Research Article

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Benzaldehyde: an alfalfa-related compound for the spring attraction of the pest weevil *Sitona humeralis* (Coleoptera: Curculionidae)

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Abstract

BACKGROUND: *Sitona* weevils (Coleoptera: Curculionidae) are a species complex comprising pests of many leguminous crops worldwide, causing damage to young plants as adults and to rootlets as larvae, resulting in significant yield losses. Timely detection of migrating adult weevils is needed to determine when deployment of control measures becomes necessary. With the aim of developing plant volatile-based lures for *Sitona* spp. detection, we investigated the responses of *S. humeralis* to host plant-related aromatic compounds.

RESULTS: In olfactometer studies, both male and female *S. humeralis* responded positively to the odour of alfalfa flowers, a source of aromatic volatiles. In single sensillum recordings, basiconic sensilla located on the third and fourth terminal segments of the antennal club of both sexes were found to respond to benzaldehyde at doses of 10^{-5} and 10^{-4} g, suggesting that the weevil is able to detect this compound at the peripheral sensory level. In field studies, *S. humeralis* was attracted to benzaldehyde in the spring, but not in the autumn.

CONCLUSION: Benzaldehyde, as described in this study, may be a suitable candidate for the development of monitoring tools for *S. humeralis*.

Keywords: *Sitona*; Curculionidae; plant volatile; chemical communication; benzaldehyde

1 INTRODUCTION

Sitona spp. weevils (Coleoptera: Curculionidae), which include ~100 species,[1] are pests of legumes worldwide. Adults damage the foliage, which can weaken or kill the plant.[2] Furthermore, soil-dwelling developing larvae chew on rootlets and thicker root parts, which leads to primary damage to the nitrogen-fixing nodules.[3,4] *Sitona humeralis* Stephens is an important pest and a dominant species of its genus in alfalfa, *Medicago sativa* L. (Fabaceae), fields in Europe, Asia Minor, Iran and Central Asia; it is also present in several other leguminous crops as a member of the *Sitona* spp. complex.[5] *S. humeralis* overwinters as an adult, becoming active again in early spring.

Until recently, the only known field attractant for *Sitona* spp. was 4-methyl-3,5-heptanedione, a male-produced aggregation pheromone component of *S. lineatus* L.[6] The compound attracted *S. macularis* Herbst and *S. humeralis* in a preliminary study.[7] More recently, a bean-derived (*Vicia faba* L.) attractant blend was described for *S. lineatus*, which in some cases increased trap catches in combination with 4-methyl-3,5-heptanedione in the autumn, but not in the spring.[8] This and other findings[9–11] suggest that within the genus *Sitona* there is scope for the development of both single- and multiple-species monitoring systems based on plant-derived attractants.

The objective of this study was to develop aromatic host volatile compound-based lures for the model species, *S. humeralis*. Such lures may become a basis for the development of more effective attractant combinations. In a previous report, alfalfa flowers – which emit aromatic compounds characteristic of their volatile profile – were tested for attractiveness to *S. humeralis* in olfactometer studies. We tested known synthetic aromatic alfalfa compounds (benzaldehyde, methyl-salicylate, 2-phenethyl alcohol and phenylacetaldehyde)[12,13] for their behavioural activity in the field. We tested the electroantennography (EAG) activity

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Table 1. Legume-related aromatic compounds field-tested for the attraction of *Sitona humeralis*, and their known electrophysiological or behavioural activity in other weevil species

Compound	Species	Type of response	Reference
2-Phenethyl alcohol	*Ceutorhychus assimilis* Payk.	Electrophysiological	Evans and Allen-Williams[14]
Phenylacetaldehyde	*Ceutorhychus assimilis* Payk.	Electrophysiological	Blight *et al.*[15] Evans and Allen-Williams[14]
	Tanymecus dilaticollis Gyllenhal	Electrophysiological	Toshova *et al.*[16]
Benzaldehyde	*Ceutorhychus assimilis* Payk.	Electrophysiological	Blight *et al.*[15]
	Myllocerinus aurolineatus Voss	Behavioural in olfactometer	Sun *et al.*[17]
	Tanymecus dilaticollis Gyllenhal	Electrophysiological	Toshova *et al.*[16]
Methyl salicylate	*Ceutorhychus assimilis* Payk.	Electrophysiological	Blight *et al.*[15] Evans and Allen-Williams[14]
	Anthonomus musculus Say	Electrophysiological	Szendrei and Rodriguez-Saona[18]
	Tanymecus dilaticollis Gyllenhal	Electrophysiological	Toshova *et al.*[16]
Eugenol	*Anthonomus grandis grandis* Boheman	Behavioural in field	Armstrong[19]

