Multivariate statistics coupled to generalised linear models reveal complex use of chemical cues by a parasitoid

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ABSTRACT

Understanding how animals integrate multiple cues, in particular complex mixtures of volatile chemicals, is a subject of current interest. Insect behavioural responses to volatile blends have traditionally been analysed separately to the changes in blends themselves, making it difficult to directly link behaviour to volatile cues. We coupled principal component analysis (PCA) and generalised linear models (GLMs) to directly link volatiles released by plants in response to pea aphid (*Acyrthosiphon pisum*) feeding to the behaviour of the aphid parasitoid *Aphidius ervi*. We used choice bioassays to compare parasitoid response to odours from aphid-damaged and undamaged plants then collected volatiles from the same plants used in bioassays and analysed volatile profiles with PCA. The principal components explaining 99% of the variation in the dataset were used as explanatory variables in a GLM to analyse the behavioural response. This was done for two plant species: alfalfa, *Medicago sativa*, and broad bean, *Vicia faba*. For each species, a single principal component was important in explaining insect attraction. In both cases it explained a small amount of variability in the volatile dataset (7.0% and 0.5% in *M. sativa* and *V. faba* respectively). For both plants, the analysis revealed the presence of volatiles that supported or inhibited parasitoid attraction. Compositional analysis of the blend revealed no major changes in either plant, highlighting that *A. ervi* can detect minor changes, ignoring the major variability in the blend. The approach could be valuable for behavioural studies on multi-sensory orientation by foraging animals.

Keywords: *Acyrthosiphon pisum*; *Aphidius ervi*; compositional analysis; foraging behaviour; induced defence; *Medicago sativa*; plant volatiles; semiochemicals; tritrophic interactions; *Vicia faba*.

45 The use of multiple behavioural cues by animals is a topic of major current interest in
46 animal behaviour and ecology (Fischer et al. 2001; Fawcett & Johnstone 2003;
47 Kroder et al. 2007). Information from multiple environmental cues can be used to
48 determine foraging behaviour (Lucchetta et al. 2007), so understanding how these
49 cues are perceived is important for a deeper understanding of how animals find and
50 select food or hosts. In chemical ecology there is increasing understanding of how
51 animals integrate multiple volatile compounds present in blends for the detection of
52 suitable hosts or food items (Blight et al. 1995; Birkett et al. 2004; Wright & Smith
53 2004) and for the discrimination of host from non-host plants (Pureswaran et al. 2004;
54 Campbell & Borden 2006). Specificity can be achieved by integrating volatile
55 information from different types of plant damage (Hoballah & Turlings 2005), and
56 the use of ratios of multiple chemical compounds is now believed to be an important
57 mechanism providing insects with the specificity needed for distinguishing suitable
58 from unsuitable hosts or plants (Bruce et al. 2005). Another possibility is that there
59 may be thresholds for some compounds, providing specificity in themselves or in
60 combination with other volatiles.
61 Insect parasitoids have long served as model organisms in ecology (Godfray
62 & Shimada 1999), and have provided some of the major advances in understanding
63 the use of chemical cues in host and habitat location. However, despite considerable
64 evidence that parasitoids respond to plant volatiles, direct links between volatile
65 profiles and insect behaviour have rarely been made (van Dam & Poppy 2008).
66 Studies usually determine insect responses to volatile blends, characterise the blends
67 and then carry out bioassays with synthetic compounds in order to determine
68 individual or multiple attractants. This approach does not provide a direct link
69 between the volatile profiles and the insects' responses, since minor changes might

appear insignificant in the blend but could contribute greatly to the specificity of the insect response. Background odour may be a very important framework within which responses are calibrated, and insects respond to volatiles in the context of this background (Mumm & Hilker 2005; Galizia & Szyszka 2008). Further, univariate analysis of individual compounds in volatile blends requires an *a priori* assumption of which compounds are likely to be most important, possibly missing other important components (van Dam & Poppy 2008). Multiple univariate testing of multivariate data obtained from the same experimental units poses problems due to ignoring the correlation structure and inflation of the experiment-wise type I error rate, α (excessive rejection of the null hypothesis) (Rencher 2002). Recently, interesting methodological advances have been proposed for studying the importance of entire blends of compounds on parasitoid/predator foraging behaviour by selective filtering of the blends (D'Alessandro & Turlings 2005), but there is still a need for appropriate statistical approaches for analysing volatile profiles and linking them to insect behavioural responses (Hern & Dorn 2001; Pareja et al. 2007b; van Dam & Poppy 2008).

The aim of this study was to understand how an aphid parasitoid distinguishes between undamaged and aphid-damaged plants by more directly linking chemical analysis of volatile profiles to the insect behavioural response. We used a system based on two legumes: alfalfa, *Medicago sativa* L., and broad bean, *Vicia faba* L., the pea aphid, *Acyrthosiphon pisum* (Harris) and the parasitoid, *Aphidius ervi* Haliday. The tritrophic chemical ecology of this system on broad bean has been well described (Guerrieri et al. 1993; Du et al. 1996; Du et al. 1997; Guerrieri et al. 1997; Du et al. 1998; Guerrieri et al. 1999), whereas the system has been less studied on *M. sativa* (Daza-Bustamante et al. 2002).

We coupled two established statistical approaches in ecology and chemical ecology: principal components analysis (PCA) and generalised linear models (GLMs). PCA is a multivariate technique used extensively in many fields, including metabolomics and chemical ecology (Ayasse et al. 2000; Sumner et al. 2003). It involves breaking down a variance-covariance matrix of *p* variables into a set of ordered *p* orthogonal principal components (PCs), in decreasing order of variability explained, such that the first few PCs explain most of the variability. Thus a few PCs can be selected, reducing the dimensionality of the dataset (Ludwig & Reynolds 1988). Generalised linear models (GLMs) have been proposed as a powerful tool for the analysis of behavioural data (van der Meer 1992; Hern & Dorn 2001; Wajnberg & Haccou 2008). Since the PCs extracted from the PCA are independent, they can be used as explanatory variables in the GLM. This provides a direct link between volatiles emitted by the experimental plants and behavioural responses by the parasitoid, because chemical characteristics of the plants tested in the bioassay are used as explanatory variables in a statistical model.

MATERIALS AND METHODS

Plants and insects

Medicago sativa (Herbiseed, Twyford, UK) were germinated on damp filter paper. When the cotyledons started expanding, they were transplanted to 8 x 8 cm plastic pots filled with garden compost and placed in a greenhouse at 18:6 L:D and 21°C ± 2°C. *Vicia faba* (variety Hangdown Grunkernig, Svalöf Weibull, Svalöf, Sweden) seeds were sown individually in 8 x 8 cm plastic pots with compost and placed in the greenhouse.

