarium* fungus. Its presence only in

472   samples containing fungus, and absence in samples containing only beetles or only diet, suggest

473   that quercivorol is not a pheromone. That evidence as well as its attraction across all three

474   species infers that quercivorol likely serves as a *Fusarium*-produced kairomone that is more

475   broadly attractive to members of the *Euwallacea* species complex. Quercivorol has recently

476   been demonstrated to attract all three species in the field (Carrillo et al. 2015; Dodge et al. 2017).

477

478   The chemical and behavioral data presented here indicates that these morphologically

479   indistinguishable beetles, carrying different species of symbiotic *Fusarium*, and originating from

480   three different invasive populations, have formed pheromone races, and further supports the

481   amassing evidence that they have speciated into three cryptic species (Kasson et al. 2013;

482   O'Donnell et al. 2015; Chen et al. 2016; Stouthamer et al. 2017). According to Stouthamer et al

483   (2017), multiple haplotypes were found of PSHB in Vietnam and Taiwan, of KSHB in Taiwan

484   and Okinawa, and of TSHB in Thailand and India, suggesting their possible native origins. All

485   three species were found to co-occur in Taiwan, PSHB and KSHB co-occured in Okinawa, and

486   PSHB and TSHB co-occurred in Thailand, however populations represented by only one

487   haplotype may represent invasions from other areas. PSHB and KSHB are less genetically

488   divergent from each other than from TSHB (O'Donnell et al. 2015; Stouthamer et al. 2017),

489   however, their pheromone ratios are the most divergent from each other, with the TSHB

490   pheromone ratio being intermediate. The fact that PSHB and KSHB naturally coexist may have

491   helped to drive the strong divergence of their pheromone component ratios, which could help

492   avoid outbreeding. Since many ambrosia beetles are haplo-diploid, inbreed as a rule, and exhibit

493   outbreeding depression (Haack 2001; Peer and Taborsky 2005), the resulting selective pressure

494   would promote traits that reduce outbreeding, and could result in the evolution of divergent

495   pheromones.

496

497   Although these pheromones may aid in avoidance of congeneric species, their ecological role

498   and function is not yet understood, and there are several hypotheses for the possible ecological

499   role they play. With respect to other pheromones, 2-heneicosanone and 2-tricosanone are

500   relatively large molecules with lower volatility than most long distance aggregation or sex

501   pheromones. Our preliminary field tests conducted in California to trap PSHB using their

502   pheromone blend did not produce long range attraction. It is likely that these compounds are

503   more akin to some insect trail pheromones such as (*Z*)-11-eicosesnal in the arboreal ant

504   *Dolichoderus thoracicus* (Morgan 2009) or (*Z*)-9-tricosene in the longhorn beetle *Anoplophora*

505   *glabripennis* (Hoover et al. 2014). Since these two ketones were found in male beetles as well as

506   both virgin and mated female beetles, and mated females were attracted to them, it is doubtful

507   they act as sex pheromones. Aggregation is a possible function, but their high abundance inside

508   galleries and their low volatility suggests they may function as trail pheromones, gallery-

509   recognition pheromones, or pheromones to facilitate communication with nest mates, and might

510   contribute to social behaviors such as cooperative brood care, as xyleborine beetles are

511   predisposed for sociality (Biedermann et al. 2009). A mechanism to avoid congeners could be

512   advantageous given that all three species share the highly attractive fungal kairomone

513   quercivorol, whereas they likely face outbreeding depression (Peer and Taborsky 2005) and

514   appear to be obligate feeders on their own *Fusarium* species (Freeman et al. 2013a). Although

515   beetles responded to their pheromone blends while walking upwind in a dual-choice

516   olfactometer, we have not demonstrated attraction to them from a distance in the field. Testing

517   for upwind flight response and close-range functions are topics for future investigation.

518

519   Although a number of pheromones are known for scolytine bark beetles, only a few examples of

520   pheromones exist in scolytine ambrosia beetles, such as species in *Gnathotrichus* (Borden et al.

521   1976), *Trypodendron* (Borden and Slater 1969), and *Xyletorus* (Francke and Heemann 1974).

522   *Gnathotrichus sulcatus* uses S-(+) and R-(-) sulcatol as an aggregation pheromone (Borden et al.

523   1976;), and *Trypodendron lineatum* (Coleoptera: Curculionidae) uses the aggregation

524   pheromone lineatin during mass attack of new hosts (Borden and Slater 1969; Borden 1988).

525   Pheromones have also been found in platypodid ambrosia beetles, such as the compounds (+)-

526   sulcatol, sulcatone, and 3-pentanol used by males of *Megalyptus mutatus* (=*Platypus mutatus*) to

527   attract females (Audino et al. 2005; Liguori et al. 2008), 1-hexanol, 3-methyl- 1-butanol, and

528 sulcatol for aggregation by *Platypus flavicornis* (Renwick et al. 1977), and quercivorol reported

529 as an aggregation pheromone for *Platypus quercivorus* (Kashiwagi et al. 2006).

