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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>	

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.

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.

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

3
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36 26
37 27 **Abstract**-The dried bean beetle, *Acanthoscelides obtectus*, is an economically important worldwide
38 28 pest of legume crops including dry beans, *Phaseolus vulgaris*. Assessment of *A. obtectus* infestation
39 29 levels in pre-harvest field crops and in post-harvest granaries is difficult to achieve, as there is no
40 30 effective monitoring tool for early detection and deployment of interventions. As *A. obtectus* is a
41 31 generic pollen and nectar feeder, we adopted an electrophysiological (EAG) screening approach
42 32 using the antennae of female *A. obtectus* to identify physiologically active plant volatile organic
43 33 compounds, which could then be investigated for their attractiveness to *A. obtectus* in laboratory
44 34 behaviour assays and preliminary field tests. Of the 27 compounds tested in the EAG screening, 5

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

46
47 **Key Words**-Bruchid beetle, Chrysomelidae, EAG, olfactometry, attractant, lure, trapping.

48 49 INTRODUCTION

50
51 This paper is dedicated to the memory of Prof. Dr. Dr. mult. h. c. Wittko Francke. In recent years, we
52 worked intensively together with him on the pheromone communication of the dried bean beetle,
53 which culminated in the complete identification of the pheromone (Vuts et al. 2015a). The present
54 paper describes our results on the beetle's non-pheromone chemical ecology, which we strongly
55 hope Prof. Francke will view with goodwill from above. Let him rest in peace, may light eternal shine
56 upon him!

57
58 Bruchids (Coleoptera: Chrysomelidae, Bruchinae) are usually small (2-5 mm), oval-shaped beetles.
59 Their larvae develop primarily in legume seeds (Fabaceae) that they infest in the field and complete
60 a single generation yearly. There are some species, however, which can infest host seeds both in the
61 field and in granaries (Southgate 1979). As their development depends often only on temperature,
62 they can have multiple generations a year, making their damage economically significant. Such
63 bruchids are mono- or oligophagous (Szentesi 1990). One of these species is the dried bean beetle,
64 *Acanthoscelides obtectus* Say, which originated in tropical America and is now a key pest of dry
65 beans (*Phaseolus vulgaris* L.) worldwide (Alvarez et al. 2005). The control of *A. obtectus* using various
66 chemical, biological, mechanical and cultural methods has met with varied success (Vétek et al.
67 2017). The ban of methyl bromide as a fumigating agent (Moultet et al. 2014) initiated attempts to
68 use alternative chemistries (Shaaya and Kostyukovsky 2010), bruchid-resistant bean varieties (Velten

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

74
75 The chemical ecology of *A. obtectus* has been studied over a number of decades. The major
76 component of a male-produced pheromone of *A. obtectus* was amongst the first insect pheromone
77 components to be identified (Horler 1970), and more recently, full characterization of other
78 pheromone components was reported (Vuts et al. 2015a, b, 2018a). Furthermore, a synthetic blend
79 of floral compounds identified from a nectar plant, *Daucus carota* L. (Apiaceae), was found to be
80 behaviourally active in lab tests (Vuts et al. 2018b). However, no attractant lure with field activity
81 has been optimised to date partially because, for example, the male pheromone blend is complex
82 and some of its constituents are difficult to synthesize.

83
84 As pollen consumption being a prerequisite for normal ovary production (Huignard and Leroi 1981),
85 *A. obtectus* visits a wide range of flowering plants for pollen and nectar (Zachariae 1958; Jarry 1987;
86 Szentesi 1990). Bruchids utilise floral volatiles to locate flowers (Bruce et al. 2011), and ubiquitous,
87 commercially available compounds, previously found to represent attractive floral cues for a number
88 of flower-visiting species (e.g. Toshova et al. 2016), have been screened for their electrophysiological
89 and behavioural bioactivity, with the most active ones having been then trialled under field
90 conditions. Our selection of putative attractants was informed by the volatile composition of flowers
91 visited by *A. obtectus* (Table 1). This approach of screening a synthetic panel of compounds in lab
92 assays to find proxies with field activity has been useful in the development of powerful lures for
93 numerous pest insect species (e.g. Beroza et al. 1961; Davis et al. 1969; Lohonyai et al. 2018). The
94 laboratory experiments presented in this paper focussed on virgin females, because it was assumed
95 that they are most attracted to flower volatiles, similar to other herbivorous insects where mating
96 induces behavioural preference changes from nectar plant to larval host (e.g. Saveer et al. 2012).
97 Females also directly determine the size of future generations, thus an attractant trap that captures
98 also females enables more precise pest forecasting and monitoring (e.g. Bruce et al. 2011). Thus, the
99 aim of the present study was to address the urgent need for an attractant that can be subsequently
100 used in trap development to detect and monitor *A. obtectus* in the field and store houses.

