Chemoecology (2013) 23:251–261
DOI 10.1007/s00049-013-0139-9

CHEMOECOLOGY

RESEARCH PAPER

Aggregation pheromones of bark beetles, *Pityogenes quadridens* and *P. bidentatus*, colonizing Scotch pine: olfactory avoidance of interspecific mating and competition

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Received: 24 May 2013 / Accepted: 21 July 2013 / Published online: 8 August 2013

Abstract The bark beetles *Pityogenes bidentatus* and *Pityogenes quadridens* (Coleoptera, Curculionidae, Scolytinae) are sibling species that feed and reproduce in bark areas on branches of Scotch pine, *Pinus sylvestris*. To identify aggregation pheromone components of both species, hindguts and head/thoraxes of males and females of both species feeding in hosts were extracted in pentane and analyzed by gas chromatography and mass spectrometry. Hindguts of male *P. bidentatus* contained grandisol as the major component along with small amounts of (4*S*)-*cis*-verbenol and other monoterpenes. Dose–response bioassays in the laboratory showed the components were attractive at 0.2 ng/min to walking beetles from a distance of ≥25 cm. In the field in southern Sweden, grandisol and (4*S*)-*cis*-verbenol were weakly attractive alone when released at rates of 0.05 and 0.5 mg/day, respectively, from a slow-rotating trap pair. Catch increased 3.6- to 13-fold when the two components were released together. The male proportion of the catch was 0.8 early in the flight period, declined to 0.5 on the peak flight day, and then declined further during the next 2 weeks to 0.2 on the last day of the flight period. Hindguts of male *P. quadridens* contained (2*S*,5*R*)- and (2*S*,5*S*)-chalcogran, as well as (*E*)-2-(3,3-dimethylcyclohexylidene)ethanol (*E*-grandlure 2) and *E/Z*-mixture of 2-(3,3-dimethylcyclohexylidene)acetaldehyde (grandlures 3 and 4), while female hindguts had only a trace amount of chalcogran. Laboratory studies proved *E*-grandlure 2 is an essential pheromone component for *P. quadridens*. Field bioassays with a slow-rotating trap pair in which the attractiveness of blends containing various candidate components were compared with that of less complete mixtures, showed that chalcogran and *E*-grandlure 2 were synergistic aggregation pheromone components of *P. quadridens*. Field tests also showed that grandisol (from *P. bidentatus*) reduced attraction of *P. quadridens* to its aggregation pheromone, whereas *E*-grandlure 2 and chalcogran (from *P. quadridens*) reduced response of *P. bidentatus* to its aggregation pheromone. Our results suggest that aggregation pheromone components from males of each species not only attract conspecifics but also aid individuals in avoiding interspecific mating and competition for food and spatial resources within the bark phloem layer.

Keywords *Pityogenes* · Host selection · Mate selection · Scolytinae · Coleoptera · *Pinus sylvestris* · Semiochemicals · Bioassays

Handling Editor: Thomas Schmitt.

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Introduction

Individuals of *Pityogenes* species are relatively small bark beetles (2- to 3-mm long) attacking conifers, usually their limbs, tops, or smaller diameter trunks of weakened hosts (Lekander et al. 1977). *Pityogenes bidentatus* (Herbst),

Pityogenes quadridens (Hartig), and *Pityogenes chalcographus* (L.) are common in northern Europe and Scandinavia and often occur in the same forest stands. *P. bidentatus* and *P. quadridens* colonize only Scotch pine, *Pinus sylvestris* L., while *P. chalcographus* attacks primarily Norway spruce, *Picea abies* (L.) and only occasionally colonizes Scotch pine (Lekander et al. 1977). Thus, these species, especially *P. bidentatus* and *P. quadridens*, may compete for the same relatively thin layer of phloem habitat as shown in other bark beetles (Anderbrant et al. 1985). Males of *Pityogenes* species initiate an entrance hole and construct a nuptial chamber in the phloem layer. Up to five or six females may join the male in response to a male-released aggregation pheromone (Francke et al. 1977; Byers et al. 1988, 1990a, 2000). After mating, females tunnel egg galleries outward from the nuptial chamber, thereby delineating an exclusive resource territory for the polygynous family (Byers 1989). Aggregation pheromones of insects are usually released by one sex that attracts both sexes, as opposed to sex pheromones released by one sex that attract only the opposite sex.

The first aggregation pheromone component for the genus *Pityogenes* was identified in *P. chalcographus* males as a mixture of the *cis*- and *trans*-isomers of 2-ethyl-1,6-dioxaspiro[4.4]nonane (chalcogran) (Francke et al. 1977)—for chemical structures of relevant compounds see Fig. 1. Enantioselective gas chromatography revealed the natural chalcogran produced by both *P. chalcographus* and *P. quadridens* shows the (2*S*)-configuration (Schurig and Weber 1984). The behaviorally active stereoisomer of *P. chalcographus* is (2*S*,5*R*)-chalcogran, whereas the (2*S*,5*S*)-stereoisomer is inactive (Byers et al. 1989). A second aggregation component for *P. chalcographus*, methyl (2*E*,4*Z*)-decadienoate, is synergistic with chalcogran (Byers et al. 1988, 1990a).

In *P. bidentatus*, verbenone, *cis*- and *trans*-verbenol, ethyl dodecanoate, and grandisol were identified as candidate semiochemicals (Francke et al. 1995). Baader (1989) investigated responses of *P. bidentatus* to synthetic compounds released in the field. He suggested that (*S*)-*cis*-verbenol, grandisol, and γ-isogeraniol comprise the aggregation pheromone of *P. bidentatus*. Only (4*S*)-*cis*-verbenol or mixtures containing this terpene were attractive, whereas the two-component blend of grandisol and γ-isogeraniol was not. In the field, the two-component blend of *cis*-verbenol and grandisol proved attractive (Byers et al. 2000); however, no identification details were published.

Pentane extracts of male *P. quadridens* contained several compounds that are behaviorally active in other bark beetles (Byers 1989; Francke et al. 1995). These were 1-hexanol, chalcogran, *trans*-verbenol, ipsdienol, verbenone, ethyl dodecanoate, and grandisol. Other compounds released by males were geraniol, γ-isogeraniol, myrtenol, (*E*)-2-(3,3-dimethylcyclohexylidene)ethanol (*E*-grandlure 2 = *E*-ochtodenol), and 2-phenylethanol. However, no behavioral response to these compounds has been demonstrated in *P. quadridens*.

The first objective of our study was to identify volatile compounds produced by the respective sexes of *P. bidentatus* and *P. quadridens* during feeding that may be possible pheromone components and compare these with those previously reported from these species (Francke et al. 1995). A second objective was to bioassay candidate components for aggregation response using a subtractive method in laboratory bioassays and in field tests with slow-rotating pairs of traps (Byers et al. 1990a; Byers 1992). Thirdly, we were interested in the role of pheromone components in interspecific communication. Better knowledge of the behavior-mediating capacity of semiochemicals of *P. bidentatus* and *P. quadridens* would provide insights into the mechanisms for maintaining reproductive isolation or limiting interspecific competition between the two species.

