Evidence of sexual attraction by pheromone in the cedar web-spinning sawfly

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Abstract—The cedar web-spinning sawfly, *Cephalcia tannourinensis* Chevin (Hymenoptera: Pamphiliidae), is a pest that has been causing serious damage to cedar (*Cedrus libani*) forests in Lebanon since 1990. The existence of a sex pheromone was shown in field experiments in a cedar forest in Lebanon and in laboratory tests in olfactometers with and without airflow. More males were caught in traps baited either with virgin females or with a hexane extract of the whole female body than in traps baited either with males alone or with mixed males and females. Male and female *C. tannourinensis* were active during the day. Mating and pheromone production were observed to occur during midday hours (1000–1400) in the field and under laboratory conditions. Olfactometer tests with extracts prepared from different body parts of the female indicated that the pheromone is produced in the abdominal region, and tests with different dilutions of female extract showed that the male response is dose-dependent.

Résumé—La thendrède du cèdre, *Cephalcia tannourinensis* Chevin (Hymenoptera : Pamphiliidae), cause de graves dommages dans les cédraies libanaises (*Cedrus libani*) depuis 1990. Des études menées sur le terrain par piégeage sexuel et au laboratoire par olfactométrie ont permis de mettre en évidence l'existence d'une phéromone sexuelle produite par les femelles et attractive pour les mâles. Les mêmes tests ont démontré que l'activité sexuelle est diurne et limitée à un moment de la journée qui se situe entre 1000 h et 1400 h; que la phéromone est extractible dans l'hexane et que l'abdomen serait le siège de la production. La réponse des mâles est dépendante de la dose.

Introduction

The cedar web-spinning sawfly (CWSS), *Cephalcia tannourinensis* (Hymenoptera: Pamphiliidae), was recently described as a new species by Chevin in 2002. Between 1990 and 1999, an infestation in the Tannourine–Hadath El Jebbeh cedar (*Cedrus libani* A. Rich. (Pinaceae)) forest in northern Lebanon increased in intensity and caused intense defoliation over 600 ha.

Adults fly in mid-April until the end of May. Mating occurs late in the morning and the females lay their eggs on new cedar buds. The larvae feed on needles, and after the last molt they drop from the crown to the ground, where they hibernate (Nemer *et al.* 2005). Larval diapause can last for more than 1 year and up to 5 years for some species in the same genus (Gruppe 1996).

Aerial spraying with the insect-growth regulator diflubenzuron was carried out in 1999–2004, resulting in considerable suppression of the CWSS population (Nemer and Nasr 2004), but for ecological reasons the development of more environmentally friendly techniques to monitor populations is required. The development of biocontrol agents against *Cephalcia* spp. that attack spruce trees has been the

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subject of many studies carried out by Battisti (1994). Various types of traps have been used for evaluating and monitoring the population of the web-spinning sawflies belonging to the genus *Cephalcia*. The most common method used in monitoring is to estimate the number of prepupae in the soil (Bogenschütz 1986; Eichhorn and Pausch 1986; Kula 1987), but this is time-consuming and labor-intensive. Traps used for visual estimation include both white buckets suspended from trees and yellow trays positioned at ground level, which were developed by Jensen (1988) and are still used for monitoring adults. Sticky bands on the trunk and sticky yellow boards suspended vertically from the branches were used by Cescatti and Battisti (1992). There was a significant linear relationship between logarithmic estimates of the catches of *Cephalcia arvensis* Panzer and adult CWSS in yellow traps and the number of prepupae ready to emerge during an outbreak in a Norway spruce (*Picea abies* (L.) Karsten (Pinaceae)) area in the southern Alps (Asiago, Italy) and in a cedar forest in northern Lebanon (Tannourine). However, visual traps were inefficient in monitoring low populations of web-spinning sawflies (Battisti and Rodeghiero 1998; Nemer *et al.* 2005).

