

Behavioural responses of the vine weevil, *Otiorhynchus sulcatus*, to semiochemicals from conspecifics, *Otiorhynchus salicicola*, and host plants

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Abstract

The vine weevil *Otiorhynchus sulcatus* is a parthenogenetic reproducing species which forages for suitable host plants at night, but is found congregated in dark places during the day. Frass of this weevil species is suspected to contain attractive compounds that are host-plant related. Using a still-air olfactometer, we tested adult vine weevils at night for their behavioural response to odours from conspecifics, feeding on a mixture of spindle tree (*Euonymus fortunei*) and yew (*Taxus baccata*), and to a sexually reproducing related species (*Otiorhynchus salicicola*), feeding on a mixture of ivy (*Hedera helix*) and cherry laurel (*Prunus laurocerasus*). Their attraction to conspecifics and *O. salicicola* appeared to be related to frass production. Freshly collected frass from *O. sulcatus* and from *O. salicicola* males and females was attractive. *Prunus laurocerasus* and *H. helix* have not been observed to be hosts of the vine weevil in the field. However, our tests showed that the vine weevil was attracted to mechanically damaged leaves of both plant species, whereas undamaged leaves were not attractive. Only undamaged young unfolding leaves of *H. helix* were also attractive. The attraction to odours from mechanically damaged host and non-host plants suggested the involvement of compounds that are commonly found in many plant species. The involvement of plant compounds and/or aggregation pheromones in attraction to frass of the vine weevil and frass of the related weevil species *O. salicicola* is discussed.

Introduction

The vine weevil, *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae), is a polyphagous insect and an important pest in the production of hardy ornamentals and small fruit world-wide (Moorhouse et al., 1992). Currently, effective pesticides against weevil larvae are not available, and the large-scale biocontrol of weevil larvae with parasitic nematodes is costly. The only economically feasible method available in The Netherlands is to control adult weevils by spraying acephate. This pesticide is not compatible with integrated control, as it kills the natural enemies of other pests. As a consequence, there is a need to spray at the right site, and at the right time. This necessitates the development of monitoring tools such as odour-baited traps.

Food-related odours may contain potential attractants. Food preference, electroantennogram (EAG) recordings,

and aggregation behaviour suggest that some plant volatiles and aggregation pheromones are involved in the attraction of *O. sulcatus* towards feeding sites (Pickett et al., 1996; van Tol & Visser, 1998, 2002; van Tol et al., 2004). For *O. sulcatus*, odour of the spindle tree [*Euonymus fortunei* (Turcz.) Hand.-Mazz.] proved to be attractive (van Tol et al., 2002), especially the young leaves and mechanically or weevil-damaged leaves. *Euonymus fortunei* is already used by growers for monitoring purposes (van der Horst & van Tol, 1995; van Tol, 1996). Another possibility is to consider pheromones, such as those involved in sexual attraction. Since *O. sulcatus* is polyploid, and reproduces by thelytokous parthenogenesis, studying sexually reproducing related species may be the only way to identify (related) sex pheromones within this weevil genus. Aggregation pheromones are another potential attractant that many weevil and beetle species produce. Aggregation pheromones have been studied in storage weevils and other beetles (Levinson & Levinson, 1995), bark beetles

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(Borden, 1982), boll and pepper weevil (*Anthonomus* spec.) (Tumlinson et al., 1969; Eller et al., 1994), and palm weevils (*Rhynchophorus* spec.) (Rochat et al., 1993).

In the search for potential attractants, even attractants used by related species may be of interest. Closely related insect species often share one or more pheromone components, differing only in their ratio of composition, in the chirality of some components, or through other small structural changes (Borden, 1984; Levinson & Levinson, 1995). To test the possibility of cross-species attraction to a sex or aggregation pheromone, we selected *Otiorynchus salicicola* Heyd., which is a pest in Pistoia, Italy (Del Bene & Parrini, 1986; Landi, 1991) and southern Switzerland. It has also recently become an increasing problem in the gardens and nurseries of northern Switzerland, partially displacing *O. sulcatus* (J.M. Grunder and R.W.H.M. van Tol, unpubl.). *Otiorynchus salicicola* prefers to feed on *Prunus laurocerasus*, *Euonymus*, *Rhododendron*, and *Hedera* species (Bassangova & Grunder, 1997).

In this article, we present the results of olfactometer studies which assessed the attractiveness of weevils, weevil excrements, and the host plants fed on by the vine weevil *O. sulcatus*, and a related weevil species, *O. salicicola*.