Materials and methods

Chemical analyses of bark beetle extracts

Limbs of Scotch pine (6- to 8-cm diameter) were observed to undergo attack by *P. quadridens* (Sjöbo, Sweden, 4 May 1994). The limbs were taken to the laboratory, and the bark was cut away to reveal nuptial chambers each containing a male (identified by presence of four large spines on the elytral declivity) and from one to three females (without spines). The females had recently arrived since the galleries were not longer than a few millimeters. Four groups, each of about 20 beetles of each sex were placed in separate bottles, agitated for 3 s with 70 % ethanol to remove resin from the bodies, and then dried on absorbent paper for 2 min. This treatment did not appear to harm the live beetles. In bark beetles and *Pityogenes* species, the midgut may produce the aggregation pheromone (Nardi et al. 2002), which then accumulates in the hindguts and is excreted with the fecal pellets (Byers 1989; Francke et al. 1995). Thus, hindguts of beetles in each group, removed by a quick pull of the abdominal tip, were pooled in a vial containing 150-µl pentane set on dry ice.

For comparison with *P. quadridens*, adult *P. bidentatus* were collected with a butterfly net near host logs in the Scotch pine forest (Sjöbo, Sweden, 16–25 May 1994) and separated by sex. The males (identified by two large spines on the elytral declivity) were introduced to freshly-cut Scotch pine logs (30-cm long × 8- to 10-cm diameter) for 24 h, allowing them to excavate nuptial chambers. Subsequently, about 60 females (one per male nuptial chamber)

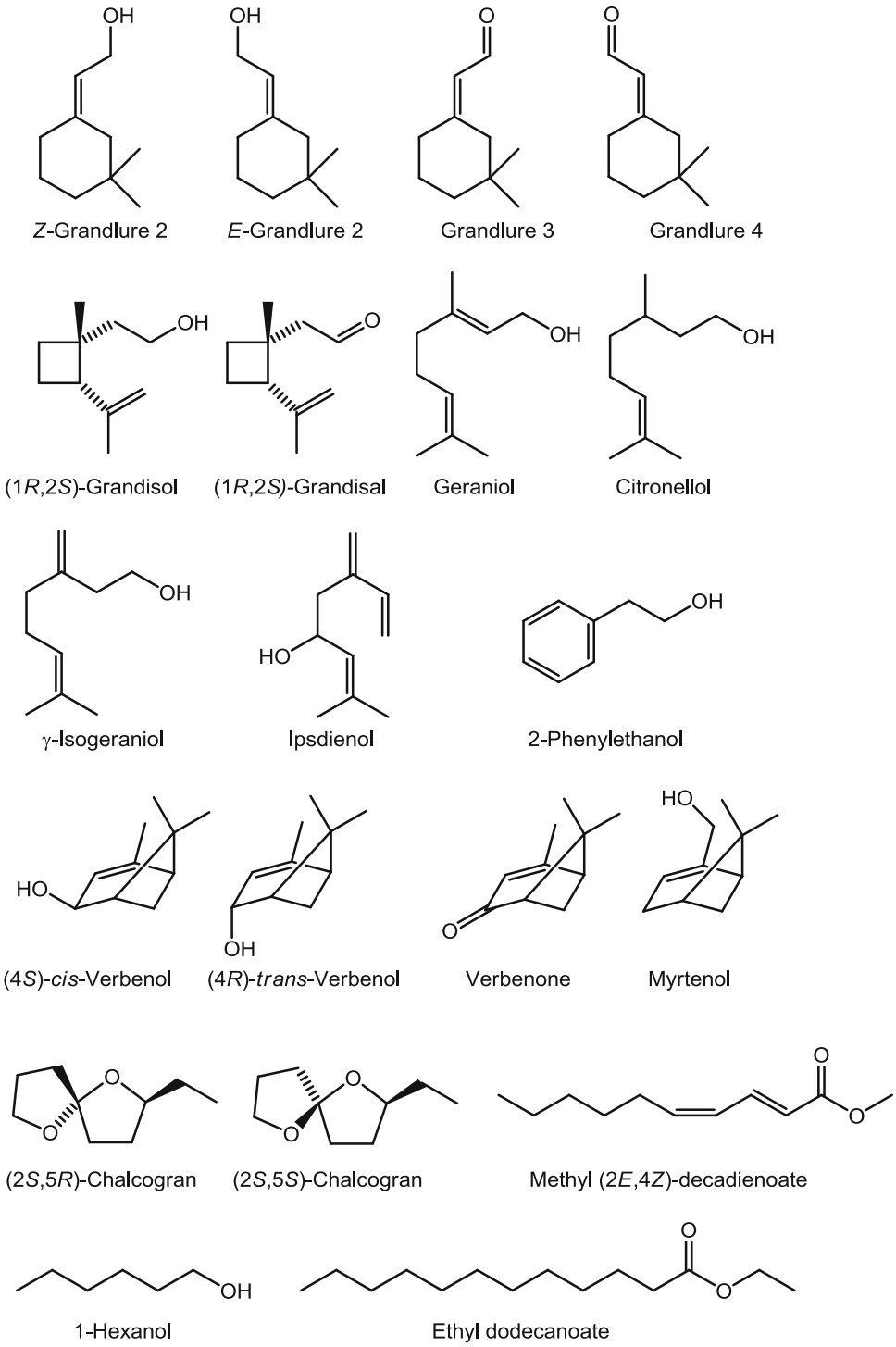

Fig. 1 Structures of volatile compounds present in *Pityogenes* bark beetles

were placed on the logs and allowed to join the males in their chambers for an additional 24 h. At the end of this procedure, the beetles were dissected from logs, washed in ethanol, and their hindguts dissected as above. The hindguts were placed in pentane extracts similarly made of each of two groups of each sex (about 20 individuals per group). The combined heads and thoraxes of these beetles were extracted similarly in groups.

The bark beetle extracts were analyzed for volatiles by a combined HP 5890 series II gas chromatograph and HP 5972 mass selective detector (GC-MSD, Hewlett-Packard, Palo Alto, USA). A fused-silica capillary column, 25 m × 0.25 mm, coated with 0.25 μm of OV351 (Ohio Valley Specialty Company, Marietta, OH, USA) was used with helium as the carrier gas at a constant flow of 30 cm/s (2 μl samples injected into injection port at 200 °C, HP

7673 autoinjector). The column was initially set at 50 °C for 2 min, followed by a 10 °C/min rise to 200° and maintained for 10 min. Eluted compounds were identified by comparing their retention times and mass spectra to those of authentic reference samples and by reference to computer data libraries (our own and the commercial NBS75K). Quantification ranges (see Table 1) were assigned by comparing peak areas of known amounts of the terpene alcohols terpinen-4-ol and myrtenol (each at 20 ng) with the peak areas of compounds extracted from the insects.

