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Gravid females of *Cephalcia chuxiongica* (Hymenoptera, Pamphiliidae) are attracted to egg-carrying needles of *Pinus yunnanensis*

Zheng-Liang Yan[1], Hui-Fen Ma[1], Yun-Ling Mao[1], Ling Liu[1]

1 *Institute of Forestry Protection, Yunnan Academy of Forestry, Kunming 650201, China*

Corresponding author: *Zheng-Liang Yan* (yan_zhengliang@126.com)

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Abstract

Cephalcia chuxiongica Xiao is one of the most dangerous defoliators of *Pinus yunnanensis* and other pine species in Yunnan province, resulting in serious losses. Its distinguishing characteristics are the females' aggregation oviposition and larvae's aggregation feeding. In order to explore the mechanism of aggregation oviposition in this sawfly, preliminary olfactory bioassay was conducted in laboratory. In in-cage choice tests, on average vast majority gravid females selected the shoots that had been loaded and oviposited by a 'pioneer' female. In one-choice tests in laboratory by a Y-tube olfactometer, the gravid females were attracted by the odors of eggs-carrying shoots (PE), shoots with one delivering female and her eggs (PGE), needles' extract (NE), and fresh eggs' eluent (EL); the virgin females were attracted by odors of fresh needles (P), PE, PGE, and NE, but repelled by odors of virgin and gravid females. In two-choice tests, the odors were tested in pairs for gravid females. When compared with odors of gravid females (G) or P, gravid females showed significantly more tendency to odors of PE or PGE. When given odors EL vs. NE, gravid females preferred the odors of NE, but they did not make obvious selection between G vs. P, and PE vs. PGE. Based on the results, our conjectures were: (1) Delivery female, as a pioneer, can summon her conspecific gravid females to aggregate in the same pine shoot; (2) Pine needles' odors were attractive for both the virgin and gravid females; (3) Gravid females could be attracted by odors released by the pioneer gravid females; (4) The olfactory sensation of the females may be changed by mating.

Keywords

Cephalcia chuxiongica, Aggregation oviposition, Host volatile, Accessory gland volatile, Olfactometer bioassay

Introduction

Cephalcia chuxiongica Xiao is an important defoliator of pine trees in Yunnan Province of southwest China. The morphology of the insect was described by Xiao Gangrou in 1984 (Xiao 1984), and its life-history was briefly reported by Liu et al. (2014). The full-grown larvae drop down to the ground from trees and drill into the soil below the canopy from August to October, and then spend two winters in the soil before pupation. Adults' emergence occurs from June to September in the third year. The females strongly prefer conifer species to lay eggs, and *Pinus yunnanensis* and *Pinus armandii* are more suitable for the oviposition than *Keteleeria evelyniana* Mast, a native conifer tree species in Yunnan Province (Cheng et al. 2014).

Our previous field investigation found that females of *C. chuxiongica* gathered on the shoots of *P. yunnanensis* to lay eggs (Liu et al. 2015). In further observation, we noticed an interesting phenomenon: the pioneer gravid female randomly laid her eggs in a pine shoot, but the subsequent gravid females selected the shoot on which the pioneer female had laid. Thus, we speculated that, besides the pine needles' volatiles, other odors may play a role in attracting the subsequent gravid females of *C. chuxiongica*. In order to verify our surmise, laboratory bioassay was conducted.

Materials and methods

Insects. Tested insects were obtained from a 20-year-old plantation of *P. yunnanensis*, located on a small mountain near Lufeng town in the Yunnan Province, in 2016. Many webbing leftovers were observed on the ground below the pine tree canopies. New adult males and females were directly dug out of the soil below the pine trees where webbing leftovers were scattered. Collected females and males were discriminated (cervical sclerites, pronotum, and scutoprescutum of the female are russet, but piceous in the male), and reared separately by 5% sugar solution in insect cages under natural condition until they were tested the next day.

Before bioassay, the candidate females were divided into two groups. One group was kept as virgin; the other group was mated with males in a transparent plastic cup, in order to get gravid females. After a round of trials was performed, the tested insects were discarded.

Crude extracts. Crude extracts of needles of *P. yunnanensis* were taken by a simple distilling apparatus. 100 g of needles were cut into small pieces and placed in a 500-mL round-bottom flask with 200 mL water plus 50 mL trichloromethane. The flask was attached to a reflux condenser. The solution was heated and refluxed for 2 h. After cooling to room temperature, the organic phases were isolated, filtrated, and dried by sodium sulfate. The solvent was removed by rotary vacuum evaporator below 40 °C.