The occurrence of sex pheromones has been demonstrated in several sawfly species (Jacobson 1972; Jewett *et al.* 1976; Anderbrant 1999). Moreover, the pheromone of the pine sawfly genus *Neodiprion* Rohwer (Hymenoptera: Diprionidae) has been used in mating-disruption experiments for population control (Anderbrant *et al.* 1995; Martini *et al.* 2002). However, no chemical identification of pheromones of the genus *Cephalcia* has been reported to date. The only two published studies on the existence of a sex pheromone in the genus *Cephalcia* demonstrated production by female *Cephalcia lariciphila* Wachtl (Borden *et al.* 1978) and female *Cephalcia abietis* L. (Gruppe and Nisselein 1996). Baker *et al.* (1983) identified one component of the sex pheromone of *C. lariciphila*, *ortho*-aminoacetophenone. This molecule is chemically very different from diprionid pheromones, the main component of which is the acetate or propionate of 3,7-dimethyl-2-pentadecanol (Anderbrant *et al.* 1992).

The objectives of this paper were to (*i*) determine whether CWSS produces a sex pheromone and which sex produces it; (*ii*) determine the diel periodicity of flight activity and sexual behavior; (*iii*) demonstrate the extractability of the pheromone in organic solvents; and (*iv*) identify the body parts that produce the pheromone. The answers to these questions are crucial for the chemical identification of the sex pheromone, which could contribute to the development of methods of monitoring and controlling this insect pest.

Methodology

Study site

Field studies were carried out in the Tannourine–Hadath El Jebbeh cedar forest about 95 km north of Beirut, Lebanon. Geographically the forest falls between 34°12′ and 34°15′N and 35°54′ and 35°56′E and lies 1430–1815 m above sea level. The area is located in the supra-Mediterranean region, with annual rainfall of 1200–1400 mm (United Nations Food and Agriculture Organization 1993). The geological substrate consists generally of limestone and the soils are generally well developed and humid (brown earth type) with a pH of 5.7–7.1.

Laboratory experiments were conducted in the Pesticides Research Laboratory at the American University of Beirut and at the Mediateurs Chimiques Laboratory at the National Institute for Agricultural Research in Versailles, France.

Occurrence of a sex pheromone

During the flight period of adult CWSS, males and females were collected every morning as they emerged from the soil and before they had a chance to mate, to ensure that they were virgins. To determine whether a sex pheromone exists and which sex produces it, four treatments using sticky Pherocon® traps (Trécé Inc., Adair, Oklahoma, USA; www.trece.com) were used: one was baited with 10 females, one with 10 males, and a third with 5 males and 5 females, and one Pherocon® trap was left empty as a control. Each trap was hung on a tree branch 2 m above the ground at 0800; the distance between traps was at least 100 m. Traps were collected at 1800 the same day. Trapped insects were sexed and scored. The insects serving as bait in the traps were not fed and were still alive at the end of the day. The experiment was replicated on 3 consecutive days in 2004 and again in 2005. One-way analysis of variance (ANOVA) was used to test for differences in the numbers of males and females caught in the different traps. Means were

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separated with Fisher's least significant difference test.

Emission of sex pheromone induces in the opposite sex a modification of the behavior that is often characterized by an increase of flight activity, attraction to the emitting source, and attempts at copulation. To determine whether either sex modifies the flight activity and orientation of the other, a laboratory experiment was carried out in a static environment (no air displacement). Ten virgin male CWSS were placed in each of three 1 L Erlenmayer flasks that were then sealed with a glass stopper. Following a 20 min acclimation, the flask was resealed with a rubber stopper through which a glass rod was inserted, and a mesh bag was suspended from the glass rod. Each mesh bag contained either five female or five male CWSS, or was empty. Each mesh bag was suspended in its flask for 2 min every hour between 1000 and 1500. After each 2 min test, the mesh bags were removed and the flasks were resealed with a glass stopper. The same males were tested 5 times throughout 1 experimental day. The same test was repeated for 3 consecutive days using new insects each day. The numbers of males exhibiting the following two behaviors were recorded while the mesh bag was in the flask: visiting or standing on the mesh bag or the glass rod, and flying or walking on the sides of the flask. All males exhibiting the above activities were regarded as responding. Of the 10 males, the total numbers responding during the day were averaged and each day was considered one replicate. All Erlenmeyer flasks were kept under laboratory conditions (17 °C and 75%–80% RH). A Kruskal–Wallis test was used to compare the numbers of male CWSS responding to the presence of males, females, or no stimulus inside the Erlenmeyer-flask olfactometer.

