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Production of Aggregating Pheromones in Re-Emerged Parent Females of the Southern Pine Beetle

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SCIENTIFIC NOTES

Production of Aggregating Pheromones in Re-Emerged Parent Females of the Southern Pine Beetle[1,2]

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Investigations of bark beetle pheromones have been concerned largely with the occurrence of these chemicals in virgin beetles. In many Scolytidae, the parent adults re-emerge from an initially attacked host tree after they have completed egg-gallery construction and oviposition in the tree. These parent adults may attack another tree and establish another brood. Very little is known about pheromone production and field attractiveness of such beetles.

The aggregating pheromone of the southern pine beetle, *Dendroctonus frontalis* Zimmerman, has been identified as a mixture of 2 compounds, the terpene alcohol *trans*-verbenol and a unique bicyclic compound known as frontalin (Kinzer et al. 1969). These substances are contained in the hindgut of emergent females and are released to the outside where, along with host odors, they bring about aggregation of both sexes at trees undergoing attack (Vité and Renwick 1968). The pheromone content of female hindguts declines rapidly as feeding and gallery construction progress (Coster 1970[4]).

PROCEDURES

Source of Beetles.—Re-emerged southern pine beetles were obtained from trees that had been mass attacked. Logs cut from such trees were placed in outdoor rearing cages and the parent beetles were collected from the cages daily. Re-emergence of parent adults began 10–15 days after the tree was mass-attacked. Virgin adults were collected in the same way as they matured and emerged from naturally attacked pine logs. Beetles were sexed according to the presence of the transverse pronotal ridge in the females (Osgood and Clark 1963).

Field Bioassay.—Beetles were introduced into loblolly pine posts (13 cm × 2.3 m) for field bioassay in the following way: a blunted 10-penny nail was driven through the pine bark to the cambium. One live female beetle was placed in each hole, and the hole was covered with a small square of plastic screen that was firmly stapled to the bark. Ten posts were prepared. Five contained 100 re-emerged females and each of the remaining contained 100 virgin females.

Bioassay of the posts was performed at forest sites adjacent to infestations of the beetles. The posts were concurrently bioassayed using tree-trunk-simulating olfactometers (Vité and Renwick 1968) modified to allow insertion of a pine post. The olfactometers consisted of a 28-cm × 2.5-m canvas sleeve fastened to a cylinder base. The sleeve was kept inflated by the air flow from an electric blower.

GLC Analysis.—Hindguts of female southern pine beetles were analyzed using gas-liquid chromatography (GLC). Frontalin and *trans*-verbenol were detected using a Varian 1200 chromatograph equipped with a flame ionization detector and a Varian A-20 recorder. The column was ⅛-in. × 5-ft stainless steel with 3% SE-30 on 100/120-mesh Varaport 30. Nitrogen and hydrogen flow rates were 25 ml/min. The column temperature was programmed from an initial temperature of 70°C to a final temperature of 140°C at a rate of 4°/min. Injector and detector temperatures were both held at 190°C. Synthetic frontalin and *trans*-verbenol were obtained from the Battelle Memorial Institute, Columbus, Ohio, for GLC verification purposes.

Six samples of re-emerged female hindguts were analyzed. Each sample consisted of 10 hindguts in 20 μliter of hexane. The solution was ground with a small amount of purified sand and then centrifuged. One μliter of each extract was subjected to GLC analysis. A sample of virgin females was prepared and analyzed in the same way.

RESULTS AND DISCUSSION

Field Bioassay.—In the field tests, live feeding virgin female southern pine beetles were 4.5 times more attractive than live feeding re-emerged females (Table 1). The total number of successful attacks by the introduced virgin females was 177. Re-emerged females made 119 successful attacks. This difference in number of attacks was significant (χ^2=11.4, 1 df), indicating perhaps less attack vigor by the re-emerged females. Evaluation of the response data of Table 1 in terms of successful attacks for each of the 2 beetle classes reveals that virgin beetles attracted 3.02 times as many beetles from the flying population as the re-emerged beetles. The sex ratio of the responding beetles was arrayed into a 2×2 contingency table and tested for independence. The ratio of sexes at the 2 classes of females did not vary significantly from each other (adjusted χ^2=1.29, 1 df).