101 102 MATERIALS AND METHODS

103

1
2 104 *Insects Acanthoscelides obtectus* beetles were obtained from a laboratory inbred population reared
3
4 105 on dry 'Cannellini' beans (*Ph. vulgaris*). The original population was established from a natural
5
6 106 infestation on *Ph. vulgaris* in Hungary ca. 50 years ago. Maintenance conditions were as follows:
7
8 107 artificial lighting with a 16:8 h L:D photoperiod, a constant temperature of 20°C, and 60% RH. In
9
10 108 order to obtain virgin insects, seeds were kept individually in wells of an Eppendorf rack and covered
11
12 109 with a piece of transparent plastic sheet until beetle emergence, at which point the sexes were
13
14 110 separated immediately (based on shape of and patterns on the pygidium; Kaszab 1967) for use in
15
16 111 experiments.

112

17
18 113 *Chemicals* 1-Phenylethanol (98%), methyl anthranilate (>98%), (*E*)-cinnamaldehyde (99%), 4-
19
20 114 methoxyphenethyl alcohol (99%), benzyl acetate (99%), phenethyl acetate (>98%), (*RS*)-lavandulol
21
22 115 (>99%), eugenol (99%), (*E*)-4-methoxycinnamaldehyde (>98%), isoamyl alcohol (>98%), (*E*)-anethol
23
24 116 (99%), 2-methyl-1-propanol (>99%), β -ionone (>97%), benzyl alcohol (>99%), methyl salicylate
25
26 117 (>99%), (*E*)-cinnamyl acetate (99%), phenylacetaldehyde (>95%), isoamyl acetate (>97%), methyl
27
28 118 eugenol (>98%), (*E*)-cinnamyl alcohol (>98%), 2-phenylethanol (>99%), (*RS*)-linalool (97%), isobutyl
29
30 119 acetate (>97%), benzaldehyde (>99%), anisyl acetone (98%) and geraniol (98%) were from Sigma-
31
32 120 Aldrich, Hungary. (*E*)-Isosafrole (>95%) was from Aurora Fine Chemicals Ltd, Austria.

121

33
34 122 *Electroantennography (EAG)* To measure the EAG responses of virgin female *A. obtectus* antennae to
35
36 123 the panel of synthetic compounds listed above (also see Table 1), an antenna freshly amputated at
37
38 124 the base from a live beetle was mounted between two glass capillaries each containing 0.1 M KCl
39
40 125 solution and connected to silver wire electrodes (0.37 mm diam., Biochrom Ltd., UK), then placed at
41
42 126 ca. 3 mm distance from a stainless steel tube (10 mm diam., teflon-coated inside) with a constant
43
44 127 charcoal-purified and humidified airflow exiting at ca. 0.7 L/min. The recording electrode was
45
46 128 connected to a high-impedance DC amplifier (IDAC-232, Ockenfels Syntech GmbH, Kirchzarten,
47
48 129 Germany). Ten μ g of each compound was delivered in 10 μ L hexane solution onto a 1 cm² piece of
49
50 130 filter paper inside a Pasteur pipette. This dose was thought to be sufficient to balance for differences
51
52 131 in volatility by saturating the air space inside the Pasteur pipettes (Roelofs 1977). Stimuli consisted
53
54 132 of pushing 1 mL of air through the Pasteur pipettes into the airstream flowing towards the antenna
55
56 133 (n=4 from four different individuals). Response amplitudes were normalized against the standard
57
58 134 (*RS*)-1-phenethyl alcohol (that elicited medium-sized, i.e. 0.3-0.4 mV, responses), which was
59
60 135 administered before and after the test compounds. Stimuli were delivered at ca. 20-30 s intervals in
61
62 136 random order.

