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# Journal of Chemical Ecology

## FIELD ACTIVITY OF A SEMIOCHEMICAL LURE FOR THE LEGUME PEST ACANTHOSCELIDES OBTECTUS SAY (COLEOPTERA: CHRYSOMELIDAE) --Manuscript Draft--

Manuscript Number:	JOCE-D-21-00084R	
Full Title:	FIELD ACTIVITY OF A SEMIOCHEMICAL LURE FOR THE LEGUME PEST ACANTHOSCELIDES OBTECTUS SAY (COLEOPTERA: CHRYSOMELIDAE)	
Article Type:	Original Research	
Keywords:	-Bruchid beetle; Chrysomelidae; EAG; olfactometry; attractant; lure; trapping	
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Abstract:	<p>The dried bean beetle, <i>Acanthoscelides obtectus</i>, is an economically important worldwide pest of legume crops including dry beans, <i>Phaseolus vulgaris</i>. Assessment of <i>A. obtectus</i> infestation levels in pre-harvest field crops and in post-harvest granaries is difficult to achieve, as there is no effective monitoring tool for early detection and deployment of interventions. As <i>A. obtectus</i> is a generic pollen and nectar feeder, we adopted an electrophysiological (EAG) screening approach using the antennae of female <i>A. obtectus</i> to identify physiologically active plant volatile organic compounds, which could then be investigated for their attractiveness to <i>A. obtectus</i> in laboratory behaviour assays and preliminary field tests. Of the 27 compounds tested in the EAG</p>	

	<p>screening, 5 compounds, i.e. methyl anthranilate, methyl eugenol, benzyl alcohol, (RS)-lavandulol and 2-phenylethanol, were shown to possess activity greater than the standard (1-phenylethanol). In 4-arm olfactometer bioassays, female <i>A. obtectus</i> preferred the arm of olfactometer containing the odour of either methyl anthranilate or benzyl alcohol compared to the solvent control. In preliminary field tests using these 2 compounds as a binary mixture, at least 5 times as many beetles were caught on baited traps compared to non-baited traps. The field data also suggested that benzyl alcohol was more responsible for the field activity of the blend. We hypothesize that the attraction of <i>A. obtectus</i> to the combined benzyl alcohol/methyl anthranilate and the single benzyl alcohol baits is connected to the species' nectar- and pollen-feeding behaviour and not to its intraspecific communication. To our knowledge, this is the first evidence that <i>A. obtectus</i> behaviour in the field can be modified by the deployment of plant-derived semiochemicals.</p>
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Dear Dr Felton,

We are very grateful for the reviewers' comments on our manuscript JOCE-D-21-00084; they helped us re-shape it in a format which will hopefully be acceptable now for publication in Journal of Chemical Ecology. Our answers in capital letters follow the reviewers' points.

Yours sincerely,

József Vuts

### Reviewer 1

Lines 103-109: Inbred population?

THIS HAS BEEN CORRECTED TO "*ACANTHOSCELIDES OBTECTUS* BEETLES WERE OBTAINED FROM A LABORATORY INBRED POPULATION REARED ON DRY 'CANNELLINI' BEANS (*PH. VULGARIS*)" (LINE 104).

Line 108: Acetate sheet may not be well known by many Readers. A short description, or simply giving a synonyme name would be useful.

`ACETATE` HAS BEEN CORRECTED TO `PLASTIC` (LINE 109).

In the discussion, I suggest to briefly reflect to the followings:

Some of those compounds, ranked as less active in EAG, could still be attractant-synergists.

THIS IS A FAIR POINT; PLEASE SEE LINES 299-304 STATING THAT COMPOUNDS DEEMED LESS EAG-ACTIVE CAN BE POTENTIAL FIELD ATTRACTANTS.

Were other bruchid species also captured in the field tests?

THERE WERE NO OTHER BRUCHID SPECIES CAUGHT.

Floral baits target unmated adults in the field. When used in granaries, probably dominated by mated beetles, would this bait still attractive enough for monitoring the population level of the pest? THANK YOU FOR THIS SUGGESTION. THE UNCERTAINTY ABOUT THE POTENTIAL INACTIVITY OF THE SYNTHETIC BLEND IN GRANARIES IS NOW DISCUSSED IN LINES 369-372.

### Reviewer 2

The title could indicate to the reader that the semiochemicals are from host plants.

Field activity of plant host semiochemicals lure for the legume pests.....

or

Lures with two host plants semiochemicals, methyl anthranilate and benzyl alcohol, are attractive to *A. obtectus* in field experiments.

THANK YOU FOR THIS SUGGESTION. WE AGREE THAT BOTH BENZYL ALCOHOL AND METHYL ANTHRANILATE ARE WIDESPREAD PLANT COMPOUNDS; HOWEVER, THEY ARE ALSO IDENTIFIED AS INSECT PHEROMONE COMPONENTS. THUS, WE FEEL THAT `SEMIOCHEMICAL` EXPRESSES THE NATURE OF THE LURE MORE PRECISELY.

Introduction

Line 61. The authors could provide a reference about the presence of the beetles in the field and

granaries.

REFERENCE 'SOUTHGATE 1979' ADDED (LINE 61).

Lines 75-81. The authors report that the pheromone components of *A. obtectus* was revisited by one of the authors of this manuscript and that new components were identified, but in the last sentence of this paragraph, the authors report that there is no attractant lure to this species. I was wondering if the pheromone of this species was not evaluated in the field. Reading the paper of Vuts et al., 2015, I observe that the pheromone of this species is complex and probably with high cost. I think it could be interesting if the authors could clarify this to the reader in the introduction. In general, pheromone are more potent for insect attraction compared to host plant volatiles. The authors comment on this on line 362-364, but I think the authors could comment about this in the introduction.

THIS POINT ABOUT THE CURRENT ISSUES AROUND FIELD USE OF THE PHEROMONE HAS NOW BEEN MADE IN THE INTRODUCTION (LINES 81-82).

