2018-11-30

Riding on the wind: volatile compounds dictate selection of grassland seedlings by snails.

Hanley, ME

http://hdl.handle.net/10026.1/12846

Riding on the wind: volatile compounds dictate selection of grassland seedlings by snails

Mick E Hanley[1*], Roger WR Shannon[1,2], Damien G Lemoine[3], Bethan Sandey[1], Philip L Newland[2], Guy M Poppy[2]

[1] *School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth. PL4 8AA. UK;* [2] *Centre for Biological Sciences, University of Southampton, Southampton SO17 1BJ, UK;* [3] *Ecologie des Hydrosystèmes Naturels et Anthropisés, Ecologie, Evolution, Ecosystèmes Souterrains (E3S) Université Lyon 1, Bât. Forel, 6, rue Raphaël Dubois, 69 622 Villeurbanne Cedex France*

* For correspondence

Dr Mick Hanley

E-mail: mehanley@plymouth.ac.uk

Orchid ID - 0000-0002-3966-8919

Running Head - Seedling volatiles and selection by snails

1 **Abstract**

2 • **Background and Aims** Seedling herbivory is an important selective filter in many

3   plant communities. The removal of preferred food plants by both vertebrate, but more

4   commonly, invertebrate herbivores can destroy entire seedling cohorts, and

5   consequently, dictate plant community assembly. Nevertheless, our understanding of

6   how and why some seedlings are more prone to herbivore attack than their neighbours

7   remains limited. For seedlings, where even minor tissue damage is fatal, avoiding

8   contact with herbivores is likely advantageous and on this basis, volatile organic

9   compounds (VOCs) are strong candidates to fulfil a primary defensive role.

10 • **Methods** We quantified seedling selection by snails (*Cornu aspersum*) for 14

11   common, European grassland species. Seedling acceptability was subsequently

12   compared with species-specific expression of constitutive secondary defence

13   metabolites (CSDMs), and VOCs to determine their relative influence on seedling

14   selection.

15 • **Results** We found no relationship between seedling acceptability and CSDMs, but

16   seedling selection was strongly associated with VOC profiles. Monoterpenes

17   (specifically β-ocimene), were identified as likely attractants, while green leaf

18   volatiles (GLVs) (3-hexen-1-ol acetate) were strongly associated with low seedling

19   acceptability.

20 • **Conclusions** By elucidating a relationship between VOCs and seedling acceptability,

21   we contradict a long-held, but poorly tested, assumption that seedling selection by

22   herbivores in (semi-) natural plant communities centres on CSDMs. Instead, our

23   results corroborate recent work evidencing how GLVs, including 3-hexen-1-ol

24   acetate, deter crop seedling selection by molluscs. Although our failure to establish

25　　any early-ontogenetic relationship between VOCs and CSDMs also suggests that the

26　　former do not 'advertise' possession of the latter, we nevertheless evidence the role

27　　that VOCs play in defending seedlings against herbivory before lethal damage occurs.

28　　**Key Words** – *Cornu aspersum*, green leaf volatiles, herbivory, monoterpenes, olfactory

29　　selection, plant defence, seedling herbivory, VOC

30

31

32 **INTRODUCTION**

33 Plants synthesize and release a varied array of volatile organic compounds (VOCs) to

34 protect themselves against abiotic stresses, communicate the availability of floral rewards

35 and fruits to pollinators and seed dispersers, and to defend themselves against pathogens

36 and herbivores (Dudareva *et al.,* 2013). Their role as herbivore feeding and oviposition

37 cues is particularly well established (Unsicker *et al.,* 2009; Hare, 2011), but as with most

38 aspects of plant-herbivore interactions, the research focus has been on established plants.