GLC Analysis.—From the GLC studies, the mean peak areas (mm^2) and the standard deviation for the 2 compounds from the 6 re-emerged female samples were: frontalin 25±24.9; *trans*-verbenol 996±491.4. The peak areas for the virgin hindguts were: frontalin 103; *trans*-verbenol 1920 mm^2. Virgin female southern pine beetles contained 4.1 times as much frontalin and 1.9 times as much *trans*-verbenol as the re-emerged beetles. This fact substantiates the field-response data where virgin females attracted about 4.5 times as many beetles as the re-emerged females.

It is clear from these results that mating does not irreversibly inhibit pheromone production in female southern pine beetles. Although the pheromone content of the females declines to a low level during gallery construction activities (Coster 1970[4]), the females again are capable of producing significant attraction upon re-emergence.

Furthermore, the occurrence of the pheromones in re-emerged females points out the inappropriateness of designating these chemical messengers solely as "sex pheromones." A 2nd mating is not required by re-emerged southern pine beetle females for them to lay viable eggs (Yu and Tsao 1967). Therefore, production of a "sex" pheromone by such females would appear to be superfluous. Aggregation has high survival value for the

[1] Coleoptera: Scolytidae.

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[4] J. E. Coster. 1970. Certain aspects of pheromone release and aggregation behavior in the southern pine beetle. (Coleoptera: Scolytidae). Ph.D. dissertation, Texas A&M University. 129 p.

Table 1.—Field response of *D. frontalis* at olfactometers baited with loblolly pine posts containing 100 re-emerged parent females or 100 virgin females each.

Trial no.	No. beetles trapped		
	Condition of feeding females		
	Re-emerged	Virgin	Total
1	21	47	68
2	7	45	52
3	7	21	28
4	6	3	9
5	1	72	73
Total	42	188	230
Sex ratio of responding beetles (males : females)	26 : 12	100 : 76	

southern pine beetle in that a host tree must be mass attacked for its oleoresin resistance mechanism to be overcome (Thatcher 1960, Vité and Pitman 1968). Failure of the beetles to attack a tree en masse may result in decimation of the beetle population, so the evolutionary value of the pheromone as an aggregant is at least as important as its value as a mating facilitant. Sexual behavior in the aggregations may have been a secondary development resulting from the greatly increased number of sexual encounters in the aggregations (Haskell 1966).

Re-emerging parent adults are an important and often overlooked consideration in southern pine beetle investigations. In the present study, 50–60% of the attacking population re-emerged in the laboratory rearing cages. These beetles form a significant portion of natural populations and may account for some of the variations in response and pheromone-producing abilities of southern pine beetles obtained from wild populations.

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Antagonism Between *Dendroctonus frontalis*[1] and the Fungus *Ceratocystis minor*[2]

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The blue-staining fungus *Ceratocystis minor* (Hedgc.) Hunt is a frequent associate of the southern pine beetle, *Dendroctonus frontalis* Zimmerman (Dixon and Osgood 1961, Francke-Grosmann 1967). The beetles introduce the fungus into tree phloem, and from there it spreads into the xylem (Bramble and Holst 1940, Hedgcock 1906, Nelson 1934, Nelson and Beal 1929) where it causes rapid wilting of the tree. Although it has been observed that miscellaneously stained phloem is unfavorable to bark beetle development (Franklin 1970), there is little information available on the role of this fungus in the complex microhabitat of the insect in the host phloem. Most *C. minor* isolations have not been made from phloem when beetles were present, but from and about older frass-filled galleries or from xylem (Rumbold 1929, 1931). Research reported here indicates that *C. minor* acting alone is detrimental to *D. frontalis* development, but that its growth and perithecia production are inhibited in the phloem when it is present with the beetle and other associated microorganisms.