of benzaldehyde (the only compound with field activity) using whole-antenna EAG and single sensillum recordings (SSR) to confirm its sensory detection. A legume-related volatile compound, eugenol,[13] was also included in the study, because it elicited behavioural responses from other weevil species (Table 1) and evoked electrophysiological responses in *S. humeralis* (Zs. Lohonyai, unpublished). Further field trapping tests were carried out to determine the level of attraction to particular candidate compounds.

2 MATERIALS AND METHODS

2.1 Four-arm olfactometry

A Perspex four-arm olfactometer was used to determine beetle behavioural responses to alfalfa floral headspace (Fig. 1),[20] which consisted of three layers held together with plastic nuts and bolts. Both the top and bottom discs had a diameter of 156 mm and a thickness of 5 mm, and were fitted with a filter paper base to provide traction for the walking insect. The middle part was 180 mm in diameter, 7 mm thick and was manufactured to include four side areas or arms (each 55 mm long × 5 mm high) situated at 90° to each other. The areas narrowed towards the perimeter and were connected to glass chambers with Teflon™ tubing, via a 3 mm diameter hole at the end of each of the four arms. One of the glass chambers contained four flower heads on a live *M. sativa* (treatment) and the other three were empty and acted as controls. The use of three empty control arms reduced the chances of accidental positive results, strengthening the statistics. Prior to each experiment, all glassware was washed with Teepol™ detergent, rinsed with acetone and distilled water, and baked in an oven overnight at 130 °C. Perspex components were washed with Teepol™ solution, rinsed with 80% ethanol solution and distilled water, and left to air dry. The olfactometer was surrounded by black paper and illuminated from above using diffuse uniform lighting from two 18 W/35 white fluorescent light bulbs shaded with a piece of red acetate. A single beetle was introduced into the middle of the olfactometer at each test period. Charcoal-filtered air was pumped into the glass chambers at a rate of 100 mL min^{-1}, then drawn through the central hole of the olfactometer at a rate of 300 mL min^{-1} using another vacuum pump (Charles Austen Pumps Ltd, Byfleet, UK), and thereby pulled through each of the four side arms (75 mL min^{-1} arm^{-1}), and subsequently exhausted from the laboratory. Each beetle was given 2 min to acclimatise, after which the experiment was run for 16 min at 24 °C, the olfactometer being rotated by 90° every 4 min to control for any directional bias. The olfactometer was divided into four regions corresponding to each of the four arms, and the time spent in each arm by a single beetle was recorded ($N = 10$/sex).

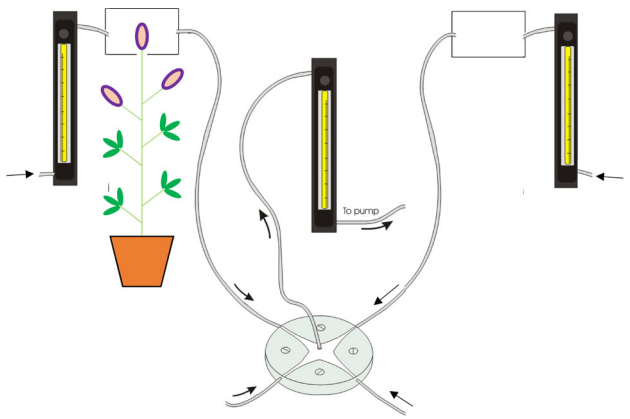

Figure 1. Diagram of four-arm olfactometer setup used in the behavioural assays.