The crude extract of fresh eggs was obtained by elution of fresh eggs with trichloromethane. The fresh eggs are bright yellow, but lose their luster after a day. Shoots with fresh eggs were cut and collected from the field, then carried back to the laboratory in sealed polyethylene taker-bags. The needles with eggs were plucked; the eggs were drip-washed with trichloromethane, taking care to avoid staining by the metabolites from the needles' wounds. A single bunch of needles (three needles in one bunch, about 85 eggs laid by each female on average) was washed by 10μL trichloromethane. The eluents were combined and stored at 4 °C until testing.

In-cage choices of oviposition. Choice tests were conducted inside 80×80×80 cm screen cages with 0.42-mm mesh openings. The cages were placed in the laboratory and the shoots of *P. yunnanensis* were tested. Fresh shoots were cut with 25-cm length, and the new cross sections were coated with white latex to prevent the shoots from withering. A pine shoot was hung upside down by a thin wire in a corner of the cage, and the other shoot in the opposite corner; both shoots were 20 cm high from the ground. One gravid female was introduced into the cage, imitating a 'pioneer' as in the field condition, and allowed to select her oviposition shoot and lay her eggs randomly. Subsequently, other 10 candidate gravid females were introduced into the cage one-by-one. Once the preceding candidate female had selected her oviposition shoot, it was taken out of the cage; and the next candidate gravid female was introduced. The number of females (except for the 'pioneer') on the shoot with vs. without eggs was counted. In order to eliminate influences of visual cues, six cages were used: in three cages, the shoots that bore the 'pioneer' females were hung in one corner of the cages, and in the other three cages the shoots bearing the 'pioneer' females were hung in the opposite corner.

Behavioral Responses with a Y-tube. Olfactory responses of gravid females were tested in a glass Y-tube olfactometer (50 mm diameter, main tube length 50 cm, arm length 25 cm), with 120° arm angles, which was placed horizontally. Incoming air was filtered through activated charcoal and humidified with doubly distilled water. The filtered air was split between two holding chambers (Blackmer et al. 2004).

Fresh pine shoots were cut at 20cm length, and the new cross sections were coated with white latex to prevent the shoots from withering. In one-way choices, one chamber of the Y-tube olfactometer held one pine shoot (P), one virgin female (V), one gravid female (G), one pine shoot each with one female and her fresh eggs on the needles (PGE), or one pine shoot with fresh eggs without protecting female (PE), as odor sources. The other chamber, for controls, held no odor source, only clean air passing through it. Air-flow through the system was maintained at 200 mL/min by an inline flow meter. A prior smoke test showed laminar airflow in both arms and throughout the olfactometer. To eliminate visual cues, the Y-tube setup was surrounded by a 100 × 80 cm black fabric enclosure. Approximately 30 min before trials were initiated, mated *C. chuxiongica* females were introduced into a separate holding container, so they would not be exposed to test odors before their release. In order to determine whether the olfactory behavior of the females changed after mating, virgin females were also tested simultaneously. The candidate mated or virgin females were tested one-by-one with 25 females as a group (one treatment), and each treatment was duplicated six times. The females were given 10 min to respond to the treatment; the females that went past the Y junction that led to the treated chamber were noted as attracted, those that remained in the main tube were noted as no-response, and those that went into the other arm tube were noted as repelled. The trend rate of females to the odor was calculated as the percentage of attracted females of the total females that were attracted or repelled. When crude extracts were tested, the needles' extract (NE) was diluted with trichloromethane to 1 mL/100 mL, the fresh eggs' eluent (EL) was used directly. 10 μL solution of NE or EL was dropped on a slip of filter paper (0.5 × 3.0 cm), and left for 10 seconds until solvent evaporation. Then, the filter paper was put into one of the chambers of the Y-tube olfactometer as a treatment; the other chamber was provided a slip of filter paper dropped with the solvent only and then left to volatize as a control. All the females were used only once for each of the treatments.

In two-way choices, the odor sources were given in pairs, and olfactory responses of gravid females were tested. Thus, the odor sources were: G vs. P, G vs. PGE, G vs. PE, P vs. PGE, P vs. PE, PGE vs. PE, and NE vs. EL. The olfactometer was washed by n-hexane; the treatment and control sides were alternated after every trial.

Data analysis. Nonparametric Wilcoxon matched pairs test was used for paired comparison (SPSS), statistical significance was accepted as $p < 0.05$. Figures were drawn by Microsoft Excel 2007.