MATERIALS AND METHODS

Six bolts, each 45 cm long, were cut from a healthy loblolly pine, *Pinus taeda* L., which was about 19 cm in diameter 1.4 m above the ground. Three bolts were inoculated with disks of *C. minor* in 4 longitudinal rows of 5 inoculations each, and 3 were left untreated (Barras and Hodges 1969). All bolts were stored in an aseptic chamber with fluorescent laboratory lighting and filtered air at 25–30°C.

After periods of 1, 2, and 3 weeks a bolt infested with *C. minor* and a control bolt were removed. In bolts stored for 1 and 2 weeks, 4 phloem samples between cork cambium and xylem were taken for determination of relative water content (RWC) (Weatherley 1950) and moisture percent on a dry weight (DW) basis. Each sample hole was flame-sterilized and covered with aseptic tape. Then each control bolt was infested with 20 pairs of *D. frontalis* at evenly spaced points (Barras and Hodges 1969). These beetles were collected from naturally infested trees and thus had a normal complement of associated microbes. In the *C. minor* bolts, the same number of pairs were introduced, but they were spaced between or within the rows of *C. minor* inoculations to test the reaction of beetles to colonized and initially noncolonized phloem. The progeny were collected as they emerged, and all bolts were examined after the completion of brood development.

RESULTS AND DISCUSSION

Beetle development was considerably better in the control bolts than in the *C. minor* bolts regardless of storage time (Table 1; Fig. 1, 2). Mating and oviposition were inhibited in the *C. minor* bolts and there was a difference in development depending on the placement of the introduced beetle pairs. Those placed between the rows of *C. minor* inoculations performed better than those within the area where the fungus was produc-

[1] Coleoptera: Scolytidae.
[2] Cost of expedited publication paid by the author.

Table 1.—Development of *D. frontalis* in control and *C. minor*-infested bolts.

Treatment	Storage (weeks)	Moisture in phloem[a]		Pairs	Initiated galleries	Egg galleries	Progeny	Progeny/egg gallery
		% DW basis	RWC					
Control	1	198[b]	51[b]	20	13	10	168	17
C. minor	1	192	68	24	14	6	29	5
Control	2	220	51	20	16	16	205	13
C. minor	2	152	55	20	17	9	9	1
Control	3	...	...	20	14	11	139	13
C. minor	3	...	...	20	15	2	0	...

[a] At time of beetle introduction; no samples taken at 3 weeks.
[b] Average of 4 samples.

ing perithecia. For example, in the infected bolt stored for 1 week most of the progeny were produced by 1 ♀ within a zone relatively free of perithecia. This female excavated an egg gallery entirely within the perithecia-free area. When she approached the area where the fungus was fruiting she turned, probably after detecting some unfavorable change in the substrate. The change in direction was repeated several times.

Larvae feeding within the perithecia-free zone developed normally. Those that began development within or moved into the perithecial areas constructed atypically elongated galleries, some up to 10 cm long. Some elongated galleries contained living larvae 1 month after the normal developmental period was over. Therefore, the substrate was quantitatively or qualitatively deficient for proper development. Similar larval and adult reactions were observed in stained shortleaf pine, *P. echinata* Mill., phloem (Franklin 1970).