#### Materials and methods

Line 132. Why was the compound 1-phenethyl alcohol used as a control? What does mean a medium-sized response? Was this response compared to all other components (26) evaluated? 1-PHENETHYL ALCOHOL WAS USED AS A POSITIVE STANDARD BEFORE AND AFTER THE PANEL OF COMPOUNDS TO FACTOR IN THE DECAY OF EAG RESPONSES OVER TIME IN THE DETERMINATION OF EAG ACTIVITY. WE EMPLOYED THIS METHOD TO NARROW DOWN THE RANGE OF COMPOUNDS TO THE MOST POTENT ONES FOR USE IN SUBSEQUENT ASSAYS, INSTEAD OF COMPARING ANTENNAL RESPONSES TO THE SOLVENT OR AIR CONTROLS. THE APPROACH WAS PUBLISHED BY ROELOFS [ROELOFS W.L. (1977): THE SCOPE AND LIMITATIONS OF THE ELECTROANTENNOGRAM TECHNIQUE IN IDENTIFYING PHEROMONE COMPONENTS. IN: CROP PROTECTION AGENTS – THEIR BIOLOGICAL EVALUATION. ED.: MCFARLANE NR. ACADEMIC PRESS, NEW YORK, PP. 147–165] AND HAS PROVEN USEFUL IN A NUMBER OF STUDIES TO DEVELOP STRONG ATTRACTANTS (FOR A FEW EXAMPLES, PLEASE SEE INTRODUCTION, LINE 93, AND DISCUSSION, LINE 304).

Line 161. The authors could provide the film thickness of the column used.  
FILM THICKNESS OF GC COLUMN HAS BEEN ADDED (LINE 164).

Figures 1 and 2. The letter are too small, difficult to read.

THANK YOU FOR POINTING THIS OUT. WE HAVE INCREASED FONT SIZE ON BOTH FIGURES.

[Click here to view linked References](#)

1 FIELD ACTIVITY OF A SEMIOCHEMICAL LURE FOR THE LEGUME PEST *ACANTHOSCELIDES OBTECTUS*  
2 SAY (COLEOPTERA: CHRYSOMELIDAE)  
3  
4

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47 27 **Abstract**-The dried bean beetle, *Acanthoscelides obtectus*, is an economically important worldwide  
48 28 pest of legume crops including dry beans, *Phaseolus vulgaris*. Assessment of *A. obtectus* infestation  
49 29 levels in pre-harvest field crops and in post-harvest granaries is difficult to achieve, as there is no  
50 30 effective monitoring tool for early detection and deployment of interventions. As *A. obtectus* is a  
51 31 generic pollen and nectar feeder, we adopted an electrophysiological (EAG) screening approach  
52 32 using the antennae of female *A. obtectus* to identify physiologically active plant volatile organic  
53 33 compounds, which could then be investigated for their attractiveness to *A. obtectus* in laboratory  
54 34 behaviour assays and preliminary field tests. Of the 27 compounds tested in the EAG screening, 5

compounds, i.e. methyl anthranilate, methyl eugenol, benzyl alcohol, (*RS*)-lavandulol and 2-phenylethanol, were shown to possess activity greater than the standard (1-phenylethanol). In 4-arm olfactometer bioassays, female *A. obtectus* preferred the arm of olfactometer containing the odour of either methyl anthranilate or benzyl alcohol compared to the solvent control. In preliminary field tests using these 2 compounds as a binary mixture, at least 5 times as many beetles were caught on baited traps compared to non-baited traps. The field data also suggested that benzyl alcohol was more responsible for the field activity of the blend. We hypothesize that the attraction of *A. obtectus* to the combined benzyl alcohol/methyl anthranilate and the single benzyl alcohol baits is connected to the species' nectar- and pollen-feeding behaviour and not to its intraspecific communication. To our knowledge, this is the first evidence that *A. obtectus* behaviour in the field can be modified by the deployment of plant-derived semiochemicals.

**Key Words**-Bruchid beetle, Chrysomelidae, EAG, olfactometry, attractant, lure, trapping.

## INTRODUCTION

This paper is dedicated to the memory of Prof. Dr. Dr. mult. h. c. Wittko Francke. In recent years, we worked intensively together with him on the pheromone communication of the dried bean beetle, which culminated in the complete identification of the pheromone (Vuts et al. 2015a). The present paper describes our results on the beetle's non-pheromone chemical ecology, which we strongly hope Prof. Francke will view with goodwill from above. Let him rest in peace, may light eternal shine upon him!

Bruchids (Coleoptera: Chrysomelidae, Bruchinae) are usually small (2-5 mm), oval-shaped beetles. Their larvae develop primarily in legume seeds (Fabaceae) that they infest in the field and complete a single generation yearly. There are some species, however, which can infest host seeds both in the field and in granaries (Southgate 1979). As their development depends often only on temperature, they can have multiple generations a year, making their damage economically significant. Such bruchids are mono- or oligophagous (Szentesi 1990). One of these species is the dried bean beetle, *Acanthoscelides obtectus* Say, which originated in tropical America and is now a key pest of dry beans (*Phaseolus vulgaris* L.) worldwide (Alvarez et al. 2005). The control of *A. obtectus* using various chemical, biological, mechanical and cultural methods has met with varied success (Vétek et al. 2017). The ban of methyl bromide as a fumigating agent (Mouttet et al. 2014) initiated attempts to use alternative chemistries (Shaaya and Kostyukovsky 2010), bruchid-resistant bean varieties (Velten

et al. 2007), natural enemies (Velten et al. 2008) and protective storage (Mutungi et al. 2015). *A. obtectus* management is still lacking sensitive and specific detection and monitoring approaches. However, similar to other stored product pest insects, semiochemical-based strategies may provide environmentally benign integrated pest management tools for pest surveillance and direct reduction of local *A. obtectus* populations (Trematerra 2012).