Laboratory and field bioassays of pheromone components

Pityogenes quadridens and *P. bidentatus* were obtained from naturally-infested Scotch pine logs taken from forests near Sjöbo (April–May 1994 and 1996) and reared in the laboratory at 27 °C (Byers et al. 1990a). After emergence, adults were maintained at 4 °C until used in experiments. The response of these unsexed beetles (about 1:1 male:female) to gut extracts taken as described above, and to various synthetic samples of compounds identified in the extracts, was tested in an open arena bioassay in the laboratory (1,000 lux, 23 °C). The bioassay arena was a plate of glass (0.8-m wide × 1-m long) covered with white paper that allowed beetles to walk. A large manifold (60-cm wide × 25 cm × 25 cm) with suction fan and flexible hose placed "downwind" of the glass plate drew air out of the room. A smaller plastic manifold (46-cm wide × 5-cm high × 8.5-cm deep) "upwind" of the glass plate blew a laminar air flow across the arena (about 0.9 m/s at the test source and 0.6 m/s at the beetle release point). A 5-µl capillary tube open at both ends and filled with one or more test chemicals diluted in diethyl ether was positioned upright 10-cm downwind of the center of the smaller manifold. The solvent solution evaporated at the bottom opening of the capillary tube, and volatiles were carried across the table by the airflow from the manifold. Beetles of approximately equal sex ratio were reared from Scotch pine logs. These beetles were released in groups of ten individuals about 25-cm downwind of the test capillary and

Table 1 Relative amounts of volatile compounds found in hindgut extracts of *Pityogenes quadridens* and *P. bidentatus* colonizing Scotch pine logs (May 1994)

	Pityogenes quadridens		*Pityogenes bidentatus*	
Compound	Male	Female	Male	Female
3-Hydroxy-2-butanone	+++	+++	−	−
1-Hexanol	++	−	++	−
cis- and *trans*-Chalcogran[a]	++ to +++	+	−	−
Grandisal	−	−	++	−
cis-Verbenol[a]	+	−	+	−
trans-Verbenol[a, b]	+	−	+	−
Verbenone[b]	−	−	+	−
Ipsdienol[a]	+	−	−	−
Grandlure 3[a]	++	−	−	−
Grandlure 4[a]	++	−	−	−
Citronellol	++	−	++	−
γ-Isogeraniol	+++	−	++	−
Grandisol[a]	−	−	++++	− or +[c]
E-Grandlure 2	++++	−	−	−
Geraniol	++	−	+	−
Dodecyl acetate	++	-?	−	−
Ethyl dodecanoate[a]	+	-?	++	−
2-Phenylethanol	++	−	++	−
Myrtenol	+	-?	+	-?

"−" (<0.1 ng/beetle), "+" (0.1–0.5 ng/beetle), "++" (0.5–5 ng), "+++" (5–10 ng), "++++" (20–50 ng/beetle), "-?" (trace amounts may have co-eluted with contaminants)

[a] Attractive pheromone component of other bark beetles or weevils (Tumlinson et al. 1969; Byers 1989; Francke et al. 1995)

[b] Inhibitory/repellent pheromone component of other bark beetles (Byers 1989; Francke et al. 1995)

[c] One sample of 23 females had no grandisol while another with 20 females had grandisol at about 5 % of male extracts (indicating that 1 male may have been included by mistake)

allowed to respond by walking to within 2 cm of the odor source. When beetles did not respond because they walked out of the arena circle (50-cm diameter), or when the diethyl ether solvent evaporated from the capillary without response, these beetles were given a second test with a newly filled capillary.

Chemicals tested in the response arena were 1-(2-hydroxyethyl)-1-methyl-2-(1-methylethenyl)cyclobutane (grandisol) (>98 %), (*Z*)-2-(3,3-dimethylcyclohexylidene) ethanol (*Z*-grandlure 2) (97 %); an *E/Z*-mixture of 2-(3,3-dimethylcyclohexylidene)acetaldehyde (*E/Z*- ochtodenal = grandlure 3 and grandlure 4) (97 %), all obtained from Frank Enterprises, Inc. (Columbus, Ohio), *E*-2-(3,3-dimethylcyclohexylidene)ethanol (*E*-grandlure 2) (95 %) (synthesized according to Bedoukian and Wolinsky 1975), a mixture of all four stereoisomers of chalcogran (54 % *cis* and 46 % *trans)* (>98 %) (Francke et al. 1977), (4*S*)-*cis*-verbenol (96 %, Borregaard, Sarpsborg, Norway), *rac*. ipsdienol (95 %, Borregaard), and ethyl dodecanoate (>99 %, Aldrich, Milwaukee, Wisconsin). Binomial confidence limits for proportions (95 % BCLP) were calculated for beetle responses in each assay (Spiegel 1961).

Blends of grandisol, (4*S*)-*cis*-verbenol, ethyl dodecanoate, chalcogran, *E*-grandlure 2, Z-grandlure 2, and ipsdienol mentioned above were formulated for field trapping tests during the spring of 1996, 1998, and 2001. The compounds were released from small glass tubes (34-mm long × 4.45-mm i.d.) with 25 µl of each compound in the bottom of a tube. Release rates of a compound as measured by weight loss were nearly constant but varied among compounds depending on their volatility. As a solid, (4*S*)-*cis*-verbenol was released similarly but from a polyethylene tube (31-mm × 6.15-mm i.d.). Unless otherwise indicated, each chemical was released from a single tube per trap. Weight loss measurements per tube gave the following mean release rates at 20 °C: ipsdienol (0.4 mg/day), ethyl dodecanoate (0.01 mg/day), chalcogran (0.8 mg/day), *cis*-verbenol (0.5 mg/day), grandisol (0.05 mg/day), *Z*- or *E*-grandlure 2 (0.1 mg/day), grandlure 3 and 4 (0.05 mg/day each). These rates were similar to release rates attractive to other bark beetles including *P. chalcographus* (Byers and Wood 1980; Schlyter et al. 1987; Byers et al. 1988; Byers 1993). A subtractive method was generally used in which each component was systematically removed from the complete blend to see if any decrease in catch resulted, indicating this particular component to be important to beetle response (Byers et al. 1990a; Byers 1992).

The various blends were tested in three slow-rotating pairs of traps; a trapping method that minimizes the catch variation due to trap position which otherwise can be large with fixed-position traps (Byers et al. 1990b, 1989, 2000). The lures were placed in rotating trap pairs, each pair consisting of two traps separated by 6 m at 1.2 m above the ground and mechanically rotated at two revolutions per hour with a gear motor. Each trap consisted of a plastic cylinder (18-cm diameter × 28-cm high), closed at the top but open at the bottom, suspended over the center of a 31-cm diameter funnel that collected falling beetles after they struck the cylinder. The funnel emptied into a plastic holding bottle with a fine metal screen bottom that allowed rain to pass through and prevented beetles from escaping. Tests were conducted for at least 1 h with baits switched between paired traps for at least one more test. Some tests were repeated three or more times. Catches for all tests were summed for each blend in a comparison, and the sums compared to the average of both sums assuming equality using a Chi square goodness of fit test. BCLPs (95 %) were calculated for male proportions of total catches in semiochemical-baited traps during April–May 1998.