Acyrthosiphon pisum colonies were started from 50 individual adults (approx.) feeding on *M. sativa* in the fields surrounding SLU, Uppsala, Sweden. The aphids were quarantined and reared in cages in the greenhouse on *V. faba*. The *A. ervi* colony was started from 50 individuals (approx.) provided by Rothamsted Research, UK, and reared on *A. pisum* on *V. faba*.

Preparation of plants and insects for bioassays and volatile collection

Medicago sativa individuals at the five-leaf stage were randomly assigned to either the undamaged (UD) or aphid-damaged (AD) treatment. AD plants received 30 mixed instar *A. pisum* and were covered with a perforated bag (Cryovac, New Jersey, USA) sealed with a rubber band around the pot. UD plants were treated in the same way but received no aphids. Plants were placed individually on plastic dishes in the greenhouse at 21°C and 18:6 L:D for three days. The plants were watered on the day of infestation with 200 ml (approx) in the plastic dish. After three days, plants were removed from the bags, and all aphids were removed from the plant using a fine paintbrush. The plastic pot and soil were covered in aluminium foil to minimise interference of volatiles from these sources in bioassays and volatile collections.

For *V. faba* the same procedure was followed with two differences; i) fifty *A. pisum* were placed on the plant, which has been shown to induce volatiles that elicit parasitoid attraction (Guerrieri et al. 1999) and ii) when aphids were removed from the plant both the UD and AD plants were swabbed with tepid water to remove traces of honeydew. This was not done for *M. sativa* because the plant was too delicate, and because the difference in architecture caused very little honeydew to accumulate.

Aphidius ervi mummies were regularly removed from the colony, separated from plant material and placed on a Petri dish in an emergence cage. Honey and water

were provided on a piece of cotton wool for adults to feed. All parasitoids used were less than four days old when tested, and were assumed to have mated.

Behavioural bioassays

Olfactory orientation of parasitoids to plant volatiles was tested in a Y-tube olfactometer (Monteith 1955) with a 0.8 cm internal diameter, 7 cm trunk and 5 cm arms. The plants were placed in glass bell jars, and charcoal-filtered air was pushed into each bell jar at 400 ml/min using Teflon tubing. The jars were connected to the Y-tube by Teflon tubing, and air was pulled out of the Y-tube at 250 ml/min through each arm. This push-pull system prevents entry of volatiles from the exterior. Bioassays were carried out in a controlled environment room at 21°C on a white bench under artificial lighting. Glass and Teflon materials were cleaned with detergent, distilled water and acetone, and baked in an oven for at least 16 hours at 175°C prior to use.

Aphidius ervi females were separated individually just before the bioassay, and introduced at the base of the Y-tube. On each Y-tube arm a choice line was drawn 1 cm after the junction. A parasitoid was observed for a maximum of five minutes, and as soon as it crossed the choice line a choice was recorded. After three insects, the Y-tube was exchanged for a clean one and the side on which UD and AD odours were presented was swapped to avoid directional bias. For each UD-AD combination a minimum of eight and a maximum of 18 parasitoids were tested on a single day. This was done to obtain a measurement of the relative attractiveness of the AD plant in each plant pair, but also to maximise the number of UD-AD pairs tested. Each UD-AD pair was used only once for bioassays and volatile collection.

166 ***Volatile collection and analysis***

167 Immediately after the bioassay, volatiles from the tested plants were collected by air-
168 entrainment (Agelopoulos et al. 1999). The whole plant was placed on a glass base
169 over which a glass vessel was fitted, and the plate and vessel were clamped together.
170 Through one of three openings at the top of the vessel, a glass liner containing 50 mg
171 of the molecular adsorbent Tenax TA (Atas GL Intl., Veldhoven, Netherlands) was
172 placed, and the other two were closed loosely with screw cap lids. A positive pressure
173 push-pull system was used, with charcoal-filtered air pushed into the vessel through
174 an aperture in the glass base, at 750 ml/min and pulled out through the adsorbent at
175 300 ml/min. The greater push rate prevents entry of contaminating volatiles, while the
176 loose caps avoid over-pressurising the vessel, which could affect the volatile release
177 by the plant (Tholl et al. 2006). All glassware and Teflon tubes were washed with
178 detergent, distilled water and acetone and baked in an oven at 175°C for at least 16
179 hours prior to the entrainment. Charcoal filters and Tenax tubes were baked at 175°C
180 and 220°C respectively under N_2 for 16 hours.

181 The UD-AD plant pairs used in bioassays were replicated 10 times for
182 *M. sativa* and nine times for *V. faba*, but for the multivariate analysis (see below)
183 more entrainment replicates were needed and an extra eight independent *M. sativa*
184 and 10 *V. faba* UD-AD pairs were entrained, giving a total of 18 replicate
185 entrainments for *M. sativa* and 19 for *V. faba*. Each pair was used only once.

186 Entrainments ran for 20 hours, after which collected volatiles were analysed
187 by gas chromatography (GC) on an Hewlett Packard 6890N (Agilent Technologies)
188 GC-flame ionisation detector (FID), mounted with an HP-1 column (100% dimethyl
189 polysiloxane, 50 m, 0.32 mm i.d. and 0.52 μm film thickness, J&W Scientific, USA),
190 fitted with an Optic 3 thermal desorption system (Atas GL Intl., Veldhoven,

191 Netherlands). As an internal standard (IS), 100 ng of octane was placed in the Tenax
192 containing the sample, which was then inserted into the unit as the inlet liner.
193 Volatiles were thermally desorbed starting at 40°C/0.5 min, and rising at 16°C/sec to
194 250°C. The GC temperature programme was 40°C/3 min, 5°C/min to 150°C/0.1 min,
195 10°C/min to 250°C/15 min, using H_2 as carrier. The amount of each compound was
196 calculated relative to the area of the IS.
197 For tentative compound identification, a sample from each treatment was
198 collected in the same way on Tenax TA and analysed by coupled GC-mass
199 spectrometry using a Thermo Finnigan MAT 95 XP instrument (ion trap mass
200 analyser, electron impact ionization 70 eV, source temperature 220°C) coupled to a
201 Finnigan Trace 2000 GC, which was fitted with an Optic 400 thermal desorption
202 system (Atas GL, UK). The column was an HP-1 (100 % dimethyl polysiloxane,
203 50 m, 0.32 mm i.d. and 0.52 μm film thickness, Hewlett-Packard, UK) and the oven
204 temperature was programmed at 30°C/5 min and 5°C/min to 250°C. The Tenax tube
205 was inserted into the unit as the inlet liner and the volatiles released directly onto the
206 GC column by thermal desorption on a programme starting at 20°C and rising to
207 220°C at a 16°C/sec. Mass spectra and retention indices were compared against a
208 NIST library and databases of retention indices from authentic standards. For
209 confirmation of compound identity, peak enhancement co-injection was carried out
210 with solvent extracts. Three solvent extracts of each treatment were collected by air
211 entrainment, as described above, but using Porapak Q (50 mg, Alltech, USA) as the
212 adsorbent. The compounds were eluted with 500 μl redistilled diethyl ether, and all
213 samples from the same treatment were bulked and concentrated down to 200 μl under
214 a gentle flow of N_2. The co-injection was carried out on the GC described above, with
215 cool-on-column injection and a temperature programme of 30°C/1 min, 5°C/min to

