Shades of yellow: interactive effects of visual and odour cues in a pest beetle

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Background. The visual ecology of pest insects is poorly studied compared to the role of odour cues in determining their behaviour. Furthermore, the combined effects of both odour and vision on insect orientation are frequently ignored, but could impact behavioural responses. **Methods.** A locomotion compensator was used to evaluate use of different visual stimuli by a major coleopteran pest of stored grains (*Sitophilus zeamais*), with and without the presence of host odours (known to be attractive to this species), in an open-loop setup. **Results.** Some visual stimuli – in particular, one shade of yellow, solid black and high contrast black-against-white stimuli – elicited positive orientation behaviour from the beetles in the absence of odour stimuli. When host odours were also present, at 90° to the source of the visual stimulus, the beetles presented with yellow and vertical black-on-white grating patterns changed their walking course and typically adopted a path intermediate between the two stimuli. The beetles presented with a solid black-on-white target continued to orient more strongly towards the visual than the odour stimulus. **Discussion.** Visual stimuli can strongly influence orientation behaviour, even in species where use of visual cues is sometimes assumed to be unimportant, while the outcomes from exposure to multimodal stimuli are unpredictable and need to be determined under differing conditions. The importance of the two modalities of stimulus (visual and olfactory) in food location is likely to depend upon relative stimulus intensity and motivational state of the insect.

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Abstract

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Methods: A locomotion compensator was used to evaluate use of different visual stimuli by a major coleopteran pest of stored grains (*Sitophilus zeamais*), with and without the presence of host odours (known to be attractive to this species) in an open-loop setup.

Results: Some visual stimuli – in particular, one shade of yellow, solid black and high contrast black-against-white stimuli – elicited positive orientation behaviour from the beetles in the absence of odour stimuli. When host odours were also present, at 90° to the source of the visual stimulus, the beetles presented with yellow and vertical black-against-white grating patterns changed their walking course and typically adopted a path intermediate between the two stimuli. The beetles presented with a solid black-on-white target continued to orient more strongly towards the visual than the odour stimulus.

Discussion: Visual stimuli can strongly influence orientation behaviour, even in species where use of visual cues is sometimes assumed to be unimportant, while the outcomes from exposure to multimodal stimuli are unpredictable and need to be determined under differing conditions. The importance of the two modalities of stimulus (visual and olfactory) in food location is likely to depend upon relative stimulus intensity and motivational state of the insect.

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Introduction

45

46 Understanding the cues used by pest insects to locate host material is an essential element of
47 devising sustainable control strategies to reduce impacts on food production and storage, as well
48 as providing insights into their ecology and evolution. While research on the olfaction of pest
49 insects is highly developed, work on the evaluation of visual preferences among pests remains
50 lacking, despite that these preferences may play a key role in host location for many pests (Reeves
51 2011). This knowledge gap in pests contrasts with extensive work on colour vision in some other
52 insect groups, in particular honeybees (Backhaus 1993; Backhaus et al. 1987; Dyer et al. 2008),
53 bumblebees (Chittka & Raine 2006; Dyer et al. 2008), Drosophila (Morante & Desplan 2008), and
54 some non-pest Lepidoptera (Bernard & Remington 1991; Eguchi et al. 1982; Kelber & Pfaff 1997;
55 Telles et al. 2014). Furthermore, it is often hard to ascertain the relative importance of visual and
56 olfactory stimuli in location of host material by some insects, as comparative work is not always
57 performed and published. However, attempting to deconstruct the use of different modalities of
58 stimulus has value in devising control strategies for pest insects as well answering questions about
59 the evolution of foraging behaviour.

60

61 Colour vision, defined as the ability to discriminate wavelengths of light independently of
62 intensity, is one of several ways insects can use visual information to orient themselves (Vorobyev
63 & Brandt 1997). There are a variety of methods that can be used to investigate insects' responses
64 to visual stimuli. Experimental setups involving tracking of the insect's movements can be
65 considered either as open-loop, in which the insect is presented with a visual stimulus controlled
66 by the experimenter and the insect's directionality or wing-movements are detected and recorded
67 (Otálora-Luna et al. 2004), versus closed-loop, in which the insect's locomotory activity is fed
68 back to drive the visual environment with which it is presented (Reiser & Dickinson 2008). Open-
69 loop setups are simpler to operate, but closed-loop setups may provide additional information
70 about the insect's dynamic responses and path control. This can be of value particularly in studies

71 relating to how insects navigate, regulate their speed, estimate distance and control pitch and yaw

72 (Maimon et al. 2008; Reiser & Dickinson 2008)}.

73

74 When studying insects during visual experiments the laboratory, larger insects can be investigated

75 in flight arenas (Arnold & Chittka 2012), Y-mazes (Dyer et al. 2007; Giurfa et al. 1996) and flight

76 tunnels (Srinivasan et al. 1996; Willis et al. 2011). When investigating the visual ecology of flying

77 insects, individuals are frequently tethered and optomotor responses can thus be investigated, as

78 has been done with Drosophila (Maimon et al. 2008; Yamaguchi et al. 2008) and locusts (Cooter

79 1979); this is amenable to investigation via both open- and closed-loop systems.

80

81 In less sophisticated setups, coloured pan traps and sticky traps can also be used to investigate

82 colour preferences, particularly in pest insects (Campbell & Hanula 2007; Han et al. 2012; Lunau

83 2014). This has value if the purpose is only to establish which of a range of colours elicits the

84 highest insect capture rates, but pan traps are imprecise for investigating colour vision itself as the

85 environment is less well controlled. A coloured trap also depends upon insects not only

86 approaching and investigating the trap, but attempting to land and being caught by it.

87 Electrophysiology can accurately evaluate the responses of individual insect photoreceptors, or the

88 whole retina, to light stimuli (Peitsch et al. 1992; Telles et al. 2014), providing information about

89 physiological capabilities to respond to visual stimuli, but does not necessarily inform about

90 behavioural preferences or inclinations.

91

92 In this study we used a locomotion compensator (Servosphere) in an open-loop setup to evaluate

93 responses of the maize weevil *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) to both

94 visual and olfactory stimuli. This is a relatively new method of investigating responses of insects

95 to coloured visual stimuli (having previously only been used to assay responses of an insect to

96 emitted light (Beattie et al. 2011; Bell et al. 1983; Otálora-Luna & Dickens 2011; Otálora-Luna et

97 al. 2013) and once to an unquantified yellow stimulus of unknown spectral composition (Van der

98 Ent & Visser 1991)). The Servosphere is a 300 mm diameter ball in a motorised support, with a

99   camera set above it. An insect placed upon the ball can run freely in any direction on the ball's

100   surface; it is tracked by the camera, and a processor controls the rotation of the ball (driven by

101   servomotors) to keep the insect always at the apex of the ball (Kramer 1976). The motion of the

102   ball is detected by the equipment, and processed to permit reconstruction of the insect's walking

103   path for analysis.

104

105   The Servosphere is thus well-suited to measuring orientation behaviour of walking insects as it

106   allows the insect to choose its direction of taxis freely and excludes the confounding factor of

107   thigmotaxis (Bell & Kramer 1980). As the insect can never reach the stimulus source, but the

108   behaviour it displays in trying to reach or avoid the stimulus is recorded, this is considered open-

109   loop and permits exploration of insect behaviour in an environment controlled by the experimenter.

110   Locomotion compensators such as the Servosphere have been used for several decades, primarily

111   to investigate use of olfactory stimuli (host odours, sex pheromones, carbon dioxide, etc.) by

112   insects. In some cases, the insect is tethered on a freely-rotating wire, especially for insects that fly

113   readily, whereas in other cases the insect is untethered.

114

115   The data generated by a locomotion compensator may include the speed and directionality of an

116   insect's movement, but also metrics such as the path straightness (which would be expected to

117   increase where a stimulus provokes a strong sensorimotor response as the insect would become

118   more directed in its behaviour). The direction is normally measured relative to either a fixed point

119   on the horizontal plane of the sphere or relative to the stimulus source (e.g. "upwind" direction).