530

531 This may be the first report of pheromones in the genus *Euwallacea,* or the use of 2-

532 heneicosanone or 2-tricosanone in scolytine beetles. However, both of these methyl ketones

533 have been reported in other arthropods. Interestingly, in social insects they occurred in the

534 mandibular glands of stingless bees where they were proposed as possible constituents of a trail

535 pheromone (Blum 1970), and trace amounts of 2-tricosanone were found in the labial and tarsal

536 glands of queen bumble bees *Bombus terrestris* (Hefetz et al. 1996). Both compounds were

537 found in the cuticle of adult male and female pecan weevils *Curculio caryae* (Coleoptera:

538 Curculionidae) (Espelie and Payne 1991). The former compound, 2-heneicosanone, was found

539 to occur on the tarsi and elytra of *Coccinella septempunctata* (Coleoptera: Coccinellidae), but

540 was not found on tarsi or elytra of 34 other beetle species in eight other families (Geiselhardt et

541 al. 2011). In Lepidoptera, traces of these two ketones occurred among 66 compounds extracted

542 from abdominal tips of male and mated female *Heliconius melpomene* butterflies, but not

543 unmated females, leading to speculation that they may be part of a complex antiaphrodisiac

544 pheromone blend (Schultz et al. 2008). Additionally, 2-heneicosanone was found in the

545 hairpencils (male scent glands) of three species of African milkweed butterflies, *Amauris ochlea,*

546 *A. damocles,* and *A. albimaculata* (Lepidoptera: Danaidae) (Schultz et al. 1993). Both ketones

547 were also found in the cuticle and web of *Tegenaria atrica* spiders (Trabalon et al. 2005). None

548 of the above arthropod studies provided behavioral evidence of the roles of these ketones. Our

549 study links these two compounds to species-specific ratios and behaviors and provides strong

550 evidence that they are pheromones in three cryptic scolytine species.

551

552   Quercivorol has been documented as an attractant for the *E. fornicatus* species complex (Carrillo

553   et al. 2015; Kendra et al. 2017; Dodge et al. 2017). It was first reported as an aggregation

554   pheromone for the ambrosia beetle *Platypus quercivorus* (Coleoptera: Platypodidae) because it

555   attracted both males and females (Tokoro et al. 2007). In that study, quercivorol was isolated

556   from droplets excreted from the anus of fed virgin males, as well as from whole body extracts of

557   fed males and newly emerged females of *P. quercivorus*, which unfortunately did not rule out the

558   possibility of it originating in the ambrosia fungus *Raffaelea quercivora* and excreted in the

559   feces. Our study found that for at least one member of the *Euwallacea fornicatus* species

560   complex, quercivorol was associated with the ambrosia fungus and not the beetles, so in this

561   system quercivorol seems to be a kairomone produced by their *Fusarium* ambrosia symbiont,

562   rather than a pheromone. That these three cryptic species are each repelled by the pheromones

563   of the other two, but are all attracted to quercivorol supports the notion of quercivorol as a

564   kairomone in this system. Further investigation is needed on the cryptic members of the *E.*

565   *fornicatus* species complex to understand the ecological role of the two ketones.

566

567   **Conclusions**

568

569   In comparisons of volatiles from three cryptic species of the *E. fornicatus* species complex we

570   found that beetles produced pheromones composed of two hydrocarbon ketones: 2-

571   heneicosanone and 2-tricosanone. These ketones were produced in unique ratios by each of the

572   three species. When presented with synthetic blends of the ketones at the three ratios, beetles

573   were attracted to their own ratio, and repelled by the ratios associated with the other two species.

574   It is unlikely that these are sex pheromones or long range attractants being that these compounds

575   are relatively high molecular weight and low volatility, both mated females and males produced

576   them and were attracted to them, they were found in greatest abundance within the galleries, and

577   the molecules are more akin to trail pheromones in other species. They may be involved in

578   social behavior inside galleries or play a role in where foundresses initiate new colonies. Future

579   work is needed to understand the full behavioral and ecological function of these pheromones.

580

581   **Acknowledgements**

582

590

591   **References**

592

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744

Table 1 (on next page)

Amounts and ratios of the two ketone pheromone components in each species.

Two hydrocarbon ketones were extracted from groups of beetles of each species and sex, and analyzed by GC-FID using an internal standard to quantify mean amount of each compound per beetle (ng ±SE).The mean ratios from mature females were subsequently used in behavioral bioassays.

PSHB, polyphagous shot hole borer from Los Angeles County, CA (*E.* sp. #1); TSHB, tea shot hole borer from Miami-Dade County, FL (*E.* sp. #2); KSHB, Kuroshio shot hole borer from San Diego County, CA (*E.* sp. #5); 2-21:Kt, 2-heneicosanone; 2-23:Kt, 2-tricosanone.

1

		FEMALES	MALES
PSHB	**Mean Ratio**	**45:55**	47:53
	2-21:Kt ng/beetle (mean ±SE)	40.9 ±8.8	18.4
	2-23:Kt ng/beetle (mean ±SE)	50.0 ±7.6	20.4
	N extractions	4	1
	Total no. of beetles extracted	81	10
TSHB	**Mean Ratio**	**68:32**	71:29
	2-21:Kt ng/beetle (mean ±SE)	32.9 ±1.2	13.3
	2-23:Kt ng/beetle (mean ±SE)	15.4 ±0.2	5.4
	N extractions	2	1
	Total no. of beetles extracted	19	3
KSHB	**Mean Ratio**	**87:13**	88:12
	2-21:Kt ng/beetle (mean ±SE)	65.3 ±8.0	20.6
	2-23:Kt ng/beetle (mean ±SE)	10.2 ±0.9	2.7
	N extractions	2	1
	Total no. of beetles extracted	31	5

2

Figure 1

Diagram of the custom Y-plate bioassay design.