150°C/0.1 min, 10°C/min to 250°C/20 min, using H_2 as carrier. Stereochemistry of linalool and germacrene D was determined using an HP5890 GC (Agilent Technologies) with FID, mounted with a β-cyclodextrin chiral column (30 m, 0.25 mm i.d. and 0.25 μm film thickness) and cool-on-column injection with a temperature programme of 40°C/1 min, 3°C/min to 150°C/0.1 min, 5°C/min to 180°C/15 min. For each compound, a 1 μl aliquot of an equal mixture of enantiomers was injected, followed by injections of solutions of each enantiomer separately. Each of the three solutions was then coinjected with the solvent extracts of the air entrainments. Peak enhancement with either enantiomer confirmed the presence of that enantiomer in the sample.

All commercially available compounds were purchased from Sigma-Aldrich (Steinheim, Germany), Fluka (Buchs., Switzerland), Botanix Ltd. (Paddock Wood, Kent, UK), Bush-Boake Allen (London, UK) or SAFC (St. Louis, MO, USA). (*E*,*E*)-4,8,12-Trimethyl-1,3,7,11-tetraene (TMTT) was synthesised from (*E*,*E*)-farnesol by oxidation to its aldehyde followed by Wittig methylenation (Leopold 1990). (S)-(-)-Germacrene D and (R)-(+)-germacrene D (both 98% purity) were obtained by incubation of farnesyl pyrophosphate with purified, expressed (R)-(+) or (S)-(−)-germacrene-D synthase and subsequent hexane extraction and purification through a short column of silica gel (BDH, 40–63 μm)/MgSO4 (10:1) (Prosser et al. 2004). 4-Oxo-(*E*)-2-hexenal was prepared from 2-ethylfuran, using aqueous N-bromosuccinimide (NBS) to promote oxidative ring opening (Moreira & Millar 2005).

Statistical analysis

Analysis of aphid-induced volatiles

The total amount of volatiles produced by each undamaged and aphid-damaged plant was calculated by summing the amount of individual compounds produced in 24 hrs. These data were logarithmically transformed and analysed by ANOVA, fitting treatment (UD or AD) as the explanatory variable and plant pair nested within date as design variables.

Changes in individual compounds were subjected to compositional analysis (Aitchison 1986), which is emerging as a powerful technique for analysing chemical blends (Martín & López 2006b; Martín & López 2006a; Pareja et al. 2007b; Moraes et al. 2008). The peak areas of all compounds obtained from the GC trace were normalised to 100%, and the area of each peak was transformed according to Aitchison (1986): $\mathrm{Ln}(A_i/A_{IS})$, where A_i is the standardised area of the *i*th peak and A_{IS} the standardised area of the internal standard. This was then analysed by multivariate analysis of variance (MANOVA) fitting the treatment (UD or AD) and plant pair as explanatory factors.

Analysis of insect responses

The amounts of individual compounds present in each plant sample were expressed as a proportion of the total amount of volatiles present in the sample. For each AD-UD pair, the proportion of each compound in the UD was subtracted from that in the AD creating a vector of differences for all compounds in each AD-UD replicate and giving a single value for each compound in each pair. A positive value indicates higher relative presence in the AD plant, while a negative value indicates higher relative presence in the UD plant. Since the insect makes a relative choice between

262 the two odours presented, this is a biologically accurate way of treating the data.
263 Combining all paired replicates gives a multivariate dataset where each compound is
264 a variable, represented in each replicate by the difference between the samples (AD-
265 UD). This was analysed by principal components analysis (PCA). From the PCA, the
266 scores for each replicate were extracted along each principal component (PC),
267 representing the position of each replicate along each PC. The PCs are orthogonal to
268 each other, allowing the use of scores along each PC as explanatory variables in the
269 GLM, eliminating the problem of collinearity (Hern & Dorn 2001).
270 *Aphidius ervi* response was analysed using a GLM with binomial errors and
271 logit link function. The response variable was the number of insects responding to the
272 AD odour as a proportion of the total number tested for each UD-AD pair. The group
273 of insects tested to each plant AD-UD pair therefore constitutes one replicate,
274 correcting for the inherent clustering of the data. The explanatory variables fitted in
275 the GLM were the PCs that explained 99% of the variability in the PCA, as well as a
276 term for the difference in total volatile amount. Significance was tested by removing
277 terms individually from the model and testing the change in deviance against a χ^2
278 distribution with one degree of freedom.
279 The loadings were then extracted from the PCA, giving the contribution of
280 each variable (compound) to each PC. Doing this for PCs that are significant in
281 explaining parasitoid attraction (from the GLM) allows determination of which
282 combination of compounds were important in explaining the attraction.
283 To test whether the response of the parasitoid was significantly different to
284 50%, a separate binomial GLM was fitted controlling for plant pair to estimate the
285 proportion response of *A. ervi*. The significance of the response to the AD plant was
286 tested using a χ^2 Wald test for departure from 50%. All analyses were carried out in R

(R Development Core Team 2007), using the princomp function for the PCA and the glm (family=binomial) function for the GLM.

RESULTS

Aphid-induced volatiles

The total amount of volatiles produced did not differ between AD and UD plants in *M. sativa* (treatment effect: $F_{1,17}$=2.93, *P*=0.105; date: $F_{9,17}$=0.71, *P*=0.697; date×plant pair: $F_{8,17}$=1.27, *P*=0.322) nor in *V. faba* (treatment effect: $F_{1,20}$=1.20, *P*=0.287; date: $F_{11,20}$=15.30, *P*<0.001; date×plant pair: $F_{9,20}$=1.32, *P*=0.289).