Interspecific avoidance of volatiles from competing species

The synthetic pheromone components of *P. bidentatus* [(4*S*)-*cis*-verbenol and grandisol] were compared with the same blend combined with *E*-grandlure 2 alone or with both *E*-grandlure 2 plus chalcogran (aggregation components of *P. quadridens*) in the paired trap rotor as described above. Similarly, the pheromone components of *P. quadridens* (chalcogran and *E*-grandlure 2) were compared with the same blend combined with grandisol (a component of *P. bidentatus* pheromone). Release rates of chemicals were as described above except in the case of *P. bidentatus* where *E*-grandlure 2 was released at a combined rate of 0.3 mg/day from three glass tubes.

Results

Chemical analyses of bark beetle extracts

In the identification of chemicals in extracts of the two *Pityogenes* species and the behavioral tests, we focused on the known pheromone components of bark beetles and weevils (Curculionidae) (e.g., Tumlinson et al. 1969; Byers and Wood 1980; Byers 1983, 1989, 1993; Byers et al. 1988, 1998, 2000; Birgersson et al. 2000; Francke et al. 1995). Hindguts of feeding male *P. quadridens* did not contain detectable levels of grandisol, but had *cis*- and *trans*-chalcogran, *E*-grandlure 2, and grandlure 3 and 4, as well as small quantities of *cis*-verbenol. In female hindguts these compounds were absent except for small amounts of chalcogran (Table 1). The presence of chalcogran in females (about 3 % of the amount in males) was probably

not caused by accidental inclusion of a male because *E*-grandlure 2, which was abundant in males, was not detected in females (Table 1). Small to moderate amounts of ipsdienol, *cis*- and *trans*-verbenol (but not verbenone) as well as 1-hexanol, 2-phenylethanol, and dodecyl acetate were found in male, but not in female, *P. quadridens* (Table 1). The pentane used for extractions did not contain any of the compounds mentioned above.

Hindguts of feeding male *P. bidentatus* contained several compounds that were not found in females, but are known to occur as pheromone components in other bark beetles or related weevils. Grandisol was produced sex specifically by males as the dominant component (Table 1). Small amounts of c*is*-verbenol and grandisal were also produced, apparently only in males. The amounts of *cis*-verbenol and grandisol were about 5–10 times higher in the hindgut compared to the head–thorax. Grandisal amounts were about equal in head–thorax and hindgut, but there was apparently a higher concentration of grandisal per unit of tissue in the hindgut based on its smaller volume compared to the head–thorax. Also, the ratio of grandisol to grandisal was about 15:1 in the hindgut but was about equal in the head–thorax. Other compounds that are known to be inhibitory/repellent or attractive to bark beetles such as 1-hexanol, verbenone, *trans*-verbenol, 2-phenylethanol, and ethyl dodecanoate were also found sex specifically in *P. bidentatus* males (Table 1; Fig. 1).

Laboratory and field bioassays of pheromone components

In 1998 trap captures of *P. bidentatus* adults were low at the end of April, but then peaked on 2 May (Fig. 2). Catches during April–May 1998 showed a pattern of more males captured than females early in the flight-dispersal and host-seeking period (Fig. 2). As host-seeking flights progressed to the peak flight day of 2 May there was an almost equal sex ratio, while near the end of the colonization period on 17 May the male proportion declined below that of females as indicated by differences in the proportions (non-overlap of 95 % BCLPs, Fig. 2). This pattern for *P. bidentatus* was similar to that known for the bark beetle *Ips paraconfusus* that is also male biased at the beginning of colonization of ponderosa pine and changes to a female-biased ratio over the colonization period (Byers 1983).

Laboratory bioassays (May 1994) showed hindgut extracts of male *P. bidentatus* (0.1 hindgut/µl extract or released at about 0.22 hindgut equivalents/min) were highly attractive to conspecifics (90 % response, $n = 40$) to the beetles (about 1:1 sex ratio). These responses were significantly higher than solvent controls (7.5 %, $n = 40$, $\chi^2 = 54.5$, df = 1, $P < 0.001$). In the laboratory, a 1:1

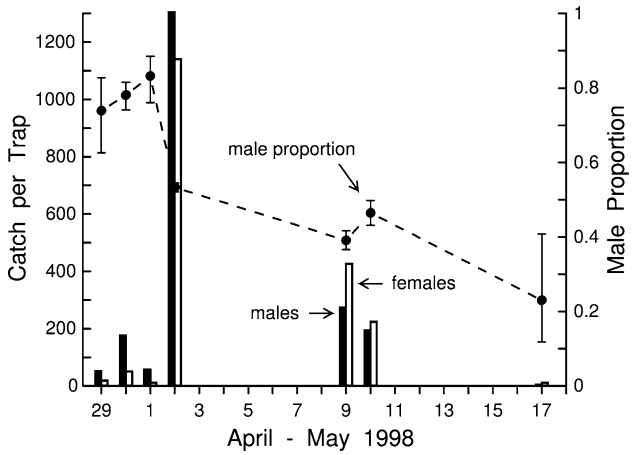

Fig. 2 Catch of *Pityogenes bidentatus* on traps baited with *rac.* grandisol and (*S*)-*cis*-verbenol during April–May 1998. Days without catch were due to daily temperatures below the flight threshold of about 17 °C. *Error bars* represent 95 % binomial confidence limits for male proportions of captured beetles

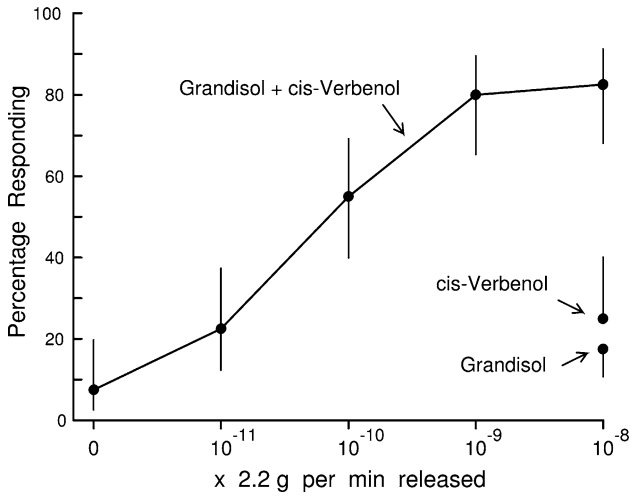

Fig. 3 Dose–responses of *Pityogenes bidentatus* to aggregation pheromone components grandisol and (4*S*)-*cis*-verbenol in laboratory bioassays (May 1996; each compound tested alone at 10^{-8} g). *Bars* represent 95 % binomial confidence limits for proportions ($N = 40$–50)

mixture of grandisol and *cis*-verbenol at several release rates was a more powerful attractant for *P. bidentatus* than either of the two compounds alone (Fig. 3), which indicated a strong synergism. The increase in response to a range of 10-fold increasing concentrations of these two components fit a kinetic formation function $Y = a - (a^{1-c} + bX(c-1))^{1/(1-c)}$, where $a = 87.34$, $b = 0.0000424$, $c = 3.035$, and $R^2 = 0.99$ (Byers 2013). This function was similar to dose–response curves observed in other bark beetles (*P. chalcographus*, *Dendroctonus brevicomis*, and *Ips paraconfusus*) in similar arenas in the laboratory (Byers 1983; Byers et al. 1990a; Byers 2013). In field tests with