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

142
143 *Preparation of Stable-Release Glass Microcapillaries* To achieve a consistency of compound
144 concentration in the olfactometer airflow and to offer the test beetles similar number of molecules
145 across all compounds during the course of olfactometer experiments, test compounds were filled
146 into glass microcapillaries (1-10 μL ; Blaubrand® intraMARK, BRAND GmbH, Wertheim,
147 Germany/Drummond MICROCAPS®, Drummond Scientific Company, Broomall, PA, USA) previously
148 heat-sealed at one end. A standard Pasteur pipette was then melted at the point where the narrow
149 half widened, and subsequently pulled apart to form a very thin thread of capillary tube, the end
150 section of which was snapped off. Neat test compounds were separately taken up with a pipetting
151 bulb and the capillary tube end of the Pasteur pipette was inserted onto the bottom of a glass
152 microcapillary. Each pure compound was injected individually into a separate glass microcapillary,
153 the end of which was snapped off at ca. 1 mm distance from the meniscus. Glass microcapillary
154 dispensers prepared this way were kept in a closed glass vial at -20°C until use.

155 To determine the release rates of compounds from the dispensers, dynamic headspace collection
156 (air entrainment) from the headspace of each dispenser type loaded with a compound was
157 undertaken ($n=3$). Charcoal-purified air was pumped into the headspace of a glass chamber (5 cm \times 9
158 cm i.d., Biochem Glass Apparatus Ltd, Milton Keynes, UK), attached to a metal plate with bulldog
159 clips, at a rate of 600 mL/min, and subsequently pulled out at 500 mL/min through 50 mg Porapak Q
160 50/80 adsorbent polymer (Sigma-Aldrich, Gillingham, UK), sandwiched between glass wool plugs in a
161 glass tube (4 mm diam.). Each collection lasted for 16 min, i.e. the duration of an olfactometer test.
162 Trapped compounds were eluted from the adsorbent with 750 μL freshly distilled diethyl ether, and
163 extracts analysed on a high-resolution GC using an Agilent 6890A gas chromatograph equipped with
164 a cool on-column injector, an FID and a 50 m \times 0.32 mm ID, 0.52 μm film thickness HP-1 column (J &
165 W Scientific). The oven temperature was maintained at 30°C for 1 min, then programmed at $5^{\circ}\text{C}/\text{min}$
166 to 150°C and held for 0.1 min, then programmed at $10^{\circ}\text{C}/\text{min}$ to 250°C and held for 20 min. The
167 carrier gas was hydrogen (3.1 mL/min flow rate). Quantification of compounds was achieved using
168 the single-point external standard method with a series of C7-C22 alkanes.

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

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

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

200 In order to account for the replication and areas within each replication as variance components in a
201 split-plot design, the method of residual maximum likelihood (REML) was used to fit a linear mixed
202 model to the time spent data, nesting the areas within each replication and testing the treatment
203 effect using an approximate *F*-test. The data were analysed on the square root scale to account for

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

208

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

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

226 PE bag dispenser: a 1 cm piece of dental roll (Celluron[®], Paul Hartmann AG, Heidenheim, Germany)
227 was placed into a tight polyethylene bag made of 0.02 mm linear polyethylene foil. The dimensions
228 of the polyethylene sachets were ca. 1.5 × 1.5 cm. The dispenser was attached to a plastic strip (8 × 1
229 cm) for easy handling when assembling the traps. For making up the lures, compounds were
230 administered onto the dental roll and the opening of the polythene bag was heat-sealed and the
231 dispensers were wrapped individually in pieces of aluminium foil. The dose of single compounds was
232 200 mg/dispenser. PE bag dispensers have successfully been used to dispense various floral
233 compounds to capture beetles (Tóth et al. 2003a), moths (Tóth et al. 2010, 2014, 2019) and
234 lacewings (Tóth et al. 2009).

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

238 aluminium foil. PE vial dispensers were found to be efficient in attracting beetles (Tóth et al. 2003b)
239 and moths (Tóth et al. 2020).
240 Release rates of PE bag dispensers for benzyl alcohol were 4.68 mg/day and for methyl anthranilate
241 4.42 mg/day (n=4 for each compound), and those of PE vial dispensers for benzyl alcohol were 1.85
242 mg/day and for methyl anthranilate 0.77 mg/day (n=4 for each compound). Release rate estimates
243 were performed by gravimetric analysis, measuring at ca. two day-intervals the weight loss of
244 dispensers for two weeks in a wind tunnel (20°C, 0.2 m/s windspeed). PE vial dispensers (supposedly
245 because their wall is much thicker) were emitting at a lower rate (and therefore would last longer in
246 the field) than PE bag dispensers (Fig. 1). Earlier experience with synthetic floral compounds showed
247 that the PE bag dispensers did not lose their activity after several weeks of field exposure (Tóth et al.
248 2010, 2019); hence, it was decided that it was safe to renew all types of lure dispensers at two-week
249 intervals. Release rate values from lab experiments also indicated that lures might not dispense all of
250 their chemical load during this time period.