120   A typical experiment will present stimuli in succession, e.g. still air, then a clean airflow, then an

121   airflow with added odour, then a final period of still or clean air, and the insect's behaviour at each

122   of these stages can be observed (Otálora-Luna et al. 2013). Most insects will orient in an upwind

123   direction when faced with clean airflow, but the speed (and thus, distance moved) will increase if

124   the insect is subsequently presented with an attractive odour. Similarly, the insect can be presented

125   with a visual stimulus such as a light or coloured item, either alone or in the presence of an odour

126   cue (either in the same angular location, or separately); the locomotion compensator will provide

127   data on the direction and nature of movement shown by the insect when such a visual cue is
128   presented (Van der Ent & Visser 1991).

129

130   However, the question of whether to use an emitted light (e.g. from a light-emitting diode (LED)
131   or a monochromator) or a non-emitting stimulus in the design of a visual assay is not always
132   straightforward. While using the light from an LED as the visual stimulus can accurately determine
133   the effects of a narrow band of wavelengths, stored cereal pests and many other insects are adapted
134   to low light conditions. Consequently, their response to a bright coloured light may not be as
135   ecologically relevant as exposing them to a non-emitting stimulus, such as coloured paper. In our
136   experiment, we elected to test a selection of quantified coloured papers with the insects, evaluating
137   responses with and without the presence of host odours.

138

139   The responses of insects to multiple or, indeed, multimodal stimuli can be diverse. Some types of
140   response to stimuli can only be observed when another stimulus is also present, as is the case for
141   the stronger response to regressive rather than progressive patterns in *Calliphora erythrocephala*,
142   observed only when georeceptors in the legs of the fly are stimulated (Horn & Knapp 1984). Some
143   insect responses to multiple stimuli can be simply additive; others can be antagonistic or
144   synergistic (Campbell & Borden 2009; Giurfa et al. 1994). When the stimuli are presented in a
145   way such that they appear to conflict or contradict one another, it becomes possible to make
146   judgements about the importance of one type of stimulus over another, as has been explored in the
147   Colorado beetle (Otálora-Luna et al. 2013) and the bumblebee (Kunze & Gumbert 2001), and
148   about factors affecting whether choices are inclined towards one stimulus, intermediate, or
149   bimodal (Horn & Wehner 1975). The relative strengths of different stimuli can be important too:
150   in *Rhagoletis pomonella*, odour cues were largely irrelevant if the visual stimulus was strong,
151   whereas if the visual stimulus was not strongly coloured, the intensity of the odour cue became
152   more important to the fly in locating a food source (Aluja & Prokopy 1993). Somewhat similarly,
153   hawkmoths (*Macroglossum stellatarum*) could learn an odour discrimination task if the scented
154   targets were of a less preferred colour, but failed to learn odours if the targets were of a more

155   preferred colour (blue), indicating that a strong, highly preferred visual stimulus interferes with

156   responses to the odour stimulus (Balkenius & Kelber 2006).

157

158   *S. zeamais* is a major pest of stored grains across sub-Saharan Africa (Kamanula et al. 2011). Both

159   adults and larvae eat cereals such as wheat, maize and rice: females bore a hole in the surface of

160   cereal grains and seal an egg within, and the larva subsequently consumes the cereal from within

161   (Dobie et al. 1991); however, the beetle will also use other food material such as pasta and dried

162   cassava when available (Dobie et al. 1991). As a stored product pest, most of their activity is

163   normally expected to take place in low light conditions, but the adults are capable of flight and

164   dispersal, so use of both visual and odour cues in host location is unsurprising. A yellow stimulus

165   has already found to be attractive to *S. zeamais* in a four-arm olfactometer, especially in

166   combination with odour (Arnold et al. 2015), and there is robust evidence showing the species is

167   attracted to various cereal odours (Arnold et al. 2015; Ukeh et al. 2010; Ukeh et al. 2012).

168   However, the viewing angle in an olfactometer makes it hard to analyse effects of contrast and

169   edges, and there is a difference between choosing to rest on an area with particular visual

170   characteristics and actively choosing to orient towards it in a free-walking scenario. Active

171   attraction (i.e. directed movement towards a stimulus) is key to host material location (Hardie

172   2012) and can be better tested in the more open-ended environment of the Servosphere.

173

174   A further advantage of the locomotion compensator is the possibility to test responses when odour

175   and colour are combined (Otálora-Luna et al. 2013). Presentation of odour and colour stimuli

176   simultaneously or successively, and from different locations relative to the insect, can help to

177   determine and quantify preferences for the different stimulus types. Otálora-Luna et al. (2013)

178   discovered that in the neotropical weevil *Diaprepes abbreviates*, a pest and a generalist, visual

179   cues took precedence over odour cues when this insect had to choose between apparently

180   conflicting visual and odour cues. They found that the presence of a visual cue in the absence

181   odour cues actually increased activity levels in male weevils, whereas the presence of odour cues

182   in the absence of light did not. Interestingly, presence of a green light cue appeared to override

183 positive attraction responses to volatiles in the perpendicular direction, indicating that the odour

184 cues are subordinate to visual cues in this species when the two appear to contradict.

185

186 In this experiment we sought to build on previous findings (Arnold et al. 2015) that the maize

187 weevil *S. zeamais* exhibits preferences for some visual stimuli more than others. Having previously

188 established that *S. zeamais* will spend time preferentially on a tested shade of yellow paper, we

189 were testing several hypotheses:

1. *S. zeamais* adults will orient towards visual stimuli
   a. particularly those with long-wavelength reflection and low short-wavelength reflection and
   b. particularly those with high achromatic contrast.
2. *S. zeamais* does not orient towards all stimuli broadly perceived as "yellow" to humans equally, and consequently it cannot be assumed that all "yellow" traps will be similarly effective.
3. *S. zeamais* responds to both visual and odour cues. When they are presented simultaneously but perpendicular to one another on a horizontal plane,
   a. *S. zeamais* will be influenced in its orientation direction by the presence of the visual cue when the cue is attractive, and will orient either towards the visual cue or intermediate between the visual and odour cue sources
   b. *S. zeamais* will orient more strongly towards the odour cue when the visual cue is not attractive.

204 Understanding the use of visual cues, when odour cues are also present, in this species, will help

205 to refine trapping technologies for monitoring populations of *S. zeamais* in grain stores. It will also

206 improve understanding of how this insect locates food sources, which may aid in future outbreak

207 prediction and better design of grain stores to reduce inwards migration by this insect species.

208

Materials and Methods

210

Insect cultures

S. zeamais adults were originally sourced from Malawi and cultured as described in previous studies (Arnold et al. 2015; Jayasekara et al. 2005) on organic whole wheat grains. The culture was maintained at 25°C and 60% r.h. in a 14:10 light:dark cycle. Individual adults of known ages and sex were used in experiments, factors which were included in the data analysis; sex was determined by inspection of the rostrum appearance under a dissecting microscope (Dobie et al. 1991). Insects were removed from cultures upon emergence and held in mixed-sex containers, so reproductively mature individuals (over around 4 days old) were assumed to be mated. Unmated and very young adult *S. zeamais* are nonetheless also motivated to forage for food as the adults feed on cereals as well as the larvae (Ukeh et al. 2012). Test insects were deprived of food for 0-72 hours before use in experiments; the specific length of time was recorded for each individual and both age of insect and duration of food deprivation were included as explanatory variables in the analysis. Each individual was used once only. Experiments took place in a separate room to the main culture, at 26°C and ambient humidity. Light was provided by high-lux plant growth lamps (irradiance in centre of room: 25.0 µmol m^{-2} s^{-1}; directly beneath camera: 6.5 µmol m^{-2} s^{-1}).

Servosphere assay

Experiments were carried out using a Syntech TrackSphere LC-300 (Syntech, Hilversum, Netherlands) Servosphere connected to a control unit. A CMOS camera set above the Servosphere provided visual tracking of the insect for locomotion compensation via servomotors. Tracks were recorded in TrackSphere 3.1 (Syntech, Hilversum, Netherlands), which provides both raw and partially-processed data.