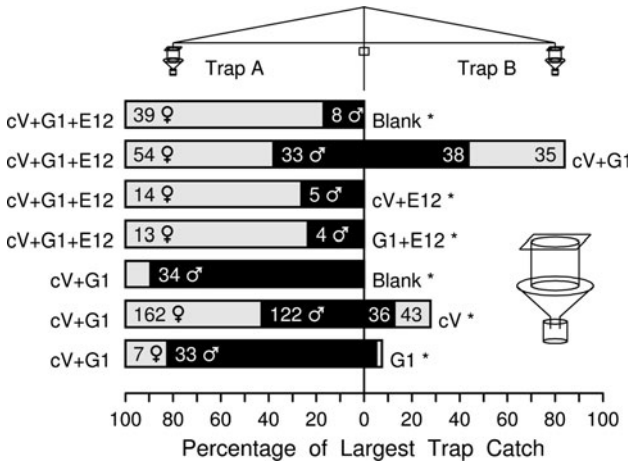

Fig. 4 Catches of *Pityogenes bidentatus* in response to various baits (*cV cis*-verbenol, *G1 rac*. grandisol, *E12* ethyl dodecanoate) in paired rotor traps. *Top four pairs of bars* (13–14 May 1996), bottom *three pairs of bars* (1–17 May 1998). *Asterisks* indicate a significant difference between paired trap baits at $P < 0.001$ (Chi square)

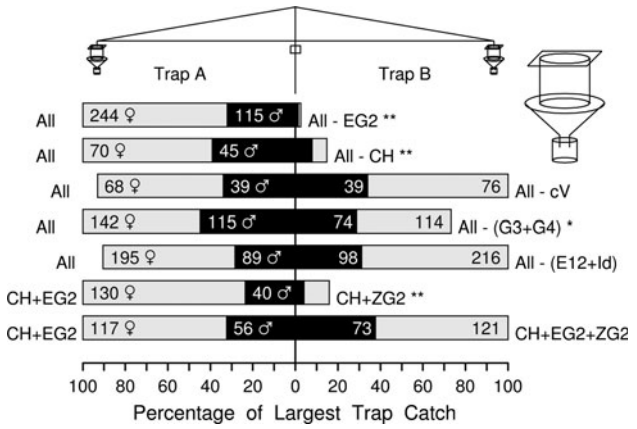

Fig. 5 Catches of *Pityogenes quadridens* in response to the full blend ("All") containing chalcogran (*CH*), *E*-grandlure 2 (*EG2*), grandlure 3 and 4 (G3+G4), (*S*)- *cis*-verbenol (*cV*), ethyl dodecanoate (*E12*), and *rac*. ipsdienol (*Id*) compared to this blend minus various components as indicated in paired rotor traps (2–10 May 2001). Z-grandlure 2 (ZG2) was added to test for substitution or inhibition. *Single* and *double asterisks* indicate a significant difference between paired trap baits at $P < 0.01$ or $P < 0.001$, respectively (Chi square)

paired traps, candidate blends of grandisol and *cis*-verbenol, intended to attract *P. bidentatus,* did catch this species, and subtraction of either of the compounds reduced attraction (Fig. 4). Subtraction of ethyl dodecanoate had no significant effect.

Laboratory bioassays of hindgut extracts of *P. quadridens* males (0.1 hindgut/µl extract, May 1994) indicated they were highly attractive to conspecifics (87.5 % response, $n = 40$) compared with solvent controls (10 %, $n = 40$, $\chi^2 = 54.5$, df = 1, $P < 0.001$). Tests with synthetic compounds proved *E*-grandlure 2 was an indispensible pheromone component, because all mixtures lacking this compound elicited little response (all 10–30 %). In the field studies using rotating paired traps, an "All" blend of seven compounds composed of *E*-grandlure 2, *cis*/*trans*-chalcogran, grandlure 3 and 4, (4*S*)-*cis*-verbenol, *rac*. ipsdienol, and ethyl dodecanoate, identified in hindguts of feeding *P. quadridens,* was extensively tested. Subtraction of various components from this blend, either *cis*-verbenol, chalcogran, *E*-grandlure 2, or both ipsdienol and ethyl decadienoate, showed that chalcogran and *E*-grandlure 2 are important components of the aggregation pheromone of *P. quadridens* (Fig. 5). Subtraction of grandlure 3 and 4 reduced attraction but appeared significant only for the females (Fig. 5). Further tests are needed to substantiate this result. Comparison of a blend of Z-grandlure 2 plus chalcogran with a mixture of *E*-grandlure 2 plus chalcogran confirmed that *E*-grandlure 2 is a pheromone component (Fig. 5). Addition of *Z*-grandlure 2 to the two-component blend of *P. quadridens* (chalcogran and *E*-grandlure 2) had no significant effect on catch (Fig. 5), revealing the *Z*-isomer is inactive.

Interspecific avoidance of volatiles from competing species

In tests to explore *P. bidentatus* avoidance of interspecific competition, addition of *E*-grandlure 2 of *P. quadridens* to the two aggregation components of *P. bidentatus* in the trap-pair rotor caused response of *P. bidentatus* to decrease 43 % (297 ♂ : 170 ♀ catch versus 520 ♂ : 301 ♀; $\chi^2 = 97$, df = 1, $P < 0.001$). Response of both sexes declined similarly. Addition of both aggregation pheromone components of *P. quadridens* (*E*-grandlure 2 and chalcogran) to the aggregation pheromone of *P. bidentatus* (*cis*-verbenol and grandisol) reduced the response of the latter species by 69 % (138 ♂ : 75 ♀ catch versus 412 ♂ : 266 ♀; $\chi^2 = 242$, df = 1, $P < 0.001$). Again, response of both sexes declined similarly. *P. quadridens* also avoided interspecific competition when grandisol (an aggregation component of *P. bidentatus*) was added to the aggregation pheromone components of *P. quadridens* (chalcogran and E-grandlure 2), causing attraction of *P. quadridens* to decrease by 23 % (78 ♂ : 146 ♀ catch versus 70 ♂ : 222 ♀; $\chi^2 = 8.96$, df = 1, $P = 0.003$). The decrease in attraction was caused by a 34 % reduction in response by females and a 10 % reduction by males.