Y-plates used for bioassays were custom designed and cut from solid blocks of Teflon. Arrows indicate the direction of airflow. Disposable clear acetate sheets were sealed against the top and bottom of the plate with a bead of electrode gel. The nozzle tips were inserted snugly into the upwind ports pushing air in the direction of the arrows.
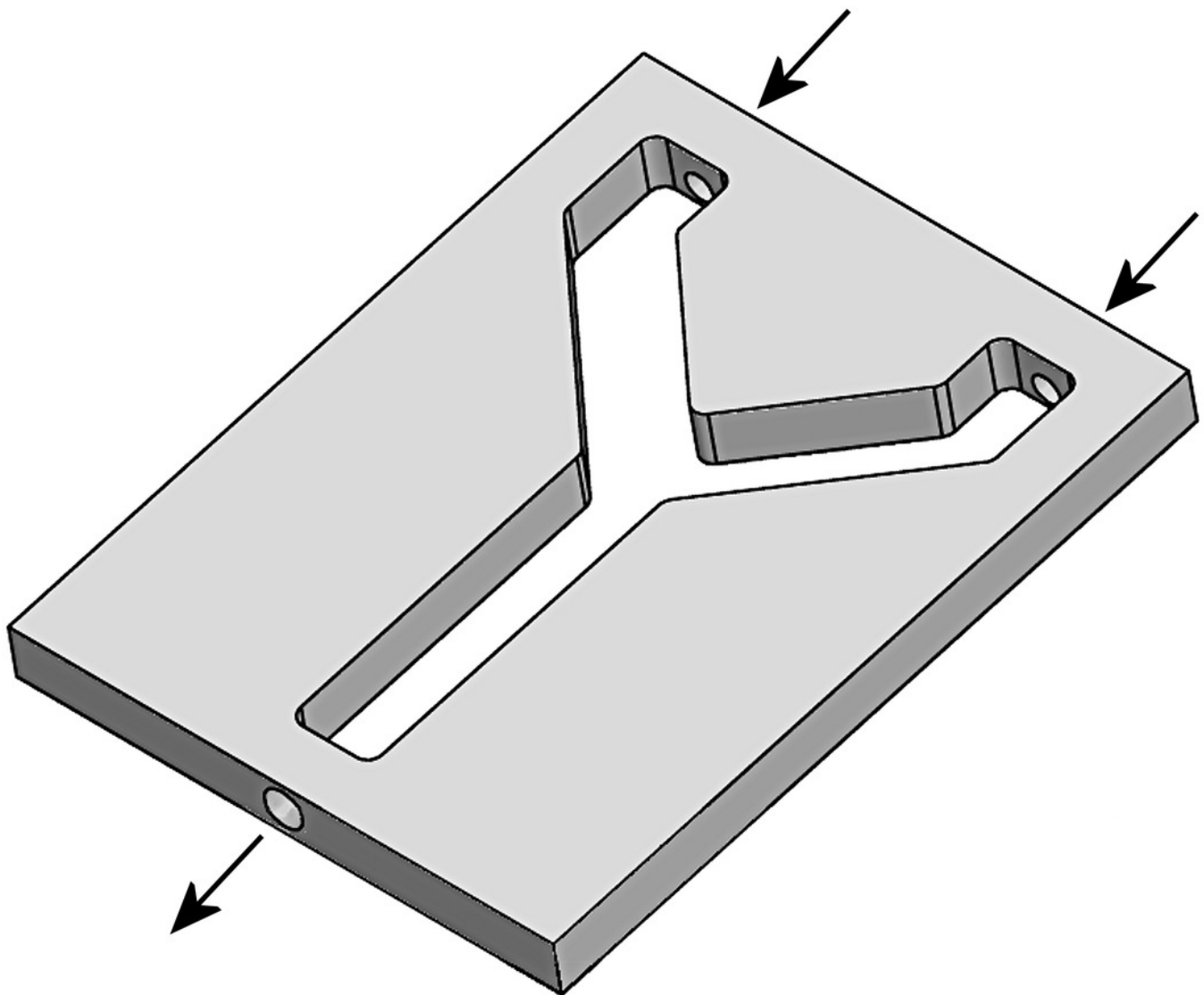
Figure 2

Nozzles for odor delivery in bioassays.

Nozzles were constructed using large and small pipette tips, respectively, cut (dotted lines) into parts (a) and (b). One eighth of a rubber septum (c) was placed into (b) which was placed into (a). Space can be seen between the two pipette tips (d) allowed clean air (dark arrows) to surround and mix with odor-laden air (light arrow). Bar measures 1 cm.