To evaluate the short-range attraction behavior of male CWSS, tests were conducted in a four-arm airflow olfactometer (Vet *et al.* 1983). The exposure chamber measured 10 cm × 10 cm and each arm was connected to a set of two 50 mL glass vials. The vial closer to the chamber contained the odor source and served as a compartment trap to catch insects reaching that vial. The farther vial in each arm contained an air purifier (XAD2, Supelco®, Bellefonte, Pennsylvania, USA) over which the incoming air passed. All connections were made of silicon tubing. One female or one male CWSS was used as a stimulus and placed in the odor-source vial in one arm, whereas the other three odor-source vials were kept empty, dispatching only pure air into the other three arms. Airflow was approximately 10 L/h. One male CWSS was placed in the exposure chamber and observed for a maximum of 4 min. For each test, the olfactometer was cleaned with ethanol, a new male was introduced into the exposure chamber, and a new male or female was used as the stimulus. To confirm that there was no bias in the experimental setup, a blank test was conducted under the same experimental conditions but with individual males released at the center of the exposure chamber, with no stimulus from any vials. All the tests were replicated 15 times. The final choice of the test male and the time required to reach the odor-source vial were recorded. All tests were conducted under laboratory conditions (17 °C and 75%–80% RH). A χ^2 test was used to determine whether there was any deviation from a normal distribution throughout the exposure chamber in the four-arm olfactometer. The times required to reach the compartment were compared by means of a Mann–Whitney test.

Diel flight periodicity and pheromone emission

To determine the diel periodicity of flight activity of CWSS an experiment was conducted in the Tannourine–Hadath El Jebbeh cedar forest with three interceptive traps consisting of rectangular white boards (20 cm × 17 cm) coated with glue and attached horizontally with aluminum wires to three different trees 2 m above the ground. Three replications were performed on 3 different days. Traps were replaced every hour from 0800 to 1700 to avoid bias due to previously caught insects, and the total numbers of males and females caught per hour in each trap were recorded.

The period of pheromone production was determined using the Erlenmeyer flask setup described above. Male and female CWSS were collected in the forest 1 day before the start of the experiment and were kept separately in small cages at 17 °C and a 11L:13D photoperiod. Ten male CWSS were placed in each of two flasks. The first treatment consisted of enclosing 5 females in the mesh bag attached to the glass rod, then introducing the bag into the flask. The second flask served as a control where males were kept exposed to an empty mesh bag. The stopper and the glass rods with their mesh bags were introduced every hour for 2 min from 0800 to 1700, and data on the behavior of the males were recorded according to

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the following criteria: the number of males visiting or standing on the mesh bag or the glass rod; the number of males flying inside and (or) actively walking on the sides of the flask. The same 10 males and 5 females were used on the same day from 0800 to 1700. Whereas the males remained in the Erlenmeyer-flask olfactometer, the females were returned to the incubator after each 2 min test. The experiment was repeated on 3 different days with new insects each day. A Mann–Whitney test was used to compare the numbers of males that became excited in the presence of a female stimulus and in the absence of any stimulus during different hours of the day and all hours of the day.

Extractability of the pheromone

Demonstration of the activity of insect extracts is required before physicochemical analysis is undertaken. Whole bodies of either 10 live virgin females or 10 live virgin males were dipped separately in 1 mL of organic solvent (hexane or pentane) for 3 min. The insects were then removed and the resulting extracts were stored at –20 °C until they were used.