2.2 Electroantennography

Feral *S. humeralis* weevils were collected at Biatorbágy and Tököl, (Pest County, Hungary). An antenna from a live female or male adult *S. humeralis* was excised at the base and mounted between two glass capillaries containing 0.1 M KCl solution. A constant humidified airflow of ~0.7 L min^{-1} was directed towards the antenna, which was placed at ~3 mm from the exiting airflow from a Teflon™-coated steel tube. One of the electrodes was grounded, and the other was connected to a high-impedance DC amplifier (IDAC-232; Ockenfels Syntech GmbH, Kirchzarten, Germany). Test compounds dissolved in 10 µL hexane were administered to a 5 × 5 mm piece of filter paper inside a Pasteur pipette. Stimulus treatments consisted of pushing 1 mL of air through the Pasteur pipette into the air stream flowing towards the antenna. Hexane was used as a solvent control, whereas pure air was the absolute control without any chemical stimulus. Response amplitudes were normalized against the means of responses to the standard (*Z*)-3-hexenol, which was tested before and after the test compounds.

Seven doses (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} g) of benzaldehyde were tested, also administered on filter paper at 10 µL volume dilutions to test for the dose-dependent responses of five male and eight female antennae.

2.3 Single sensillum recordings

The same source of beetles was used for SSR studies as for EAG studies. SSR were performed with sharp tungsten microelectrodes using standard equipment (Syntech). The beetle was inserted into a plastic pipette tip to immobilize the body, after which the head was moved forward outside the pipette tip and the antennae were fixed on a microscopy glass slide covered with glue (Tanglefoot, Planet Natural Ltd., Bozeman, MT, USA). A sharpened tungsten wire reference electrode was inserted into the head between the eyes. The basiconic sensilla on the immobilized antenna were localized under a light microscope (Olympus BX51WI) at ×750 magnification. The electrolytically sharpened tungsten-recording electrode was placed into the base of the sensillum using a micromanipulator (DC-3K, Märzhäuser-Wetzlar Gmbh & Co Kg, Wetzlar, Germany). Using a pre-amplification probe (Universal Single Ended AC/DC Probe PRS-1, Syntech), the extracellular analogue signal was amplified ×10, which was sampled using an integrated digital–analogue converter (IDAC-4, Syntech) and filtered with 50–60 Hz suppression. During the test, the antenna was kept under a humidified and charcoal-filtered air stream (1 L min^{-1}).

Seven dilutions (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} g) of benzaldehyde were prepared using mineral oil (Sigma-Aldrich Kft., Budapest, Hungary) as a solvent, and 10 µL of each dilution was applied on filter paper discs (12.7 mm diameter; Schleicher & Schnell GmbH, Dassel, Germany) and placed in a Pasteur pipette. Mineral oil alone was used as a control stimulus. A continuous air flow (1 L min^{-1}) delivered a 0.5 s stimulus using a stimulus controller (CS-55, Syntech). The number of action potentials (spikes) before stimulation (pre-stimulus) showed the spontaneous activity of the neuron involved. Thus, spike frequency was calculated by deducting the number of spikes before the stimulus onset (0.5 s) from the number of spikes during the stimulation (0.5 s) and calculating the net number of spikes per second observed.

2.4 Field experiments

2.4.1 Bait dispensers

Polyethylene sachet dispensers[21] were prepared putting a 1 cm piece of dental roll into a tight polyethylene bag (1.5 × 1.5 cm, wall thickness: 0.02 mm). Test compounds, all obtained from Sigma-Aldrich (> 95% pure) were administered on the dental roll and the sachet was heat sealed. For polyethylene vial dispensers, a 0.5 cm piece of dental roll was put into a 0.7 mL polyethylene vial with a lid (no. 730; Kartell S.p.a., Noviglio, Italy). Each polyethylene vial dispenser was loaded with 25 µL (~ 20 mg) of 4-methyl-3,5-heptanedione obtained from Rothamsted Research (Harpenden, UK). Dispensers were wrapped singly in aluminium foil and were stored at −30 °C prior to use.