The chemical ecology of *A. obtectus* has been studied over a number of decades. The major component of a male-produced pheromone of *A. obtectus* was amongst the first insect pheromone components to be identified (Horler 1970), and more recently, full characterization of other pheromone components was reported (Vuts et al. 2015a, b, 2018a). Furthermore, a synthetic blend of floral compounds identified from a nectar plant, *Daucus carota* L. (Apiaceae), was found to be behaviourally active in lab tests (Vuts et al. 2018b). However, no attractant lure with field activity has been optimised to date partially because, for example, the male pheromone blend is complex and some of its constituents are difficult to synthesize.

As pollen consumption being a prerequisite for normal ovary production (Huignard and Leroi 1981), *A. obtectus* visits a wide range of flowering plants for pollen and nectar (Zachariae 1958; Jarry 1987; Szentesi 1990). Bruchids utilise floral volatiles to locate flowers (Bruce et al. 2011), and ubiquitous, commercially available compounds, previously found to represent attractive floral cues for a number of flower-visiting species (e.g. Toshova et al. 2016), have been screened for their electrophysiological and behavioural bioactivity, with the most active ones having been then trialled under field conditions. Our selection of putative attractants was informed by the volatile composition of flowers visited by *A. obtectus* (Table 1). This approach of screening a synthetic panel of compounds in lab assays to find proxies with field activity has been useful in the development of powerful lures for numerous pest insect species (e.g. Beroza et al. 1961; Davis et al. 1969; Lohonyai et al. 2018). The laboratory experiments presented in this paper focussed on virgin females, because it was assumed that they are most attracted to flower volatiles, similar to other herbivorous insects where mating induces behavioural preference changes from nectar plant to larval host (e.g. Saveer et al. 2012). Females also directly determine the size of future generations, thus an attractant trap that captures also females enables more precise pest forecasting and monitoring (e.g. Bruce et al. 2011). Thus, the aim of the present study was to address the urgent need for an attractant that can be subsequently used in trap development to detect and monitor *A. obtectus* in the field and store houses.

## MATERIALS AND METHODS



*Insects* *Acanthoscelides obtectus* beetles were obtained from a laboratory inbred population reared on dry ‘Cannellini’ beans (*Ph. vulgaris*). The original population was established from a natural infestation on *Ph. vulgaris* in Hungary ca. 50 years ago. Maintenance conditions were as follows: artificial lighting with a 16:8 h L:D photoperiod, a constant temperature of 20°C, and 60% RH. In order to obtain virgin insects, seeds were kept individually in wells of an Eppendorf rack and covered with a piece of transparent plastic sheet until beetle emergence, at which point the sexes were separated immediately (based on shape of and patterns on the pygidium; Kaszab 1967) for use in experiments.

*Chemicals* 1-Phenylethanol (98%), methyl anthranilate (>98%), (*E*)-cinnamaldehyde (99%), 4-methoxyphenethyl alcohol (99%), benzyl acetate (99%), phenethyl acetate (>98%), (*RS*)-lavandulol (>99%), eugenol (99%), (*E*)-4-methoxycinnamaldehyde (>98%), isoamyl alcohol (>98%), (*E*)-anethol (99%), 2-methyl-1-propanol (>99%),  $\beta$ -ionone (>97%), benzyl alcohol (>99%), methyl salicylate (>99%), (*E*)-cinnamyl acetate (99%), phenylacetaldehyde (>95%), isoamyl acetate (>97%), methyl eugenol (>98%), (*E*)-cinnamyl alcohol (>98%), 2-phenylethanol (>99%), (*RS*)-linalool (97%), isobutyl acetate (>97%), benzaldehyde (>99%), anisyl acetone (98%) and geraniol (98%) were from Sigma-Aldrich, Hungary. (*E*)-Isosafrole (>95%) was from Aurora Fine Chemicals Ltd, Austria.

*Electroantennography (EAG)* To measure the EAG responses of virgin female *A. obtectus* antennae to the panel of synthetic compounds listed above (also see Table 1), an antenna freshly amputated at the base from a live beetle was mounted between two glass capillaries each containing 0.1 M KCl solution and connected to silver wire electrodes (0.37 mm diam., Biochrom Ltd., UK), then placed at ca. 3 mm distance from a stainless steel tube (10 mm diam., teflon-coated inside) with a constant charcoal-purified and humidified airflow exiting at ca. 0.7 L/min. The recording electrode was connected to a high-impedance DC amplifier (IDAC-232, Ockenfels Syntech GmbH, Kirchzarten, Germany). Ten  $\mu$ g of each compound was delivered in 10  $\mu$ L hexane solution onto a 1 cm<sup>2</sup> piece of filter paper inside a Pasteur pipette. This dose was thought to be sufficient to balance for differences in volatility by saturating the air space inside the Pasteur pipettes (Roelofs 1977). Stimuli consisted of pushing 1 mL of air through the Pasteur pipettes into the airstream flowing towards the antenna (n=4 from four different individuals). Response amplitudes were normalized against the standard (*RS*)-1-phenethyl alcohol (that elicited medium-sized, i.e. 0.3-0.4 mV, responses), which was administered before and after the test compounds. Stimuli were delivered at ca. 20-30 s intervals in random order.

A log-to-base 10 transformation on normalised EAG data was used to account for some heterogeneity of variance over chemical stimuli. ANOVA, providing an F-test for the overall difference between stimuli, was followed by application of Fisher's least significant difference (LSD) test ( $p < 0.05$ ) for the statistical separation of means. The Genstat (2015, 18th edition, VSN International Ltd, Hemel Hempstead, UK) statistical package was used for the analysis.