To ascertain that attraction of the male by the female was due to emission of a pheromone and that the chemicals are extractible in solvent, attraction tests were conducted in the cedar forest. Two dilutions, 1/10 and 1/20, were prepared from the initial extract from 10 females. The extracts and the resulting dilutions were deposited onto rubber septa (Z124354–100EA, Sigma–Aldrich, Milwaukee, Wisconsin, USA) and used after all the solvent had evaporated. Four treatments consisting of crude female extract, a 1/10 and a 1/20 dilution of this crude female extract, and crude male extract were used. Pherocon® traps were used, hung and spaced as described for the first trapping experiment. Four replicates of each treatment were carried out during the insect's flight period in May 2004. Insect catches were collected and sexed 24 h after installation of the traps. Numbers of trapped male and female CWSS were analyzed by one-way ANOVA and treatment means were separated with Fisher's least significant difference test.

To confirm the results of the field experiment, laboratory tests were conducted in the four-arm airflow olfactometer. Aliquots of 10 µL, 100 µL, and 500 µL of the crude female extract corresponding to 0.1, 1, and 5 female equivalents, respectively, were tested separately. Each solution was deposited on a filter paper before its introduction into one of the four compartments of the olfactometer. Another filter paper infused with a corresponding volume of solvent was placed in another compartment and a filter paper with no solvent was placed in a third compartment; and the fourth compartment was left empty. A single male CWSS was placed in the exposure chamber and its choice was recorded during a 4 min test. Ten replications were performed, with new males, for each of the three female equivalents. The olfactometer was cleaned with alcohol, the glass vials were replaced between tests, and new extracts were loaded each time. All tests were conducted under laboratory conditions (17 °C and 75%–80% RH). A χ^2 test was used to determine whether there was any deviation from a random distribution over the four compartments in the four-arm olfactometer with the different female equivalents.

Identification of the body parts producing the pheromone

Organic extractions were carried out with different female body parts. Each female body was cut into three parts, head, thorax, and abdomen, using dissecting scissors. Each sample, consisting of 10 heads, thoraxes, or abdomens, was soaked in 1 mL of solvent for 3 min. The body parts were then removed and the extracts stored at –20 °C until they were used.

An experiment with the same protocol as above was carried out with female body parts. Aliquots of 100 µL, equivalent to one head, one thorax, and one abdomen, were tested separately on individual males by means of the four-arm olfactometer. The total number of replications for each body part was 15. Data were analyzed by a χ^2 test to determine whether males were randomly distributed throughout the exposure chamber.

Results

The occurrence of a sex pheromone

Virgin female CWSS were significantly more attractive to males than any other baits tested (Table 1) (ANOVA, $F_{3,23} = 19.30$, $P < 0.0001$). The number of females trapped was very low and did not differ significantly among all treatments for the years 2004 and 2005 (ANOVA, $F_{3,23} = 0.48$, $P > 0.05$). It is worth noting that traps baited with pairs attracted some males, though statistically fewer than those baited with virgin females.

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Table 1. Numbers of male and female cedar web-spinning sawflies, *Cephalcia tannourinensis*, caught in Pherocon® traps baited with live *C. tannourinensis* of different sexes in the Tannourine–Hadath El Jebbeh cedar forest in northern Lebanon in 2004 and 2005.

Trap baited with:	No. of males per trap	No. of females per trap
virgin females	28.5±4.94*a*	1.50±0.76*a*
virgin males	5.50±1.09*c*	1.5±0.52*a*
virgin males and females	12.00±3.30*b*	1.5±1.00*a*
nothing (control)	3.50±1.86*c*	0.67±0.60*a*

Note: Values are given as the mean ± SE and represent the means of six replicates. Values within a column followed by a different letter are different (ANOVA, $P < 0.05$, Fisher's least significant difference test).

The mean number of male CWSS responding to the presence of the caged females in the Erlenmeyer-flask olfactometer was higher (5.27 ± 0.95 (mean ± SD), $n = 3$) than in the presence of either caged males (2.67 ± 0.61, $n = 3$) or an empty mesh bag (1.73 ± 0.50, $n = 3$). Results showed statistical differences between the number of responding males to the caged females and caged males or empty cage (Kruskal–Wallis test, $H_{[2]} = 6.49$, $P < 0.05$).