Results

Results of in-cage oviposition tests of the gravid females are given in Figure 1. The pioneer gravid females' selection of oviposition pine shoot was random, no obvious preference between the two shoots in the opposite corners was observed. However, the majority of delivery females selected the shoots that bore a 'pioneer' female (and her eggs) as their oviposition sites (9.00±1.26 females/shoot on average); only sporadic females (0.33±1.26) selected the shoots without the 'pioneer' female.

Trend rates of gravid females to odors from PE, PGE, NE, and EL were significantly larger than those to clean air; there was no significant difference between the trend rates to the odor of P, V, and G and to clean air (Figure 2). The trend rates of virgin females to P, PGE, PE, and NE were obviously larger than that to clean air; the virgin females did not make choice between odor of EL and clean air. Compared with the odors of V and G, the virgin females were more inclined to clean air (Figure 3).

In two-way choice tests, only gravid females were tested (Figure 4). When compared with odors of G or P, gravid females were significantly more attracted to odors of PE or PGE. Gravid females did not make obvious choice between P vs. G, and PE vs. PGE; but preferred to the odors of NE than EL.

Discussion

Delivery female can summon the conspecific gravid females to aggregate in the same pine shoot. In in-cage test of oviposition choices, vast majority of gravid females selected the

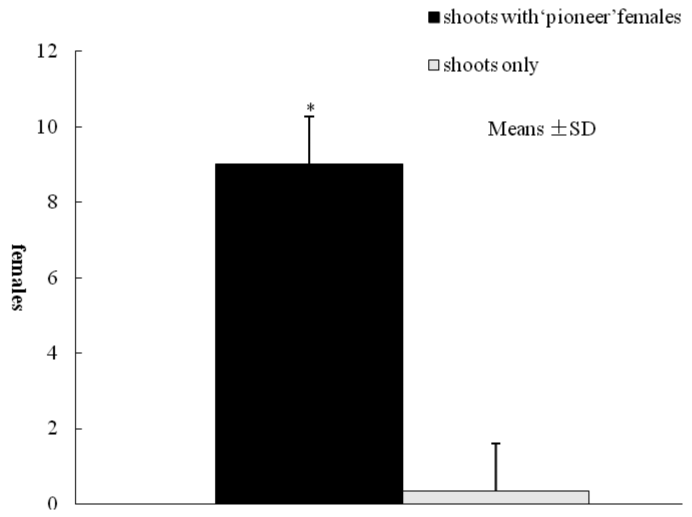

Figure 1. Oviposition selection of gravid females of *Cephalcia chuxiongica* in pine shoots with vs. without 'pioneer' females. *Indicates a statistically significant difference ($P < 0.05$) between mean numbers of delivery females on the shoots with and without 'pioneer' females (non-parametric Wilcoxon matched pairs test, two-tailed).

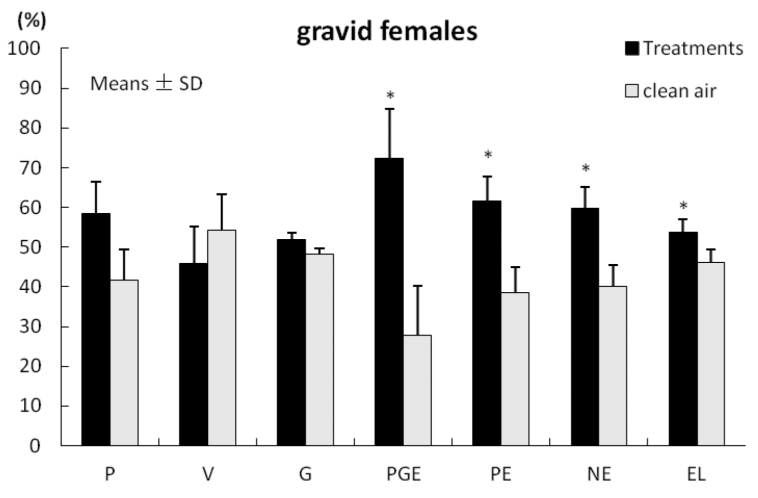

Figure 2. Trend rates of gravid females of *Cephalcia chuxiongica* to volatiles in one-way choice tests. **P** one pine shoot **V** one virgin female **G** one gravid female **PGE** one pine shoot with one female and her fresh eggs on the needles **PE** one pine shoot with fresh eggs but no female **NE** needles' extract **EL** eggs' eluent. *Indicates a statistically significant difference ($P < 0.05$) between the trend rates of the gravid females to the odor and to clean air (nonparametric Wilcoxon matched pairs test, two-tailed).

shoot in which the 'pioneer' females had laid eggs (Figure 1). This affirmed our surmise that the delivery females of *C. chuxiongica* in pine shoots can attract more other conspecific gravid females to aggregate in the same pine shoot for oviposition.