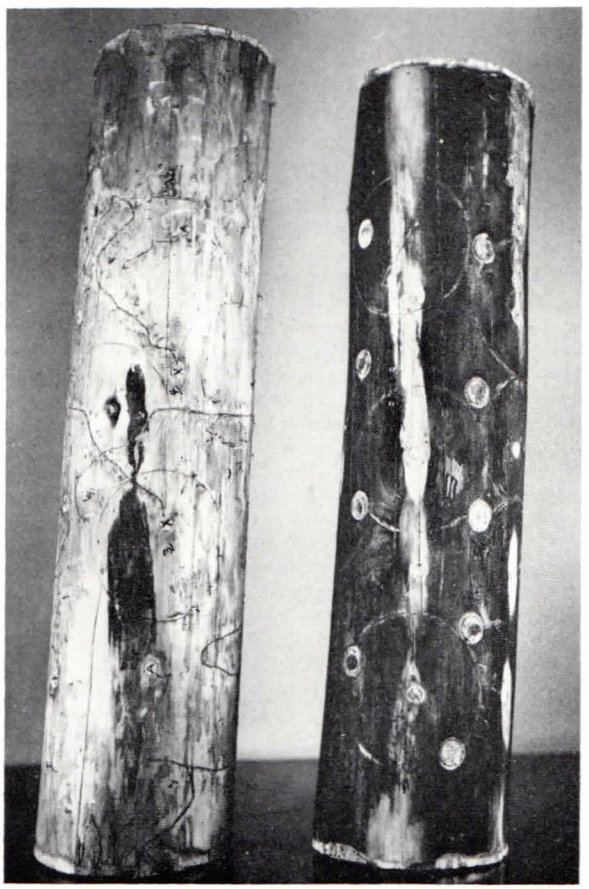

FIG. 1—Development of *D. frontalis* in control bolt (left) and *C. minor* bolt (right).

Adults introduced within the fruiting areas produced short, numerously forked galleries. The majority of the galleries were without nuptial chambers and most beetles soon rejected the substrate and emerged from the bolts. In a somewhat similar experiment, Yearian (1967) found oviposition was inhibited when *Ips* spp. were introduced into *P. echinata* bolts previously colonized with *C. ips* (Rumb.) C. Moreau for 8 days.

The performance of the beetles and moisture contents in the bolts (Table 1) should be compared with the report by Gaumer and Gara (1967) that southern pine beetle broods in loblolly pine bolts develop best when moisture percent falls below 200%, and as low as 150%, for 10 days. Although a decrease in moisture percent (DW) may be beneficial to the beetle, it apparently is not beneficial when caused by *C. minor*. Thus, *C. minor* rendered the phloem unsuitable not through moisture changes, but through some other factor(s). This conclusion is supported by the data in Table 1, which show that the beetle developed well in control phloem at 198% moisture (DW) but poorly in infected tissue at 192% moisture (DW). Although it appears that RWC had no effect on beetle development, the low values encountered correspond closely to those (51–62%) reported by Lorio and Hodges (1968) in flooded trees attacked by the southern pine beetle.

Several other chemical factors in the phloem that are influenced by the growth of *C. minor* may alone or together have caused poor beetle development. The fungus lowers the contents of reducing and total sugars, which are important sources of energy (Barras and Hodges 1969). Hodges and Pickard (1970) showed that there is a correlation between carbohydrate content of inner bark and the number and size of *D. frontalis* progeny emerging from lightning-struck trees. The fungus also causes a decrease in the quantity of free amino acids and and an increase in protein-bound amino acids (Hodges et al. 1968). These changes, and others (e.g., in sterols, lipids, and vitamins) that have not been investigated, may also be detrimental for beetle development.

Whatever the cause of poor development, inhibition of *C. minor* fruiting in galleries would be advantageous to the beetle. And such a process apparently occurs. In numerous active galleries in naturally infested trees *C. minor* fruiting was sparse and did not approach that in bolts infested artificially by hand or beetles. Oberle[3]

[3] A. Oberle. 1966. The microflora isolated from galleries of southern pine bark beetles, *Dendroctonus frontalis* Zimm. M.S. thesis. Northwest. State Coll., Natchitoches, La.