Similarly, when the males were introduced into the exposure chamber of the four-arm olfactometer, 60% walked directly to the compartment containing the female and 40% moved to an empty compartment (observed, 9:4:2:0; expected, 3.75:3.75:3.75:3.75; $\chi^2 = 11.93$, $P = 0.0076$). When one male was used as the stimulus in one of the four compartments, or when all the compartments were empty, the compartment choice made by introduced males did not differ statistically from random. However, the time required to reach the compartment varied with the nature of the stimulus. The time required to reach the compartments was longer when the stimulus was either one male (3.1 ± 0.96 min (mean ± SD) or absent (3.04 ± 0.88 min) than when one female was used as the stimulus (1.82 ± 0.48 min) (Mann–Whitney test, $U = 44.5$, $n_1 = 4$, $n_2 = 9$, $P = 0.0133$; $U = 59.0$, $n_1 = 5$, $n_2 = 9$, $P = 0.005$).

Diel flight periodicity and pheromone emission

The flight-activity pattern of CWSS peaked between 1000 and 1200. Males and females followed similar flight patterns; the only differences were in the time when flight activity began and the number of each sex caught (Fig. 1). Males fly earlier than females and more males than females were caught. The average sex ratio, or sex index, *i.e.*, number of females / total number of females and males, ranged from 0.35 to 0.41. Observations made in the field during data collection showed that mating took place on cedar branches and was generally observed after 1100 and until 1500.

The flight activity of males in the presence of female stimuli differed significantly from that of males in the absence of stimuli inside the Erlenmeyer-flask olfactometer (Mann–Whitney test, $U = 947.5$, $n_1 = 27$, $n_2 = 27$, $P = 0.0004$; Fig. 2). Activity reached a peak (53%) between 1000 and 1430 and decreased thereafter, indicating that pheromone emission peaked between 1000 and 1430. The activity of males in the absence of any stimulus never reached more than 33% between 1000 and 1430.

Extractability of the pheromone

Traps baited with extracts of female CWSS attracted higher numbers of males in the field, showing that the chemicals released by the female were extractible in organic solvent (Table 2). Attraction was found to be dose-related: the highest number of males were caught in the trap with the female crude extract (10 females in 1 mL hexane), followed by the 1/10 dilution and then by the 1/20 dilution. The crude male extract attracted very few males, the numbers being significantly different from those in the trap with the female crude extract and its 1/10 dilution (ANOVA, $F_{3,15} = 7.31$, $P < 0.01$). In all the treatments, the numbers of females caught were very low and did not differ statistically among the different traps (ANOVA, $F_{3,15} = 1.46$, $P > 0.05$).

When tested in the four-arm olfactometer, the female extracts elicited male responses but the level of attraction was only weakly related to the dose. With the lower doses (1/10 dilution and 1 female equivalent), there was no significant difference between the observed frequency

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Fig. 1. Flight-activity patterns (hourly catches (mean ± SD) in nine traps) of male and female cedar web-spinning sawflies, *Cephalcia tannourinensis*, measured using sticky white rectangular boards in the Tannourine–Hadath El Jebbeh cedar forest, Lebanon, in spring 2005.