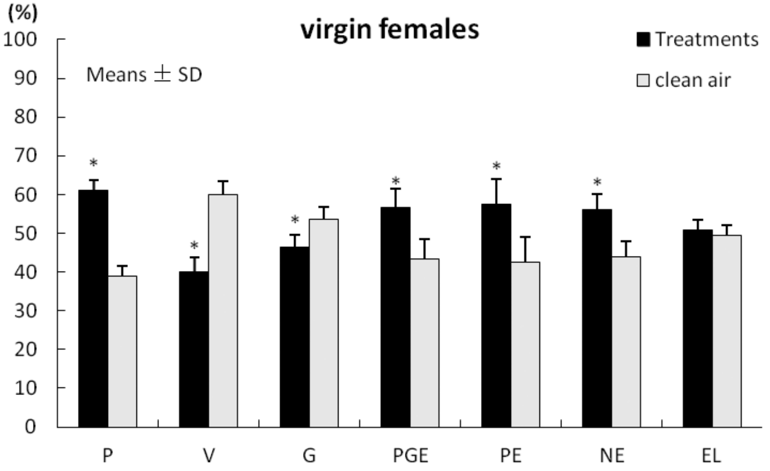

Figure 3. Trend rates of virgin females of *Cephalcia chuxiongica* to volatiles in one-way choice tests. **P** one pine shoot **V** one virgin female **G** one gravid female **PGE** one pine shoot with one female and her fresh eggs on the needles **PE** one pine shoot with fresh eggs but no female **NE** needles' extract **EL** eggs' eluent. *Indicates a statistically significant difference ($P < 0.05$) between the trend rates of the virgin females to the odor and to clean air (nonparametric Wilcoxon matched pairs test, twotailed).

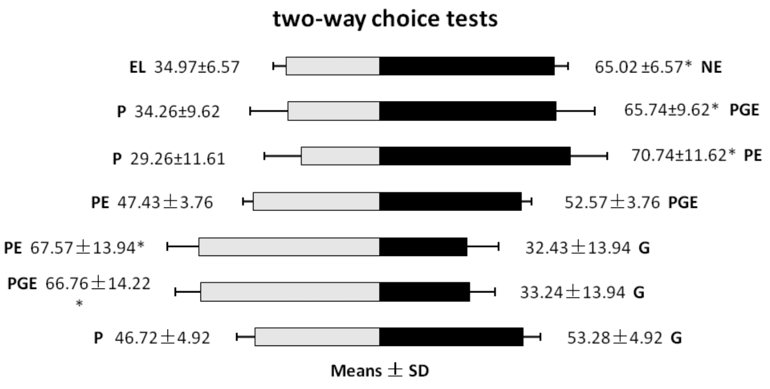

Figure 4. Paired comparison of trend responses of gravid females of *Cephalcia chuxiongica* to attractive odors **P** one pine shoot **G** one gravid female **PGE** one pine shoot with one female and her fresh eggs on the needles **PE** one pine shoot with fresh eggs but no female **NE** needles' extract **EL** eggs' eluent. *Indicates that the trend rate to the odor is statistically significantly higher than that to the other odor in this pair ($P < 0.05$, nonparametric Wilcoxon matched pairs test, two-tailed).

Pine needles' odors were attractive for both the virgin and gravid females. Host volatiles and their chemical profiles play an indispensable role in oviposition preference in phyllophagous sawflies. Females of the sawfly *Diprion pini* can discriminate between pine species for their oviposition sites (Barre et al. 2002); *Pinus banksiana* and *Pinus strobus*, whose needles release significantly higher relative concentrations of limonene, β-pinene and myrcene than needles of *Pinus sylvestris* and *Pinus nigra*, are not suitable for *D. pini* oviposition (Buda 2011); the high-carene chemotype of *P. sylvestris* captured more numbers of *Sirex noctilio* females than the low-carene chemotype (Böröczky 2012); In an olfactometer bioassay, a low amount of limonene was attractive for *Neodiprion sertifer* females, while a repellent effect was evident when higher amounts were used (Martini et al. 2010). Trend rates of virgin females (Figure 3) of *C. chuxiongica* to odors from P or NE were significantly larger than those to clean air; gravid females did not show obvious tendency to odors of P, but significantly inclined to odors of NE. This indicates that volatiles of needles are attractive for both the virgin and gravid females. The reason for the inconspicuous tendency of gravid females to odors of P (P = 0.0789) may be due to the lower concentration of odors of undamaged needles. However, the crude extracts of needles of *P. yunnanensis*, or the monoterpene β-pinene or myrcene captured a few of females in field tests (data unpublished). There are huge differences between compositions of volatile extracts obtained by steam distillation (Yang, 2009) and by head-space adsorption (Wu et al. 2010); the volatile composition of crude extract of needles did not simulate the chemical profile of needles' odor in the natural state adequately. This maybe a probable reason of the low captures. In fact, the pioneer females randomly selected their oviposition shoots in cages and in the field; but the subsequent gravid females were summoned to the shoot that had born delivery females. Thus, we surmise that, in addition to host volatiles, other factors may also play a role in the aggregation oviposition in this sawfly.