1000 μl tip

a

100 μl tip

b

a

c

b

c

1   1/2   1/4   1/8

d
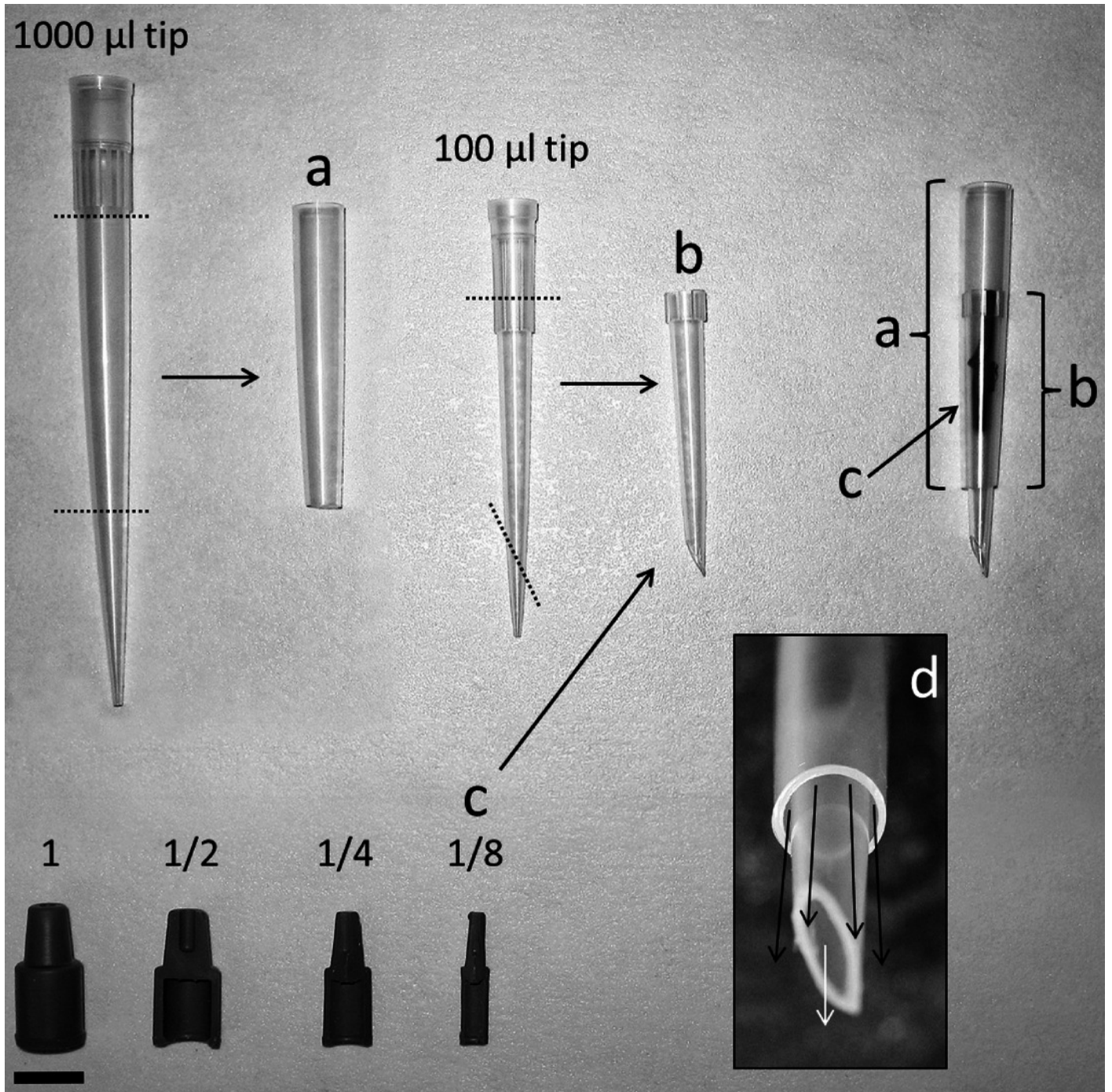
Figure 3

Frequency detected in volatile collections.