Materials and methods

Insects

A population of *O. sulcatus* was kept at 22 °C in a climate room under long-day conditions of L16:D8. For practical reasons the night conditions were created from 06.00 hours to 14.00 hours. The weevils were reared on *Astilbe chinensis* Franch. and Say. plants cultivated in 3 l pots. The plants were inoculated with eggs of *O. sulcatus* in the summer, and stored at a low temperature (4 °C). Three months before the start of the trials, they were transferred to a greenhouse at 20 °C. Emerging weevils were fed with a mixture of yew (*Taxus baccata* L.) and spindle tree (*E. fortunei* cv. 'Dart's Blanket'). In summer, the weevils were additionally collected from an infested field with *Taxus*, *Rhododendron*, and *Euonymus* at the Research Station in Boskoop. Weevils collected from greenhouse pots or from the field were kept separately in a room controlled for temperature and humidity. Trials were carried out with both populations in order to correct for any influence of their origin. In all trials, weevils were used that had emerged from the soil not more than 6 months previously. The weevils were returned to their stock population after each test and re-used in tests done at least a week later.

Weevils of *O. salicicola* were collected from gardens in Zürich, Switzerland, in May, and used for tests in August. They were kept at 22 °C in a controlled temperature and

humidity room under long-day conditions L16:D8, and fed with leaves of *P. laurocerasus* and *H. helix*. Male and female weevils were kept in separate boxes. As for *O. sulcatus*, the weevils were returned to stock populations after each test.

Plants

The test plants were ivy (*H. helix*) and cherry laurel (*P. laurocerasus*). Stems were cut from stock plants and leaves from these cuttings were used for the trials within 24 h.

Extracts from weevil excrements

Weevil frass (30 ml) of *O. sulcatus* and *O. salicicola* were collected over several weeks from the weevils held in the controlled temperature and humidity room. The frass of either male or female *O. salicicola* (30 ml) was collected and mixed with 50 ml of tap water. The suspensions were kept at 4 °C in a climate room, and filtered shortly before use. The filtrate was used for the olfactometer tests.

Fresh *O. sulcatus* frass (15 ml) was collected from a group of young weevils fed with *T. baccata* for 2 days. A 5 ml sample of the collected excrements was suspended in 50 ml of tap water. The suspension was kept at 4 °C in a climate room and filtered shortly before use. The filtrate was used for the olfactometer tests.

Bioassay set-up

A still-air olfactometer (van Tol et al., 2002) adapted from Prokopy et al. (1995) was used for all tests. The olfactometer consisted of a glass Petri dish (140 mm diameter × 20 mm high) with two holes (12 mm diameter and 95 mm apart) in the Petri dish lid. Small glass tubes (length 25 mm; diameter 12 mm) were positioned on top of the holes in the dish lid. Two glass cylinders (length 65 mm; diameter 60 mm) were placed on the dish lid such that the small glass tubes were in its centre. On top of each glass cylinder was placed a glass cup (length 60 mm; diameter 60 mm) with a rim (length 15 mm; diameter 67 mm), which interlocked the cylinder. A rigid nylon gauze (mesh 1 mm) was clamped between the cylinder and the cup. Weevils were deprived of food for 24 h prior to testing. Several olfactometers were placed in a non-air-tight black box. For each test, 10 weevils were released into a Petri dish. Each test lasted 2 h, starting when the weevils were in their active period for food searching (3–4 h after sunset). The weevils could choose to remain in the dish or to enter the cylinder parts through the inner surface of the glass tubes on the Petri dish lids. The nylon gauze prevented the weevils from entering the glass cups on top of the cylinders.

For each test, we determined 'Activity' and 'Preference' (choice) of the weevils. Activity was measured as the number of weevils that showed behavioural activity in the

olfactometer (they were trapped in the left or right glass cylinder) compared to weevils not responding (not entering the glass cylinders and remaining in the Petri dish). Preference was measured as the number of active weevils choosing one or the other side in the olfactometer.