2.4.2 Trap design

CSALOMON® TAL modified pitfall traps[22] were used. The catch container of the trap is a plastic tray (7 × 17.5 × 11.5 cm) sunk into a shallow hole in the soil. A roof made of a folded transparent plastic sheet is placed above the container, and two vertical side sheets on the ground are attached at soil level, thus providing a smooth surface for weevils entering the trap and leading them into the container. A sticky plastic sheet (16 × 10 cm, CSALOMON®, Plant Protection Institute, CAR, HAS, Budapest, Hungary) was placed at the bottom of the container to prevent weevils from escaping. Also, a piece of a household insecticide strip (Chemotox®, Sara Lee; Temana Intl. Ltd, Slough, UK; active ingredient 15% dichlorvos) was applied on the inner surface of the catch container of the trap to reduce chance escapes from the traps. The attractant dispenser was hung inside the roof of each treated trap, whereas control traps contained no dispenser.

2.4.3 Experimental sites

Experiments were conducted at two alfalfa field sites in Hungary during 2012–2016 (Table 2). Initially, experiments were carried out at Biatorbágy, but later a site at Tököl was used due to the higher abundance of *S. humeralis*. Four replicates of each trap/bait combination were set up at each experimental site, including baited and unbaited (control) traps of the respective designs. Traps within a replicate were ~ 10 m apart in a randomized complete block design. Traps were inspected twice weekly; captured weevils were collected and sticky inserts were replaced if necessary. Dispensers were replaced every second week. Collected specimens were identified to species level, as described by Endrődi.[23]

2.5 Statistics

For olfactometer experiments, the time spent in each arm by a single beetle was recorded using special software (OLFA, Udine, Italy). To account for the replication and areas within each replication as variance components in a split-plot design, the residual maximum likelihood method was used to fit a linear mixed model to the time spent data, nesting the areas within each replication and testing the treatment effect using an approximate *F*-test. The data

Table 2. Location, experimental period, volatile load details and the specific aims of single field experiments

Experiment	Site (GPS coordinates)	Experimental period	Aromatic compound load on one PE bag dispenser	Aim
1	Biatorbágy (47.470686, 18.821101)	10 April–8 May 2013	100 µL	Spring attraction
2	Tököl (47.318796, 18.970266)	29 March–23 May 2016	400 µL	Release rate-dependent attraction in spring
3	Biatorbágy (47.470686, 18.821101)	27 March–22 April 2014	300 µL	Spring attraction in combination with 4-methyl-3,5-heptanedione
4	Tököl (47.318796, 18.970266)	22 October–17 November 2015	400 µL	Autumn attraction
5	Biatorbágy (47.470686, 18.821101)	22 October–17 November 2015	400 µL	Autumn attraction
6	Tököl (47.318796, 18.970266)	28 October–10 November 2016	400 µL	Autumn attraction

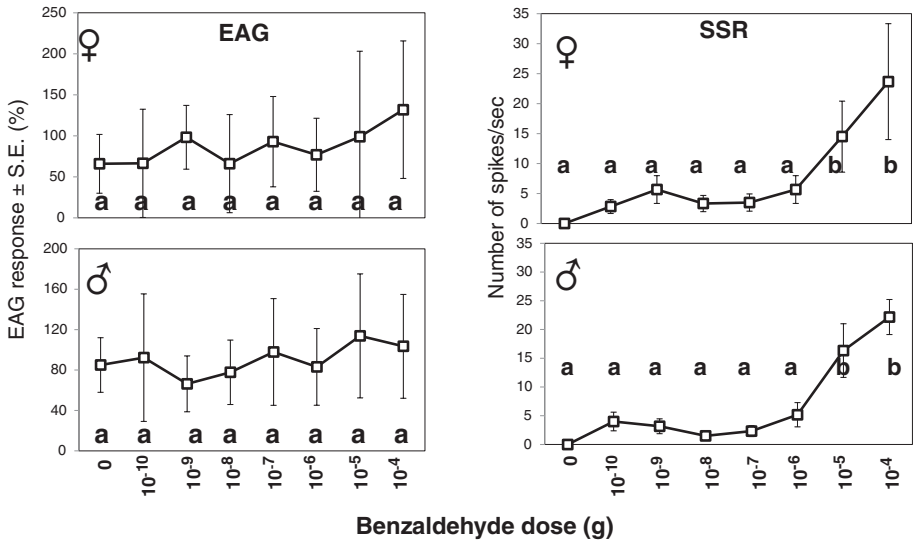

Figure 2. Electrophysiological responses of *Sitona humeralis* to different doses of benzaldehyde. (A) Electroantennogram responses: means of five males and eight females. (B) Single sensillum recordings: means of 6-6 sensilla of 6-6 male and female antennae. Means followed by the same letter are not significantly different; ANOVA and Fisher's protected LSD test ($P < 0.05$).