*Preparation of Stable-Release Glass Microcapillaries* To achieve a consistency of compound concentration in the olfactometer airflow and to offer the test beetles similar number of molecules across all compounds during the course of olfactometer experiments, test compounds were filled into glass microcapillaries (1-10  $\mu$ L; Blaubrand® intraMARK, BRAND GmbH, Wertheim, Germany/Drummond MICROCAPS®, Drummond Scientific Company, Broomall, PA, USA) previously heat-sealed at one end. A standard Pasteur pipette was then melted at the point where the narrow half widened, and subsequently pulled apart to form a very thin thread of capillary tube, the end section of which was snapped off. Neat test compounds were separately taken up with a pipetting bulb and the capillary tube end of the Pasteur pipette was inserted onto the bottom of a glass microcapillary. Each pure compound was injected individually into a separate glass microcapillary, the end of which was snapped off at ca. 1 mm distance from the meniscus. Glass microcapillary dispensers prepared this way were kept in a closed glass vial at  $-20^{\circ}\text{C}$  until use.

To determine the release rates of compounds from the dispensers, dynamic headspace collection (air entrainment) from the headspace of each dispenser type loaded with a compound was undertaken ( $n=3$ ). Charcoal-purified air was pumped into the headspace of a glass chamber (5 cm  $\times$  9 cm i.d., Biochem Glass Apparatus Ltd, Milton Keynes, UK), attached to a metal plate with bulldog clips, at a rate of 600 mL/min, and subsequently pulled out at 500 mL/min through 50 mg Porapak Q 50/80 adsorbent polymer (Sigma-Aldrich, Gillingham, UK), sandwiched between glass wool plugs in a glass tube (4 mm diam.). Each collection lasted for 16 min, i.e. the duration of an olfactometer test. Trapped compounds were eluted from the adsorbent with 750  $\mu$ L freshly distilled diethyl ether, and extracts analysed on a high-resolution GC using an Agilent 6890A gas chromatograph equipped with a cool on-column injector, an FID and a 50 m  $\times$  0.32 mm ID, 0.52  $\mu$ m film thickness HP-1 column (J & W Scientific). The oven temperature was maintained at  $30^{\circ}\text{C}$  for 1 min, then programmed at  $5^{\circ}\text{C}/\text{min}$  to  $150^{\circ}\text{C}$  and held for 0.1 min, then programmed at  $10^{\circ}\text{C}/\text{min}$  to  $250^{\circ}\text{C}$  and held for 20 min. The carrier gas was hydrogen (3.1 mL/min flow rate). Quantification of compounds was achieved using the single-point external standard method with a series of C7-C22 alkanes.

*Olfactometer tests* A four-arm olfactometer was used to measure female *A. obtectus* responses to synthetic compounds. Five highly EAG-active compounds, as well as (*E*)-anethole because of its attractiveness to the closely related *A. pallidipennis* Motschulsky in a field trapping trial (I. Szarukán and M. Tóth, unpublished data), were selected for behavioural tests (Table 2). Glass microcapillary dispensers containing the same compound were fed through holes made on a 1 cm diam. PTFE septum (Thermo Scientific, Waltham, MA, USA).

The olfactometer consisted of three layers of Perspex, held together with plastic nuts and bolts. Both the top and bottom discs had a 156 mm diameter and 5 mm thickness, and the bottom disc was fitted with a filter paper base to provide traction for the walking insect. The middle part was 180 mm in diameter and 7 mm thick and was manufactured to embody four side areas or arms (55 mm in length × 5 mm height each) situated at 90° to each other. The side areas narrowed towards the perimeter. Glass arms (narrow part: 50 mm length × 2.5 mm diam., wide part: 90 mm length × 20 mm diam.) were attached through a 3 mm diameter hole to the end of each of the four arms. Prior to each experiment, all glassware was washed with Teepol (Orpington, UK) detergent, rinsed with acetone and distilled water and baked overnight at 160°C. Perspex components were washed with Teepol solution, rinsed with 80% ethanol solution and distilled water, and left to air-dry. The olfactometer was illuminated from above by diffuse uniform lighting from two 18W/35 white fluorescent light bulbs screened with red acetate. The device was surrounded by black paper to remove any external visual stimuli.

Only one glass arm was treated with loaded glass microcapillary dispensers at a time, whereas each of the three control arms contained an empty glass capillary. This setup ensured the robustness of the experiment by making it less likely for an insect to accidentally walk in or out of the treated region. A single 3-6-day old virgin female was introduced through a hole in the top of the olfactometer. Air was drawn through the central hole by a vacuum pump and, consequently, pulled through each of the four side arms (75 mL/min/arm) and subsequently exhausted from the room. Each beetle was given 2 min to acclimatize in the olfactometer (the room temperature was 20°C and RH 60%), after which the experiment was run for 16 min. The olfactometer was rotated 90° every 2 min to control for any directional bias. The olfactometer was divided into five regions that corresponded to each of the four glass arms and the central compartment, and the time spent in each area was recorded using specialist software (OLFA, Udine, Italy) (n=10/compound).

In order to account for the replication and areas within each replication as variance components in a split-plot design, the method of residual maximum likelihood (REML) 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 were analysed on the square root scale to account for

some heterogeneity of variance over the treatments. Means are presented with standard error of the difference (SED) values for their comparison, and the least significant difference (LSD) at the 5% ( $P=0.05$ ) level of significance was used for separation of means. Genstat (18<sup>th</sup> edition; VSN International Ltd, Hemel Hempstead, UK) was used for the analysis.

*Field tests* Preliminary trials aimed at assessing field activity of synthetic benzyl alcohol and methyl anthranilate (the two compounds preferred by female *A. obtectus* in olfactometer studies) were conducted at Nagydobrony in the Kárpátalja region of the Ukraine between 10 August – 17 September 2020, using accepted methods for trapping experiments of the same nature (Roelofs and Cardé 1977). In the tests, 23 × 36 cm light green (described as fluorescent yellow; for reflectance spectrum, see Schmera et al. 2004; Tóth et al. 2004; Jenser et al. 2010) PVC sheets (sticky on one side with Tangle Trap Insect Glue, Tanglefoot Co. Grand Rapids, MI 49504) were used as traps. Bait dispensers were suspended in the middle of the upper part in front of the sticky surface. Traps were suspended from poles at a height of 100-120 cm along the edge of a *Ph. vulgaris* bean field during the main flowering period (BBCH scale 65; Feller et al. 1995). Traps were arranged in a randomised complete block design, so that each block contained one trap of each treatment. Traps within blocks were separated by 8 – 10 m and blocks were at least 30 m apart. Traps were inspected twice weekly, when captured *A. obtectus* were recorded and removed.