Frequency (percent of samples) in which compounds were detected in volatile collections exploring for presence of potential pheromones. Samples were collected from different combinations of beetles, fungus, and diet, as listed on the x-axis. N indicates the number of volatile collections made and analyzed in each treatment.
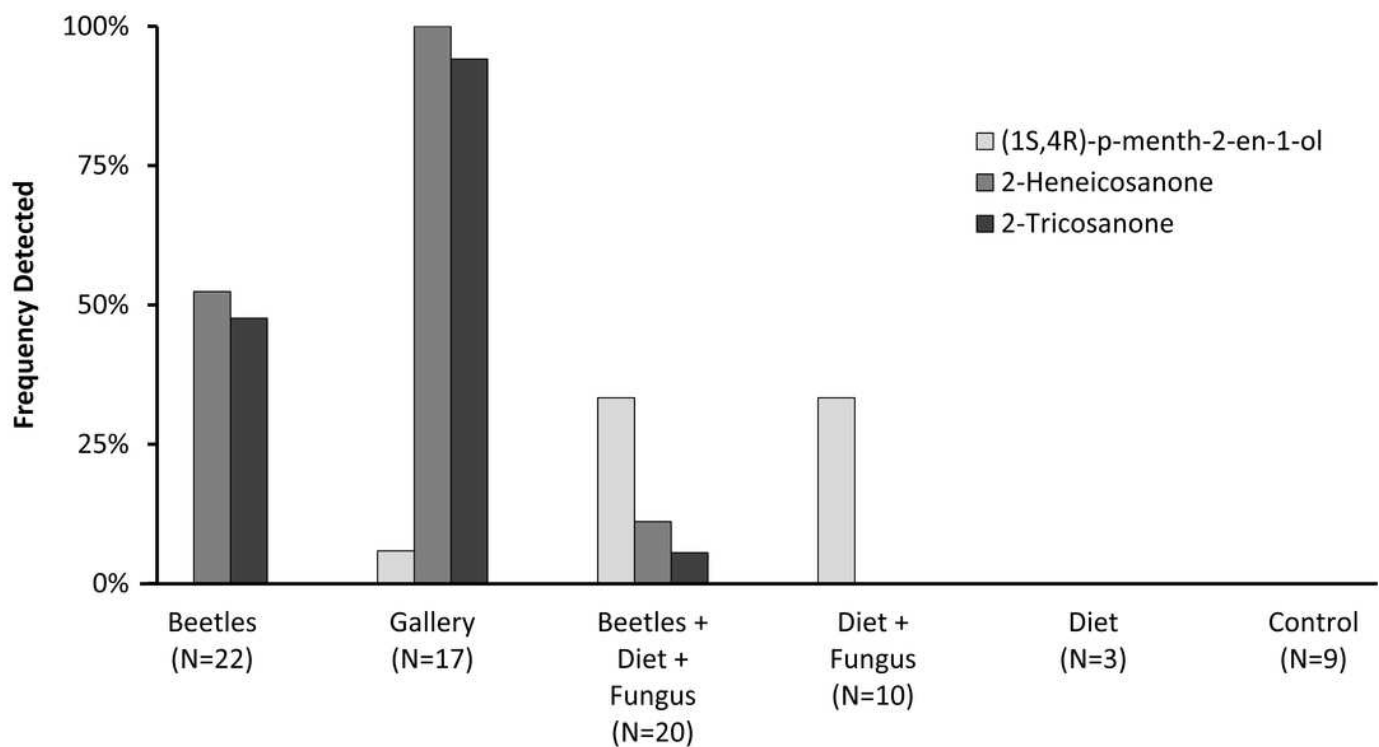
Figure 4

Representative gas chromatograms for volatiles collected from different treatments.

GC traces showing volatiles from different PSHB treatments and controls eluting between 22 and 32 min. Note the Y-axes differ in abundance.

Labeled compounds are 2-heneicosanone (I) and 2-tricosanone (II). PSHB, polyphagous shot hole borer (*E. sp #1*), the population from Los Angeles Co. a) SPME fiber exposed in a control Pasteur pipette with glass wool for 120 min; b) SPME fiber exposed to boxelder diet only (no fungus) inside of a Pasteur pipette for 960 min; c) SPME fiber exposed to boxelder diet and fungus (non-gallery) from a PSHB colony tube inside of a Pasteur pipette for 75 min; d) SPME inserted into gallery from the same PSHB colony tube for 1.5 min; e) SPME fiber exposed to eleven mature female PSHB in a Pasteur pipette with glass wool for 60 min; f) SPME fiber exposed to seven virgin teneral PSHB females in a Pasteur pipette with glass wool for 40 min; g) 1 μl of extract of six PSHB males soaked in hexane for 2 d; h) 50 ng each of synthetic 2-heneicosanone (I) and 2-tricosanone (II).
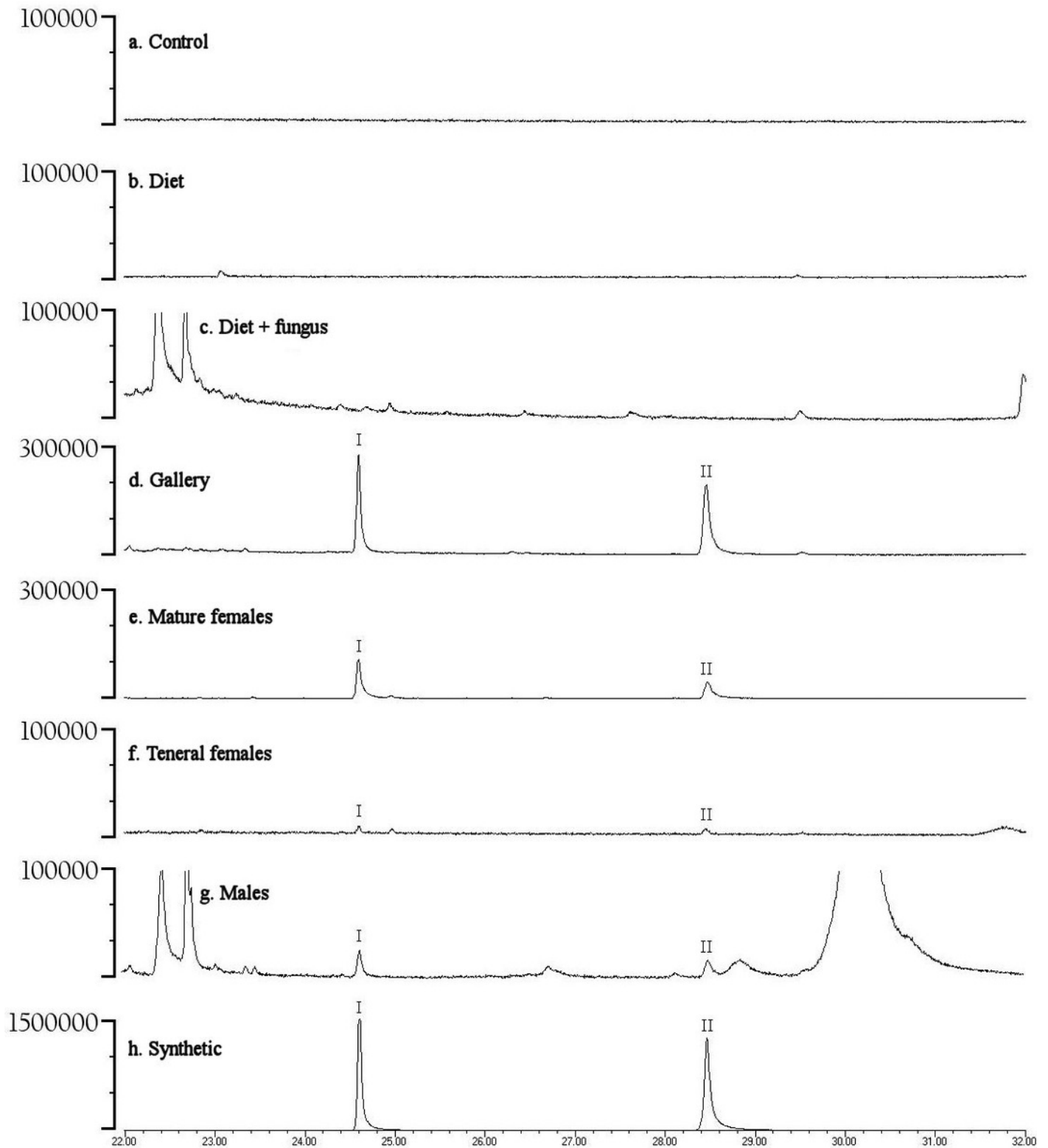
Figure 5