The volatile blend of *M. sativa* did not differ significantly between UD and AD plants when analysed by compositional analysis (Wilks' $\Lambda=8.21\times10^{-2}$, approx. $F_{17,1}$=0.66, *P*=0.766). Plant pairs differed significantly in their blend (Wilks' $\Lambda=1.36\times10^{-13}$, approx. $F_{289,55.7}$=2.05, *P*<0.001), highlighting the importance of controlling for variability in design factors. Benzyl alcohol and methyl salicylate showed increases in the AD plant (Table 1). With univariate ANOVA a significant increase was detected for both benzyl alcohol ($F_{1,17}$=4.53, *P*=0.048) and methyl salicylate ($F_{1,17}$=13.33, *P*=0.002).

The volatile blend detected in *V. faba* is very similar to previous reports (Webster et al. 2008) (Table 2). Compositional analysis did not reveal any change in the overall blend upon aphid damage (Wilks' $\Lambda=2.50\times10^{-2}$, $F_{19,2}$=4.104, *P*=0.214). The effect of plant pair was significant (Wilks' $\Lambda=5.39\times10^{-16}$, $F_{380,87.4}$=2.70, *P*<0.001). (*Z*)-3-Hexenal ($F_{1,20}$=6.53, *P*=0.019) and 6-methyl-5-hepten-2-one ($F_{1,20}$=5.28, *P*=0.032) showed decreases in the AD plant when the univariate breakdown was carried out (Table 2).

Benzaldehyde, nonanal and decanal were detected in both plants, but could not be analysed quantitatively because preliminary studies showed they are produced as breakdown products by thermal desorption of Tenax TA, as reported previously (Tholl et al. 2006).

Response of A. ervi

In total, 162 *A. ervi* females were tested for attraction to AD against UD *M. sativa*, of which 146 (90%) made a choice. Of these, 78 (53%) chose the AD plant, which in the GLM controlling for plant pair was not significantly different from 50% (Wald χ^2=1.95, P=0.162). However this hid much useful information, since attraction to the AD varied among plant pairs from 29% to 75%.

In the PCA for *M. sativa*, the principal components that explained 99% of the variability were PC1-PC6, but only PC4 was significant (Table 3) in explaining the proportion response of *A. ervi* in the GLM (Fig. 1), even though this component only explained 7% of the variation in the multivariate dataset (Table 3). PC2 was marginally non-significant (Table 3). However, this might have been a spurious effect due to the presence of an outlier, which also reduced the influence of PC4 (Fig. 1). The compounds contributing most to PC4 were benzyl alcohol and, to a lesser extent, (*E*)-ocimene, (*Z*)-3-hexenyl acetate and methyl salicylate (positively) and (*Z*)-3-hexen-1-ol (negatively) (Table 1). This indicates that a high positive difference in benzyl alcohol and (*E*)-ocimene (greater amounts in the AD plant) combined with a large negative difference in the amount of (*Z*)-3-hexen-1-ol (lower amounts in the AD plant) elicit parasitoid attraction to the AD plant (right side in Fig. 1), while the opposite elicits attraction to the UD plant (left side of Fig. 1). This is illustrated by representative GC traces from samples of two pairs of *M. sativa* used in biossays (Fig. 2). It can be seen that the plants with a high relative presence of benzyl alcohol were

335 attractive, while those with a high presence of (*Z*)-3-hexen-1-ol were unattractive,
336 even if they were aphid-infested. In pair A (Fig. 2A) the AD plant has a larger amount
337 of benzyl alcohol, (*E*)-ocimene and methyl salicylate, relative to the UD plant, and
338 was highly attractive. On the other hand for pair B (Fig. 2B), even though benzyl
339 alcohol was higher in the AD plant, the increase in (*Z*)-3-hexen-1-ol, coupled to
340 similar amounts of (*E*)-ocimene and methyl salicylate made the AD plant unattractive.
341 When the two plants were roughly equal in terms of these compounds, the parasitoids
342 did not discriminate between the test plants (Fig. 2C).
343 For *V. faba*, a total of 136 *A. ervi* females were tested, with 124 making a
344 choice (91%). Of these, 73 (59%) chose the AD plant, and this was significant when
345 tested with the GLM controlling for plant pair (χ^2=3.98, *P*=0.046). Variability in
346 attraction to the AD plant between plant pairs was large, and varied between 30% and
347 76%.
348 Principal components PC1-PC7 explained 99% of the variability in the
349 multivariate dataset, and only PC7 was significant in explaining *A. ervi* response,
350 despite this component only explaining 0.5% of the variability (Table 3). The effect
351 of PC7 was negative (Fig. 3), and therefore the interpretation of the importance of
352 compounds in explaining *A. ervi* response is inverted: negative loadings indicate
353 positive effects on attraction, while positive loadings indicate negative effects on
354 attraction. Thus, the attraction of *A. ervi* was stronger with large differences (i.e. more
355 in the AD plant) in benzyl alcohol, (*E*)-caryophyllene and methyl salicylate, coupled
356 to large negative differences (less in the AD plant) in (*Z*)-3-hexenal, (*S*)-(-)-
357 germacrene D, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tetraene (TMTT), hexan-1-ol and (*R*)-
358 linalool (Table 2).

DISCUSSION

We have presented an analysis that directly links behavioural cues to which foraging insects were exposed with the insects' response in a simplified environment (olfactometer). When the mean response was tested, *A. ervi* responded significantly to volatiles from *V. faba* but not *M. sativa*. The response to *V. faba* is in accordance with previous studies showing the attraction of this parasitoid to *A. pisum*-damaged plants (Guerrieri et al. 1993; Du et al. 1996; Guerrieri et al. 1999). Previous studies showing attraction to *M. sativa* (Daza-Bustamante et al. 2002) used individuals reared on *M. sativa*. Thus, the similarity in the importance of individual compounds to the response to both plants may be due to the insect being conditioned to *V. faba* upon eclosion from the mummy (van Emden et al. 1996; Storeck et al. 2000), and subsequently responding to those plants most similar to *V. faba*. The ecological relevance of this may be that individuals of a rare plant species in a given habitat may attract more parasitoids when they are more chemically similar to dominant plants.