were analysed at the square root scale to account for some heterogeneity of variance over the treatments. Means were presented with standard error of the difference (SED) values for comparison, and the least significant difference (LSD) at the 5% ($P = 0.05$) level of significance was used for separation of means. The Genstat 18th Edition (VSN International Ltd, Hemel Hempstead, UK) was used for the analysis.

EAG and SSR data were subjected to analysis of variance (ANOVA) and the means were separated using Fisher's protected LSD test (StatView® v. 4.01 and Super ANOVA® v. 1.11; Abacus Concepts Inc., Berkeley, CA, USA).

For field data, statistical analyses were conducted using the software packages StatView® v. 4.01 and Super ANOVA® v. 1.11. Because transformation of the data did not allow the assumptions of a parametric test to be met, the non-parametric Kruskal–Wallis test was used. When the Kruskal–Wallis test indicated significant differences, pairwise comparisons by the Mann–Whitney *U* test were conducted.

3 RESULTS

3.1 Olfactometry

In four-arm olfactometer experiments, both female and male *S. humeralis* spent more time in the arm containing odour from *M. sativa* flowers, compared with the control arms [for females, mean ± SE 3.16 ± 0.52 *versus* 1.69 ± 0.16 min, $P < 0.001$, predicted means on square root scale: floral headspace = 1.72 ($n = 10$), control = 1.24 ($n = 30$), SED = 0.12, on 38 df; for males 2.1 ± 0.46 *versus* 0.65 ± 0.16 min, $P < 0.001$, predicted means on square root scale: floral headspace = 1.36 ($n = 10$), control = 0.56 ($n = 30$), SED = 0.12, on 38 df].

3.2 Electrophysiology

In EAG experiments, there was a slight increase in the response of male and female *S. humeralis* antennae to higher doses of benzaldehyde compared with controls, but there were no statistically significant trends due to high variability (Fig. 2). However, in SSR experiments on both male and female *S. humeralis* antennae, olfactory sensory neurons (OSN) were stimulated successfully by benzaldehyde in a clear dose-dependent manner. The male and female sensilla gave significantly greater responses to benzaldehyde doses of 10^{-5} and 10^{-4} g compared with lower doses (Fig. 2), and mineral oil as a solvent showed no activity in any of the OSNs tested. *Sensillum basiconicum* was identified as a morphological type of sensilla involved in detecting benzaldehyde on both male and female *S. humeralis* antennae. These sensilla are located on the third and fourth terminal segments of the antennal club.

3.3 Field experiments

In field experiment 1, only traps baited with benzaldehyde caught significantly more *S. humeralis* than unbaited traps ($P = 0.0026$, Mann–Whitney *U* test), indicating an attractant effect (Fig. 3A). There were no apparent differences between the sexes. In field experiment 2, *S. humeralis* female catches increased with increasing numbers of dispensers applied, catches with three and nine dispensers being significantly greater than in traps baited with a single dispenser or in unbaited traps (Fig. 3B). In males, a similar trend occurred, but only the greatest number of dispensers (nine) caught significantly more than unbaited traps. In field experiment 3, traps baited with benzaldehyde alone, 4-methyl-3,5-heptanedione alone and their combination resulted in significantly more *S. humeralis* catches in comparison with the unbaited traps. Catches of the three treatments containing baits were not significantly different from each other (Fig. 3C). In field experiments 4–6 in late October to early November, there was no effect of benzaldehyde treatment upon trap catches, whereas sweep netting indicated the presence of *S. humeralis* in large numbers at the site (Table 3).