Quantitative comparison of pheromone component ratios between each species.

Mean ratios (±SE) of the two hydrocarbon ketones, 2-heneicosanone and 2-tricosanone, found in extracts of three members of the *E. fornicatus* species complex invasive in the U.S. Beetles were extracted in pentane for 30 min and 2-tridecanone (internal standard) was added. N = 6, 3, and 4, extractions of groups of PSHB, TSHB, and KSHB beetles, respectively. Letters indicate significant differences (ANOVA and Tukey means separation F=179.93, *P* <0.0001, α=0.05). PSHB, polyphagous shot hole borer, the population from Los Angeles Co. TSHB, tea shot hole borer, the population from Miami Dade Co. KSHB, Kuroshio shot hole borer, the population from San Diego Co. 2:21-Kt, 2-heneicosanone. 2:23-Kt, 2-tricosanone.
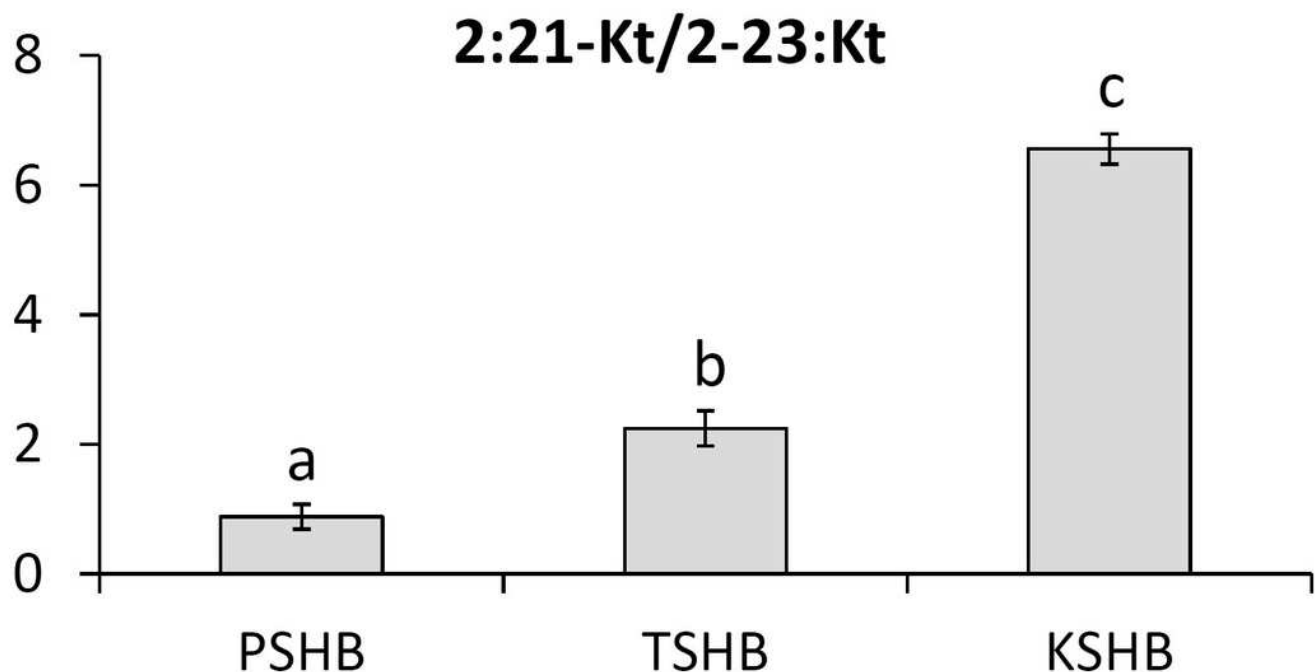
Figure 6