The decision on dispenser type to be used in the field trapping tests was difficult, since no attractant with field activity was known, hence no previous information was available on field attraction behaviour of *A. obtectus*. Therefore, we decided to formulate synthetic compounds in two dispenser types:

PE bag dispenser: a 1 cm piece of dental roll (Celluron®, Paul Hartmann AG, Heidenheim, Germany) was placed into a tight polyethylene bag made of 0.02 mm linear polyethylene foil. The dimensions of the polyethylene sachets were ca. 1.5 × 1.5 cm. The dispenser was attached to a plastic strip (8 × 1 cm) for easy handling when assembling the traps. For making up the lures, compounds were administered onto the dental roll and the opening of the polythene bag was heat-sealed and the dispensers were wrapped individually in pieces of aluminium foil. The dose of single compounds was 200 mg/dispenser. PE bag dispensers have successfully been used to dispense various floral compounds to capture beetles (Tóth et al. 2003a), moths (Tóth et al. 2010, 2014, 2019) and lacewings (Tóth et al. 2009).

PE vial dispensers: bait dispensers were prepared by adding 100 mg of synthetic compounds into 0.7 mL polyethylene vials with lid (No. 730, Kartell Co., Italy, wall thickness ca 0.5 mm). After loading, the lid of the dispensers was closed and the dispensers were wrapped individually in pieces of

aluminium foil. PE vial dispensers were found to be efficient in attracting beetles (Tóth et al. 2003b) and moths (Tóth et al. 2020).

Release rates of PE bag dispensers for benzyl alcohol were 4.68 mg/day and for methyl anthranilate 4.42 mg/day (n=4 for each compound), and those of PE vial dispensers for benzyl alcohol were 1.85 mg/day and for methyl anthranilate 0.77 mg/day (n=4 for each compound). Release rate estimates were performed by gravimetric analysis, measuring at ca. two day-intervals the weight loss of dispensers for two weeks in a wind tunnel (20°C, 0.2 m/s windspeed). PE vial dispensers (supposedly because their wall is much thicker) were emitting at a lower rate (and therefore would last longer in the field) than PE bag dispensers (Fig. 1). Earlier experience with synthetic floral compounds showed that the PE bag dispensers did not lose their activity after several weeks of field exposure (Tóth et al. 2010, 2019); hence, it was decided that it was safe to renew all types of lure dispensers at two-week intervals. Release rate values from lab experiments also indicated that lures might not dispense all of their chemical load during this time period.

Experiment 1 assessed the field attractiveness of lures containing both compounds vs unbaited control traps. Because there was no information on the optimal dose of test compounds for *A. obtectus* field attraction, the combined use of both dispenser types in the same trap was thought to compensate for 1) possible too slow release from the PE vial by initial higher release from the PE bag, or 2) volatile release from the PE bag quickly running below the detection threshold of beetles compensated for by a lower, but more stable release from the PE vial. The baited treatment thus consisted of benzyl alcohol and methyl anthranilate in both PE vial and PE bag dispensers, with all 4 dispensers applied in the same trap. The test was run in a 0.25 ha bean field in four replicates.

Experiment 2 compared field activity of single compounds. The following treatments were compared: 1) benzyl alcohol in both PE vial and PE bag dispensers applied in the same trap; 2) methyl anthranilate in both PE vial and PE bag dispensers applied in the same trap; 3) unbaited control traps. The test was run in a 0.3 ha bean field in three replicates.

As it is frequently found in field trapping experiments, the catch data (even after transformation) did not fulfil requirements for a parametric analysis. Therefore, data were analysed by the non-parametric Kruskal-Wallis test. When the Kruskal-Wallis test showed significance (p=5%), differences between treatments were analysed by pairwise comparisons with Mann-Whitney U test. All statistical procedures were conducted using R 3.6.2 (R Core Team 2019), dplyr (v0.8.3) (Wickham et al. 2020) and ggplot2 (v3.2.1) (Wickham et al. 2019) packages.

## RESULTS

Methyl anthranilate, methyl eugenol, benzyl alcohol, (*RS*)-lavandulol and 2-phenylethanol elicited stronger EAG responses from female *A. obtectus* antennae than the solvent hexane or blank air ( $p < 0.001$ , ANOVA) (Fig. 2). EAG amplitudes were in the 0.1-0.5 mV range.

Of the five compounds chosen based on their EAG activity [plus (*E*)-anethole], virgin female *A. obtectus* showed positive behavioural responses only towards methyl anthranilate (*F*-test:  $F = 10.78$ ;  $df = 1, 38$ ;  $P = 0.002$ ) and benzyl alcohol (*F*-test:  $F = 8.16$ ;  $df = 1, 38$ ;  $P = 0.008$ ) when tested against blank air in the four-arm olfactometer, the beetles staying on average ca. twice as long in the treated arm than the control arms (Fig. 3A and C, resp.). Methyl eugenol (*F*-test:  $F = 0.11$ ;  $df = 1, 38$ ;  $P = 0.746$ ), (*RS*)-lavandulol (*F*-test:  $F = 2.61$ ;  $df = 1, 38$ ;  $P = 0.115$ ), 2-phenethyl alcohol (*F*-test:  $F = 1.79$ ;  $df = 1, 38$ ;  $P = 0.192$ ) and (*E*)-anethole (*F*-test:  $F = 0.87$ ;  $df = 1, 38$ ;  $P = 0.356$ ) did not elicit any behavioural preference from the beetles (Fig. 3B, D, E and F, resp.).

