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Olfactometric Evidence for Aggregation Pheromone Production by Females of the Four-Eyed Fir Bark Beetle *Polygraphus proximus* Blandf. (Coleoptera, Curculionidae: Scolytinae)[1]

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Abstract—Chemical communication of the four-eyed fir bark beetle *Polygraphus proximus* Blandf., an aggressive invasive pest of the Siberian fir *Abies sibirica* Ledeb., was experimentally studied using a four-way olfactometer of a modified design, in which the tested insects were allowed to move on their own from a lightproof plastic container onto the lighted arena. Young hibernated adults of *P. proximus* were offered four variants of odor: fir log segments infested with (1) 10 males, (2) 10 females, (3) 10 couples of *P. proximus*, and (4) clean air as the control. The pheromone of *P. proximus* was shown to be produced by females; a similar response of both sexes characterized it as an aggregation pheromone. All the three variants with infested fir logs were much more attractive than the control variant with clean air. Volatiles from fir tissues not only seem to serve as markers facilitating host selection by the first-to-arrive beetles before direct contact with the tree bark, but also to be used as pheromone precursors or synergists. The absence of difference in response to the logs infested with males and couples of the bark beetle indicated that pheromone synthesis was inhibited after couple formation.

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The ecological specialization of most species of bark beetles (Coleoptera, Curculionidae: Scolytinae) is that of natural destructors of dying or heavily stressed trees (Raffa and Gregoire, 2015). In this case, the advantages of colonizing trees with weakened defense are to a considerable extent balanced by severe intra- and interspecific competition and the rapidly decreasing nutritional value of the substrate. There are about thirty species of the so-called aggressive bark beetles (Ohmart, 1989; Raffa and Gregoire, 2015) which use a different strategy: they kill trees on their own by suppressing the plant defense mechanisms, damaging the resin ducts, and transferring phytopathogens (Kirkendall et al., 1997). For this reason, of crucial importance for the bark beetle species colonizing healthy trees is the possibility of individual insects to cooperate in a massed attack on the same plant; this possibility is realized with the help of aggregation pheromones (Lindgren and Raffa, 2013).

The four-eyed fir bark beetle *Polygraphus proximus* Blandf. is a species of the Far Eastern origin which has become a new aggressive phytophage of the Siberian fir *Abies sibirica* Ledeb. as the result of its recent invasion (Baranchikov and Krivets, 2010; Krivets et al., 2015). Our previous experiments revealed an important feature of the reproductive behavior of this species, namely its monogyny (Kerchev, 2014a). Considering the previously published data (Nobuchi, 1966; Tokuda et al., 2008), in our experiments the males were released onto the substrate before the females. Although the females successfully joined the males and started the colonies under such conditions, it was realized that appearance of the sexes on the substrate in this particular order was not typical of monogynous mating systems (Kirkendall et al., 1997; Kerchev, 2014a). Field observations in the summer of 2015 showed that females were the first to bore into the substrate during colonization of both apparently healthy trees and freshly windthrown ones. This fact raised some doubts as to whether the sex pheromone was really produced by males of *P. proximus*, as had been reported earlier from its native range (Nobuchi, 1966; Tokuda et al., 2008). Thus, the goal of our study was to demonstrate the presence of chemical communication in *P. proximus* and to determine the type of the pheromone and the sex producing it.

[1] This article was originally submitted by the authors in Russian and is first published in translation.