The experimental design permitted individual beetles to be tested with one particular visual cue per beetle, under conditions with no odour or blown air; with a clean airstream; and with host odours. A separate cohort of beetles were also tested without any visual stimulus, with otherwise identical no odour/clean air/host odour conditions. Therefore, all condition combinations (with/without visual; with/without odour) were tested.

239

240 Odour stimuli were delivered using a vacuum pump that pushed charcoal-filtered air (Agilent

241 Technologies, Wokingham, Berks, UK), through a gas-washing bottle that was either empty or

242 contained 50 g roughly crushed yellow maize (crushed by placing in a plastic bag and crushing

243 with a hammer for 2 minutes to simulate recently damaged grain (Arnold et al. 2015)). Silicone

244 Tygon tubing (Ø internal 0.6 mm) (Tygon, Sigma-Aldrich, St Louis, MO, USA) was used

245 throughout and the flow rate was set at 150 ml/minute for all odour sources.

246

247 Odours were introduced to the insect at 180° relative to the azimuth of the camera recording display

248 (termed the "upwards" direction by the TrackSphere software) (Fig. 1). Previous research has

249 demonstrated that maize volatiles are attractive to *S. zeamais* (Arnold et al. ; Ukeh et al. 2012),

250 and that the components causing positive chemotaxis include hexanal, (E)-2-heptenal, and octanal,

251 particularly when those components are presented as a three-odour blend (Ukeh et al. 2012).

252 Furthermore, *Sitophilus* sp. have a preference for maize as a host material, even when individuals

253 were themselves raised on wheat (Trematerra et al. 2013).

254

255 The Servosphere was surrounded by a screen of white paper (height 270mm) on all sides to exclude

256 conflating visual distractions originating from the room. Light was provided by high-lux plant

257 growth lamps (irradiance in centre of room: 25.0 μmol m-2 s-1; directly beneath camera: 6.5 μmol

258 m-2 s-1); the spectral composition is provided in Fig. S1. Tested visual stimuli consisted each time

259 of a circle of paper (Ø 153mm). Use of paper visual stimuli rather than emitting stimuli (e.g. LEDs)

260 was chosen because *S. zeamais* are considered to do the majority of their activities in low-light

261 conditions, and therefore emitting stimuli would be ecologically atypical for this species. The

262 paper circles presented one of the following appearances:

263 1. Coloured circle (one of five colours, with spectral reflectance profiles as in Fig. 2 – referred to

264 from here onwards for simplicity as yellow, yellow textured, sand, amber and orange according to

265 their appearance to human eyes). A range of yellow stimuli were tested because yellow has

266 previously been shown to be attractive to *S. zeamais* (Arnold et al. 2015) and we sought to test

267 whether this applied to all shades, or only stimuli with particular spectral properties.

268 2. Black circle – "black".

269   3. Circle patterned with black and white vertical grating (width of black and white bands equal, 6

270   mm) – "vertical grating". This explores the concept of high-contrast edges facilitating attraction,

271   and previous studies have shown that vertical black stimuli can elicit attraction behaviours from

272   stored product Coleoptera (Semeao et al. 2011).

273   3. No visual stimulus – "control". This permitted a comparative data set in which the response to

274   odour in absence of visual stimulus could be tested.

275

276   HSV (hue, saturation, value) figures are provided for all the stimuli, and also for wheat and maize,

277   in Table 1, to provide human-relevant context for the appearance of these stimuli. Values for

278   reflectance at 366 nm, 520 nm and 564 nm are also given for these stimuli, as these are the

279   published peaks in spectral sensitivity in the eyes of another pest weevil, *Rhynchophorus*

280   *ferrugineus* (Ilić et al. 2016), and are consequently a potential indication of the colour vision a

281   maize weevil may possess. Two spectral receptor types with sensitivities >500 nm implies the

282   beetles are likely to be able to discriminate green, yellow and orange hues well. While the sand-

283   coloured paper appears superficially most similar to the colours of white and yellow maize, the

284   yellow textured and yellow papers are closer to maize in terms of the hue and value (brightness)

285   measures compared to the other coloured stimuli. The yellow paper also has the highest reflectance

286   at 564 nm out of all the coloured stimuli.

287

288   Visual stimuli were presented at 90° or 270° (randomised) to the direction on the Servosphere (Fig.

289   1). These were positioned at 154 mm from the weevil's location, meaning that the solid stimuli

290   would subtend a visual angle of 52.4°. Previous research on bees indicates that honeybees and

291   bumblebees can detect the colour of a stimulus subtending 15° (Dyer et al. 2008; Giurfa et al.

292   1996; Spaethe et al. 2001), and therefore even with the low-resolution eyes of small insects it can

293   be confidently argued that these stimuli were not only visible to the weevils, but that they should

294   also have been able to detect the colour.

295

296   Each insect was placed on the Servosphere and allowed 1 minute to acclimate to the new location

297   before commencing motion recording. The insect was then recorded for 15 minutes in total

298   (sequence below) consisting of five periods of three minutes, in which combinations of visual

299   and/or odour stimuli were presented. The presentation order was: visual stimulus alone, visual

300   stimulus with clean airstream, two periods of visual + odour stimulus, then a final "recovery"

301   period with the visual stimulus alone (it is not practical to alter visual stimuli during the recording

302   period). If the insect flew away (*S. zeamais* can fly but rarely chooses to) the recording was

303   abandoned. The surface of the Servosphere was only handled while wearing gloves and was

304   cleaned regularly using 70% ethanol to prevent chemical residues of previous test animals

305   influencing subsequent animal behaviours.

306

307   *Predicted behaviours*

308   Predictions of the angle relative to the camera azimuth that one might expect the beetle to average

309   in its orientation in the case of different visual stimuli are shown in Table 2, based on the principle

310   that the insect will walk towards an attractive stimulus, and when two equally attractive stimuli (of

311   any modality) are presented at different angles, the insect would be expected to choose a path that

312   (on average) is intermediate between them. The odour stimulus used is a known attractant for *S.*

313   *zeamais* (Ukeh et al. 2010; Ukeh et al. 2012).

314

315   *Statistics*

316   The TrackSphere software provides information about each insect's distance walked (both in total

317   and towards the odour stimulus), direction walked and path straightness. Generalised Linear

318   Models were performed on linear data, using age, sex and period of food deprivation as well as

319   treatment as explanatory variables (Table S1). For comparisons of movement towards the odour

320   source, if a beetle did not move during a particular recording period, a zero distance value was

321   recorded and the beetle was included in the analysis. Period 4 was chosen over period 3 to evaluate

322   responses in the presence of both odour and visual cues, as this assessed the beetle's final choice

323   after having had time to settle on a behavioural response to the dual stimuli and was therefore

324   judged to be more representative of the behavioural preference.

325

326   Vectors of movement were calculated from the total X and Y displacement of the insect during

327   each recording period. Statistical analyses of these vectors were performed in SPSS version 20

328   (IBM, NY, USA), RStudio version 0.97 running R version 3.0.2 (R Development Core Team
329   2008), and Microsoft Excel for Windows 7, using circular statistics techniques described in
330   Batschelet (Batschelet 1981). Vectors for each beetle during each of the five exposure periods
331   (control 1, airstream, odour 1, odour 2, control 2) were calculated relative to the azimuth values,
332   and "mirror-reversed" in the case of replicates where the insect was presented with the visual cue
333   at 270°, so that all vectors could be directly compared. Mean vectors for each period for each visual
334   stimulus were calculated and tested for significant clustering around the mean via the modified
335   Rayleigh's V-test (Batschelet 1981). This is a standard method of analysis employed in previous
336   Servosphere studies (Bell & Kramer 1980). Using the "circular" package in R, differences between
337   the directional responses to the different colours of stimuli, with and without odour present, were
338   compared using the Watson-Wheeler test. Bonferroni corrections were applied to outputs as
339   appropriate.
340
341   To test for directionality, we categorised angular deviation for each insect as "towards the
342   stimulus" or "not towards the stimulus" for Periods 1 and 4. "Towards the stimulus" was
343   considered to be any direction between 60° and 120° for the visual stimulus and 150° to 210° for
344   the odour stimulus when present. For period 4 we also calculated the number of insects displaying
345   an "intermediate direction" of movement, meaning any angular deviation between 90° and 180°.
346   For each visual stimulus type, we then used a binomial test to consider whether the insects were
347   more likely than random (i.e. more than one sixth of the insects for the stimulus/not stimulus or
348   more than one quarter of the insects for the intermediate/not intermediate directionality) to select
349   that direction of movement, with a Bonferroni correction for multiple comparisons.
350

Results

352
353   In total, 147 individuals were tested, (74 males and 73 females). 31 individuals were tested with
354   the yellow stimulus, 16 yellow textured, 16 sand, 15 amber, 15 orange, and 19 with the black
355   stimulus, 19 with the grating and 17 in the control setup with no visual stimulus. This was expected
356   to provide suitable power to detect differences in orientation angle of 20° between treatments, and
357   differences in distance moved of 13 mm towards odour sources.