In pilot field trials, traps baited with benzyl alcohol plus methyl anthranilate caught significantly more *A. obtectus* than unbaited traps, which caught only single specimens sporadically (Experiment 1, Fig. 4A). In Experiment 2, traps baited with benzyl alcohol caught significantly more *A. obtectus* than traps baited with methyl anthranilate alone or unbaited traps. The latter two treatments did not differ from each other (Fig. 4B). Again, only single sporadic catches were recorded in unbaited traps.

## DISCUSSION

In this study, the field attractiveness of two volatile compounds was demonstrated for the first time in the legume pest *A. obtectus*. Both methyl anthranilate and benzyl alcohol showed antennal electrophysiological and behavioural activity in lab experiments, which suggested that they may be active also in the field. It is important to note, however, that EAG activity of a compound does not indicate whether it is behaviourally active (Roelofs 1977), implying that compounds deemed as less active by our EAG screening and not included in subsequent tests might actually be powerful behavioural synergists. Previous work using a similar experimental regime, i.e. selection of highly EAG-active compounds for behavioural tests, may provide confidence that this approach bears value in the discovery of field attractants (e.g. Vuts et al. 2010a, 2010b).

Methyl anthranilate is reported to be emitted by ca. 20 plant families (Knudsen et al. 2006), among which by those *A. obtectus* visits for pollen and nectar (Zachariae 1958), and it is an attractant of hymenopteran, dipteran and coleopteran species (Ruther 2004; Toshova et al. 2016). The compound is also used in the intraspecific chemical communication of ants possibly as an alarm pheromone constituent (Duffield et al. 1980). Benzyl alcohol is a ubiquitous plant volatile (Knudsen et al. 2006) and is emitted by *Fragaria* spp. that are visited by *A. obtectus* (Zachariae 1958). It is an attractant for a range of species in the Hymenoptera, Lepidoptera, Diptera and Homoptera, as well as for *Conotrachelus nenuphar* Herbst (Coleoptera: Curculionidae) (Prokopy et al. 2001). Interestingly, it was also described to be a male pheromone component of *Podisus maculiventris* Say (Heteroptera: Pentatomidae) (Aldrich et al. 1986).

We hypothesize that the attraction of *A. obtectus* to the combined benzyl alcohol/methyl anthranilate and the single benzyl alcohol baits is connected to the species' nectar- and pollen-feeding behaviour and not to its intraspecific communication. The male-produced sex pheromone consists of methyl (2*E*,4*Z*,7*Z*)-2,4,7-decatrienoate, methyl (2*E*,4*Z*)-2,4-decadienoate, (3*Z*,6*E*)- $\alpha$ -farnesene, (3*E*,6*E*)- $\alpha$ -farnesene, methyl (*E*,*R*)-2,4,5-tetradecatrienoate and octadecanal, and no other chemistries with pheromone function have so far been identified from the beetles (Vuts et al. 2015a). Laboratory feeding experiments with female *A. obtectus* have demonstrated that pollen consumption stimulates ovary production (Huignard and Leroi 1981). Similarly, obligatory pre-copulation feeding on pollen was reported in *Bruchus pisorum* L. on *Pisum sativum* L. (Fabaceae) (Pajni 1981), as well as nectar feeding to obtain a readily available source of energy to sustain flight (Clement 1992). The chemically guided relationships between *A. obtectus* and one of its nectar plants, *D. carota*, was recently studied (Vuts et al. 2018b). Six EAG-active flower headspace constituents [ $\alpha$ -pinene *S*:*R* 16:1, sabinene, myrcene, limonene *S*:*R* 1:3, terpinolene and (*S*)-bornyl acetate] were isolated and identified from *D. carota* and their synthetic blend found to induce behavioural preference in virgin females in laboratory olfactometer tests. Another bruchid, *Bruchus rufimanus* L., also uses flower volatiles to locate nectar plants. It is often found within flowers of *Vicia faba* L. (Fabaceae) and is attracted in the field to a synthetic mixture of the *V. faba* floral scent constituents (*R*)-linalool, cinnamyl alcohol and cinnamaldehyde, identified from flower headspace extracts (Bruce et al. 2011).

It is important to note that there is a significant lack of knowledge in our understanding of the semiochemicals that govern larval host location of *A. obtectus* females for egg-laying. Only a few studies characterised the volatile profile of *Ph. vulgaris*, showing that, for example, benzyl alcohol is

released, alongside over 60 compounds, in small amounts from dry seeds of certain bean cultivars (Oomah et al. 2007), and whole plants subjected to leaf herbivory emit a complex bouquet of 25 compounds (Wei et al. 2006). Interestingly, one of the volatiles was identified as methyl salicylate, which is structurally similar to methyl anthranilate except the alcohol moiety, but with weaker EAG activity in our study. Thus, a focus of future research should be to characterise volatile profiles of bean plants bearing dry seed pods, which is the preferred stage of pod maturity for ovipositing females (Szentesi 1990).

Further tests are underway to optimise synthetic lure composition (binary or single blend) and dose, as well as dispenser and trap design, to maximise *A. obtectus* catches and develop a potent detection and monitoring trap for field and store house use. It will be important to determine the sex ratio of catches in future trap optimisation experiments, because the synthetic blend of benzyl alcohol/methyl anthranilate is expected to lure both sexes. To date, the only monitoring tools in existence for bruchids are an attractant, (*Z*)-3-hexenyl acetate (Frérot and Leppik 2015), and an attractant trap for *B. rufimanus* (Bruce et al. 2011), which completes its life cycle in a year and does not infest stored legumes. The synthetic blend constituents of the trap developed by Bruce et al. (2011) were identified from *V. faba* flowers and thus the trap needs to compete with the flowering crop for beetles which move in to feed and stay until green pods develop. More recently, attraction of *Callosobruchus chinensis* L. to a synthetic mixture of benzaldehyde and (*E*)-2-hexenal was reported (Wang et al. 2020).