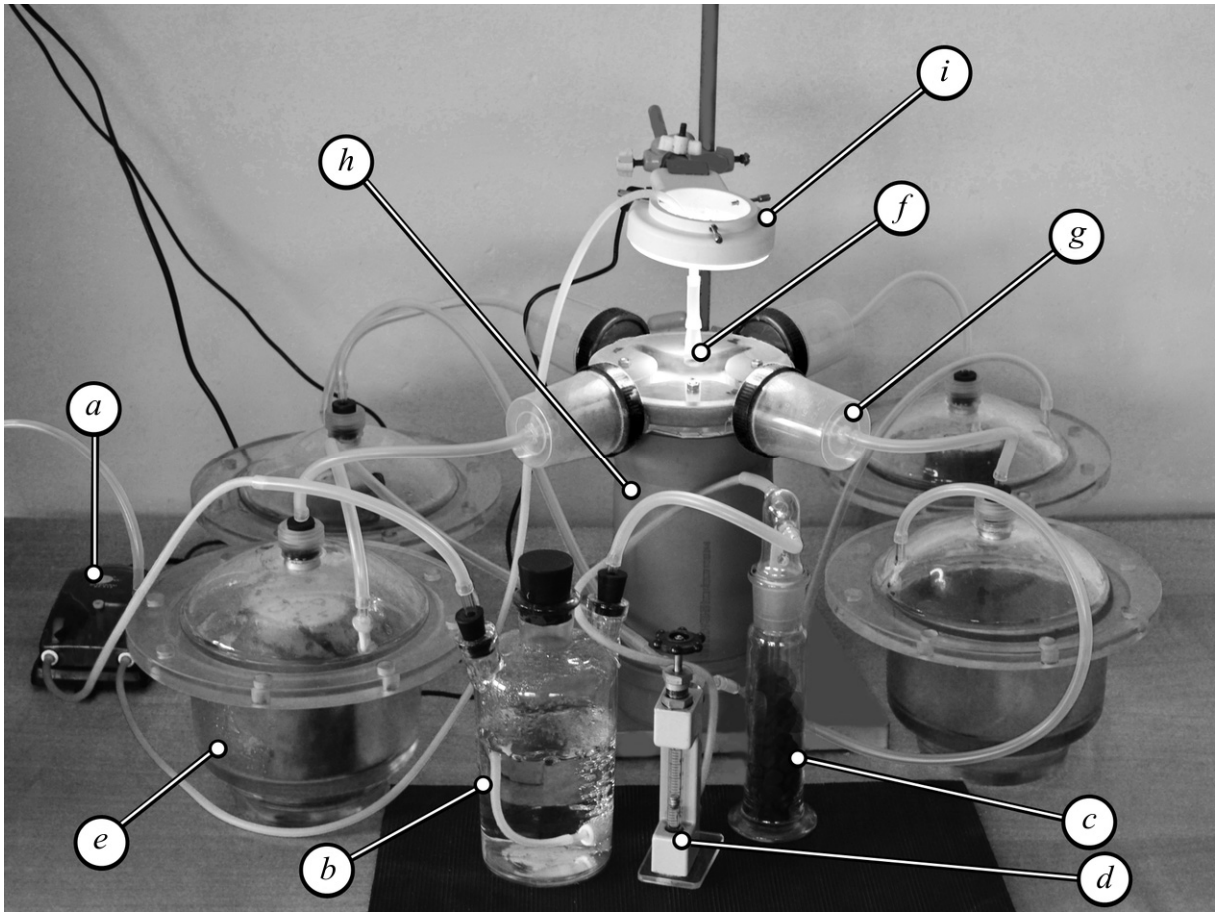

Fig. 1. The olfactometer: (*a*) 2-channel air pump; (*b*) air washing bottle with distilled water; (*c*) Drechsel bottle with activated charcoal; (*d*) flow rate meter; (*e*) container with odor source; (*f*) 4-way olfactometer; (*g*) trap for insects; (*h*) plastic container with insects; (*i*) circular LED light source.

MATERIALS AND METHODS

The Biological Material

Experiments were performed with young adults of the four-eyed fir bark beetle *P. proximus* collected in the environs of Tomsk (56°27'45"N, 85°05'25"E) in September 2015. These beetles completed their development and hibernated in the trees that were killed at the end of summer. The insects were collected together with fragments of tree bark and stored in plastic bags under the ambient conditions. One week before the experiments, the fragments of bark with beetles were transferred into a temperature controlled chamber and kept there at 15°C.

Log segments 130 mm long and 70 mm in diameter were obtained from the basal trunk portion of a freshly cut 16-year old Siberian fir tree on December 24, 2015. The segments were kept at room temperature for 24 h before their infestation with beetles.