39 Despite the pivotal role seedling herbivores play in shaping the dynamics and structure

40 of established vegetation (Barton and Hanley, 2013), remarkably few studies have set out

41 to examine how VOCs influence seedling herbivory. This omission may in part be

42 ascribed to the view that physiological and biochemical constraints limit the development

43 and expression of seedling defences (Boege and Marquis, 2005). Indeed, studies on

44 ontogenetic shifts in plant chemical defence show, that in general, seedlings are less well

45 defended than older conspecifics (Elger *et al.,* 2009; Barton and Hanley, 2013; Hanley *et*

46 *al.,* 2013; but see Goodger *et al.,* 2013).

47 There are, nevertheless, good reasons to expect seedlings to defend themselves.

48 Herbivores like molluscs prefer herbaceous plants during the early ontogenetic stages

49 compared to older plants (Barton and Koricheva, 2010). Moreover, unlike mature plants,

50 seedlings often suffer immediate and total destruction following herbivore attack with

51 limited opportunity to compensate for extreme tissue loss (Hanley *et al.,* 1995; Hanley *et*

52 *al.,* 2004; Barton, 2016). Like mature plants, however, there is species-specific variation

53 in seedling selection by herbivores (see Hanley *et al.,* 1996; Hanley, 2004; Barlow *et al.,*

54 2013), with concomitant implications for the composition of established vegetation

55 (Barton and Hanley, 2013; Hanley and Sykes, 2014). Given the limited opportunity for

56   the development of structural defences during early ontogeny (Hanley et al., 2007), the

57   assumption is that seedling selection is based on variation in the expression of constitutive

58   secondary defence metabolites (CSDMs) (Hulme, 1994; Barlow *et al.,* 2013; Barton and

59   Hanley, 2013). Nevertheless, we are aware of only one study that has actually tested this

60   hypothesis; Hanley and Lamont, (2001) reported a strong negative relationship between

61   phenolic concentrations and herbivory in cotyledon-stage Proteaceae seedlings in

62   Western Australia. This study focussed however, on seedlings exposed to (unknown)

63   herbivores in established Mediterranean-climate heathlands. In fact, seedling

64   regeneration in this system is generally restricted to the immediate post-fire period when

65   herbivore abundance is much reduced; a scenario very different from temperate

66   grasslands where invertebrate herbivores like molluscs are numerous and particularly

67   active when seedlings appear.

68   Possession of CSDMs alone may however, be insufficient to protect a very young

69   seedling from herbivory. If the herbivore is unaware of the defence until it actually

70   removes tissue, significant negative repercussions for immediate seedling survival, or

71   even subsequent longer-term growth and reproductive potential, may ensue (Hanley and

72   Fegan, 2006; Hanley, 2012; Barton, 2013). Consequently, there seems to be a compelling

73   reason why seedlings might advertise defensive capabilities before substantial damage

74   occurs. Indeed, observation of snail feeding behaviour in experimental arenas suggests

75   that some seedlings (i.e. *Jacobaeae vulgaris*) are avoided even before physical contact is

76   made (Hanley, 1995). Moreover, olfactory detection of seedling VOCs by molluscs is

77   likely given that slugs and snails exhibit strong physiological and behavioural responses

78   to volatiles from established plants (Birkett *et al.,* 2004; Kiss, 2017).

79   Empirical evidence for VOC-linked defence in seedlings is nonetheless extremely

80   limited. Hanley *et al*., (2011) reported that snail (*Cornu aspersum* syn. *Helix aspersa*)

81   olfactory preferences were strongly correlated with seedling (gustatory) acceptability, but

82   based the former on the use of macerated seedlings whose volatile profiles are likely very

83   different from intact or even partially damaged seedlings. A similar limitation is also true

84   of Hanley *et al.,* (2013) study on ontogenetic shifts in olfactory selection of *Plantago*

85   *lanceolata*. In fact, the only studies on herbivore (*Spodoptera frugiperda*) response to

86   seedling VOCs have been conducted on crop species (Carroll *et al*., 2006, 2008), and

87   reveal little about how species-specific seedling volatile profiles affect herbivore

88   selection. Recently however, Shannon *et al.,* (2016) not only reported considerable

89   variation in VOC profiles from six different oilseed rape (OSR - *Brassica napus*)

90   cultivars, but that these VOC profiles corresponded to mollusc gustatory preferences;

91   specifically, monoterpenes acting as attractants and GLVs as repellents. Consequently,

92   for this crop plant at least, there is evidence that terrestrial molluscs use volatile signals

93   to detect and select preferred seedlings.