358

359   In the absence of a discrete coloured visual stimulus, insects oriented towards the odour when it

360   was present (mean angular deviation 155°, r = 0.409, p = 0.006) and randomly when it was not

361   (mean angular deviation 137°, r = 0.241, p = 0.177). The control confirms that in absence of a

362   visual stimulus, the beetles do not orient towards the 90° direction on the Servosphere: in the

363   absence of all stimuli the mean angular deviation is random, whereas when a food odour or

364   airstream is present, it is consistently at 180° to the azimuth of the camera display (i.e. towards the

365   odour source).

366

367   Table 3 shows the results of a General Linear Model analysis of the whole dataset, with treatment,

368   age, sex and period of food deprivation as independent variables and the distance walked towards

369   the odour source during periods 1-5 as a response variable each time. While responses to odour

370   when colour stimuli were present were not fully consistent, treatment was a significant factor

371   overall in determining odour response (Tables 3 and S1; GLM, Hotelling's Trace, $F_{30,117}$ = 2.196

372   , p = 0.002). Effects were particularly notable during period 4, when visual and food odours were

373   both present. In period 4, stimuli that appeared attractive in terms of mean vectors also elicited

374   movements towards odour stimuli (with the exception of the vertical grating) (Fig. 3 a, b),

375   suggesting that combination of odour and colour, even when locations differ, may increase

376   motivation. In addition, the yellow textured stimulus was also associated with movement towards

377   the stimulus. In comparison, amber, orange and sand coloured stimuli were not associated with

378   movement towards the odour stimulus. There was a significant difference in distance walked

379   towards the odour source in the presence of yellow versus orange stimuli (difference = 252 mm,

380   yellow greater, p = 0.026) and yellow versus amber stimuli (difference = 273 mm, yellow greater,

381   p = 0.013). Mean distances walked in periods 1 and 4 for the control visual stimulus and yellow,

382   an attractive stimulus, are shown in Fig. 3 c.

383

384   With respect to mean angular deviations, the directions of insects with and without odour are

385   shown in Fig. 4, with Rayleigh test results presented in Table S2. The black (mean vector 84.4°

386   without odour (Rayleigh test, z = 15.0, p < 0.0001); 98.5° with odour (z = 9.49, p < 0.0001)),

387   vertical grating (mean vector 80.1° without odour (z = 3.42, p = 0.0003); 95.1° with (z = 3.40, p <

388   0.0001)) and yellow (mean vector 93.6° without odour (z = 6.67, p < 0.0001); 145.6° with (z =

389   10.2, $p < 0.0001$)) stimuli all showed significant nonhomogeneity (i.e. the insects were not
390   choosing random directions). The binomial analysis of proportion of insects in each case tending
391   to choose a direction towards the stimuli indicated that, in absence of odour, the black stimulus
392   elicited significant clustering of the angular deviations around 90° (i.e. towards the stimulus
393   source, binomial test, $p < 0.0001$) (clustering towards the yellow stimulus was no longer significant
394   after Bonferroni correction). When the odour was introduced as well, while insects with the black
395   stimulus present continued to be clustered significantly in their orientation towards it (binomial
396   test, $p < 0.0001$), the insects presented with the yellow stimulus instead were significantly clustered
397   around the odour source (binomial test, $p = 0.0005$) and, in fact, in a direction intermediate between
398   the odour and visual stimuli (binomial test, $p = 0.0002$).

399

400   The Watson-Wheeler tests revealed that there were differences between the beetles' responses to
401   different colours in terms of the mean vector they chose, both without the presence of any odour
402   cues (Watson-Wheeler test, $W_{12} = 33.31$, $p = 0.00087$) and with both odour and visual cues present,
403   but at right angles to each other ($W_{12} = 27.64$, $p = 0.00624$). This indicates that some colours elicit
404   a stronger behavioural response than others, and in some cases this response is strong enough to
405   override or interfere with the response to odour cues. Pairwise comparisons of colour responses
406   indicate that both with and without the presence of odour, beetles responded significantly
407   differently to black stimuli compared with most others stimuli, and that without an odour cue, the
408   beetles also responded differently to the sand-coloured cue, with the mean vector leading away
409   from the stimulus source; however, as noted above, the vectors are not significantly clustered in
410   the presence of this stimulus, indicating that this was probably random.

411

412   Based on the results, black was most consistent with the "attractive" prediction; i.e. the high-
413   contrast, achromatic stimulus produced attraction with and without food odours present. Yellow
414   and vertical grating targets were also attractive stimuli: beetles oriented towards these stimuli in
415   absence of any confounding odour stimulus. However, when the odour was presented
416   perpendicular to the visual cue, the two stimulus modalities affected beetle behaviour and the path
417   chosen by the beetle was more intermediate between the two sources. This suggests that the odour
418   and visual cues, at this intensity, are of comparable attractiveness. The behaviour of the beetles
419   towards these three visual cues was consistent between individuals, indicated by significant

420   clustering of mean vectors. These data indicate that different shades of yellow are not equally

421   attractive, and high achromatic contrast appears to be equally or more attractive to *S. zeamais* than

422   the chromatic cues presented.

423

Discussion and conclusions

425

426   Locomotion compensators have been used to study the behaviour of insects in response to

427   attractive odour stimuli (host odours, pheromones, plant volatiles, etc.) (Becher & Guerin 2009;

428   Otálora-Luna et al. 2004). They are of limited use to study repellent odours (we could only find

429   two incidences in the literature (McMahon et al. 2003; Zermoglio et al. 2015)) as insects may not

430   always respond to a repellent odour by simply walking in the downstream direction relative to the

431   odour; they may instead attempt to move laterally, stop dead or take flight and abandon the

432   Servosphere. It can therefore be difficult to characterise repellency as a behavioural response on

433   this apparatus. However, as attractive odours will induce a walking insect to orient in an upwind

434   direction, locomotion compensators such as the Servosphere can be used to examine responses to

435   host volatiles and pheromones.

436

437   Servospheres have rarely been used to study spectral preferences in visual orientation of walking

438   insects (Beattie et al. 2011; Otálora-Luna & Dickens 2011; Otálora-Luna et al. 2013; Van der Ent

439   & Visser 1991) and never previously used with spectrally quantified non-emitting stimuli such as

440   coloured paper that might present a controlled but more ecologically relevant motivation. We

441   demonstrate its utility in this context for the first time, presenting evidence that attractive responses

442   to non-emitting visual stimuli can be at least as strong as for odours. Future work could incorporate

443   instantaneous remote control of visual stimuli, permitting increased complexity and evaluation of

444   the effect of adding or removing a visual cue mid-recording. However, the value of using non-

445   emitting stimuli in tests must be highlighted, as coloured lights may elicit unusual behaviour in

446   insects that often forage in dark conditions. While a 360° LED display cylinder around the

447   Servosphere could provide maximal real-time ability to control an insect's visual environment and

448   could permit detailed studies of visually-guided orientation and navigation behaviour in pest

species such as *Sitophilus zeamais* (as well as insects such as ants or carabids), the ecological relevance of such a setup must be considered. The dispersal behaviour of *S. zeamais* is not fully characterised, so the timing of it (day versus night) and the visual cues used for navigation by dispersing individuals remain to be discovered. How they respond to point sources of light when dispersing and how often they would be expected to be active when sufficient light is available to make use of colour cues are not known, but this and our previous study (Arnold et al. 2015) indicate that the capacity to use colour information in this species is present.