Placing 10 weevils in the glass cup on the treatment side and no weevils on the control side tested the attractiveness of starved and non-starved weevils of *O. salicicola* and *O. sulcatus* for *O. sulcatus*. A wet cotton ball was placed on both sides of the olfactometer to even out any humidity differences. Starved weevils were deprived of food for 24 h prior to testing. A filtered suspension of weevil frass of *O. sulcatus* and *O. salicicola* in water was tested by placing small glass tubes filled with 1 ml of the suspension in the treatment glass cup, and 1 ml of tap water in glass tubes in the control treatment cup. For these tests we did not use glass cylinders and gauze to separate the odour source from the weevils, but placed the glass cups directly on to the dish lids of the Petri dish. For tests with fresh vine weevil frass in water, small filter papers (diameter 42.5 mm) were dosed with 150 µl of the suspension and placed in the treatment cup. A filter paper with 150 µl of water was used as a control. Preference for odours from plants was tested by placing leaves with petioles wrapped in wet cotton balls in the cups, separated by gauze from the connecting glass cylinders, several minutes prior to the start of the test. To compensate for humidity effects, wet cotton balls were also placed in the control treatment cups. For each replicate, the Petri dishes were rotated 90° (four different orientations of the treatment and control side).

Statistics

Data from all the trials were analysed by a Generalised Linear Model (GLM) with binomial distribution and logit link and with the different test combinations as independent variables ($P = 0.05$; one-tailed analysis for experiments where an odour source was compared to a control, and two-tailed analysis for all other experiments), where response was measured as the fraction of active weevils (Activity), and the fraction of weevils choosing one treatment side (Preference), using the Genstat 5 computer program. After GLM analysis, the estimated fractions for Activity were compared. For Preference, the estimated fractions that were different from zero (indicating the fractions that were different from 50%) were determined with a t-test, performed on logit transformed data.

Results

In all trials we determined the activity and preference of the weevils. Activity was the number of weevils that showed a behavioural activity in the olfactometer (trapped

into the left or right glass cylinders) compared to weevils not responding (remaining in the Petri dish). Preference provided information about the choice of active weevils for one or the other side in the olfactometer. Both activity and preference provided us with different information about the behaviour of the weevils. Excluding the non-responding weevils (those that remained in the Petri dish), the preference analysis provided us with correct information about the preferences that weevils have in actively responding to the test. We thereby excluded 'bad' weevils and/or weevils that were not conditioned to search for food or other sources during the test. 'Activity' gave us information about how well or badly the batch of weevils were conditioned to responding to odours when tested in the olfactometer and, more important, information about any increased but undirected responsiveness by the weevils because of the presence of a certain odour source. Some odours may just function as an activator to disperse or move more actively around, and may not attract the weevils specifically to a source. The results of all the trials are presented in Table 1.

Preference of *Otiorhynchus sulcatus* for starved and non-starved weevils

More weevils responded (Activity) in tests with odours from non-starved *O. sulcatus* and *O. salicicola* than in tests with odours from starved weevils (57% for starved and 75% for non-starved *O. sulcatus*; 67% for starved and 81% for non-starved male *O. salicicola*; Table 1).

Otiorhynchus sulcatus showed a significant preference for the odour of non-starved male and female *O. salicicola* (68 and 69%, respectively) but not towards the odour of starved male *O. salicicola*. *Otiorhynchus sulcatus* showed no preference for starved or non-starved *O. sulcatus* over clean air.

Preference of *Otiorhynchus sulcatus* for extracts of weevil frass

Undiluted suspensions of male and female *O. salicicola* frass triggered significantly more *O. sulcatus* weevils to move into the cups (88% and 93%, respectively) compared to controls (75%). The 10-fold diluted suspensions of male and female *O. salicicola* frass, and both undiluted and diluted suspensions of *O. sulcatus* frass did not differ from controls. *Otiorhynchus sulcatus* did not show any preference for the frass suspension of conspecific insects. In contrast, *O. sulcatus* was attracted to the frass suspension of males (preference to undiluted 67%, and to 10-fold dilution 72%), and females (preference to undiluted 74%, and to 10-fold dilution 69%) of *O. salicicola*. Preference for suspensions of 1-day-old excrements of the vine weevil suspended in tap water was found when tested undiluted (64%), but the attraction was lost for a 10-fold diluted suspension.

Table 1 Preference and activity of *Otiorhynchus sulcatus* in a still-air olfactometer to odours from conspecifics, *Otiorhynchus salicicola*, frass, and host plants of *O. salicicola*