Pine sawfly *D. pini* oviposition can change the volatile composition of host *P. sylvestris* twigs quantitatively or constitutively (Hilker et al. 2002, Mumm et al. 2003, Mumm et al. 2004); and the terpenoid volatile pattern of systemically oviposition-induced pine twigs changes quantitatively after an induction time of 3 d compared to controls (Mumm et al. 2003). The difference of volatiles of *P. yunnanensis* needles before and after *C. chuxiongica* females' oviposition remains to be analyzed.

Gravid females could be attracted by odors released from the fresh eggs. The results showed (Figure 2) that the trend rates of gravid females to odors from EL were significantly larger than those to clean air. Based on the results of Günthardt-Goerg (2010), the volatile of the needles' eluent contains negligible monoterpenes. All these indicate that odors released from fresh eggs are attractive for the gravid females. This can be further corroborated by the results in Figure 4; the trend rate of the gravid females to odors of PE was significantly larger than to odors of P; also, the trend rate to odor PGE was significantly larger than to odor of P. Adult's aggregation oviposition and larvae's aggregation feeding is the obvious feature in *C. chuxiongica* (Fang, 2010); the former is a necessary premise for the latter. These prompted us to further confirm that the volatiles released from fresh eggs contain some attractive components, which are not the monoterpenes themselves. Blümke and Anderbrant (1997) mentioned that the presence of *N. sertifer* eggs and extracts obtained from eggs had no inhibitory effect on the females' egg-laying, but they did not record whether the odor of eggs' extract was attractive for females. However, reproductive accessory gland secretions of some insect species, such as in *Delia radicum* (Gouinguené et al. 2006) and *Schistocerca gregaria* (Kahoro et al. 1997, Torto et al. 2015), which are attached to the surface of the eggs, contain stimulating pheromone that induces the same female adult to aggregate and lay eggs. The constituents of the secretion of *C. chuxiongica* remain to be analyzed and the attractive components remain to be identified.

The gravid females did not show a significant tendency to odors of G (Figure 2). However, this did not mean that the gravid females did not release odors attractive for the conspecific females, since the odors of fresh eggs were attractive; we suspect that females, as odor sources in chamber of the Y-tube olfactometer, were constantly disturbed in the bioassay, consequently the odor release was disturbed.

The olfactory sensation of the females may be changed by mating. Firstly, there was no difference between the trend rates of virgin females to EL and to clean air (Figure 3), while an obvious large trend rate of gravid females to EL (Figure 2). Secondly, a relatively low trend rate to V was observed in virgin females (Figure 3), but gravid females did not express any selectivity between V and clean air (Figure2). This means that odor of V was repellent for the virgin females, whereas odor of EL was attractive for the gravid females. Thirdly, the odor of P (Figure 3) was significantly attractive for the virgin females, but its attraction to gravid females was not obvious (Figure 2). Therefore, we infer that the physiological state, such as olfactory receptor of the *C. chuxiongica* females in this case, had changed. The truth remains to be confirmed by further research.

On the whole, we can imagine a scene for *C. chuxiongica*: the females select their suitable host by distinguishing the differences among volatiles of different tree species (Cheng et al. 2014). The pioneer female randomly locates a pine shoot as its oviposition site; after mating, she informs her conspecific gravid females to gather in the same pine shoot by sending some signals (such as attractive chemicals) through her reproductive accessory gland; the attractive chemicals maybe part of the components of secretion attached on the surfaces of fresh eggs. The aggregated females lay their eggs on the same shoot; the later aggregation feeding of the low instar larvae might be beneficial to resist the resistance of the host. This conjecture is consistent with the behaviors of this sawfly observed in the field (Liu et al. 2014, 2015). If this conjecture does indeed reflect the truth about the mechanism by the sawfly's aggregation oviposition, many details in the process of insect damage remain to be studied in depth.

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