Visual experiments involving the Servosphere have an additional advantage of being able to present multiple visual stimuli simultaneously. It can be used to test relative importance of different stimuli in orientation. Future work could also test additive effects, in a setup where odour and visual stimuli both originate from the same source, though we found that presentation of the visual stimulus can be obstructed by the odour administering tube. The most useful variables for these studies appear to be the mean angular deviation of the insect's movement during each recording period (indicating overall direction of movement), and the upwind distance walked. As the periods are of set duration, this is determined by the mean velocity of the insect's motion in that direction.

Our experiment shows that a coloured stimulus (a yellow circle with some UV-reflectance and a mid-point of the step function around 525 nm) is attractive to *S. zeamais*, but that other shades of yellow are not. Furthermore, monochromatic stimuli (black circle, or black and white grating) are more attractive than the yellow stimulus. This is consistent with many other studies of insects, including pests and animal disease vectors, showing contrast is a key cue for orientation behaviours (Rockstein 1974; Semeao et al. 2011). Therefore, it is likely that *S. zeamais* responds both to coloured stimuli and to high-contrast stimuli (black-on-white). This is similar to Semeao et al. (2011)'s findings that another pest of stored cereal products, *Tribolium castaneum*, orients towards tall, vertical black shapes. How this behaviour is mediated at the neural level remains unknown. Bees are known to do much of their visual processing using an achromatic channel mediated by the green receptor (e.g. motion, distance vision) (Giurfa et al. 1996) and it is likely that similar mechanisms underpin the vision of other insects, including weevils. In this experiment, the most attractive colour (yellow) had a relatively high ratio of green to blue reflectance, but also moderate

480 UV reflectance. This suggests that attractiveness of a chromatic stimulus to this species may be

481 influenced by the relative intensities of green, blue and UV reflection of the surface, but further

482 investigation and modelling are required. It is also possible that the intense yellow of this type of

483 paper serves as a supernormal stimulus related to food, eliciting similar positive orientation

484 behaviours to that of yellow in hoverflies (thought perhaps to be a supernormal stimulus response

485 that aids pollen-seeking behaviours) (Kelber 2003) or leafhoppers (thought to aid in seeking

486 foliage) (Todd et al. 1990). Responding strongly to exaggerated stimuli that considerably exceed

487 the intensity of the natural material encountered in nature has been hypothesised as a way to

488 increase success in locating the food substance.

489

490 Insect responses to the odour were not entirely consistently attractive. We hypothesised that this

491 may depend on the motivational state of the insect. While period of food deprivation was not a

492 significant factor in determining distance walked towards the odour during period 4 (colour +

493 odour), other factors including interactions with other cues, reproductive state of females or some

494 effect of larval conditions (Rietdorf & Steidle 2002) could also influence the level of motivation a

495 beetle has for orientating towards host material. There also appears to be an interaction with visual

496 cues, as the distance walked towards host odour was typically greater when an attractive visual

497 cue was present – even in a different location to the odour source – than when a less attractive

498 colour was presented. Otálora-Luna et al. (2013) also found that the visual environment could

499 stimulate higher levels of walking activity, but in that case where the visual and odour cues were

500 presented perpendicularly, the presence of an attractive visual cue overrode the attraction to the

501 odour source. Our result could imply that the presence of an attractive, coloured stimulus may

502 enhance the overall motivation of beetles to seek food.

503

504 This study underlines the importance of visual cues in host location by pest insects, both in terms

505 of contrast and chromaticity – even in insects normally associated with poorly lit environments.

506 The interaction between odour and colour in orientation towards targets is very important in

507 various insect taxa (Raguso & Willis 2005; Wackers & Lewis 1994); optimising both types of cue

508 can be used to enhance the efficacy of trapping and monitoring devices. Visual and odour cues can

509 be synergistic when presented together; they can also operate at different distances from the source,

510 with insects responding to visual cues further away and odour at closer range (Frye et al. 2003), or

511   vice versa. It is evident that while some visual appearances may enhance the effectiveness of traps

512   or, conversely, deterrents, other colours or patterns will be less effective. The most attractive

513   colours may not necessarily correspond perfectly to the colours of host material.

514

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Figure legends

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Fig. 1 Schematic of top view of Servosphere setup, showing two alternative positions for visual stimulus (A = 90°; B = 270°) and direction of odour delivery. Note that odour and visual stimuli are presented perpendicular to each other.

707

Fig. 2 Spectral reflectance curves for the coloured stimuli presented to the beetles, as measured on an Avantes AvaSpect-2048 using an Avantes AvaLight-DH-S-BAL relative to a $BaSO_4$ white standard.

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Fig. 3 Distance travelled (mean ± s.e.m.) in direction of odour tube: A: by beetles presented with no visual stimulus, with and without odour (N = 17 insects) (without = neither blown air nor odour); B: by beetles presented with the yellow visual stimulus during Period 1 (visual stimulus present, no odour/blown air) and Period 4 (both visual and odour stimuli present) (N = 31); C: distance moved towards odour tube by all beetle cohorts, during Period 4 (both odour and visual stimuli present) (N = 147 insects) (negative values indicate net movement away from the tube). ("Control" bar = no visual stimulus presented.)

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Fig. 4 Mean angles (°) of beetle orientation in the presence of A: a visual stimulus and no odour or blown air or B: with both the visual stimulus and an airstream containing host (maize) odour. ("no stimulus" = odour conditions as for other cohorts, but with no visual stimulus presented.) (N = 147 insects in total)