The approach we present provides insight into the balance of cues *A. ervi* uses when foraging for hosts, which is only possible when the same plants used in bioassays are analysed chemically. Thus, in *M. sativa,* AD plants that released high levels of benzyl alcohol and (*E*)-ocimene, and low levels of (*Z*)-3-hexen-1-ol relative to the UD control elicited higher proportional responses from the parasitoid, which was supported by inspection of individual GC traces. In *V. faba,* AD plants that released high levels of benzyl alcohol and (*E*)-caryophyllene and low levels of (*Z*)-3-hexenal, germacrene D and TMTT relative to the UD control elicited higher proportional responses from the parasitoid. Several interesting patterns emerge from these results. First, benzyl alcohol appears to be of major importance in eliciting positive responses in *A. ervi* in both plant species. Second, this positive effect is

coupled to a negative effect of green leaf volatiles: (*Z*)-3-hexen-1-ol in *M. sativa* and (*Z*)-3-hexenal in *V. faba*. Third, methyl salicylate appears to be an important compound, with smaller quantitative importance, but qualitatively consistent in both plants, serving to enhance responses. *Aphidius ervi* therefore appears to be integrating all these chemical cues when foraging for aphid-infested plants. Methyl salicylate has been shown to be repellent to aphids (Glinwood & Pettersson 2000), and the results presented here add to the evidence that methyl salicylate plays an important role in plant-aphid-natural enemy interactions (Ninkovic et al. 2003; Zhu & Park 2005; Glinwood et al. 2007). We could not detect any effect of 6-methyl-5-hepten-2-one in this study as has been previously reported (Du et al. 1998). This could be due to conditioning to very minor differences in varietal odours of *V. faba*, since Du et al. (1998) reported an increase in parasitoid response to this compound after adult experience. Laboratory bioassays have shown *A. ervi* responses to synthetic standards of many of the compounds detected here (Du et al. 1998; Sasso et al. 2007), but care should be taken in extrapolating these bioassays because the same compound can have both negative and positive effects on parasitoid response depending on the concentration presented (Pareja et al. 2007b) and the background blend can modify these responses (Mumm & Hilker 2005). Insects will respond to a wide range of plant compounds presented at detectable concentrations if the alternative is clean air (Bruce et al. 2005), though the minimal blends necessary for insect attraction have been recreated (Piñero & Dorn 2007; Piñero et al. 2008).

It is likely that in many tritrophic systems chemical mediation is due, not to failsafe foraging cues, but to complex integration of multiple chemical cues (van Dam & Poppy 2008), so to advance understanding of how organisms respond to chemical and non-chemical cues, study of this complex integration is needed. Recent advances

409 in the study of insect neural processing of olfactory responses reveal that odorants are
410 probably handled in a combinatorial fashion, with different quantitative combinations
411 of odorants triggering different responses (Galizia & Szyszka 2008). A recent review
412 has highlighted the need for statistical techniques that confront this complexity, in
413 order to obtain a greater understanding of how multiple cues are used by animals (van
414 Dam & Poppy 2008). The recent application of compositional analysis to the study of
415 semiochemical variation is a promising development (López & Martín 2005; López et
416 al. 2006; Martín & López 2006a; Martín & López 2006b; Pareja et al. 2007a; Pareja
417 et al. 2007b; Moraes et al. 2008), but does not directly link changes to the relevant
418 insect responses, since the volatile blends are analysed separately to the plants used in
419 bioassays.

420 Multivariate statistics and GLMs have been proposed individually for the
421 analysis of volatile blends (van Dam & Poppy 2008) and animal responses (van der
422 Meer 1992), but to our knowledge only one study has used both PCA and GLMs for
423 the study of semiochemicals (Hern & Dorn 2001). The cited study used PCA to select
424 compounds that were independent from each other, thus reducing collinearity. Here
425 we have included the principal components directly as explanatory variables in the
426 GLM with the advantage of capturing most of the variability in the dataset with fewer
427 explanatory variables. This approach directly analyses the effect of the entire blend on
428 insect attraction, in a manner analogous to many other ecological experiments, which
429 aids in assigning relevant biology to the detected chemical compounds. The analysis
430 removes the emphasis on large changes, and considers how even minor changes
431 might affect foraging behaviour (Piñero et al. 2008). The principal components
432 explaining attraction of *A. ervi* accounted for only 7% and 0.5% of the variation in the
433 dataset in *M. sativa* and *V. faba*, respectively. It appears that the parasitoid avoids the

major variability in the volatile blend, which is likely a source of "noise", and thus uninformative. These results agree with the suggestions that small, reliable changes in in plant chemistry could explain changes in insect responses (Bukovinszky et al. 2005; Pareja et al. 2007b). Some of the variability observed in our results could be due to age differences in the parasitoids tested, and future work should study how parasitoid age affects these responses. With the same methodology presented, but using mixed effects models, parasitoid age can be introduced as a fixed effect to study how this interacts with the volatile blend.

The analyses for aphid-induced changes were carried out on the relative contributions of each compound to the blend, correcting for differences in total amounts in different replicates. (*Z*)-3-Hexenyl acetate in both plants appeared to have large changes in concentration (Table 1 and Table 2), but the compositional analysis did not reveal differences in the contribution of this compound to the overall blend. In *M. sativa*, benzyl alcohol and methyl salicylate showed an increase in AD plants, corresponding to the effect found for PC4, in which both of these compounds had a large loading, and the resulting behavioural importance for *A. ervi* (Fig. 1). However, for *V. faba*, only a partial correspondence was found, with the decrease in (*Z*)-3-hexenal corresponding to the negative effect this compound had on attraction of *A. ervi*. This emphasises that studying the volatiles as a blend, and not as individual compounds released in isolation, is more biologically realistic, since the production of compounds is not independent due to shared biochemical pathways and frequent crosstalk between the pathways (Bostock 2005).

This study shows volatile blends can be used as explanatory variables to elucidate insect foraging, thus directly linking induced plant defence and insect behaviour. Furthermore, widely-used techniques can be adapted for the simultaneous

459 analysis of multiple cues and animal foraging behaviour with only minor
460 experimental modifications. When applied to parasitoid foraging in complex volatile
461 blends, the technique revealed that *A. ervi* uses similar volatile cues when foraging for
462 two different plant species, and that the parasitoid can detect minor changes in
463 volatile blends. The approach could be of use in behavioural studies aimed at
464 understanding the integration of different sensory modalities by foraging animals
465 through standardisation of variables measured in different units, a technique widely
466 used in multivariate statistics. For example, the egg parasitoid *Telenomus podisi* uses
467 semiochemical cues from its host (Borges et al. 2003), its host plant (Moraes et al.
468 2005; Moraes et al. 2008) and vibratory cues on the plant (Laumann et al. 2007).
469 These have been studied separately, but studies could collect information on these
470 three types of cue during or just after bioassays, generating a multivariate dataset, and
471 the technique presented could be used to analyse the insects' repsonses. This type of
472 approach could contribute to a more integrated understanding of how animals
473 perceive and respond to the complexity of their environment.