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

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

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

271 RESULTS

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1
2 273 Methyl anthranilate, methyl eugenol, benzyl alcohol, (*RS*)-lavandulol and 2-phenylethanol elicited
3
4 274 stronger EAG responses from female *A. obtectus* antennae than the solvent hexane or blank air
5
6 275 ($p < 0.001$, ANOVA) (Fig. 2). EAG amplitudes were in the 0.1–0.5 mV range.

7 276

8
9 277 Of the five compounds chosen based on their EAG activity [plus (*E*)-anethole], virgin female *A.*
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11 278 *obtectus* showed positive behavioural responses only towards methyl anthranilate (*F*-test: $F = 10.78$;
12
13 279 $df = 1, 38$; $P = 0.002$) and benzyl alcohol (*F*-test: $F = 8.16$; $df = 1, 38$; $P = 0.008$) when tested against blank air
14
15 280 in the four-arm olfactometer, the beetles staying on average ca. twice as long in the treated arm
16
17 281 than the control arms (Fig. 3A and C, resp.). Methyl eugenol (*F*-test: $F = 0.11$; $df = 1, 38$; $P = 0.746$), (*RS*)-
18
19 282 lavandulol (*F*-test: $F = 2.61$; $df = 1, 38$; $P = 0.115$), 2-phenethyl alcohol (*F*-test: $F = 1.79$; $df = 1, 38$; $P = 0.192$)
20
21 283 and (*E*)-anethole (*F*-test: $F = 0.87$; $df = 1, 38$; $P = 0.356$) did not elicit any behavioural preference from
22
23 284 the beetles (Fig. 3B, D, E and F, resp.).

24 285

25 286 In pilot field trials, traps baited with benzyl alcohol plus methyl anthranilate caught significantly
26
27 287 more *A. obtectus* than unbaited traps, which caught only single specimens sporadically (Experiment
28
29 288 1, Fig. 4A). In Experiment 2, traps baited with benzyl alcohol caught significantly more *A. obtectus*
30
31 289 than traps baited with methyl anthranilate alone or unbaited traps. The latter two treatments did
32
33 290 not differ from each other (Fig. 4B). Again, only single sporadic catches were recorded in unbaited
34
35 291 traps.

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37 293

38 294 DISCUSSION

39 295

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41
42 296 In this study, the field attractiveness of two volatile compounds was demonstrated for the first time
43
44 297 in the legume pest *A. obtectus*. Both methyl anthranilate and benzyl alcohol showed antennal
45
46 298 electrophysiological and behavioural activity in lab experiments, which suggested that they may be
47
48 299 active also in the field. It is important to note, however, that EAG activity of a compound does not
49
50 300 indicate whether it is behaviourally active (Roelofs 1977), implying that compounds deemed as less
51
52 301 active by our EAG screening and not included in subsequent tests might actually be powerful
53
54 302 behavioural synergists. Previous work using a similar experimental regime, i.e. selection of highly
55
56 303 EAG-active compounds for behavioural tests, may provide confidence that this approach bears value
57
58 304 in the discovery of field attractants (e.g. Vuts et al. 2010a, 2010b).