The field behaviour of *A. obtectus* is little known. Adults are thought to leave overwintering sites in Hungary in May and feed on pollen and nectar of a range of plants in and around bean fields until late summer, when pods mature and oviposition begins (Szentesi 1990). It is therefore reasonable to assume that ubiquitous flower volatiles at least in part are exploited by beetles to find nectar and pollen sources and are thus suitable candidates for attractant development. Based on our preliminary field results, benzyl alcohol with or without methyl anthranilate appears to be a potent lure constituent. It is as yet unclear if these compounds represent an ecologically relevant stimulus for *A. obtectus* flying into or moving within bean fields or if their blend is perceived as a novel, unusual stimulus (Bernays et al. 1992), which may be attributed to a high excitatory state of the central nervous system (Dethier et al. 1965), causing increased reactivity to this blend. At the same time, it will need to be carefully evaluated if this bait would still be attractive enough for monitoring the population level of the pest in granaries probably dominated by mated beetles, because mating can induce behavioural preference changes from nectar plant to larval host (e.g. Saveer et al. 2012).



The male-produced pheromone may synergise the activity of an optimised floral attractant, but its use is currently impractical because of its complexity and the difficulties with synthesizing enantiomerically pure constituents.

Trap development for *A. obtectus* needs to consider the specific behaviour of the species. Many chrysomelids, including *A. obtectus*, start climbing upwards after landing on a vertical surface, suggesting that a trap design described in Tóth et al. (2006) might be a good initial type. Specificity of the trap is an important consideration to reduce possible by-catches, which is the function of design, lure specificity and potentially colour cues. The latter may certainly be important in bruchid orientation behaviour whilst searching for inflorescences. Attraction of flower-visiting insects can be enhanced by the combination of odour and colour stimuli (Toshova et al. 2016); however, little is known about colour preference in *A. obtectus*. Zachariae (1958) lists nectar plants that all appear white or light yellow to the human eye, and traps coloured in white or yellow were found to be more attractive than other colours. Thus, trapping trials will need to assess a range of colours, including those mimicking the reflectance spectra of common inflorescences visited by the species.

**Author contributions** JV, MT and MAB conceived the study, JV conducted EAG experiments and analysed data statistically, JV performed air entrainment and GC analysis, LK performed the 4-arm olfactometry and JV analysed data statistically, SzSz, KSz, AN and MT ran field experiments, ZI analysed field data statistically, JV and MAB wrote the first draft and all authors reviewed and approved of the final draft.

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#### COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest Declaration** The authors declare they have no conflict of interest.

**Ethics Approval** Not applicable.

**Consent to Participate** Not applicable.

**Consent for Publication** All authors approved of the submission of the manuscript.

**Code Availability** Not applicable.

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Compound	Plant genera and families visited by <i>Acanthoscelides obtectus</i>						
	<i>Ranunculus</i> a	<i>Fragaria</i> b	<i>Potentilla</i> c	<i>Daucus</i> d	Apiaceae e	Ranunculaceae e	Rosaceae e
(E)-anethol						x	
anisyl acetone							
benzaldehyde		x	x				
benzyl acetate							x
benzyl alcohol		x					
(E)-cinnamaldehyde					x		x
(E)-cinnamyl alcohol							x
eugenol							x
geraniol				x			
β-ionone				x			
isoamyl acetate					x		x
isoamyl alcohol						x	x
isobutyl acetate					x		
(RS)-lavandulol				x			
(RS)-linalool	x		x	x			
methyl anthranilate					x	x	
methyl eugenol							x
2-methyl-1-propanol							x
methyl salicylate	x	x	x				
2-phenethyl acetate				x			
phenylacetaldehyde				x			
2-phenylethanol	x	x					

Table 1. Occurrence of compounds used for EAG tests in plant genera and families visited by *Acanthoscelides obtectus* (Zachariae 1958). No such data could be found for (E)-cinnamyl acetate, (E)-isosafole, (RS)-1-phenylethanol, 4-methoxycinnamaldehyde and 4-metoxypheethyl alcohol.

*Petroselinum* and *Torilis* spp., also listed by Zachariae (1958) to be visited by *A. obtectus*, could not be identified to contain any of the EAG test compounds.

<sup>a</sup>*R. acris*, *R. inamoenus* (Bergström et al. 1995; Dobson 1991; Raguso and Roy 1998)

<sup>b</sup>*F. ananassa* (Hamilton-Kemp et al. 1990; Innocenzi et al. 2001)

<sup>c</sup>*P. recta* (Burkle and Runyon 2016)

<sup>d</sup>*D. muricatus*, *D. crinitus*, *D. carota* (Bendiabdellah et al. 2012; Dib et al. 2010; Nehlin et al. 1996)

<sup>e</sup>Knudsen et al. (2006)

compound	MW	microcapillary inner diam. in mm (type of capillary)	mean ng amount in sample	n (nmol)	no. molecules ( $\times 10^{14}$ )	no. capillaries used
benzyl alcohol	108	0.22 (1 $\mu$ L Drummond)	235.72	2.18	13.1	1
2- phenylethanol	122	0.3 (1-5 $\mu$ L BlauBRAND)	221.98	1.82	10.92	1
(E)-anethole	148	0.22 (1 $\mu$ L Drummond)	151.74	1.03	6.15	2
methyl anthranilate	151	0.46 (10 $\mu$ L BlauBRAND)	260.27	1.72	10.34	1
(RS)- lavandulol	154	0.46 (10 $\mu$ L BlauBRAND)	63.12	0.41	2.46	5
methyl eugenol	178	0.3 (1-5 $\mu$ L BlauBRAND)	94.95	0.53	3.2	4

Table 2. Details of molecular and microcapillary parameters used to standardise release rates in olfactometer tests