724 **Figures**

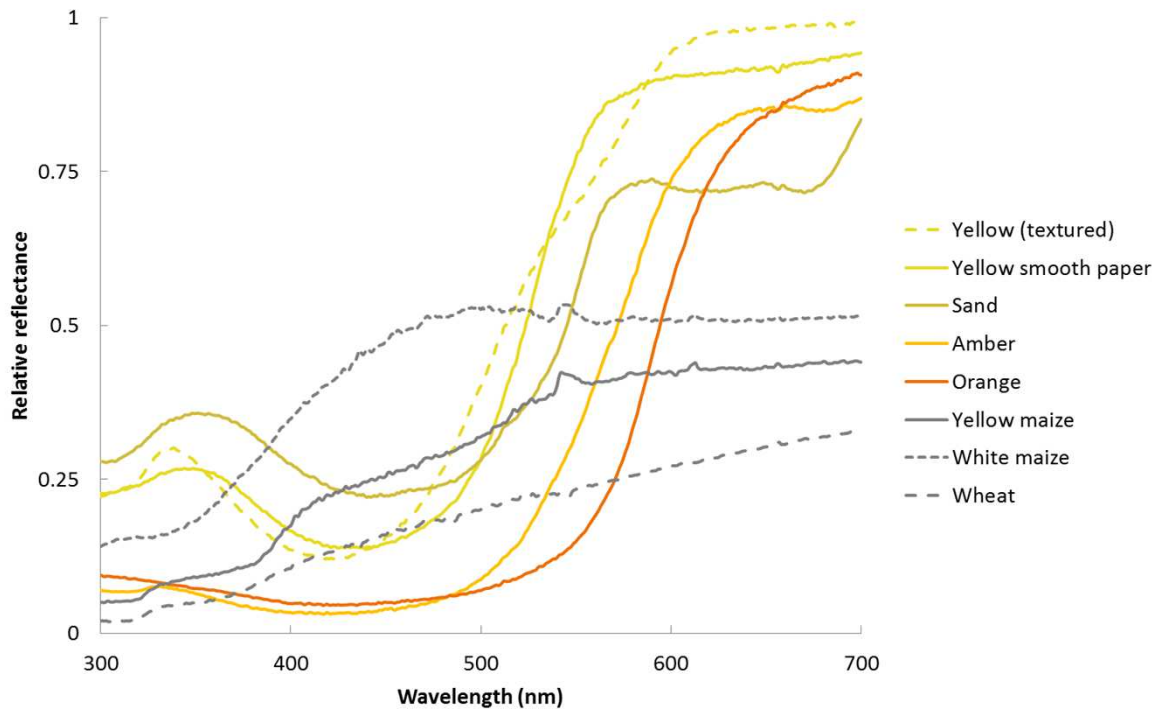725 Fig. 1
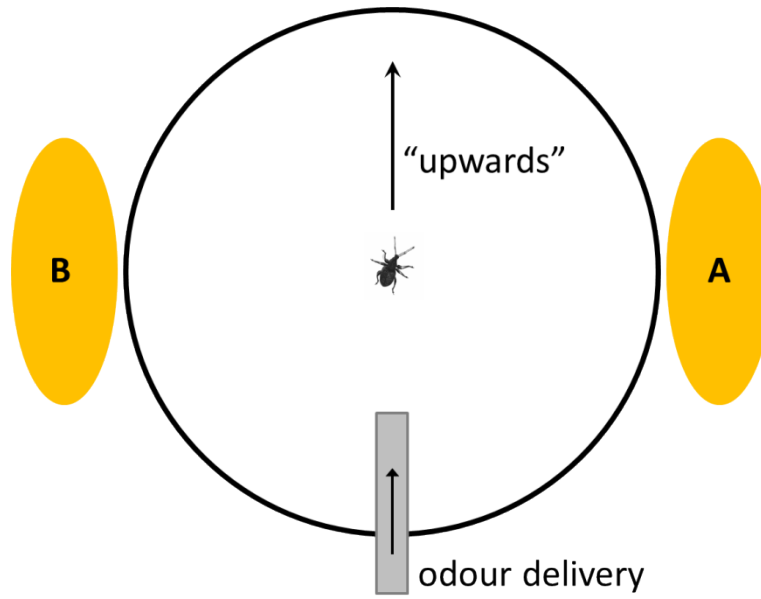
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729 Fig. 2
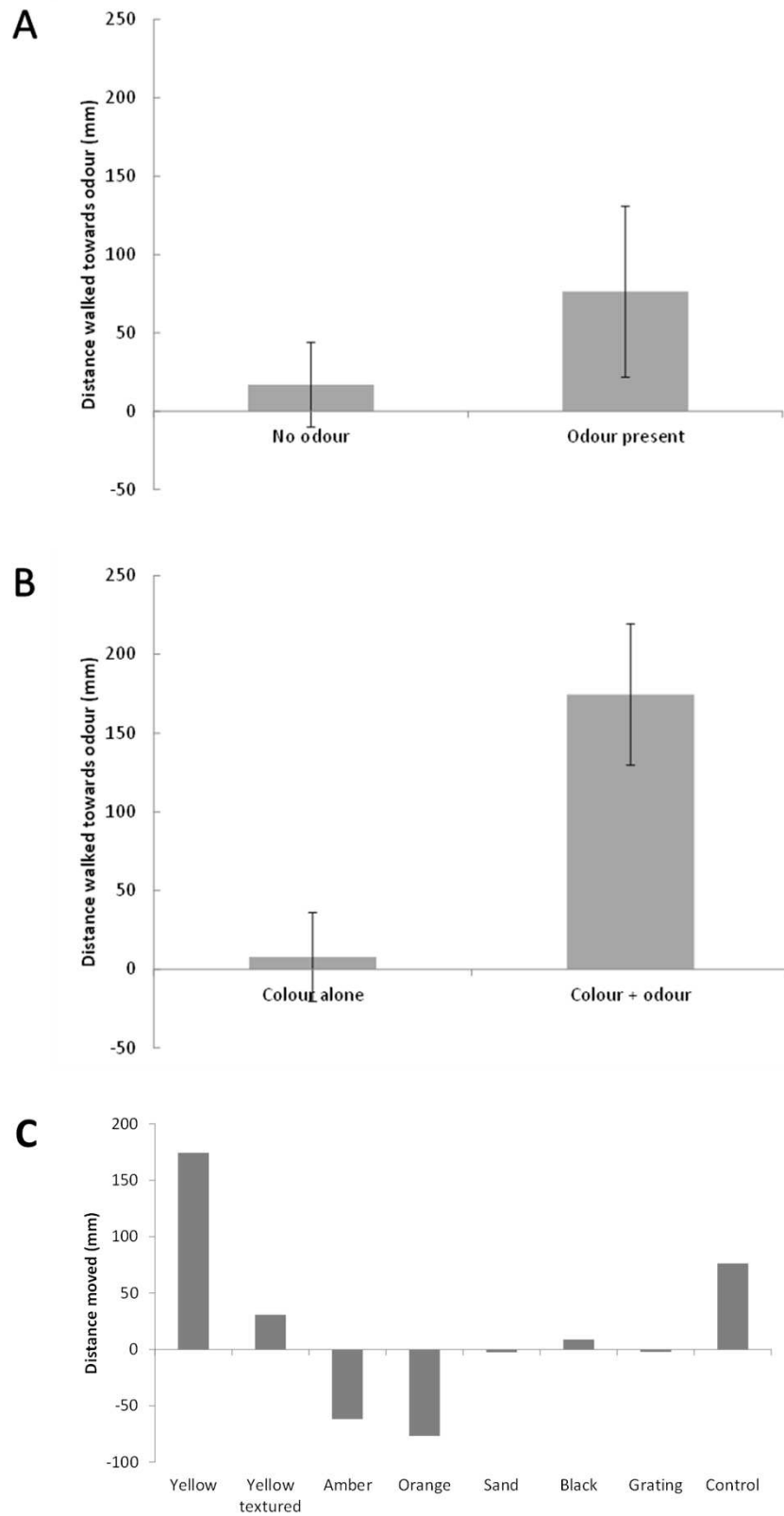

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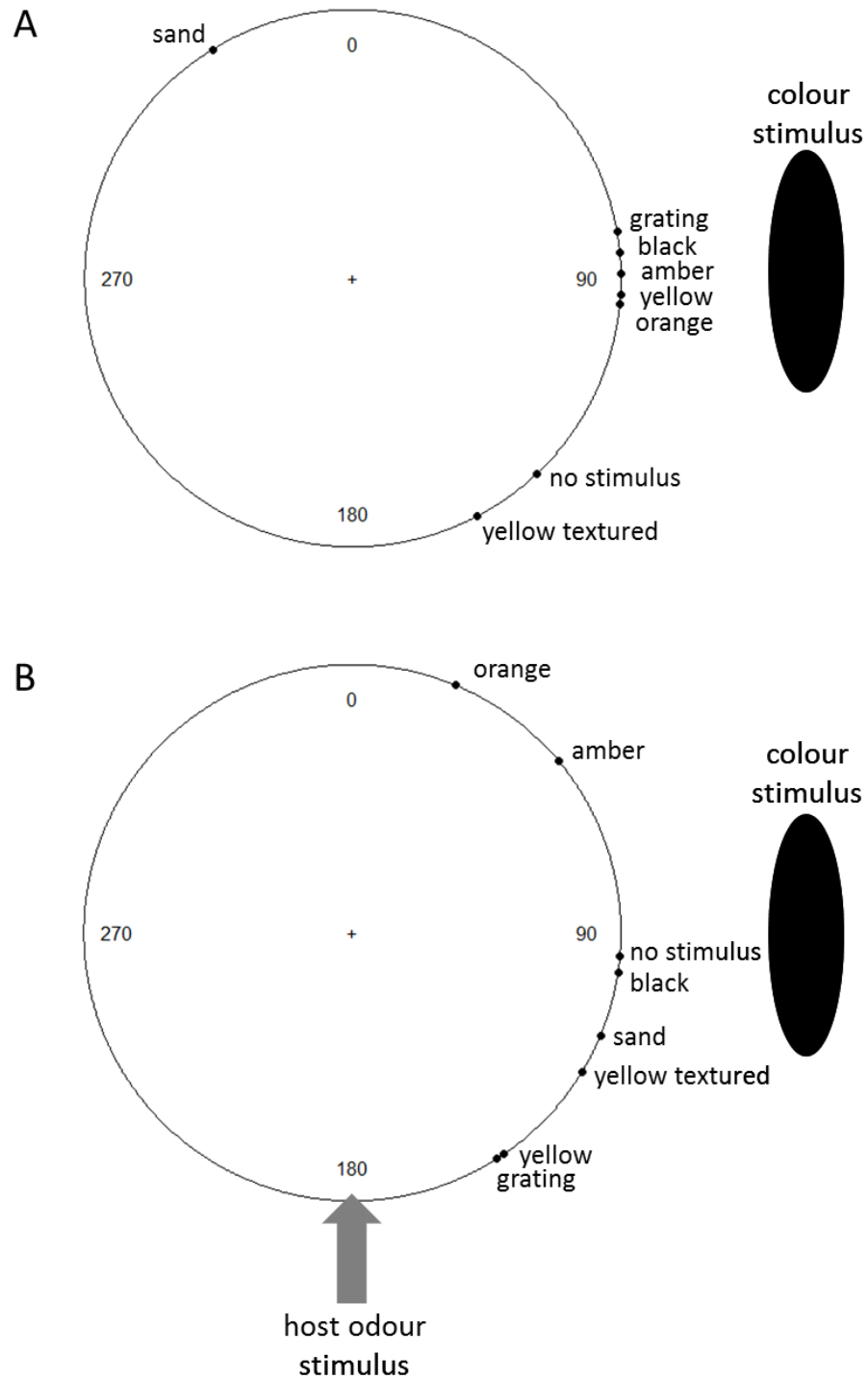734