Odour source (+)	Odour source (-)	n	% preference for (+) ^{1,2}	P	% activity ^{3,4}
Control	Control	15	46.8	0.57	76 bc
Starved <i>O. sulcatus</i>	Control	8	36.5	0.06	57 a
Non-starved <i>O. sulcatus</i>	Control	14	52.4	0.34	75 bc
Starved male <i>O. salicicola</i>	Control	10	43.5	0.18	67 ab
Non-starved male <i>O. salicicola</i>	Control	16	67.5***	0.0005	81 c
non-starved female <i>O. salicicola</i>	Control	12	69.3***	0.001	77 bc
Control	Control	17	48.5	0.78	75 a
Male <i>O. salicicola</i> frass (undiluted)	Control	12	66.9**	0.002	88 bc
Male <i>O. salicicola</i> frass (10-fold diluted)	Control	12	72.3***	0.0002	78 ab
Female <i>O. salicicola</i> frass (undiluted)	Control	12	74.3***	< 0.0001	93 c
Female <i>O. salicicola</i> frass (10-fold diluted)	Control	12	69.0***	0.0008	86 abc
<i>O. sulcatus</i> old frass (undiluted)	Control	12	52.5	0.34	79 ab
<i>O. sulcatus</i> old frass (10-fold diluted)	Control	12	48.8	0.42	83 ab
Control	Control	3	50.0	1.0	93 a
<i>O. sulcatus</i> fresh frass (undiluted)	Control	16	63.6**	0.006	94 a
<i>O. sulcatus</i> fresh frass (10-fold diluted)	Control	16	56.0	0.14	89 a
Control	Control	15	46.9	0.57	76 a
<i>P. laurocerasus</i> (undamaged full-grown leaves)	Control	26	54.0	0.16	83 ab
<i>P. laurocerasus</i> (undamaged young leaves)	Control	12	54.6	0.23	77 a
<i>P. laurocerasus</i> (mechanically damaged full-grown leaves)	Control	26	59.0*	0.01	85 ab
<i>P. laurocerasus</i> (mechanically damaged young leaves)	Control	12	69.6***	0.0005	87 ab
<i>P. laurocerasus</i> (mechanically damaged full-grown leaves)	<i>P. laurocerasus</i> (undamaged full-grown leaves)	20	59.4*	0.02	89 b
<i>H. helix</i> (undamaged full-grown leaves)	Control	22	52.3	0.30	78 a
<i>H. helix</i> (undamaged young leaves)	Control	12	64.5**	0.006	82 ab
<i>H. helix</i> (mechanically damaged full-grown leaves)	Control	22	61.9**	0.003	88 b
<i>H. helix</i> (mechanically damaged young leaves)	Control	12	66.0**	0.003	80 ab
<i>H. helix</i> (mechanically damaged full-grown leaves)	<i>H. helix</i> (undamaged full-grown leaves)	16	63.2**	0.004	91 b

¹Asterisks indicate significant differences from even distribution at *P ≤ 0.05, **P ≤ 0.01, or ***P ≤ 0.001.

²Preference is the percentage of responding (active) weevils choosing odour source (+).

³Activity is the total percentage of weevils responding [choosing odour source (+) and (-)].

⁴Values followed by a different letter in the same column group (each column group is separated by a horizontal dashed line) indicate significant different activity of the weevils at the 5% level.

Preference of *Otiorhynchus sulcatus* for host plants of *Otiorhynchus salicicola*

The number of *O. sulcatus* that responded (activity) was significantly different from the control test (control vs. control: 76%) when the weevils were offered a choice between fully grown mechanically damaged or undamaged leaves of *P. laurocerasus* (89%). Preference for *P. laurocerasus* only occurred in tests with mechanically damaged leaves. Preference for mechanically damaged, young unfolding leaves vs. clean air was significant (70%), as well as preference for mechanically damaged fully grown leaves compared with clean air (59%). Fully grown mechanically

damaged leaves of *P. laurocerasus* were preferred over undamaged fully grown leaves of *P. laurocerasus* (59%).

With *Hedera* as a test plant, the results were somewhat different. The number of weevils responding (activity) was significantly higher than the control (control vs. control: 76%) in tests where the weevils were offered a choice between fully grown, mechanically damaged leaves and undamaged *Hedera* (91%) or clean air (88%). The treatments where weevils could choose between undamaged fully grown *Hedera* and clean air also differed in activity (78%) from the treatments where weevils could choose between fully grown, mechanically damaged *Hedera* and

undamaged *Hedera* (91%) or clean air (88%). The weevils preferred both fully grown (62%) and young unfolding (66%) mechanically damaged leaves of *Hedera* when clean air was the alternative. Mechanically damaged fully grown leaves were also preferred over undamaged fully grown leaves (63%) in the olfactometer test. Undamaged *Hedera* leaves were only preferred when they were in a young unfolding stage (65%). Fully grown leaves lost their attractiveness for the weevils.