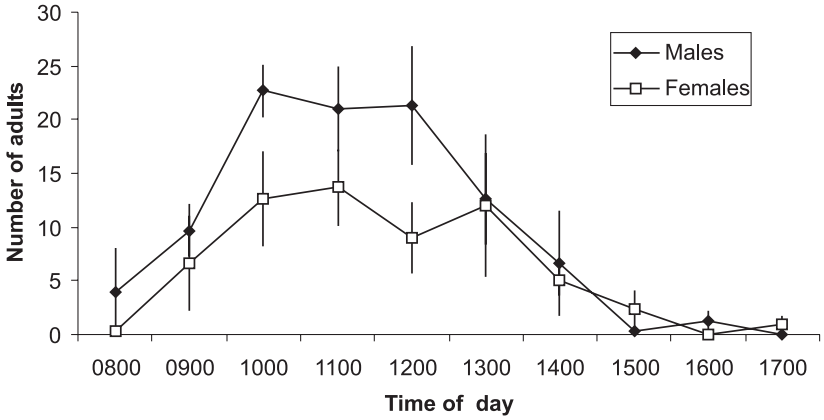
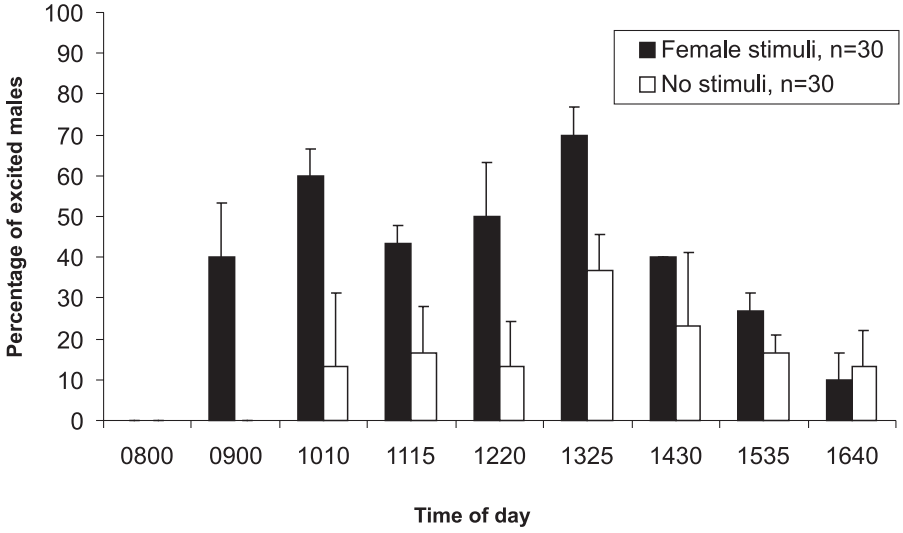


Fig. 2. Flight activity of male *Cephalcia tannourinensis* (*i.e.*, percentages (mean + SD) of excited males) in the presence and absence of female stimuli during different hours of the day.



of males in the compartments and the expected frequency (25%). A significant statistical difference was observed when a higher dose (5 female equivalents) was used (observed, 6:3:1:0; expected, 2.5:2.5:2.5:2.5; $\chi^2 = 8.40$, $P = 0.078$).

Identification of the body parts producing the pheromone

Male CWSS tested with the extract of female abdomen in one compartment of the four-arm olfactometer made a highly significant choice of that compartment (observed, 12:0:2:1; expected, 3.75:3.75:3.75:3.75; $\chi^2 = 24.73$, $P = 0.000017$). Distributions that did not differ statistically from random were obtained in tests using thorax and head extracts of female CWSS.

Discussion

Observations of sexual behavior and the results of trapping experiments (Tables 1 and 2), as well as the results of the olfactometer tests, clearly indicated that treatments with virgin female CWSS attracted more males than any other treatment. An extract of female whole body in organic solvent induced the same male behavior as that elicited by the females. These results confirmed that mate finding in CWSS is

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Table 2. Numbers of male and female cedar web-spinning sawflies, *Cephalcia tannourinensis*, caught in Pherocon® traps baited with different dilutions of female extract and with male extract in the Tannourine–Hadath El Jebbeh cedar forest in northern Lebanon in 2004.

Treatment	No. of males per trap	No. of females per trap
Female extract (10 ♀♀ in 1 mL hexane)	22.75±5.38*a*	1.75±0.85*a*
Female extract (1/10 dilution)	14.00±3.75*b*	0.75±0.29*a*
Female extract (1/20 dilution)	4.50±2.53*bc*	0.25±0.25*a*
Male extract (10 ♂♂ in 1 mL hexane)	1.75±0.63*c*	0.75±0.25*a*

Note: Values are given as the mean ± SE and represent the means of four replicates. Values within a column followed by a different letter are different (ANOVA, $P < 0.05$, Fisher's least significant difference test).

guided by the emission of a female sex pheromone. Borden *et al.* (1978) and Gruppe and Nisselein (1996) demonstrated in two other species belonging to the genus *Cephalcia*, *C. lariciphila* and *C. abietis*, respectively, that the female was the pheromone-releasing sex. Thus, production of sex pheromone by females appears to be the rule in this genus.