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681 **TABLES**

682 Table 1 Volatile compounds identified from *M. sativa* headspace, with the mean amount detected in 24 hrs in undamaged (UD) and *A. pisum*-
683 damaged (AD) plants. The six principal components (PC) listed are those explaining 99% of the variability in the PCA, and used in the GLM for
684 *A. ervi* response to AD vs UD plants. For each PC only the largest loadings are presented. PC4 (in bold) was the only significant PC in
685 explaining *A. ervi* choice responses; (+) and (-) next to the loadings indicate a positive and negative effect (respectively) on *A. ervi* attraction.
686 nq-not quantified because the compound is a known breakdown product of the molecular absorbent (Tholl et al. 2006)

	Amount in 24 hrs (mean in ng ± s.e.)		Loadings					
Compound	UD	AD	PC1	PC2	PC3	**PC4**	PC5	PC6
Hexanal	8.49 (± 3.36)	11.96 (± 5.72)						
(*Z*)-3-Hexenal	15.84 (± 1.64)	15.75 (± 1.79)						
(*E*)-2-Hexenal	6.93 (± 2.66)	10.26 (± 6.67)			0.162		-0.228	
(*Z*)-3-Hexen-1-ol	40.50 (± 12.25)	60.76 (± 17.54)	0.195	0.409	0.584	**-0.603 (-)**		-0.110
6-Methyl-5-hepten-2-one	15.55 (± 3.69)	9.30 (± 1.95)		-0.159			-0.673	-0.655
Sabinene	1.03 (± 0.45)	0.79 (± 0.33)						
Myrcene	28.21 (± 3.32)	25.41 (±2.82)		-0.122				

	Amount in 24 hrs (mean in ng ± s.e.)		Loadings					
Compound	UD	AD	PC1	PC2	PC3	**PC4**	PC5	PC6
(*Z*)-3-Hexenyl acetate	150.88 (± 37.54)	243.62 (± 42.01)	0.965		-0.189	**0.155 (+)**		
Benzyl alcohol	72.88 (± 19.83)	120.60 (± 33.89)			0.661	**0.725 (+)**		0.114
(*Z*)-Ocimene	1.19 (± 0.49)	1.94 (± 0.86)						-0.262
(*E*)-Ocimene	21.82 (± 7.61)	36.98 (± 13.01)	-0.143	0.767	-0.318	**0.231 (+)**	0.148	
(*E*)-2-Octen-1-ol	8.30 (± 0.93)	7.07 (± 0.93)						
(*R*)-Linalool	10.24 (± 4.84)	12.47 (± 6.26)		0.379			-0.659	
Methyl salicylate	8.64 (± 1.77)	24.26 (± 4.34)			-0.194	**0.125 (+)**		
Indole	5.26 (± 1.34)	3.99 (± 0.68)					0.129	-0.196
(*E*)-Caryophyllene	6.62 (± 2.42)	5.73 (± 2.08)		0.196				-0.357

687

688

689 Table 2 Volatile compounds identified from *V. faba* headspace, with the mean amount detected in 24 hrs in undamaged (UD) and *A. pisum*-
690 damaged (AD) plants. The seven principal components (PC) listed are those explaining 99% of the variability in the PCA, and used in the GLM
691 for *A. ervi* response to AD vs UD plants. For each PC only the largest loadings are presented. PC7 (in bold) was the only significant PC in
692 explaining *A. ervi* choice responses; (+) and (-) next to the loadings indicate a positive and negative effect (respectively) on *A. ervi* attraction.
693 nq-not quantified because the compound is a known breakdown product of the molecular absorbent (Tholl et al. 2006)

	Amount in 24 hrs (mean in ng ± s.e.)		Loadings						
Compound	UD	AD	PC1	PC2	PC3	PC4	PC5	PC6	**PC7**
Hexanal	55.47 (± 15.90)	39.85 (± 10.12)						0.211	
(*Z*)-3-Hexenal	13.48 (± 2.66)	9.39 (± 2.95)						-0.245	**0.452 (-)**
(*E*)-2-Hexenal	21.87 (± 8.85)	16.81 (± 7.01)						0.102	
(*Z*)-3-Hexen-1-ol	649.71 (± 209.78)	575.82 (± 139.86)	0.743	-0.139	0.610	-0.124	-0.145		
Hexan-1-ol	31.97 (± 11.67)	31.53 (± 8.82)						0.232	**0.115 (-)**
4-Oxo-(*E*)-2-hexenal	26.73 (± 5.59)	26.37 (± 3.82)						-0.124	
6-Methyl-5-hepten-2-one	32.06 (± 7.50)	21.20 (± 3.54)							
(*Z*)-3-Hexenyl acetate	795.40 (± 206.06)	391.88 (± 87.33)	-0.211	-0.966					
Benzyl alcohol	41.26 (± 6.51)	47.95 (± 8.40)				0.184	0.239	0.278	**-0.707 (+)**

Compound	Amount in 24 hrs (mean in ng ± s.e.)		Loadings						
	UD	AD	PC1	PC2	PC3	PC4	PC5	PC6	**PC7**
(*Z*)-Ocimene	8.06 (± 2.81)	7.35 (± 2.54)							
(*E*)-Ocimene	163.19 (± 45.94)	160.42 (± 55.82)	-0.622	0.154	0.739		-0.139		
(*R*)-Linalool	13.94 (± 4.31)	13.60 (± 5.63)							**0.104 (-)**
Methyl salicylate	13.81 (± 3.65)	14.63 (± 4.21)			-0.223		-0.920		**-0.118 (+)**
Benzothiazole	15.75 (± 1.91)	18.28 (± 5.02)							
Indole	16.54 (± 2.67)	16.52 (± 1.92)					0.123	-0.115	
(*E*)-Caryophyllene	59.66 (± 13.37)	49.88 (± 11.12)			-0.129	-0.880		-0.271	**-0.303 (+)**
(*S*)-(-)-Germacrene D	10.54 (± 2.11)	16.96 (± 7.67)				-0.111			**0.263 (-)**
(*E*,*E*)-4,8,12-Trimethyl-1,3,7,11-tetraene (TMTT)	71.58 (± 17.43)	63.58 (± 17.03)				-0.366		0.790	**0.223 (-)**
Methyl jasmonate	6.13 (± 1.13)	5.68 (± 1.71							

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Table 3 Results of the statistical analyses for *M. sativa* and *V. faba*. The top line for each species presents the variation explained from the PCA by each principal components (PC), together explaining 99% of the variability. The bottom line presents the χ^2 and *P*-value from the GLM model simplification (see Methods) including the PCs and the difference in total volatile amount as explanatory variables for the proportion of *A. ervi* choosing the aphid-damaged plant.