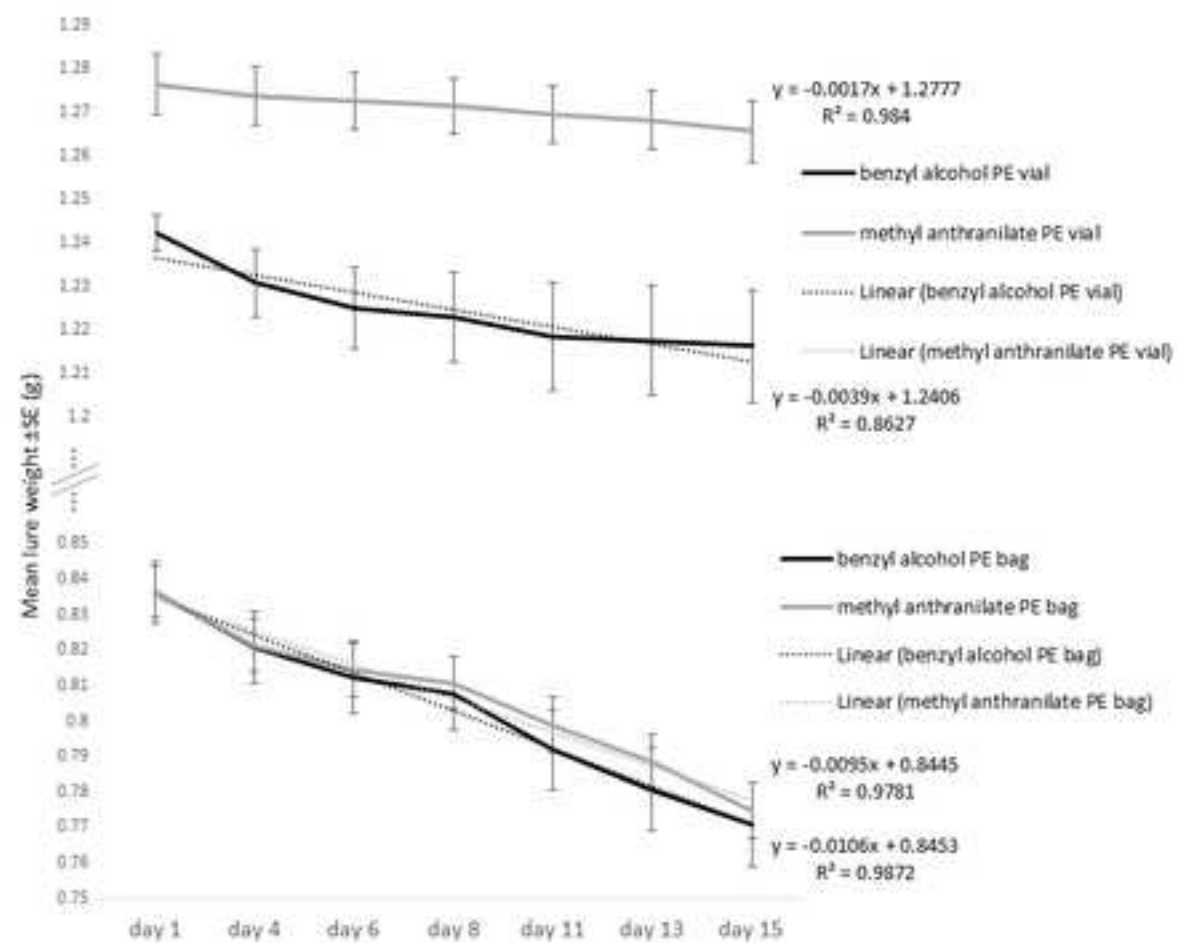
Figure legends

Fig. 1. Weight loss measurement over two weeks of PE bag and PE vial dispensers, loaded with either benzyl alcohol or methyl anthranilate (n=4 for each compound and dispenser type). A 200 mg load was used for PE bag dispensers, whereas it was 100 mg for the PE vials

Fig. 2. EAG responses of virgin female *Acanthoscelides obtectus* antennae to a panel of synthetic compounds (10 µg each; n=4). The EAG amplitudes were normalised to antennal responses given to (RS)-1-phenethyl alcohol (=100%). Significance from Fisher's LSD following ANOVA (P < 0.05). Compounds with the same letter are not significantly different

Fig. 3. Behavioural response of individual virgin female *Acanthoscelides obtectus* (n=10 for each compound) to synthetic standards of EAG-active compounds, each released separately in one arm (treatment) of a four-arm olfactometer against blank air in the three control arms. Volatiles were dispensed into the air stream at similar rates from glass microcapillaries. A: methyl anthranilate [predicted means on square root scale: control=1.282 (n=30); synthetic compound=2.036 (n=10); SED=0.229; df=38], B: methyl eugenol [predicted means on square root scale: control=1.333 (n=30); synthetic compound=1.431 (n=10); SED=0.3; df=38], C: benzyl alcohol [predicted means on square root scale: control=1.046 (n=30); synthetic compound=1.593 (n=10); SED=0.191; df=38], D: (RS)-lavandulol [predicted means on square root scale: control=1.57 (n=30); synthetic compound=1.193 (n=10); SED=0.234; df=38], E: 2-phenethyl alcohol [predicted means on square root scale: control=1.44 (n=30); synthetic compound=1.711 (n=10); SED=0.203; df=38], F: (E)-anethole [predicted means on square root scale: control=1.227 (n=30); synthetic compound=0.935 (n=10); SED=0.313; df=38]. \*: significant difference

Fig. 4. Catches of both sexes of *A. obtectus* on sticky traps baited with synthetic benzyl alcohol and methyl anthranilate alone or in combination vs unbaited control traps. Nagydobrony, 10 August – 17 September 2020, edge of bean fields. A: total catch 87 beetles, p=0.0093 (Mann-Whitney U test). B: total catch 59 beetles, Kruskal-Wallis test  $\chi^2 = 10.698$ , df = 2, p=0.0047; Mann-Whitney U test pairwise comparisons: benzyl alcohol – methyl anthranilate p=0.0115, benzyl alcohol – unbaited p=0.0037, methyl anthranilate – unbaited p=0.9648



Figure