94   It remains the case, however, that the role of both VOCs and CSDMs, in influencing

95   patterns of seedling selection by herbivores is unclear for the majority of plant species.

96   Here we examine the relative roles of VOCs and CSDMs in dictating patterns of seedling

97   selection of 14 common grassland species by terrestrial molluscs (the snail *Cornu*

98   *aspersum* Müller). Specifically, and based on an assumption that seedlings deter

99   herbivore before any damage occurs, we test the following hypotheses:

100   1: seedling selection by herbivores is more closely associated with VOC profiles

101   than CSDMs.

102   2: if seedlings possess effective CSDM deterrents, they advertise them with

103   distinct VOC profiles.

104   **MATERIALS AND METHODS**

105   *Study species*

106   Seeds of eleven dicotyledonous herb species (*Achillea millefolium* L., *Centaurea nigra*

107   L., *Cerastium fontanum* Baugm., *Jacobaeae vulgaris* Gaertn., *Leontodon hispidus* L.,

108   *Lotus corniculatus* L., *Leucanthemeum vulgare* Lam., *Plantago lanceolata* L.,

109   *Taraxacum officinale* F.H. Wigg, *Trifolium pratense* L. and *T. repens* L.), and three

110   grasses (*Dactylis glomerata* L.*, Festuca rubra* L.*, Holcus lanatus* L.) were obtained from

111   a commercial supplier (Herbiseed Ltd, Twyford, UK). These species are common

112   components of many European grassland ecosystems and include a relatively wide cross-

113   section of dominant Asteraceae, Fabaceae and Poaceae species, along with

114   representatives from two other common grassland plant families (Caryophyllaceae and

115   Plantaginaceae). Many of these species have been included in field and laboratory feeding

116   trials with molluscs (Hulme, 1994; Fenner *et al.,* 1999; Hanley, 2004; Barlow *et al.,* 2013;

117   Hanley and Sykes, 2014), such that they offer a broad range of likely seedling

118   acceptability. Following Hanley *et al.,* (2004) definition of the term 'seedling', all assays

119   were performed only on plants still dependent on their cotyledons for nutrition and use of

120   the term hereafter is restricted to this definition.

121   *Snail collection and culture*

122   As the principal seedling herbivore in temperate grasslands (Hulme, 1994; Allan and

123   Crawley, 2011), molluscs are particularly important in shaping interactions between

124   component plant species (Barton and Hanley, 2013). In addition, not only is the feeding

125   activity of both snails and slugs influenced (negatively) by CSDMs such as phenolics

126   (Fritz *et al.,* 2001), cyanogenic glycosides (Horrill and Richards, 1986), and alkaloids

127   (Speiser *et al.,* 1992), terrestrial molluscs also detect and respond to a range of common

128   plant VOCs (Birkett *et al.,* 2004; Kiss, 2017).

129   Three hundred snails (*Cornu aspersum)* were collected from sites around Plymouth, UK

130   and subsequently retained in large plastic containers in controlled conditions (15ºC $\pm$

131   0.2ºC, 12 hr day/night illumination) and fed on a diet of lettuce (supplemented with

132   cuttlefish to provide calcium) for at least 1 month prior to experimental use.

133   Consequently, all snails experienced the same environmental and dietary conditions for

134   several weeks prior to the start of the experiment, reducing the potential for individual

135   preference and hunger to confound seedling selection (Clark *et al.,* 1997; Hanley *et al.,*

136   2003). As a generalist herbivore, *Cornu* diet is highly varied, likely further reducing

137   selective bias and making the species ideal for plant selection trials (Hanley 1995).