Dose response testing of synthetic pheromone blend.

Walking responses of female PSHB in a Y-plate behavioral bioassay to three concentrations of the 45:55 blend of 2-heneicosanone and 2-tricosanone.

Bars represent proportion of beetles making choices towards and away from the volatile source with respect to N beetles tested, using 1/8 rubber septa loaded with the compounds (shaded bars) or solvent controls (white bars). Numbers inside bars represent number of female beetles making each choice. Asterisks indicate significant difference from 50:50 (Sign test; *** α=0.02).
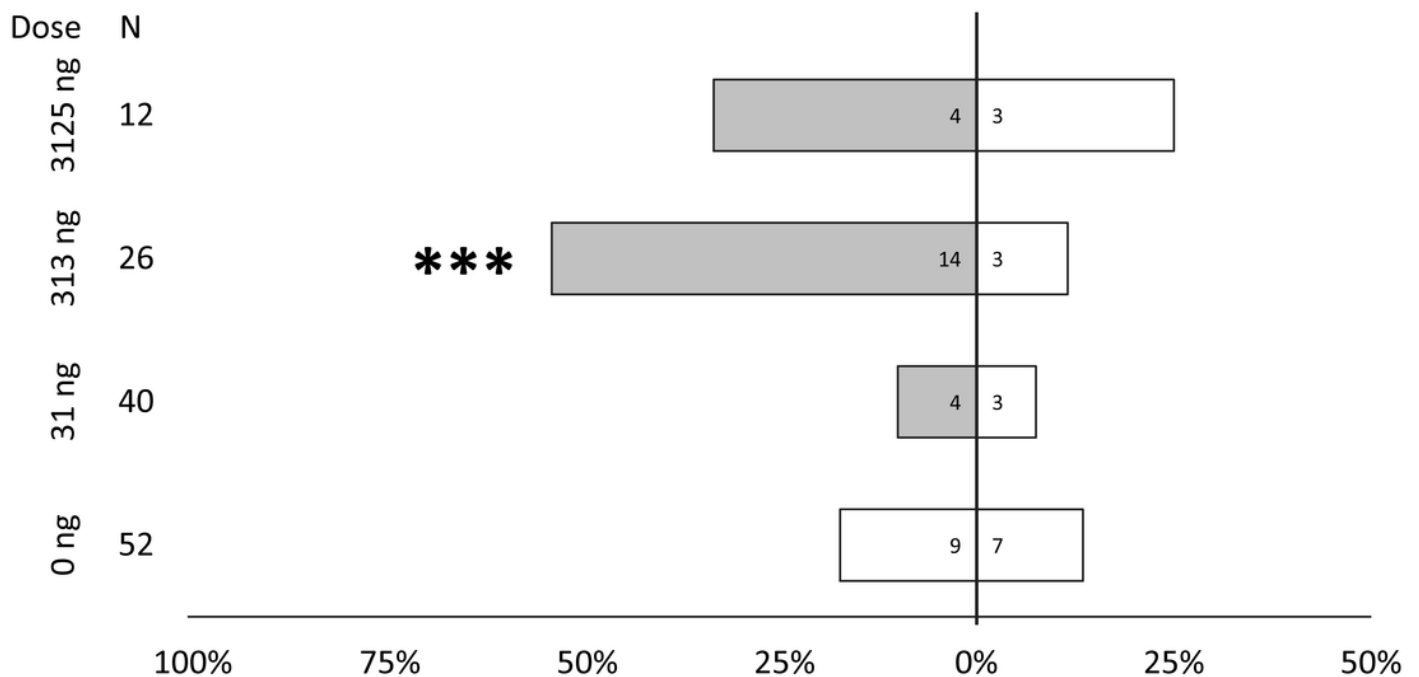
Figure 7

Female walking responses to synthetic pheromone components at different ratios.

Female walking responses of the three *Euwallacea* species (KSHB, TSHB, and PSHB) in a Y-plate behavioral bioassay to three ratios of the two ketone pheromone components, 2-heneicosanone and 2-tricosanone, corresponding to the three species (K, T, P). Beetles were offered a choice between 313 ng of a synthetic pheromone blend (positive choice) and no odor (negative choice).