4 DISCUSSION

In this study, olfactometer tests showed that both male and female *S. humeralis* responded positively to the odour of alfalfa flowers, suggesting that one or more of the compounds emitted could be attractive to the beetles. This notion was supported when among alfalfa related aromatic volatile compounds tested we

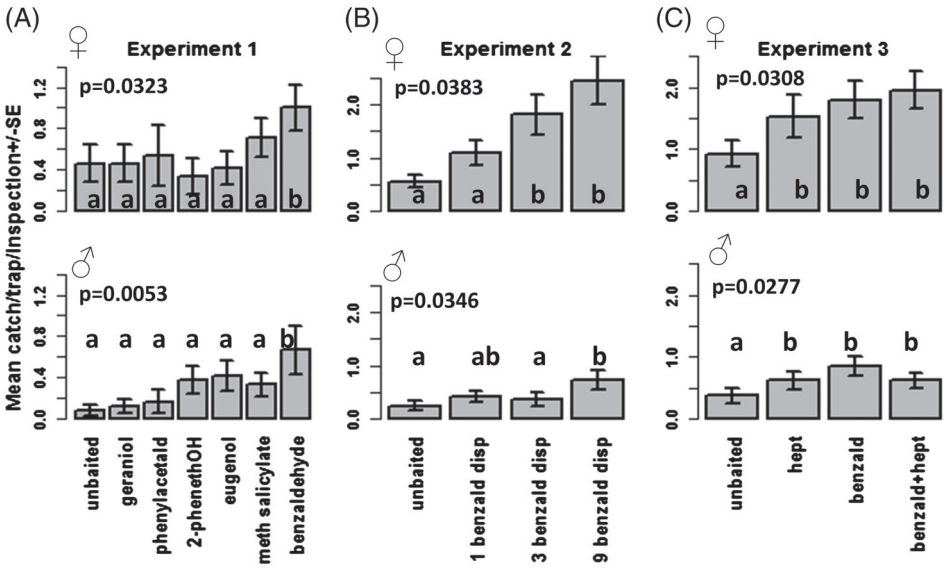

Figure 3. Mean numbers (±SE) of female or male *Sitona humeralis* in baited and unbaited traps. Results of: (A) experiment 1 (total numbers caught were 94 female and 56 male weevils), (B) experiment 2 (total numbers caught were 307 female and 92 male weevils) and (C) experiment 3 (total numbers caught were 248 female and 99 male weevils). *P*-values are derived from a Kruskal–Wallis test. Columns with the same letters within each diagram are not significantly different at $P = 0.05$ by Mann–Whitney *U* tests. Experiment 1: 2-phenethOH, 2-phenethyl alcohol; meth salicylate, methyl salicylate; phenylacetald, phenylacetaldehyde; Experiment 2: 1, 3, 9 benzald disp, 1, 3, or 9 benzaldehyde dispensers were used in the traps respectively. Experiment 3: hept, 4-methyl-3,5-heptanedione; benzald, benzaldehyde.

Table 3. Mean number (±SE) of *Sitona humeralis* in TAL traps with or without benzaldehyde between 22 October–17 November 2015 and 28 October–10 November 2016

Mean catch/trap/insp. ± S.E.	Experiment 4	Experiment 5	Experiment 6
Benzaldehyde	0.73 ± 0.03a	1.13 ± 0.10a	0.00 ± 0.00a
Unbaited	0.77 ± 0.04a	1.07 ± 0.09a	0.08 ± 0.08a
Total trap catch:	35	3	0
Total catch with 10 × 10 sweep netting:	107	103	883

Columns with the same letters within a row are not significantly different at $P = 0.05$ by Mann–Whitney *U*-tests.

found synthetic benzaldehyde attracting *S. humeralis* in the field. This compound is known to be released from alfalfa flowers and pods,[12,24] and is also present in leaves and flowers of white and red clover (*Trifolium repens* L., *Trifolium pratense* L.) (Fabaceae) used in forages (green or hay), as well as in their essential oil.[25,26]