138   Individuals ranged between 20 mm – 30 mm shell diameter, although individual size has

139   little effect on patterns of seedling selection in the short-duration experiments such as

140   those conducted here (Hanley *et al.,* 2003).

141   *Seedling Acceptability*

142   Seeds were set to germinate in large plastic trays (350-mm x 215-mm x 70-mm deep)

143   filled with John Innes No2 potting compost and maintained in a controlled plant growth

144   room (mean daily temp = 15.0ºC $\pm$ 0.2ºC; 12-hr day: night). All species germinated within

145   3 – 5 days. Immediately following radicle appearance, seedlings were transferred in to 50

146   mm diameter plastic plant pots containing John Innes No. 2 potting compost. Two newly-

147   germinated, conspecific 'Test' seedlings were planted 45 mm apart and grown in

148   controlled plant growth room conditions (mean daily temp = 15.0ºC ± 0.2ºC, 12-hr day:

149   night) for 7 days. At this time two newly emerged lettuce seedlings (cv Little Gem) were

150   planted 45 mm apart in the same pot, perpendicular to the 'Test' seedlings (such that all

151   4 seedlings were arranged in a square). Lettuce seedlings, cultivated simultaneously in

152   large plastic trays containing commercial potting compost, were used to ascertain the

153   relative acceptability of the 'Test' species (Fenner *et al.,* 1999). Rapid development of

154   lettuce seedlings compared with the test species meant that 7 d-old seedlings were at

155   approximately the same ontogenetic stage as 14 d-old test seedlings (i.e. cotyledons with

156   initiation of first true leaf).

157   When the 'Test' seedlings were 14 d-old they were exposed to snails. Five replicate pots

158   for each 'Test' species were sunk into large plastic propagator trays (350 mm x 215 mm

159   x 70 mm deep) filled with commercial potting compost, such that the top of each pot was

160   flush with the level of the compost. One pot was placed into the centre of each tray, with

161   the remaining four pots located in the tray corners. This arrangement was replicated 10

162   times for each 'Test' species. Three snails were then added to each tray and retained

163   overnight (≈ 16hr) using a clear plastic propagator lid (350 mm x 215 mm x 115 mm

164   deep). The total number of 'Test' species and lettuce index seedlings attacked by snails

165   was determined for each replicate tray. These values were used to calculate an

166   acceptability index (AI) for 'Test' species seedlings within individual trays, based on the

167   formula given by Fenner *et al.,* (1999):

168

$$\text{AI per tray} = \frac{\text{Mean number of 'test' seedlings attacked}}{\text{Mean number of 'test' + index seedlings attacked}}$$

169   Average seedling AI for each species was then calculated across all replicate trays for

170   each species. AI ranges between 0 (highly unacceptable) and 1 (highly acceptable) where

171   a value of 0.5 represents equal acceptability to the lettuce control. Acceptability trials

172   were conducted between October 2014 and January 2015 in such a way that the timing of

173   trials for each species was interspersed at random with all other species.

174   *Seedling constitutive secondary defence metabolites*

175   For each species, 20 newly emerged seedlings were cultivated in each of twenty replicate

176   90 mm diameter pots filled with John Innes No. 2 potting compost maintained in a

177   controlled plant growth room (mean daily temp = 15.0ºC ± 0.2ºC; 12 hr day: night).

178   Fourteen day-old seedlings were cut at ground level and immediately flash frozen in tin

179   foil packets in liquid nitrogen, before storage at -80^0C. Samples were subsequently placed

180   in an Edwards Modulyo freeze drier (Edwards Ltd., Sussex, UK) for 48 hr until dry before

181   being transferred into airtight tubes. Seedlings from individual pots were 'bulked up' in

182   order to generate sufficient sample replication (4) for subsequent CSDM analysis.