Medicago sativa	PC1	PC2	PC3	PC4	PC5	PC6		Diff. total amount
Variation explained (%)	63.7	14.5	11.7	7.0	1.5	0.7	-	-
χ^2 (*P*-value)	1.22 (0.270)	2.89 (0.089)	0.87 (0.350)	5.17 (0.023)	0.21 (0.648)	0.85 (0.356)	-	0.52 (0.470)
Vicia faba	PC1	PC2	PC3	PC4	PC5	PC6	PC7	Diff. total amount
Variation explained (%)	46.6	37.8	9.0	2.7	1.5	0.9	0.5	-
χ^2 (*P*-value)	0.08 (0.777)	<0.01 (0.964)	0.26 (0.611)	0.48 (0.490)	0.64 (0.423)	0.26 (0.609)	4.59 (0.032)	1.54 (0.215)

FIGURE LEGENDS

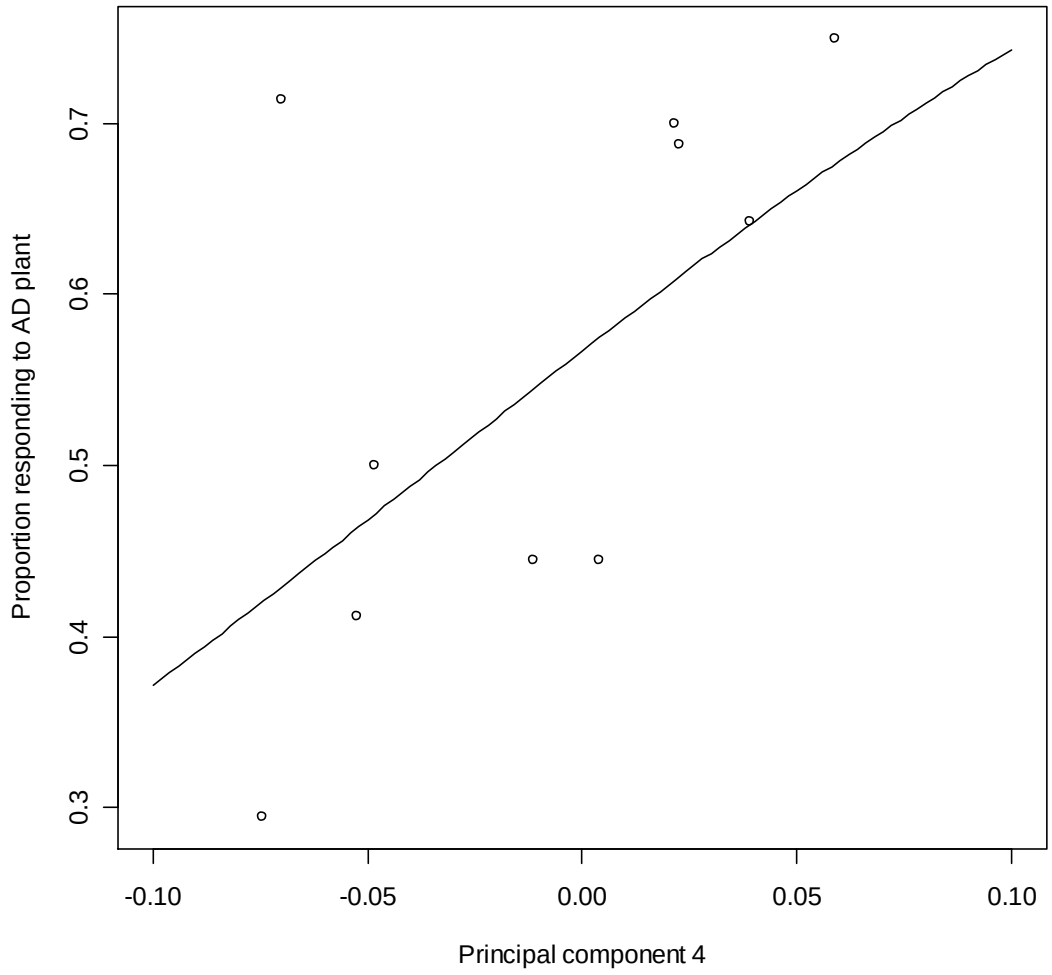
Fig. 1 Relationship between principal component PC4 and the proportion of *A. ervi* responding to *M. sativa* damaged by *A. pisum* (AD plant). The effect of PC4 in explaining parasitoid attraction was significant in a generalised linear model (χ^2==5.17, *P*=0.023). The line is the fitted GLM model: p= exp(0.27+7.95×*PC4*)/(1+(exp(0.27+7.95×*PC4*)), where p is the proportion response.

Fig. 2 Representative examples of GC traces from entrainments of *M. sativa* previously tested in a Y-tube olfactometer for *A. ervi* attraction. For the top pair (A) the parasitoid preferred the aphid-damaged (AD) plant over the undamaged (UD) plant, with 75% choosing the AD plant. For the middle pair (B), the parasitoid preferred the UD plant, with only 29% choosing the AD plant. When tested with the bottom pair (C) the parasitoid did not discriminate between the UD and AD plant, as shown by paired PCA-GLM analysis, the parasitoid chose plants with higher levels of (*Z*)-3-hexenyl acetate (**2**), benzyl alcohol (**3**), (*E*)-ocimene (**4**) and methyl salicylate (**5**) relative to (*Z*)-3-hexen-1-ol (**1**). IS-internal standard: 100 ng octane. See text for further discussion.

Fig. 3 Relationship between principal component PC7 and the proportion of *A. ervi* responding to *V. faba* damaged by *A. pisum* (AD plant). The effect of PC7 in explaining parasitoid attraction was significant in a generalised linear model (χ^2==4.59, *P*=0.032). The line is the fitted GLM model: p= exp(0.43-36.48×*PC7*)/(1+(exp(0.43-36.48×*PC7*)), where p is the proportion response.