Discussion

Chemical analyses of male hindguts reported here largely confirm those reported earlier (Francke et al. 1995). We have added comparative analyses of hindguts and head–

thoraxes as well as corresponding female tissues. There appear to be some minor differences between the two studies, which may be due in part to the preparation of extracts, particular host tree, and bark beetle population sampled. Compared with Francke et al. (1995), in *P. bidentatus* males, 1-hexanol, citronellol, geraniol, and grandisal were additionally detected (Table 1). In *P. quadridens* males, we found 3-hydroxy-2-butenone, *cis*-verbenol, grandlure 3 and 4, and dodecyl acetate not previously reported. Earlier studies by Baader (1989) of *P. bidentatus* implicated (4*S*)-*cis*-verbenol as part of the aggregation pheromone. Grandisol plus γ-isogeraniol caught no beetles whereas this combination plus (4*S*)-*cis*-verbenol caught more than (4*S*)-*cis*-verbenol alone. However, his uncertain results needed confirmation and expansion. We did not test γ-isogeraniol because it is found in both species (Francke et al. 1995; Table 1) and not known to be behaviorally active in bark beetles. We found that grandisol and (4*S*)-*cis*-verbenol caught large numbers of *P. bidentatus* (Figs. 2, 4). In the laboratory, the synergism of the two components was clearly shown (Fig. 3).

Grandisol and grandlures are pheromone components of the boll weevil *Anthonomus grandis* (Tumlinson et al. 1969), the pecan weevil (Hedin et al. 1997) and several other weevil species (Francke and Dettner 2005). Grandisol was also identified as an aggregation pheromone component in two pine weevils, *Pissodes strobi* and *P. nemorensis*, which also use grandisal as a component (Booth et al. 1983; Hibbard and Webster 1993). As a component of a bark beetle pheromone, grandisol was first identified in *Pityophthorus pityographus* Ratz. (Francke et al. 1987). In the boll weevil, only (1*R*,2*S*)-(+)-grandisol is behaviorally active (Dickens and Mori 1989), and in the insects in most of the above studies it is produced as a pure (1*R*,2*S*)-enantiomer. However, grandisal identified in the two *Pissodes* species was by no means enantiomerically pure, and its enantiomeric composition may well play a role in the communication systems of the two species. Whether this is also true for the grandisol in *P. bidentatus* is not currently known. In contrast, *E*-grandlure 2 is relatively rare as a semiochemical, and other than our report for *P. quadridens* it has been identified only in *Anthonomus eugenii* (Eller et al. 1994) and *Sternechus subsignatus* (Ambrogi et al. 2012).

Both *P. quadridens* and *P. chalcographus* produce a mixture of C5-epimers of chalcogran in a nearly equal ratio (Schurig and Weber 1984). In *P. chalcographus*, only (2*S*,5*R*)-chalcogran is behaviorally active, but it is not known whether the same stereoisomer or its C5-epimer (2*S*,5*S*) is behaviorally active in *P. quadridens*. In *P. chalcographus* (2*S*,5*S*)-chalcogran is inactive (Byers et al. 1989), and thus might play a role in *P. quadridens* to maintain species specificity. Different responses of the two species to the two stereoisomers would be ecologically adaptive, allowing both species to avoid wasting time and energy in competition during colonization events. Whereas *P. chalcographus* has methyl (2*E*,4*Z*)-decadienoate as a second component (Byers et al. 1988, 1990a), *P. quadridens* uses *E*-grandlure 2 in addition to chalcogran. Chalcogran, which alone elicited only weak behavioral activity both in the laboratory (30 % response) and in the field (Fig. 5), is strongly synergistic with *E*-grandlure 2, both produced by *P. quadridens*. The presence of *Z*-grandlure 2 did not block or inhibit this response (Fig. 5). Racemic ipsdienol, known as an important pheromone

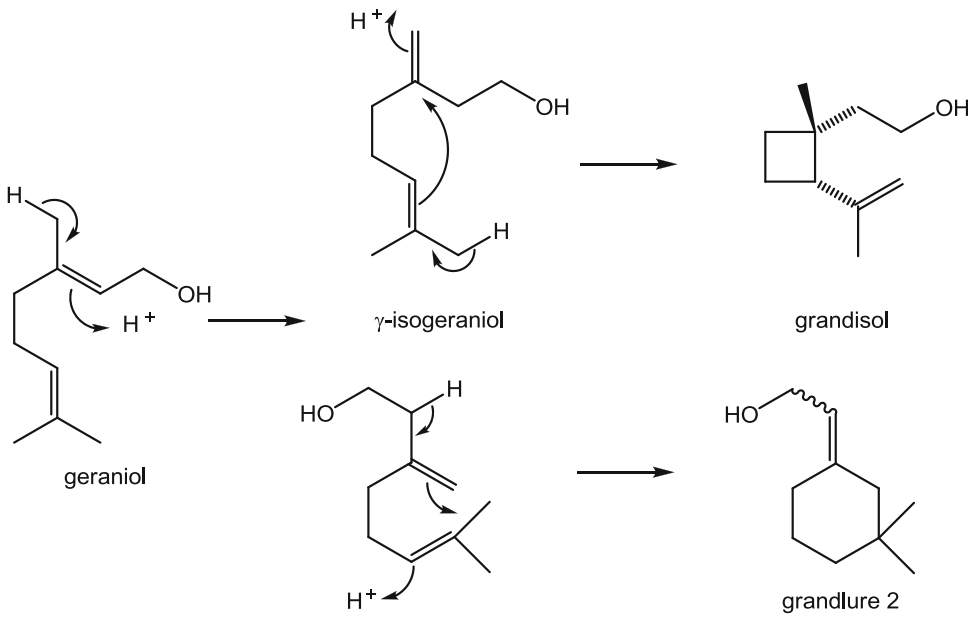

Fig. 6 Hypothesized biosynthesis pathway of grandisol and grandlure 2 from geraniol via γ-isogeraniol

component of *P. knechteli* (Savoie et al. 1998), had no effect on *P. quadridens* in our field tests (Fig. 5).

It is interesting to note that the three species, *P. chalcographus, P. quadridens,* and *P. bidentatus* use different two-component blends as pheromones: While the pheromone of the first species comprises two acetogenins with unbranched carbon chains (which may even be biogenetically related), that of the second one is a mixture of an acetogenin and a terpene. Thus, the species producing chalcogran show relations in their potential to biosynthesize certain specific secondary metabolites. The pheromone blend of the third species is made up of two terpenes. Both bark beetles *P. quadridens* and *P. bidentatus* use pheromone components similar or identical to those used by the closely related weevils, very likely originating from γ-isogeraniol. It has been shown that the boll weevil pheromones can be produced de novo (Taban et al. 2006). In a biomimetic approach, the six-membered ring skeletons of the grandlures 2 were synthesized from γ-isogeraniol (Bedoukian and Wolinsky 1975), and there is no reason to exclude the possibility of an enzymatic formation of grandisol from this precursor or directly from geraniol (Fig. 6). In fact, we detected geraniol in males of *P. quadridens*, whereas γ-isogeraniol was found in both species.