183   Phenolic content was determined using the Hagerman and Buttler method (see Smolders

184   *et al.,* 2000). Three hundred milligrams of lyophilized tissues were ground and extracted

185   with 1 ml of 80% methanol for 60 min. The samples were centrifuged at 800 g for 2 min

186   and the supernatant analysed. Five hundred µl of 10 mM $FeCl_3$ solution was added to 500

187   µl of supernatant. The Fe III reduction into Fe II by the phenolic compounds was

188   measured at a wavelength of 510 nm (Shimadzu spectrophotometer UV-1280). Tannic

189   acid was used as a standard. Alkaloids extraction was performed using the method

190   described by Tikhomiroff and Jolicoeur (2002). Three hundred mg of lyophilized tissues

191   were ground and extracted at room temperature in 1 ml of methanol for 60 min. The

192   extract was centrifuged at 15 000 rpm for 5 min at room temperature and the supernatant

193   was filtered through a PTFE 0.45 mm filter and desalted on a Sephadex G25 column with

194   elution medium (100 mM HEPES-KOH, pH 7.5, 2 mM DTT, 10% (v/v) glycerol, 5 mM

195   $MgCl_2$) and then used to determine total alkaloids quantities. The determination of total

196   alkaloids used the Dragendorff's reactive method (Pothier *et al*., 1983). Alkaloid

197   concentration was assessed by the absorbance of the solution using a Shimadzu UV-1280

198   spectrophotometer measured at a wavelength of 555 nm after having added the reactive.

199   Veratrine hydrochloride was used as a standard.

200   Cyanogenic glycosides were quantified using the method by Bradbury and Egan (1992),

201   based on the natural liberation of volatile HCN after plants were crushed and CN- reacted

202   with picrate paper. Three hundred mg of the lyophilized potential cyanogenic sample

203   were ground and placed in a glass vial, followed by 2 ml of phosphate buffer at pH 6. A

204   strip of filter paper was previously prepared by dipping the paper in 0.02 M picric acid,

205   drying in air and cutting to two cm^2 pieces. The picrate paper was suspended above the

206   cyanogenic sample and the vial immediately tightly stoppered. The vials were placed in

207   an oven at 30°C for 12 h. The paper was immersed in distilled water for about 30 min and

208   the absorbance measured using a Shimadzu UV-1280 spectrophotometer at 510 nm

209   against a similarly prepared blank developed in the absence of cyanogen. Standards were

210   made with commercial products of linamarin (α-Hydroxyisobutyronitrile β-D-

211   glucopyranoside) and linamarase (β-Glucosidase EC 3.2.1.21).

212   *Seedling VOC profiles*

213   For each species, 20 newly emerged seedlings were grown in 90 mm diameter pots

214   filled with John Innes No. 2 potting compost as described above. Due to the high

215   number of seedlings required to provide enough VOCs to be detectable, it was not

216   possible to isolate the aerial parts of the plants from the soil and pot to eliminate

217   background volatiles. Instead, when 14 day-old, the seedlings and compost were gently

218   removed from the pots and soil carefully washed away to avoid damage. Up to 140

219   seedlings per replicate (i.e. seven individual pots) were placed together in a 200 ml glass

220   beaker with 100 ml of distilled water (see Rohloff and Bones 2005; Shannon *et al*.,

221   2016). This process was repeated four times for each species. We had previously

222   established that while this approach eliminated volatiles from the soil and the pot, it did

223   not alter the plant VOC profile (Shannon *et al*., 2016) and allowed us to quantify a 1.1%

224   to 75.3% cultivar-specific range of the major constituent GLV, 3-hexen-1-ol acetate, for

225   *Brassica napus*.