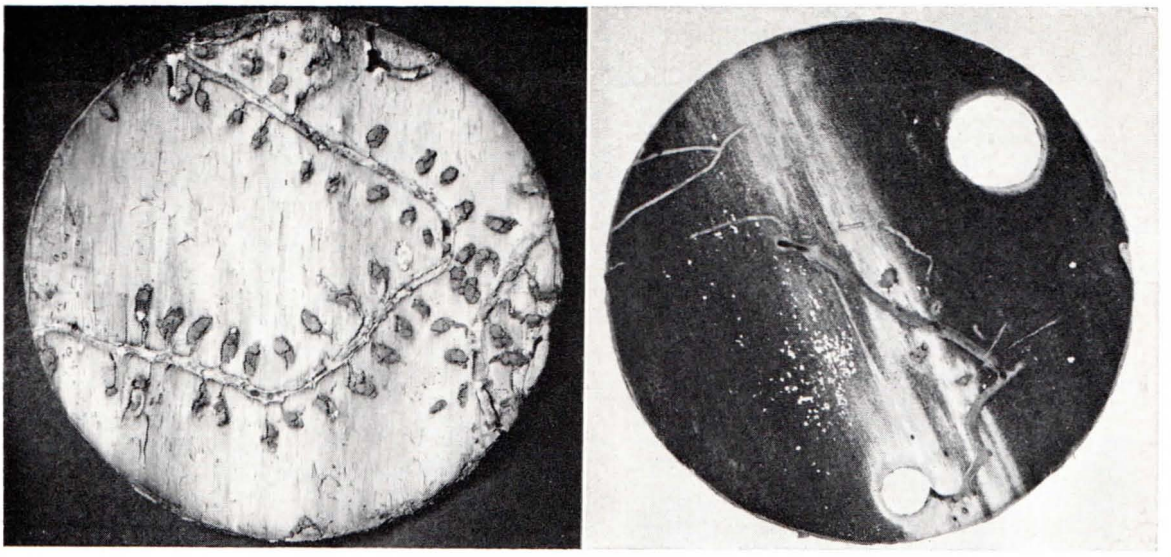

FIG. 2.—Normal *D. frontalis* larval development in control bolt (left) and abnormal development in C. *minor* bolt (right).

reported that *C. minor* could not be isolated from active beetle galleries. Only after the brood had completed development were perithecia found in scattered patches. In my laboratory only 1 of 100 isolation attempts from phloem in and about active adult and larval galleries yielded *C. minor,* even though the fungus may be isolated directly from the body of attacking adults (Nelson and Beal 1929, Rumbold 1931). The inhibition mechanism is unknown, but oleoresins (Cobb et al. 1968), associated bacteria,[3] or mycangial fungi (Barras and Hodges 1969, Hodges et al. 1968), may be directly involved.

It is also possible that live phloem is initially unsuitable for perithecia production, but no inhibition is observed in cut bolts. Chemical alterations caused by the development of associated microbes and possibly the beetle itself may be necessary for perithecia production in naturally infested trees. This argument would explain why *C. minor* perithecia can readily be found in old galleries where other microbes and the beetles are well developed. *Ceratocystis* is known to produce various types of imperfect stages, and *C. minor* may be present in new galleries in an obscure imperfect form. For example, one of the *D. frontalis* mycangial fungi is a *Candida* sp. which at times forms what appear to be rudimentary perithecia. No asci have been observed in association with these structures.

In any event it appears that a complex but biologically efficient facultative relationship exists between *C. minor* and *D. frontalis.* The beetle provides transport and entry to the xylem where the fungus rapidly colonizes the nutritious ray parenchyma. This fungus and possibly others cause rapid wilting and drying (Bramble and Holst 1940, Mathre 1964, Nelson 1934) of the tree. These physiological changes may be beneficial to the beetle. In a mass attack, once the beetles have overcome the initial force of oleoresin exudation pressure and flow, it is possible that enough *C. minor* inoculum is present to adversely affect subsequent production of oleoresins. Thus, beetle development can proceed without the flooding of egg and larval galleries with oleoresins.

The complex relationships among *D. frontalis,* its various microbial associates, and host phloem offer an excellent opportunity for study of intimate interactions of diverse organisms—a case of symbiosis as perceived by DeBary (1879) and Leach (1940). Those authors defined symbiosis simply as the living together of dissimilar organisms, regardless of the possible results of the association.

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Two Techniques for Dissecting and Mounting Genitalia of Male Butterflies (Lepidoptera)[1]

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The 1st technique described is an efficient variation of an accepted method of dissecting butterfly genitalia; the 2nd technique is a new general method for mounting genitalia. The latter technique is described for butterfly genitalia but has proved useful for other insects as well.