Ethyl dodecanoate was initially of interest as a behavior-mediating compound because it is an aggregation pheromone component for *P. hopkinsi* (Birgersson et al. 2000) and is produced by *P. quadridens*, *P. bidentatus*, and *P. calcaratus* (Francke et al. 1995). However, we found no evidence of activity in the field for *P. quadridens* or *P. bidentatus* (Fig. 5). It should be mentioned that another *Pityogenes* species, *P. conjunctus*, contains relatively large amounts of grandisol and traces of *trans*-verbenol as male specific constituents, whereas small amounts of methyl octanoate and methyl decanoate were found in both sexes (Francke, unpublished). Whether ethyl or methyl esters of mid-chain carboxylic acids function as intra- or inter-specific semiochemicals will need further investigation.

It is interesting that 1-hexanol has been found in males of *P. chalcographus* (Francke et al. 1977), *P. hopkinsi* (Birgersson et al. 2000), *P. quadridens* (Francke et al. 1995; Table 1) and *P. bidentatus* (Table 1) but not in females. 1-Hexanol is known to inhibit response of several species of bark beetles, including *P. chalcographus* (Byers et al. 1998), *P. hopkinsi* (Birgersson et al. 2000), and *P. bidentatus* (Byers et al. 2000). It is possible that males of these species, which make the entrance attack, produce 1-hexanol as a short-range spacing inhibitor to deter other males from boring too close and thereby reduce competition. The males could have evolved the ability to produce 1-hexanol because the inhibitory function of the compound was already used evolutionarily as a signal to avoid non-host deciduous trees such as birch which release 1-hexanol (Byers et al. 1998). Similar effects have been described for cone beetles, *Conophthorus* spp. and conophthorin, another non-host volatile (Birgersson et al. 1995).

In *P. calcaratus*, *cis*-verbenol, an essential aggregation pheromone component of *P. bidentatus*, is missing (Francke et al. 1995). Thus, it would seem a reasonable hypothesis that *P. calcaratus* has evolved an avoidance response to *cis*-verbenol to reduce competitive interactions with *P. bidentatus*. Byers et al. (2000) showed that addition of chalcogran, a major pheromone component of *P. quadridens*, to the aggregation pheromone of *P. bidentatus* reduced the responses of *P. bidentatus* to 40 % of response to its pheromone alone. Addition of *E*-grandlure 2 of the *P. quadridens* pheromone to the two components of *P. bidentatus* in our field tests likewise reduced response significantly (51 % of pheromone alone). Addition of both chalcogran and *E*-grandlure 2 to *P. bidentatus* pheromone had even greater effect in reducing the response of *P. bidentatus* (31 % compared to aggregation pheromone alone).

Addition of *P. bidentatus* pheromone components to the *P. quadridens* pheromone also diminished response of *P. quadridens* in our field tests. When grandisol was added to the two components of *P. quadridens*, attraction of *P. quadridens* was reduced to 77 % of attraction to the *P. quadridens* pheromone. Therefore, grandisol might signal to *P. quadridens* the presence of the competing *P. bidentatus.* Olfactory recognition of the semiochemicals of a cohabiting species to avoid interspecific competition is suggested by a significant reduction in catch by traps releasing combined aggregation pheromone components of both species. This phenomenon was observed for species colonizing the same host tree such as *Ips paraconfusus* and *Dendroctonus brevicomis* in ponderosa pine in California (Byers and Wood 1980), scolytid species in loblolly pine in the USA (Birch et al. 1980; Birgersson et al. 2012), and *Ips typographus* and *P. chalcographus* in Norway spruce in Europe (Byers 1993).

The olfactory inhibition of heterospecific compounds was also suggested by Birch and Wood (1975) as a means to achieve reproductive isolation (avoidance of interspecific mating). It is difficult to determine the relative importance of selective pressures for mating isolation and/or avoiding competition since knowledge of interspecific mating mistakes is needed. In cases of intra-specific avoidance, mating isolation can be ruled out and competition is the only selective force in evolution of olfactory avoidance. For example, males of *I. paraconfusus* and *I. typographus*, which space colonization sites on trees and regulate competition, avoid high concentrations of male aggregation pheromone while females do not (Byers 1983; Schlyter et al. 1987). Perhaps males of *Pityogenes* species,

the sex responsible for initiating colonization territories, are primarily concerned with avoiding competition (as the male does not seek females). However, he could make mating mistakes if colonizing in areas with many heterospecific males because arriving heterospecific females may try to join him and mate. Females, the sex joining males in their gallery, may be concerned with both avoiding resource competition and mating mistakes with resident heterospecific males.

In summary, the major aggregation pheromone components of *P. bidentatus* are *cis*-verbenol and grandisol, whereas *E*-grandlure 2 and chalcogran are the major aggregation components of *P. quadridens*. Evolution of unique components by each *Pityogenes* species as described above would allow increased spatial and temporal segregation among these species with regard to host colonization, thereby reducing competition and increasing mating success. Identification of the aggregation pheromones of *P. bidentatus* and *P. quadridens* provides a basis for the development of trapping systems to monitor population levels during the spring flight season.

Acknowledgments The study was supported by grants from the Swedish Council for Forestry and Agricultural Research (SJFR) to the Swedish University of Agricultural Sciences, Alnarp, Sweden. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture. USDA is an equal opportunity provider and employer.

References

Ambrogi BG, Palacio Cortés AM, Zarbin PHG (2012) Identification of male-produced aggregation pheromones of the curculionid beetle Sternec*hus subsignatus*. J Chem Ecol 38:272–277

Anderbrant O, Schlyter F, Birgersson G (1985) Intraspecific competition affecting parents and offspring in the bark beetle *Ips typographus*. Oikos 45:89–98

Baader VEJ (1989) *Pityogenes* spp. (Col., Scolytidae): Untersuchungen über verhaltenssteuernde Duftstoffe und deren Anwendung im Waldschutz. J Appl Entomol 107:1–31

Bedoukian RH, Wolinsky J (1975) A biogenetic-type synthesis of the cyclohexyl constituents of the boll weevil pheromone. J Org Chem 40:2154–2156

Birch MC, Wood DL (1975) Mutual inhibition of the attractant pheromone response by two species of *Ips* (Coleoptera: Scolytidae). J Chem Ecol 1:101–113

Birch MC, Svihra P, Paine TD, Miller J (1980) Influence of chemically mediated behaviour on host tree colonization by four cohabiting species of bark beetles. J Chem Ecol 6:395–414