The Olfactometer

We used a modified version of the four-way olfactometer designed by Pettersson (1970). In the original construction and later modifications the tested insects were individually placed onto the arena through the airflow connector opening (Pettersson, 1970; Leahy et al., 2007; Pope et al., 2012). By contrast, we used the principle of the first olfactometer proposed by McIndoo (1926) and developed the variant in which insects were allowed to move from a darkened container onto the arena on their own while attracted by light. In our apparatus (Fig. 1), hibernated adults of *P. proximus* were placed together with bark fragments in a lightproof plastic container 200 mm tall and 110 mm in diameter. The cover of the container was the arena or the testing zone; it had a central opening 1.5 mm in diameter through which only one beetle at a time could pass. The circular LED-64T light source was fixed 15 cm above the opening. The size of the

arena was 27.2 cm^2, the measurements of the arms were 5 × 1 × 1.2 cm. The experimentally determined optimum air flow was 125 ml/min for each arm. Before pumping the air into the odorant containers it was purified in a washing flask with distilled water and a Drechsel bottle with activated charcoal. The second channel of the pump was used to draw air (at a rate of 500 ml/min) through an outlet above the center of the arena and to remove it from the room. The lids of the olfactometer and the odorant containers were made airtight with petroleum jelly. Before the experiment the apparatus was tested with smoke.

The beetles were offered odors from four sources: (1) a fir log segment infested with 10 conspecific females; (2) a log segment infested with 10 males; (3) a log segment infested with 10 couples of the bark beetle; (4) clean air.

Every other day, the captured beetles were counted, the arena was cleaned with 70° ethanol, and the relative position of the variants was changed. The captured beetles were not tested again. The arrangement of the variants was changed 15 times; after the 7th rotation the odorants were replaced and the whole set of equipment was rinsed.

The attractiveness of variants was statistically assessed based on the total captures for two-day intervals, using the χ^2 test with Benjamini–Yekutieli correction (Benjamini and Yekutieli, 2001). The differences were considered to be significant at $P \leq 0.05$. The agreement between samples was tested using Kendall's coefficient of concordance ($W = 0.57$).

RESULTS

Our experiment showed that adults of the four-eyed fir bark beetle responded differently to the odor variants offered to them ($\chi^2 = 27.37$, $N = 16$, df = 3; $P < 0.00001$), the most attractive variant being the Siberian fir log segment infested with conspecific females (Fig. 2).

Pairwise comparison of attractiveness of the tested odors revealed highly significant ($P < 0.001$) differences between all the variants, except for the log infested with males and that in which colonies were formed. In all the cases the insects preferred the variants loaded with the odor of their host plant over the control variant with clean air (table).

During the whole experiment, 258 individuals (131 females and 127 males) moved from the container onto the arena and selected one of the four odor variants. Most of the females chose the fir logs infested with conspecific females ($N = 57$) and couples ($N = 38$) (Fig. 3). However, our analysis revealed no differences in the sex ratio of beetles attracted by different variants ($\chi^2 = 0.42$, df = 3, $\chi^2_{crit.} = 7.8$; $P < 0.05$); therefore, the two sexes responded to the offered odors in the same way.

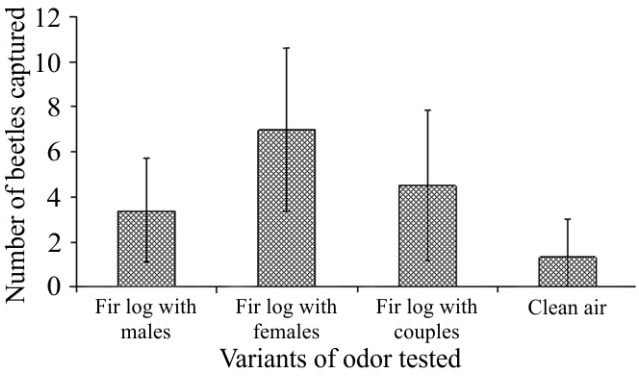

Fig. 2. Preference of the odor variants by adults of *Polygraphus proximus* Blandf. in the olfactometer experiment (mean ± SE).

DISCUSSION

Our experiment showed that all the variants containing the odor of the host plant were significantly more attractive for the beetles than clean air; this result confirms that *P. proximus* is attracted by the odors of the trophic object. Although this species was repeatedly shown to be able to feed on various plants of the family Pinaceae, it absolutely prefers firs of the genus *Abies* Er. (Kerchev, 2014b). An important feature distinguishing firs from other conifers growing within the range of *P. proximus* is the presence of specific triterpenoids with a modified lanostane-type carbon skeleton in their resin (Raldugin, 2004). This peculiar chemical trait of firs may allow the beetles to identify their host plants among other trees in the forest stand.

Pairwise comparison of odor attractiveness for adults of *Polygraphus proximus* Blandf. ($\chi^2_{crit.} = 3.84$).