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737   Fig. 3

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744   **Tables**

Table 1. Hue, saturation and value figures for coloured paper stimuli and natural host materials, alongside proportional reflectance at 366, 520 and 564 nm (these being the published peaks of photoreceptor sensitivity in another weevil species, *Rhynchophorus ferrugineus* (Ilić et al. 2016)).

745

Stimulus	H	S	V	366nm	520nm	564nm
Yellow	28	92.7	74.9	0.248018	0.461077	0.84916
Yellow (textured)	29	94.3	75.3	0.226178	0.547739	0.76652
Sand	22	83.3	70.6	0.349127	0.355651	0.685641
Amber	16	100	100	0.050626	0.145598	0.439424
Orange	9	97.3	100	0.066094	0.090617	0.211301
Yellow maize	30	58.9	50.6	0.099471	0.362622	0.408071
White maize	25	30.1	40.4	0.229231	0.523592	0.505379
Wheat	24	61.3	53.7	0.059449	0.217541	0.242793

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Table 2. Predictions of insect walking vectors, if visual stimulus is presented at 90° and odour at 180°, and assuming beetle is motivated to seek food. All angles in degrees

Nature of visual stimulus	Period 1 (visual alone)	Period 2 (visual + air)	Period 3 (visual + odour 1)	Period 4 (visual + odour 2)	Period 5
Attractive	90	Between 90 and 180 but closer to 90	Between 90 and 180	As period 3	90
Neutral	Random	180	180	180	Random

Repellent	270	180-270	180-270	180-270	270

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Table 3 Effect of different parameters and their interactions on distance walked by *S. zeamais* during Servosphere recording periods 1, 2 and 4 (analysed via General Linear Model with listed parameters included as explanatory variables)

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Factor	Has effect?	Colour alone (*p*-value if significant)	Colour + airstream (*p*-value if significant)	Colour + odour (*p*-value if significant)
Treatment	Yes	**0.005**	**0.019**	--
Sex	No	--	--	--
Age	No	--	--	--
Time food deprived	Yes	**0.016**	--	--
Treatment * Sex	No	--	--	--
Treatment * Age	No	--	--	--
Age * Sex	No	--	--	--
Age * Time food deprived	No	--	--	--

Too few degrees of freedom present to return results for: Treatment * Time food deprived; Sex * Time food deprived; Treatment * Sex * Age; Treatment * Sex * Time food deprived; Treatment * Age * Time food deprived; Sex * Age * Time food deprived; Treatment * Sex * Age * Time food deprived

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752 **Supplementary information**

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754 **Table S1 a) Inputs and b) full outputs for the statistical analyses performed on walking**

755 **vectors and distance moved.**

756 **A**

757 **Model design:**

758 Intercept + Treatment + Sex + Age + Starv + Treatment * Sex + Treatment * Age + Treatment * Starv +

759 Sex * Age + Sex * Starv + Age * Starv + Treatment * Sex * Age + Treatment * Sex * Starv + Treatment *

760 Age * Starv + Sex * Age * Starv + Treatment * Sex * Age * Starv

761 Where "Starv" is the period of food deprivation

762 **B**

Effect	Value	F	Hypothesis df	Error df	p-value
Intercept	.356	1.782b	5.000	25.000	.153
Treatment	2.724	2.124	30.000	117.000	.002
Sex	.077	.385b	5.000	25.000	.854
Age	5.780	1.288	105.000	117.000	.091
Period of food deprivation	3.377	2.258	35.000	117.000	.001
Treatment * Sex	.415	2.073b	5.000	25.000	.103
Treatment * Age	.228	1.141b	5.000	25.000	.365
Treatment * Period of food deprivation	.000	.b	.000	2.000	.
Sex * Age	1.068	.999	25.000	117.000	.474
Sex * Period of food deprivation	.000	.b	.000	2.000	.
Age * Period of food deprivation	.000	.b	.000	2.000	.
Treatment * Sex * Age	.000	.b	.000	2.000	.

Treatment * Sex * Period of food deprivation	.000	.b	.000	2.000	.
Treatment * Age * Period of food deprivation	.000	.b	.000	2.000	.
Sex * Age * Period of food deprivation	.000	.b	.000	2.000	.
Treatment * Sex * Age * Period of food deprivation	.000	.b	.000	2.000	.

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Source	Recording period	Type III Sum of Squares	df	Mean Square	F	p-value
Corrected Model	P1	2870588.684a	86	33378.938	1.678	.058
	P4	5209959.817d	86	60580.928	1.223	.275
Intercept	P1	646.826	1	646.826	.033	.858
	P4	216459.546	1	216459.546	4.371	.045
Treatment	P1	473732.575	6	78955.429	3.969	.005
	P4	218772.401	6	36462.067	.736	.625
Sex	P1	11191.776	1	11191.776	.563	.459
	P4	55057.259	1	55057.259	1.112	.300
Age	P1	656711.864	21	31271.994	1.572	.128
	P4	454057.445	21	21621.783	.437	.973
Period of food deprivation	P1	422895.831	7	60413.690	3.037	.016
	P4	724672.927	7	103524.704	2.091	.077
Treatment * Sex	P1	4382.440	1	4382.440	.220	.642
	P4	97281.610	1	97281.610	1.965	.172
Treatment * Age	P1	55855.808	1	55855.808	2.807	.105
	P4	5292.000	1	5292.000	.107	.746
Treatment * Period of food deprivation	P1	.000	0	.	.	.
	P4	.000	0	.	.	.
Sex * Age	P1	81738.101	5	16347.620	.822	.544
	P4	294876.107	5	58975.221	1.191	.338
Sex * Period of food deprivation	P1	.000	0	.	.	.