Birgersson G, Debarr GL, de Groot P, Dalusky MJ, Pierce HDP Jr, Borden JH, Meyer H, Francke W, Espelie KE, Berisford CW (1995) Pheromones in white pine cone beetle, *Conophthorus coniperda* (Schwarz) (Coleoptera: Scolytidae). J Chem Ecol 21:143–167

Birgersson G, Dalusky MJ, Berisford CW (2000) Identification of an aggregation pheromone for *Pityogenes hopkinsi* (Coleptera: Scolytidae). Can Entomol 132:951–963

Birgersson G, Dalusky MJ, Espelie KE, Berisford CW (2012) Pheromone production, attraction, and interspecific inhibition among four species of Ips bark beetles in the southwestern USA. Psyche Entomol J vol. 2012, ID 532652 p 14

Booth DC, Phillips TW, Claesson A, Silverstein RM, Lanier GN, West JR (1983) Aggregation pheromone components of two species of *Pissodes* weevils (Coleoptera: Curculionidae): Isolation, identification, and field activity. J Chem Ecol 9:1–12

Byers JA (1983) Sex-specific responses to aggregation pheromone: Regulation of colonization density in the bark beetle *Ips paraconfusus*. J Chem Ecol 9:129–142

Byers JA (1989) Chemical ecology of bark beetles. Experientia 45:271–283

Byers JA (1992) Optimal fractionation and bioassay plans for isolation of synergistic chemicals: the subtractive-combination method. J Chem Ecol 18:1603–1621

Byers JA (1993) Avoidance of competition by spruce bark beetles, *Ips typographus* and *Pityogenes chalcographus*. Experientia 49:272–275

Byers JA (2013) Modeling and regression analysis of semiochemical dose-response curves of insect antennal reception and behavior. J Chem Ecol (in press)

Byers JA, Wood DL (1980) Interspecific inhibition of the response of the bark beetles, *Dendroctonus brevicomis* and *Ips paraconfusus*, to their pheromones in the field. J Chem Ecol 6:149–164

Byers JA, Birgersson G, Löfqvist J, Bergström G (1988) Synergistic pheromones and monoterpenes enable aggregation and host recognition by a bark beetle, *Pityogenes chalcographus*. Naturwissenschaften 75:153–155

Byers JA, Högberg HE, Unelius CR, Birgersson G, Löfqvist J (1989) Structure-activity studies on aggregation pheromone components of *Pityogenes chalcographus* (Coleoptera: Scolytidae): All stereoisomers of chalcogran and methyl 2,4-decadienoate. J Chem Ecol 15:685–695

Byers JA, Birgersson G, Löfqvist J, Applegren M, Bergström G (1990a) Isolation of pheromone synergists of bark beetle, *Pityogenes chalcographus*, from complex insect-plant odors by fractionation and subtractive-combination bioassay. J Chem Ecol 16:861–876

Byers JA, Schlyter F, Birgersson G, Francke W (1990b) *E*-Myrcenol in *Ips duplicatus*: An aggregation pheromone component new for bark beetles. Experientia 46:1209–1211

Byers JA, Zhang QH, Schlyter F, Birgersson G (1998) Volatiles from nonhost birch trees inhibit pheromone response in spruce bark beetles. Naturwissenschaften 85:557–561

Byers JA, Zhang QH, Birgersson G (2000) Strategies of a bark beetle, *Pityogenes bidentatus*, in an olfactory landscape. Naturwissenschaften 87:503–507

Dickens JC, Mori K (1989) Receptor chirality and behavioral specificity of the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae), for its pheromone; (+)-grandisol. J Chem Ecol 15:517–528

Eller FJ, Bartelt RJ, Baruch SS, Schuster DJ, Riley DG, Stansly PA, Müller TF, Shuler KD, Johnson B, Davis JH, Sutherland CA (1994) Aggregation pheromone for the pepper weevil *Anthonomus eugenii* Cano (Coleoptera: Curculionidae): Identification and field activity. J Chem Ecol 20:1537–1555

Francke W, Dettner K (2005) Chemical signalling in beetles. Top Curr Chem 240:85–166

Francke W, Heemann V, Gerken B, Renwick JAA, Vité JP (1977) 2-Ethyl-1,6-dioxaspiro[4.4]nonane, principal aggregation pheromone of *Pityogenes chalcographus.* (L.). Naturwissenschaften 64:590–591

Francke W, Pan M-L, König WA, Mori K, Paupoomchareon P, Heuer H, Vité JP (1987) Identification of 'pityol' and 'grandisol' as

pheromone components of the bark beetle, *Pityophthorus pityographus*. Naturwissenschaften 74:343–345

Francke W, Bartels J, Meyer H, Schröder F, Kohnle U, Baader E, Vité JP (1995) Semiochemical from bark beetles: new results, remarks, and reflections. J Chem Ecol 21:1043–1063

Hedin PA, Dollar DA, Collins JK, Dubois JG, Mulder PG, Hedger GH, Smith MW, Eikenbary RD (1997) Identification of male pecan weevil pheromone. J Chem Ecol 23:965–977

Hibbard BE, Webster FX (1993) Enantiomeric composition of grandisol and grandisal produced by *Pissodes strobi* and *P. nemorensis* and their electroantennogram response to pure enantiomers. J Chem Ecol 19:2129–2141

Lekander B, Bejer-Peterson B, Kangas E, Bakke A (1977) The distribution of bark beetles in the Nordic countries. Acta Entomol Fenn 32:1–36

Nardi JB, Young AG, Ujhelyi E, Tittiger C, Lehane MJ, Blomquist GJ (2002) Specialization of midgut cells for synthesis of male isoprenoid pheromone components in two scolytid beetles, *Dendroctonus jeffreyi* and *Ips pini*". Tissue Cell 34:221–231

Savoie A, Borden JH, Pierce HD Jr, Gries R, Gries G (1998) Aggregation pheromone of *Pityogenes knechteli* and semiochemical-based interactions with three other bark beetles. J Chem Ecol 24:321–337

Schlyter F, Byers JA, Löfqvist J (1987) Attraction to pheromone sources of different quantity, quality, and spacing: Density-regulation mechanisms in bark beetle *Ips typographus*. J Chem Ecol 13:1503–1523

Schurig V, Weber R (1984) Use of glass and fused–silica open tubular columns for the separation of structural, configurational and optical isomers by selective complexation gas chromatography. J Chromatogr 289:321–332

Spiegel MR (1961) Statistics. McGraw-Hill Book Co., New York

Taban AH, Fu J, Blake J, Awano A, Tittiger C, Blomquist GJ (2006) Site of pheromone biosynthesis and isolation of HMG-CoA reductase cDNA in the cotton boll weevil, *Anthonomus grandis*. Arch Insect Biochem Physiol 62:153–163

Tumlinson JH, Hardee DD, Gueldner RC, Thompson AC, Hedin PA, Minyard JP (1969) Sex pheromones produced by male boll weevil: Isolation, identification and synthesis. Science 166:1010–1012