Discussion

Preference of *Otiorhynchus sulcatus* for starved and non-starved weevils

During the daytime, vine weevils often congregate in small groups in the field. We tested the aggregation behaviour of vine weevils at night in an olfactometer. Weevils were repelled from their conspecifics when they were starved for 24 h prior to testing, and neither attraction nor repellence was found when these weevils were not starved prior to testing. This, as well as the high number of non-responding weevils, verified the anti-aggregation behaviour of the vine weevil during night.

However, we demonstrated an attraction of *O. sulcatus* to the related species *O. salicicola*. Since *O. sulcatus* was attracted to non-starved *O. salicicola* males and females, and to the frass of *O. salicicola*, we suggest that frass from non-starved weevils is involved in attraction, whereas the absence of frass in experiments with starved *O. salicicola* explains the lack of attraction. This makes it unlikely that this response is due to pheromones. Extracts of *O. sulcatus* frass in water was attractive when collected within 1 day after excretion by the weevils, but frass collected later had lost its attractiveness. In contrast, older frass of *O. salicicola* suspended in tap water attracted large numbers of *O. sulcatus*. Frass of *O. salicicola* remained attractive independent of the collection time.

Preference of *Otiorhynchus sulcatus* for host plants of *Otiorhynchus salicicola*

There was no difference in the attraction of *O. sulcatus* to male or female weevils of *O. salicicola* and their frass, and we therefore hypothesise that the attractive compounds released by the frass are closely related to food plant odours. Attraction to the host plants of *O. sulcatus*, *Taxus*, and *Euonymus*, has been demonstrated in earlier tests (van Tol et al., 2002), but attraction to the host plants of *O. salicicola* has not been tested before. We therefore tested the attraction of *O. sulcatus* to cherry laurel (*P. laurocerasus*) and ivy (*H. helix*). Weevils of *O. salicicola* in the stock population were fed with these two host plants. In general, the results showed an attraction of *O. sulcatus* to damaged

leaves of *Hedera* and *Prunus*, and no attraction to undamaged leaves. For *Hedera* only, we also found an attraction to very young undamaged leaves. These results are in accordance with our field observations on weevils in infested nurseries. Bassangova & Grunder (1997) reported that in Switzerland *O. salicicola* were abundant on the shrubs of *P. laurocerasus*, and only in these cases were they found in association with *O. sulcatus* and *O. crataegi*. It is possible that these two last-mentioned weevil species were attracted to odours released from plants damaged by *O. salicicola*, despite the fact that these plants were not their hosts, and have been shown to be poor plants for the reproduction and survival of *O. sulcatus* (threefold higher mortality and 70% reduced oviposition compared to that on *Taxus*; van Tol et al., 2004). This attraction to non-hosts is an interesting phenomenon because it may have consequences for competitive displacement and speciation.

Otiorhynchus sulcatus is attracted to the frass of its own species as well as to the frass of the related species *O. salicicola*, which feeds primarily on other host plants in the field. Since various plants – even several non-host plant species – seem to release attractive compounds for *O. sulcatus* when damaged, we hypothesise that host plant and frass odours have attractive compounds in common. This, of course, does not exclude the possibility that weevil-damaged plants (van Tol et al., 2002) and frass release different compounds which have a synergistic effect on the weevil's response to other compounds. In the field, the weevils are observed to aggregate on host plants and also damaged non-host plants. The question is whether aggregation is solely caused by the response to (damaged) plant odours, or whether there is an aggregation pheromone which is intrinsic to the weevil's physiology.

Release of aggregation pheromones with frass and/or during plant-feeding is shown for a range of coleopteran species such as the boll weevil (*Anthonomus grandis*), palm weevil (*Rhynchoporus palmarum*), banded fruit weevil (*Phlyctinus callosus*), sugarcane rootstalk borer weevil (*Diaprepes abbreviatus*), mountain pine beetle (*Dendroctonus ponderosae*), and the Japanese beetle (*Popillia japonica*) (Tumlinson et al., 1969; Pitman, 1971; Billings et al., 1976; Barnes & Capatos, 1989; Loughrin et al., 1996; Sánchez et al., 1996; Harari & Landolt, 1997). All these examples support the idea that feeding-induced odours may serve as an indicator of conspecifics feeding on a high-quality host.

The electrophysiological responses of *O. sulcatus* towards compounds from bio-active extracts of *Euonymus* and weevil frass can be recorded by gas chromatography with electro-antennographic detection (GC-EAD) in order to identify the compounds involved in attraction and aggregation. Using these attractive compounds, we hope to develop a monitoring instrument that can be used by growers

in their pest management programmes. Furthermore, we hope that combining the attractant with a chemical or biopesticide in baits can be used in an 'attract and kill' strategy for pest control.

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