Variants of odor	Observed χ^2 values
Log with males / clean air	14.52**
Log with females / clean air	61.36**
Log with couples / clean air	27.97**
Log with males / log with females	19.69**
Log with males / log with couples	2.57*
Log with females / log with couples	8.06**

* $P > 0.05$; ** $P < 0.001$

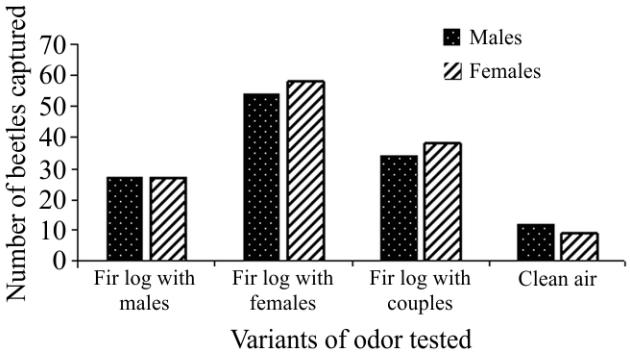

Fig. 3. Preference of the odor variants by adults of *Polygraphus proximus* Blandf. of different sexes (total values for the whole experiment).

It was supposed that pheromones of bark beetles of the genera *Ips* De Geer and *Dendroctonus* Er. were synthesized in the hindgut (Blomquist, 2010). It is highly probable that pheromones of *P. proximus*, similar to those of the above genera, are based on the compounds produced by the host plant, which are treated with enzymes and mixed with some compounds synthesized de novo by the beetles themselves to increase their attractiveness for conspecific individuals (Raffa et al., 2015). In the aggressive pests, active resistance of the tree increases the production of attractants since the defensive compounds of the plant are used as synergists or precursors of the pheromone. As the tree gets colonized by pests, terpenes in its tissues are oxidized while the developing symbiotic fungi start to produce the anti-aggregation pheromone. Correspondingly, the infested tree becomes less attractive for the newly arriving beetles, which reduces intraspecific competition (Kirkendall, 1997; Kirkendall et al., 2015).

Since both sexes responded in the same way to the odor of the female frass, we may conclude that the pheromone in question acts as an aggregation pheromone rather than a sex attractant. Similar results were obtained by laboratory observations of a closely related North American species *P. rufipennis* (Kirby), in which both sexes responded to the odor of the male frass (Bowers and Borden, 1990). At the same time, the two species have an important behavioral difference: *P. proximus* is monogynous while *P. rufipennis* forms polygamous colonies (Rudinsky, 1978). They also cardinally differ in their ways of colony initiation: colonies of *P. rufipennis* are typically started by males while in *P. proximus*, as we ascertained, the female is usually the first to bore into the substrate. These results support the hypothesis of Kirkendall (1983) that colony initiation and pheromone production by females is more typical of monogynous bark beetles.

Considerable differences in attractiveness between the fir log infested with females only and that infested with beetle colonies indicate that females possess a mechanism that inhibits synthesis of their aggregation pheromone after they are joined by males. Such a mechanism was described in bark beetles of the genus *Dendroctonus* Er. (Ryker, 1988); most probably, chemical communication in the four-eyed fir bark beetle is also inhibited in response to acoustic signals produced by males (Kerchev, 2015).

Using olfactometer experiments, we were able to study the mechanisms of pheromone communication in *P. proximus* not on the individual but only on the population level. The ability of both females and males to start the colony indicates a high level of behavioral plasticity of this species. The male may choose its behavioral model (joining the female or starting its own tunnel) depending on a number of factors, such as the state of the tree, the stage of its colonization, the sex ratio of the arriving beetles, the frequency of contacts with individuals of the same sex, and the season.

CONCLUSIONS

Our experiment confirmed the existence of chemical communication in the four-eyed fir bark beetle.

Pheromones in *P. proximus* are produced by females.

The similar rates of response of both sexes to the female pheromone of *P. proximus* characterize it as an aggregation pheromone. This chemical signal allows the beetles to reach the optimum density for overcoming the defenses of a healthy tree.

The similar levels of attractiveness of the fir logs infested with males and couples of *P. proximus* indicate that pheromone synthesis is inhibited after the males join the females, so that attraction of additional individuals is stopped and the risk of severe intraspecific competition is reduced.

Preference for variants with the Siberian fir odor over clean air indicates that volatile components of the fir resin contain some markers which allow the beetles to detect suitable host plants before direct contact with the bark. These components may also be synergists and/or precursors of the aggregation pheromones.

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