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

315
316 We hypothesize that the attraction of *A. obtectus* to the combined benzyl alcohol/methyl
317 anthranilate and the single benzyl alcohol baits is connected to the species` nectar- and pollen-
318 feeding behaviour and not to its intraspecific communication. The male-produced sex pheromone
319 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*)- α -
320 farnesene, (3*E*,6*E*)- α -farnesene, methyl (*E*,*R*)-2,4,5-tetradecatrienoate and octadecanal, and no
321 other chemistries with pheromone function have so far been identified from the beetles (Vuts et al.
322 2015a). Laboratory feeding experiments with female *A. obtectus* have demonstrated that pollen
323 consumption stimulates ovary production (Huignard and Leroi 1981). Similarly, obligatory pre-
324 copulation feeding on pollen was reported in *Bruchus pisorum* L. on *Pisum sativum* L. (Fabaceae)
325 (Pajni 1981), as well as nectar feeding to obtain a readily available source of energy to sustain flight
326 (Clement 1992). The chemically guided relationships between *A. obtectus* and one of its nectar
327 plants, *D. carota*, was recently studied (Vuts et al. 2018b). Six EAG-active flower headspace
328 constituents [α -pinene *S*:*R* 16:1, sabinene, myrcene, limonene *S*:*R* 1:3, terpinolene and (*S*)-bornyl
329 acetate] were isolated and identified from *D. carota* and their synthetic blend found to induce
330 behavioural preference in virgin females in laboratory olfactometer tests. Another bruchid, *Bruchus*
331 *rufimanus* L., also uses flower volatiles to locate nectar plants. It is often found within flowers of
332 *Vicia faba* L. (Fabaceae) and is attracted in the field to a synthetic mixture of the *V. faba* floral scent
333 constituents (*R*)-linalool, cinnamyl alcohol and cinnamaldehyde, identified from flower headspace
334 extracts (Bruce et al. 2011).

335
336 It is important to note that there is a significant lack of knowledge in our understanding of the
337 semiochemicals that govern larval host location of *A. obtectus* females for egg-laying. Only a few
338 studies characterised the volatile profile of *Ph. vulgaris*, showing that, for example, benzyl alcohol is

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

346

347 Further tests are underway to optimise synthetic lure composition (binary or single blend) and dose,
348 as well as dispenser and trap design, to maximise *A. obtectus* catches and develop a potent
349 detection and monitoring trap for field and store house use. It will be important to determine the
350 sex ratio of catches in future trap optimisation experiments, because the synthetic blend of benzyl
351 alcohol/methyl anthranilate is expected to lure both sexes. To date, the only monitoring tools in
352 existence for bruchids are an attractant, (*Z*)-3-hexenyl acetate (Frérot and Leppik 2015), and an
353 attractant trap for *B. rufimanus* (Bruce et al. 2011), which completes its life cycle in a year and does
354 not infest stored legumes. The synthetic blend constituents of the trap developed by Bruce et al.
355 (2011) were identified from *V. faba* flowers and thus the trap needs to compete with the flowering
356 crop for beetles which move in to feed and stay until green pods develop. More recently, attraction
357 of *Callosobruchus chinensis* L. to a synthetic mixture of benzaldehyde and (*E*)-2-hexenal was
358 reported (Wang et al. 2020).

359

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

373 The male-produced pheromone may synergise the activity of an optimised floral attractant, but its
1 use is currently impractical because of its complexity and the difficulties with synthesizing
2
3 375 enantiomerically pure constituents.
4

5 376

6
7 377 Trap development for *A. obtectus* needs to consider the specific behaviour of the species. Many
8
9 378 chrysomelids, including *A. obtectus*, start climbing upwards after landing on a vertical surface,
10
11 379 suggesting that a trap design described in Tóth et al. (2006) might be a good initial type. Specificity
12
13 380 of the trap is an important consideration to reduce possible by-catches, which is the function of
14
15 381 design, lure specificity and potentially colour cues. The latter may certainly be important in bruchid
16
17 382 orientation behaviour whilst searching for inflorescences. Attraction of flower-visiting insects can be
18
19 383 enhanced by the combination of odour and colour stimuli (Toshova et al. 2016); however, little is
20
21 384 known about colour preference in *A. obtectus*. Zachariae (1958) lists nectar plants that all appear
22
23 385 white or light yellow to the human eye, and traps coloured in white or yellow were found to be more
24
25 386 attractive than other colours. Thus, trapping trials will need to assess a range of colours, including
26
27 387 those mimicking the reflectance spectra of common inflorescences visited by the species.
28

29 388

30 389

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32 analysed data statistically, JV performed air entrainment and GC analysis, LK performed the 4-arm
33
34 392 olfactometry and JV analysed data statistically, SzSz, KSz, AN and MT ran field experiments, ZI
35
36 393 analysed field data statistically, JV and MAB wrote the first draft and all authors reviewed and
37
38 394 approved of the final draft.
39

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COMPLIANCE WITH ETHICAL STANDARDS

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58 406 **Conflict of Interest Declaration** The authors declare they have no conflict of interest.
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1
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4 409

5 410 **Consent to Participate** Not applicable.