Traps baited with pairs attracted some males. The fact that only 5 females were added to the 5 males used in this trap, compared with 10 females in the trap baited with virgin females, may have caused some confusion as to whether the number of males caught was directly related to the number of females used. Our objective in using a trap baited with pairs was to determine if pairs are attractive as virgin females or as virgin males alone. However, because traps baited with virgin males were the least attractive, indicating that females are the producers of the sex pheromone, the number of males caught in the trap baited with pairs can be explained by the fact that the females had emitted pheromone before mating or remained virgin. In both cases, the pheromone could have been adsorbed onto the glue. Also, the female can remain attractive to males for a short time after mating as suggested by Borden *et al.* (1978): female *C. lariciphila* in a larch forest in Britain continued to be attractive to males for approximately 10 min after mating. The evidence of pheromone production by female CWSS is a new piece of information about sawfly biology and points to a certain homogeneity in this inadequately studied group.

The flight-pattern experiments showed a sex index that is biased in favor of males. A sex ratio favoring males was also reported for *C. abietis*, based on catches of adults (Eichhorn and Bogenschütz 2000). The abundance of males could be explained by the fact that females in the genus *Cephalcia* may lay their eggs without mating (arrhenotoky), producing a sex ratio favoring the male population (Eidt 1969). According to Lyytikäinen-Saarenmaa *et al.* (1999), sawflies have an ability to adjust the progeny sex in response to resources, *e.g.*, host quality, environmental conditions, population density, or population structure. More studies are needed to determine whether or not the CWSS population is male-biased.

CWSS flight activity is diurnal and male flight activity is stimulated by the presence of females. Maximum flight and walking activities of males when exposed to females were recorded between 1000 and 1400. In a study on the weather factors influencing catches of the European pine sawfly *Neodiprion sertifer* (Geoffroy), Jönsson and Anderbrant (1993) showed also that 98% of the insects were caught between 1040 and 1820, with peak activity around 1400. Those authors demonstrated that weather factors such as temperature and rainfall affect the ability of *N. sertifer* to fly. In the case of CWSS, we observed that temperatures below 15 °C inhibit flight (N. Nemer, unpublished data). Zhang *et al.* (2005) demonstrated a diurnal activity pattern of the male Chinese pine sawfly *Diprion jingyuanensis* Xiao and Zhang, with a peak around noon. Diurnal flight activity may be a common biological trait in sawflies. This finding indicated that it would be more efficient to carry out trials utilizing pheromones during daylight hours.

Testing different dilutions of female extract under field conditions demonstrated a dose-related response of male CWSS. Anderbrant *et al.* (1992) and Lyytikäinen-Saarenmaa *et al.* (1999) also found a dose-related response of *N. sertifer* to synthetic pheromones.

The results of the experiment with extracts of different body parts of female CWSS indicated that the abdomen is the region of pheromone production. Wassgren *et al.* (1992) showed that about one third of the total amount of the pheromone of *N. sertifer* was found in the head

and thorax, whereas two thirds was found in the abdomen. Our results suggest that pheromone biosynthesis takes place in the abdomen, and that the body part producing pheromone could be the cuticle or the hind gut, although no anal secretion was observed. Further studies should be undertaken to assess these hypotheses.

The positive orientation of male CWSS to female-produced pheromones could be practically exploited. Pheromone-baited traps would find obvious use in the survey and detection of CWSS populations in cedar forests. Now that we know that female pheromone can be extracted in solvent, identification of the chemicals can be undertaken. The synthesis and use of the pheromone in forests would certainly lead to a better understanding of the sexual behavior of males of the genus *Cephalcia*.

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