	P4	.000	0	.	.	.
Age * Period of food deprivation	P1	.000	0	.	.	.
	P4	.000	0	.	.	.
Treatment * Sex * Age	P1	.000	0	.	.	.
	P4	.000	0	.	.	.
Treatment * Sex * Period of food deprivation	P1	.000	0	.	.	.
	P4	.000	0	.	.	.
Treatment * Age * Period of food deprivation	P1	.000	0	.	.	.
	P4	.000	0	.	.	.
Sex * Age * Period of food deprivation	P1	.000	0	.	.	.
	P4	.000	0	.	.	.
Treatment * Sex * Age * Period of food deprivation	P1	.000	0	.	.	.
	P4	.000	0	.	.	.
Error	P1	576970.148	29	19895.522		
	P4	1436058.109	29	49519.245		

Table S1 Analysis of insects' directions of movement a) Results of Rayleigh test analysis (z-scores) of *S. zeamais* orientation vectors and b) binomial test probabilities (Bonferroni-corrected to a significance value of 0.0016) in the presence and absence of host odours. Visual stimuli were presented at 90° to the camera azimuth and odour stimuli at 180° to the camera azimuth. (In a), * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; in b) # indicates significance after correction.)

A

Colour	*N*	*z* without odour	*z* with odour
Amber	15	2.169624*	2.01539*
Orange	15	0.533501	1.595403
Yellow	31	6.673843***	10.2351***
Yellow (textured)	16	2.077826*	0.155136
Sand	16	2.702122*	1.064188
Black	19	14.98742***	9.486128***
Grating	19	3.419676***	3.399831***

Control	17	0.986605	1.816808

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771 **B**

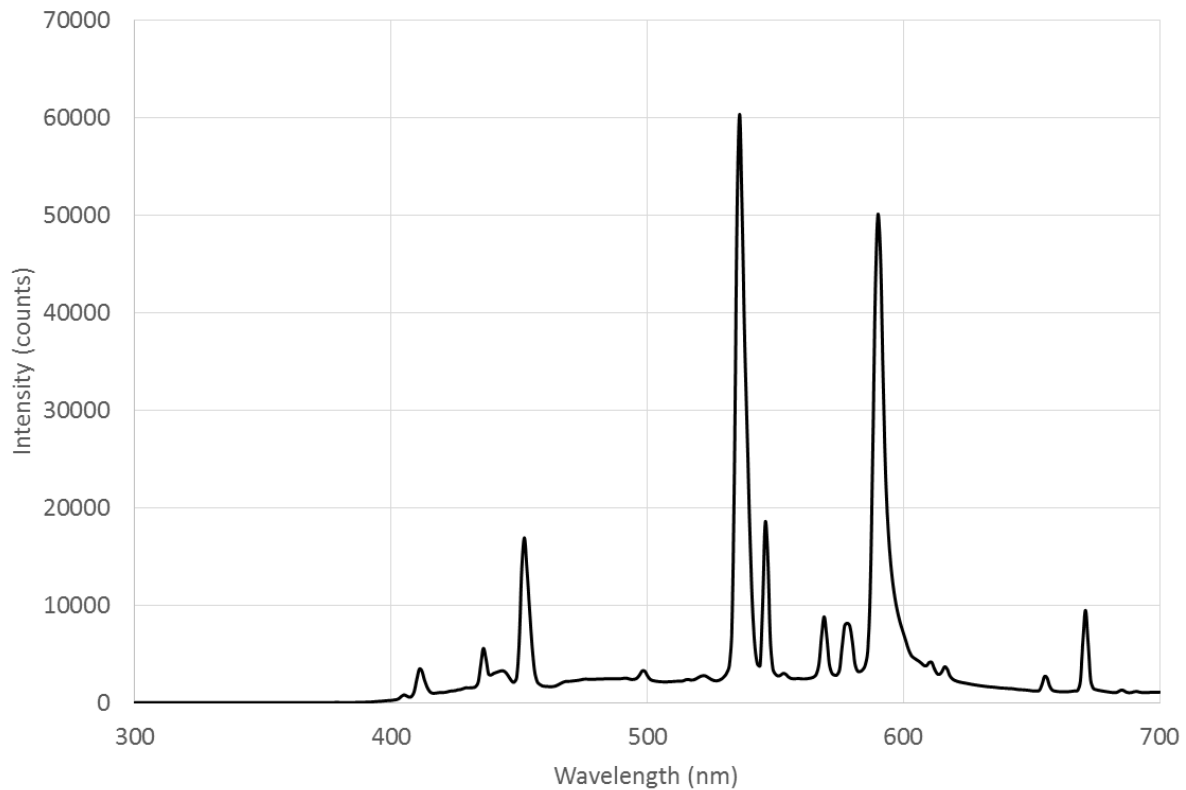| Colour	Period 1, visual direction	Period 4, visual direction	Period 4, odour direction	Period 4, intermediate visual/odour
Amber	0.1134	0.01908	0.92212	0.25847
Orange	0.23153	0.9351	0.0274	1
Yellow	0.00282	0.22348	0.00054#	0.00022#
Yellow (textured)	0.23153	0.77284	0.51322	0.36981
Sand	0.9351	0.70404	0.42054	0.11167
Black	<0.0001#	<0.0001#	0.74039	0.14837
Grating	0.02808	0.31129	0.31129	0.42611
Control	0.77284	0.27091	0.27091	0.80289

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Fig. S1 Spectral composition of light source in the experimental room a) Over the whole insect-visible spectrum; b) expanded view of the near-UV region (with an increased integration time) showing the availability of < 400nm light in addition to human-visible light. Spectra were measured with an Avantes AvaSpec-2048 with an integration time of a) 15ms and b) 50ms respectively, in the centre of bench areas where work took place.
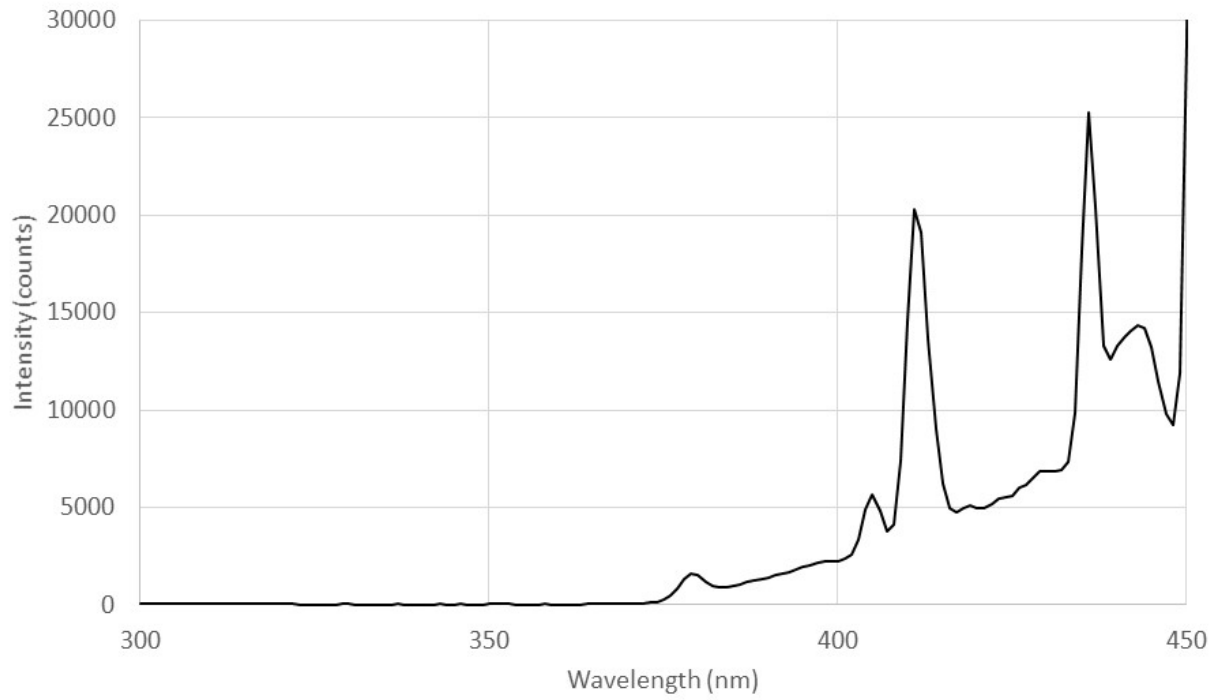
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781 A

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784 B

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