226   All collections took place within an environment-controlled room (ECR) at 15°C. Each

227   beaker was placed inside a 46 x 56 cm polyester (PET) oven bag (Lakeland, Cumbria,

228   UK) (Stewart-Jones & Poppy, 2006) with one corner cut off, through which a Teflon

229   tube was inserted before being tied shut. Air was drawn from the ECR air inlet via

230   Tygon tubing (Saint-Gobain S.A., Paris, France), passed through an activated charcoal

231   filter and pumped into the bag at a rate of 1000 ml min^{-1} using a Neuberger KNDC B

232   pump (Neuberger, Freiburg, Germany). Three samples and one control (a bag

233   containing a beaker with distilled water) were taken simultaneously, under two racks of

234   compact fluorescent bulbs, giving approximately 200 μmol photons m^{-2} s^{-1} of

235   photosynthetically active radiation at canopy height, equivalent to an overcast day. The

236   open end of the bag was tied around a manual solid-phase microextraction (SPME) fibre

237   holder (Supelco Inc., Bellefont, PA, USA) until the bag inflated, after which the SPME

238   holder was removed. The bag was left for 1 hr with the pump running to completely

239   purge unfiltered air and allow the plants to acclimatise. The SPME holder was then

240   replaced, the bag allowed to fully inflate and the SPME fibre exposed (Blue

241   PDMS/DVB 65 μm fibre 57310-U, all from the same lot, Supelco Inc., Bellefont, PA,

242   USA). The airflow to each bag was reduced to 100 ml min^{-1} to maintain positive

243   pressure, preventing contamination over the 2 hr of VOC collection. As the volume of

244   each tied and inflated bag was approximately 14 l, this small airflow would only result

245   in one change of air every 140 min (over a 120 min collection time). The SPME fibres

246   were never saturated with any particular VOC. The maximum amount collected was a

247   TIC of around 10,000,000 – five to ten times less than typically collected when running

248   standards. We had established previously that the proportion of each volatile in the

249   profile remained constant as collection time increased, so there was no effect of volatile

250   exclusion from a saturated fibre due to a long collection time (Shannon *et al.,* 2016).

251   VOCs were detected using an Agilent 7890 gas chromatography (GC) system fitted

252   with an HP Innowax column (polyethylene glycol, 30.0 m x 250 μm i.d. x 0.25 μm

253   film) coupled to an Agilent 5977A Mass Selective Detector (MS) (Agilent Technologies

254   Inc., CA, USA) run in EI mode. Immediately following collection, the VOCs were

255   thermally desorbed for 10 minutes from the fibre in the injector port. The GC was

256   operated in splitless mode, with helium carrier gas at 7.45 psi and the inlet temperature

257   at 250°C. The oven was maintained at 50°C for the first 2 min, then increased by 5°C

258   min^{-1} for 4 min, followed by 10°C min^{-1} for 17 min, ending at 240°C. The GC-MS was

259   controlled by Agilent Mass Hunter software and data analysed by Agilent Qualitative

260   Analysis version B.06.00 software (Agilent Technologies Inc., CA, USA). VOCs were

261   initially identified using the NIST database, and confirmed by comparing retention time

262   with standards (Sigma-Aldrich Ltd., UK) and calculating their Kovats retention index

263   (by reference to the retention times of an alkane series (C7 – C17) analysed using the

264   same method as our samples – see Hanley *et al.,* (2013))

265   As several SPME fibres from the same manufacturing batch were used in collection of

266   the VOCs, we used the percentage of each volatile in the total VOCs collected for each

267   sample rather than using the Total Ion Count to quantify VOCs, after first removing

268   artefacts identified from our controls as arising from the SPME fibres or the GC

269   column. We did not impose a lower limit for the percentage of total VOCs for

270   individual compounds, as this would have excluded some that we had reason to expect

271   might influence snail behaviour (Hanley *et al*., 2013; Shannon *et al*., 2016). However,

272   we only included VOCs that appeared in the majority of samples from at least one

273   species, to ensure consistency (Van Dam and Poppy, 2008).