725 **FIGURES**

726 Fig. 1



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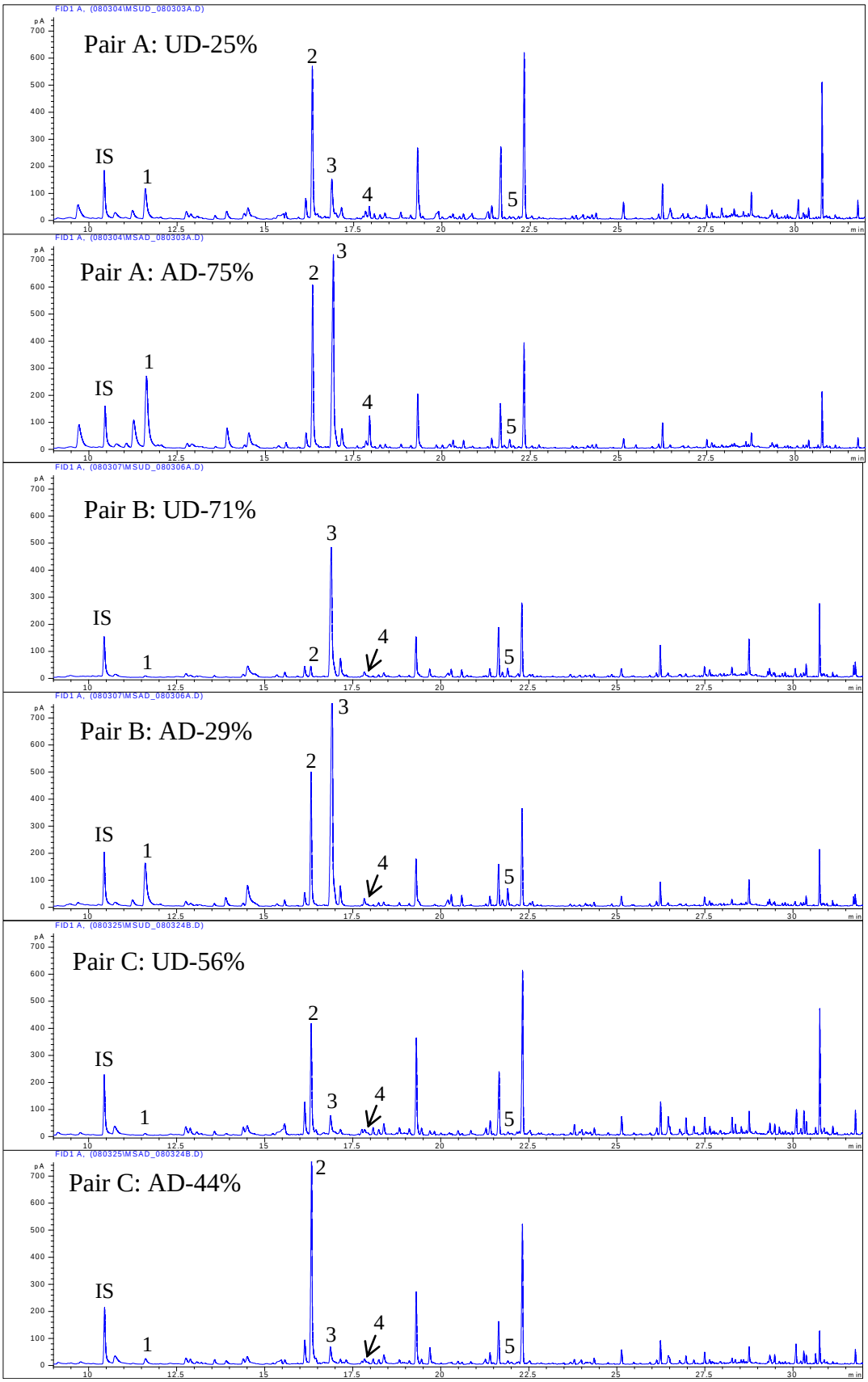
729 Fig. 2



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733 Fig. 3

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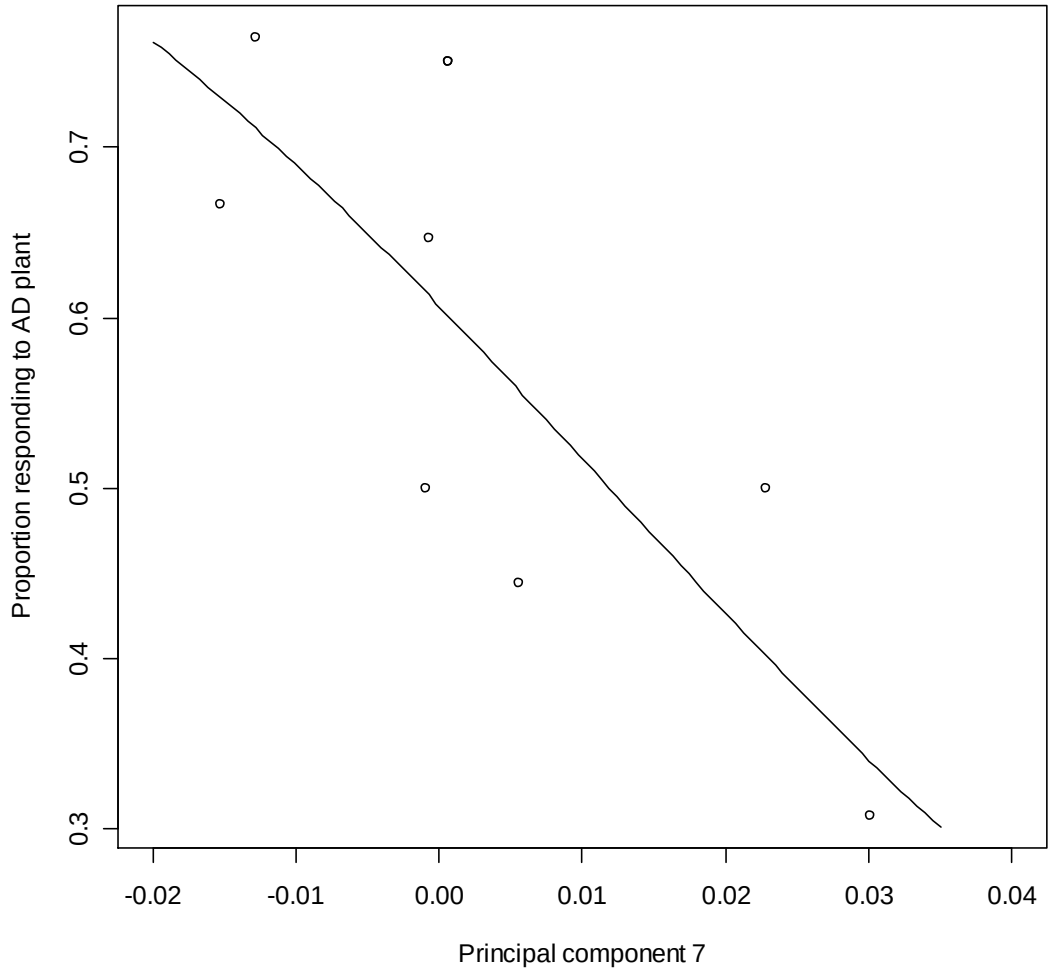

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