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9 412 **Consent for Publication** All authors approved of the submission of the manuscript.

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12 414 **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					

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

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

601 ^a*R. acris*, *R. inamoenus* (Bergström et al. 1995; Dobson 1991; Raguso and Roy 1998)

602 ^b*F. ananassa* (Hamilton-Kemp et al. 1990; Innocenzi et al. 2001)

603 ^c*P. recta* (Burkle and Runyon 2016)

604 ^d*D. muricatus*, *D. crinitus*, *D. carota* (Bendiabdellah et al. 2012; Dib et al. 2010; Nehlin et al. 1996)

605 ^eKnudsen et al. (2006)

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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 μ L Drummond)	235.72	2.18	13.1	1
2- phenylethanol	122	0.3 (1-5 μ L BlauBRAND)	221.98	1.82	10.92	1
(<i>E</i>)-anethole	148	0.22 (1 μ L Drummond)	151.74	1.03	6.15	2
methyl anthranilate	151	0.46 (10 μ L BlauBRAND)	260.27	1.72	10.34	1
(<i>RS</i>)- lavandulol	154	0.46 (10 μ L BlauBRAND)	63.12	0.41	2.46	5
methyl eugenol	178	0.3 (1-5 μ L BlauBRAND)	94.95	0.53	3.2	4

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

609 Figure legends

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2 610

3 611 Fig. 1. Weight loss measurement over two weeks of PE bag and PE vial dispensers, loaded with either
4
5 612 benzyl alcohol or methyl anthranilate (n=4 for each compound and dispenser type). A 200 mg load
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7 613 was used for PE bag dispensers, whereas it was 100 mg for the PE vials

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10 615 Fig. 2. EAG responses of virgin female *Acanthoscelides obtectus* antennae to a panel of synthetic
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12 616 compounds (10 µg each; n=4). The EAG amplitudes were normalised to antennal responses given to
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14 617 (RS)-1-phenethyl alcohol (=100%). Significance from Fisher's LSD following ANOVA (P < 0.05).

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16 618 Compounds with the same letter are not significantly different

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18 619

19 620 Fig. 3. Behavioural response of individual virgin female *Acanthoscelides obtectus* (n=10 for each
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21 621 compound) to synthetic standards of EAG-active compounds, each released separately in one arm
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23 622 (treatment) of a four-arm olfactometer against blank air in the three control arms. Volatiles were
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25 623 dispensed into the air stream at similar rates from glass microcapillaries. A: methyl anthranilate
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27 624 [predicted means on square root scale: control=1.282 (n=30); synthetic compound=2.036 (n=10);
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29 625 SED=0.229; df=38], B: methyl eugenol [predicted means on square root scale: control=1.333 (n=30);
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31 626 synthetic compound=1.431 (n=10); SED=0.3; df=38], C: benzyl alcohol [predicted means on square
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33 627 root scale: control=1.046 (n=30); synthetic compound=1.593 (n=10); SED=0.191; df=38], D: (RS)-
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35 628 lavandulol [predicted means on square root scale: control=1.57 (n=30); synthetic compound=1.193
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37 629 (n=10); SED=0.234; df=38], E: 2-phenethyl alcohol [predicted means on square root scale:
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39 630 control=1.44 (n=30); synthetic compound=1.711 (n=10); SED=0.203; df=38], F: (E)-anethole
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41 631 [predicted means on square root scale: control=1.227 (n=30); synthetic compound=0.935 (n=10);
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43 632 SED=0.313; df=38]. *: significant difference

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46 634 Fig. 4. Catches of both sexes of *A. obtectus* on sticky traps baited with synthetic benzyl alcohol and
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48 635 methyl anthranilate alone or in combination vs unbaited control traps. Nagydobrony, 10 August – 17
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50 636 September 2020, edge of bean fields. A: total catch 87 beetles, p=0.0093 (Mann-Whitney U test). B:
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52 637 total catch 59 beetles, Kruskal-Wallis test $\chi^2 = 10.698$, df = 2, p=0.0047; Mann-Whitney U test
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54 638 pairwise comparisons: benzyl alcohol – methyl anthranilate p=0.0115, benzyl alcohol – unbaited
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56 639 p=0.0037, methyl anthranilate – unbaited p=0.9648