Bars represent the proportion of female beetles making choices towards (shaded bars) and away from (white bars) the odor source with respect to N beetles tested. Numbers inside bars represent number of female beetles making each choice. Asterisks indicate significant difference from 50:50 (Sign test, **** α=0.01; *** α=0.02; ** α=0.05; * α=0.1). PSHB, polyphagous shot hole borer, the population from Los Angeles Co. TSHB, tea shot hole borer, the population from Miami Dade Co. KSHB, Kuroshio shot hole borer, the population from San Diego Co.
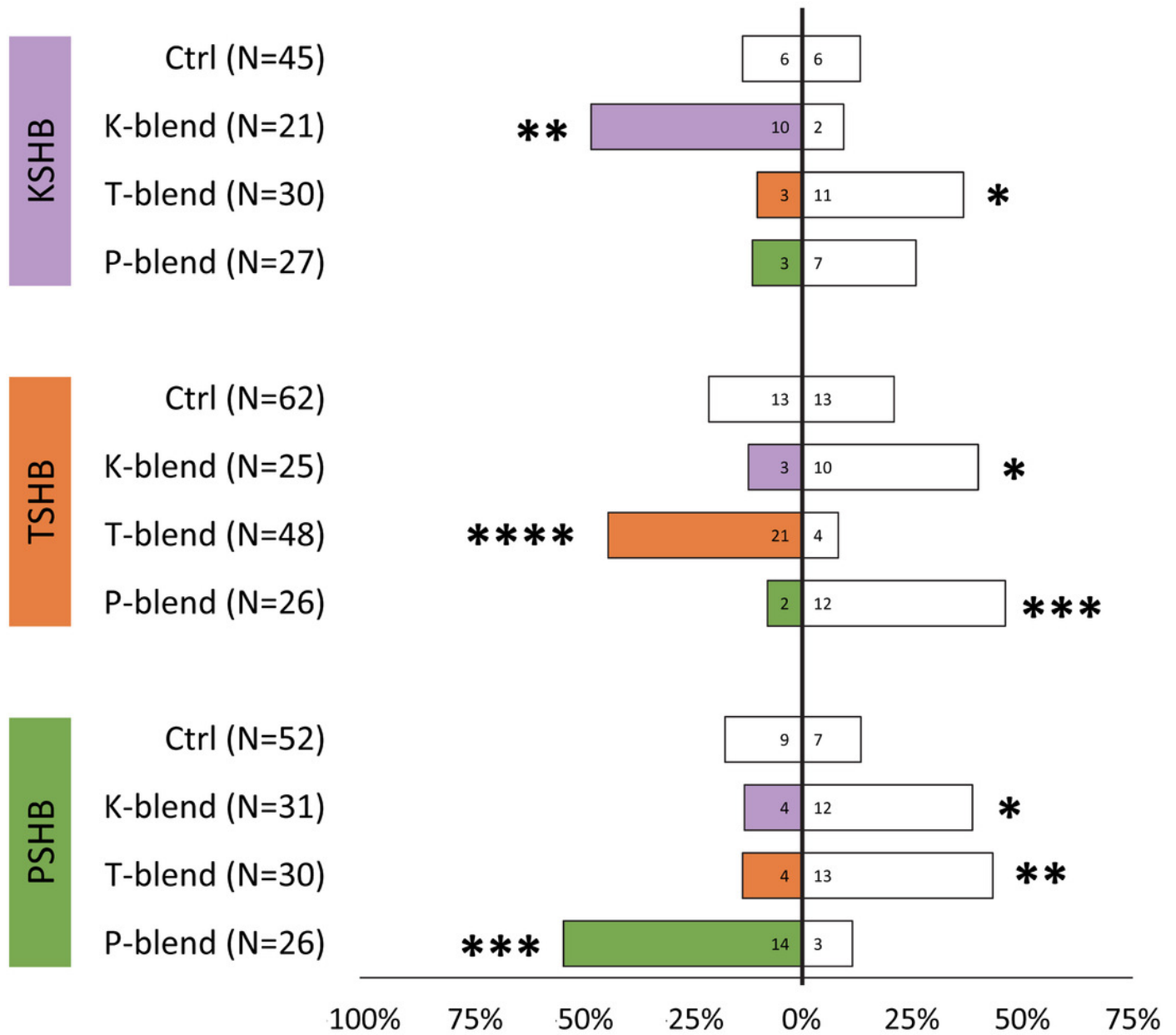
Figure 8

Male walking responses to synthetic pheromone components at different ratios.

Male walking responses of the three *Euwallacea* species (KSHB, TSHB, and PSHB) in a Y-plate behavioral bioassay to three ratios of the two ketone pheromone components, 2-heneicosanone and 2-tricosanone, corresponding to the three species (K, T, P). Beetles were offered a choice between 313 ng of a synthetic pheromone blend (positive choice) and no odor (negative choice).

Bars represent the proportion of male beetles making choices towards (shaded bars) and away from (white bars) the odor source with respect to N beetles tested. Numbers inside bars represent number of male beetles making each choice. Asterisks indicate significant difference from 50:50 (Sign test, **** α=0.01; *** α=0.02; ** α=0.05; * α=0.1). PSHB, polyphagous shot hole borer, the population from Los Angeles Co. TSHB, tea shot hole borer, the population from Miami Dade Co. KSHB, Kuroshio shot hole borer, the population from San Diego Co.