Two-thirds of the abdomen of a male butterfly was cut off and placed in a shallow Stender dish. To relax the genitalia and macerate the soft, extraneous tissue, the abdomen was soaked in a solution of 20% cold KOH for 10–24 hr, depending on the length of time the specimen had been in the collection. Some specimens after 10 hr or less turned the KOH solution brown; if left in the solution any longer, they disintegrated into separate components. This disintegration made mounting and study of genitalia more difficult. Occasionally an older specimen required longer exposure to the KOH (up to 26 hr). After the treatment the specimen was rinsed 3 times (1 min in each rinse) in tap water to wash away the KOH. The specimen was then placed in a Syracuse watch glass and covered with 50% ethyl alcohol.

With 2 dissecting needles, one straight and the other hooked, the terminal segments were detached from the abdomen; the remaining extraneous tissue and scales were carefully removed from the genitalia. The genitalia were transferred to another Syracuse watch glass with 70% ethyl alcohol for further cleaning. The valvae were opened to expose the harpes, and any loose tissue was removed from the space within each valva. The inside of the body of the superuncus also needed careful cleaning. The rectum was cut off with fine curved forceps.

The last delicate operation was the removal of the lower part of the tegumen. With the valvae closed the genitalia were held with the hooked needle and with iridectomy scissors the tegumen was cut at a slant to the vinculum at the point of attachment of the valvae. Then with fine curved forceps, the lower part of the tegumen was removed from the vinculum.

The study of the vesica required its evagination from the aedeagus. The 2 needles and a glass tube, 4 mm diam and 15 mm long, with a capillary end the same diameter as the phallobase, were used. The other end of the glass tube was attached to a plastic tube ca. 30 mm long. The genitalia were held with 1 needle. The 2nd hooked needle was inserted in the phallobase and moved so as to separate the end of the vesica from phallobase to which it was attached. The glass tube was filled with 50% ethyl alcohol and carefully inserted in the phallobase. When air was blown through the plastic tube, the jet of alcohol was forced through the aedeagus, ejecting the vesica, which was evaginated. A tuberculin syringe to which a hypodermic needle was attached was used as a Vaseline® gun to fill up the vesica to study its shape and spines. Needles of various diameters were used for phallobases of different sizes. The use of the tuberculin syringe and needle permitted greater control over the introduction of vaseline into the vesica than was possible with the standard vaseline gun. The dissected and cleaned genitalia were stored in small (30×7 mm) cork-stoppered glass vials half filled (10 drops) with a mixture of 70% ethyl alcohol and glycerine (4:1).

To facilitate study and comparison, the genitalia were mounted between 2 plates of glass inside the vial. The glass plates were 25×7 mm and were cut from cover slips. A piece of paper 10×5 mm bearing the number of the specimen was glued at 1 end of 1 plate. The genitalia were placed in a small drop of glycerine in the 3rd quarter of the plate. The other plate was positioned exactly on top of the first. With the aid of a forceps the whole preparation was slid into the horizontally held vial so that the genitalia were at the lower portion of the vial and the label was near the open top. The 2 long sides of the mount rested securely against the sides of the vial at its diameter, and the base of the mount was closely pressed against the bottom of the vial. Ten drops of the alcohol-glycerine mixture were introduced into the vial. Then, with the vial in the vertical position, the cork stopper was pushed into the open end of the vial until it touched the end of the glass plates (ca. 3 mm). The mount was now immobile. The vials were stored in a vertical position; the alcohol-glycerine mixture covered the genitalic portion of the mount.

This type of mounting has several advantages over permanent mounting. First, by placing the vial under the microscope and rotating the vial, the genitalia can be observed from different angles. The mobility of the vial is particularly useful when studying the teeth of the harpe. Mount mobility is not possible to this degree in permanent preparations. Second, when the vial is half filled (10 drops) the surface of the alcohol-glycerine mixture coincides with the horizontally held mount. This helps avoid any distortion of the image that follows if the vial is completely filled. Distortion may arise when permanent mounts are used. Third, the mounting can easily be taken apart if necessary, thereby facilitating comparison of specific structures among butterflies of the same or different species. Permanent mounts cannot be so quickly and efficiently taken apart.

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