The presence of olfactory sensilla in both sexes of *S. humeralis* that were responsive to benzaldehyde suggests that *S. humeralis* is able to detect this compound at the peripheral olfactory level. Because EAG responses are a summation of sensillum responses from the entire antenna,[27] the lack of significant EAG responses to this compound may be a consequence of an overall low frequency of benzaldehyde-sensitive sensilla on the antenna. Similar cases are well known from earlier literature on Lepidopteran pheromones. For example, in male *Agrotis segetum* Den. et Schiff., very high EAG responses could be recorded to the pheromone components (*Z*)-5-decenyl acetate and (*Z*)-7-dodecenyl acetate, whereas no significant response could be recorded to (*Z*)-9-tetradecenyl acetate, which was explained by a lack of trichoid sensilla that were tuned for this compound.[28]

In the closely related *S. lepidus* Gyllenhal, four types of olfactory sensilla have been described in males and five in females,[29] which contain OSNs responsive to plant volatile compounds. The club-shaped terminal of the seventh flagella segment consisted of four subsections, which harbour the highest numbers of trichoid and basiconic sensilla, mostly on the circumferential bands of the first three subsections. Some 110 sensilla were found to contain OSNs responsive to plant volatile compounds, which showed specialization in their responsiveness, and responded only to a narrow range of plant volatile compounds. Benzaldehyde and phenylacetaldehyde elicited significant responses from OSNs of one type of sensilla on *S. lepidus* males, whereas a compound with a closely related structure, 2-phenethyl alcohol, elicited a strong response from the OSNs of two other types of sensilla in both males and females.[29]

Interestingly, in our experiments, *S. humeralis* responded to benzaldehyde in the spring only, but not in the autumn. Such season-dependent behavioural responses are not unprecedented in *Sitona* spp., as a mixture of bean plant-derived compounds, namely (*Z*)-3-hexen-1-yl acetate, (*Z*)-3-hexen-1-ol and linalool,[30] enhanced catches of *S. lineatus* in traps baited with the aggregation pheromone, 4-methyl-3,5-heptanedione, in the autumn, but not in spring.[8] In this study, we confirmed the attractiveness of 4-methyl-3,5-heptanedione for *S. humeralis*, described previously by Tóth *et al.*[7] However, when presented together with benzaldehyde, neither compound enhanced the activity of the other, suggesting that they are involved in different chemical communication channels.

To date in *Sitona* (Entiminae), there has been only one attractant combination tested successfully for the attraction of *S. lineatus*.[8,30] However, in this study a single alfalfa related aromatic compound alone attracted *S. humeralis* in the field.

The additional or even synergistic effect of combining either plant-derived compounds or natural plant material with pheromone components has been reported in a number of distinct weevil subfamilies including Rhynchophorinae,[31–35] Calendrinae,[36] Dryophthorinae[37] and Curculioninae.[10] Some weevil species have been found to be attracted to host plant volatiles alone in the field, including the cabbage seed weevil, *Ceutorhychus assimilis* Payk. (Ceutorhynchinae),[11,38] and the banana weevil, *Cosmopolites sordidus* Germar (Dryophthorinae).[39] For *S. lineatus*, attraction to host plant volatiles was shown in laboratory olfactometer bioassays.[40]

Benzaldehyde, the attractant described in this study, was shown previously to synergise the response of plum curculio, *Conotrachelus nenuphar* Herbst (Molytinae) to the male-produced pheromone grandisoic acid.[41] Using a Y-tube olfactometer, benzaldehyde and the structurally close benzyl alcohol were attractive to both males and females of another species in the Entiminae subfamily, *Myllocerinus aurolineatus* Voss.[17] Furthermore, Blight *et al.*[15] showed that benzyl alcohol increased trap catches of *C. assimilis* compared with the unbaited treatment.

5 CONCLUSION

The primary aim of *Sitona* spp. pest management is to prevent foliar damage of young plants by beetles or of root damage by larvae, both of which may result in a significant decrease in plant density or yield. Suitable tools for detection of the seasonal appearance of adults could help to mitigate severe damage, and semiochemical-baited traps might form the basis of such a tool for plant protection monitoring systems.[42,43] Benzaldehyde, as described in this study, may be a suitable candidate for the development of such monitoring tools for *S. humeralis*.

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CONFLICTS OF INTEREST

The authors declare that they have no competing financial and/or non-financial interests in relation to the work described.

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