274   *Statistical analysis*

275   To test the hypothesis (1a) that AI was influenced by CSDMs, we conducted Kendall's

276   Tau correlation tests between the mean values of the three groups of chemicals (phenolics,

277   alkaloids and cyanogenics) and the AI. To test the Hypothesis (1b) that seedling

278   acceptability (AI) was linked with identifiable VOC profiles we first established that each

279   species had a distinct VOC profile using a Canonical Discriminant analysis. Due to the

280   great diversity of VOCs collected from 14 species this was not suitable to subsequently

281   use to compare with the AI. Having first performed a logit transformation on the VOCs

282   percentage data, we used Pearson's product-moment correlations to establish whether AI

283   was influenced by any of the major classes of VOCs, namely monoterpenes,

284   sesquiterpenes, and green leaf volatiles. To test hypothesis 2, that seedlings expressing

285   high concentrations of CSDMs advertise defensive capability with distinct VOC profiles,

286   we conducted Kendall's Tau correlation tests between the three groups of CSDMs and

287   the three major groups of VOCs.

288   **RESULTS**

289   *Seedling Acceptability*

290   The 14 plant species exhibited a very broad range of seedling acceptability (Table 1),

291   extending from an AI score of zero for *Jacobaeae vulgaris* (where no 'Test' seedlings

292   were attacked) to highly acceptable *Centaurea nigra*. Overall, of the total 424 seedlings

293   attacked and damaged by snails, 300 (71%) were consumed completely most likely

294   immediately after initial contact was made. For seedlings of two species (*Centuarea nigra*

295   - 38% killed and *Leucanthemum vulgare* – 35% killed) however, the likelihood of

296   complete consumption after contact appeared to be markedly lower than the average for

297   all other species, suggesting that snails were deterred from further attack after inflicting

298   initial damage to seedlings.

299   *Constitutive Secondary Defence Metabolites*

300   All species contained phenolic compounds (Table 1), the highest concentrations in the

301   Asteraceae, while the Poaceae contained relatively low phenolic concentrations (and with

302   no other compounds detected). Alkaloids were detected in six species, cyanogenic

303   compounds in only four, the latter being most prevalent in the Fabaceae. There were

304   however, only very weak relationships between seedling AI and the amount of phenolic

305   (Kendall's Tau correlation $r^2 = 0.07$; $\tau = 0.990$ $P = 0.322$), alkaloid ($r^2 = 0.05$; $\tau = -0.187$,

306    $P = 0.392$), or cyanogenic ($r^2 = 0.04$; $\tau = 0.094$, $P = 0.677$) compounds present in seedling

307   leaf tissue. Indeed, a number of species with the highest concentrations of single (e.g.

308   cyanide – *Lotus*, phenolics – *Centaurea*), or even multiple (phenolics & alkaloids -

309   *Plantago*) CSDMs were amongst species with the highest (> 0.68) AI. Consequently, we

310   conclude that species-specific seedling selection by snails was unrelated to the expression

311   of major CSDMs.

312   *Volatile compounds*

313   A large number of different VOCs were collected, including many that could be

314   categorised as monoterpenes, GLVs, or sesquiterpenes (Table S1). In any investigation

315   of this nature (i.e. 14 different species), a number of unknown VOCs are likely to be

316   detected. Most unknowns could however, be classified as mono- or sesqui-terpenes, by

317   virtue of their molecular weights, retention times, and Kovats indices. Each species had

318   a distinct VOC profile and the CDA categorised each sample into its correct species with

319   100% accuracy (Fig. 1). The GLV 3-hexen-1-ol acetate was for most species, the

320   dominant VOC (Table 2), only *Cerastium fontanum* (6.9% of total profile) and *Lotus*

321   *corniculatus* (4.2%) had profiles where this compound contributed less than 10% of all

322   VOCs. Other GLVs made a relatively minor contribution, except *Jacobea vulgaris*, where

323   3-hexen-1-ol comprised 16.4% of total VOCs, although as with the other species 3-hexen-

324   1-ol acetate was still the dominant GLV, with 75.7% of total VOCs. *Jacobea vulgaris*

325   was also noteworthy in that its volatile profile was dominated (>96%) by GLVs. Of the

326   monoterpenes, β–ocimene (>15% of all VOCs) dominated species volatile profiles.

327   Although sesquiterpenes were detected in most species, they were generally present at

328   only low amounts, and none were detected in *Festuca rubra*, *Taraxacum officinale* and

329   *Trifolium repens*. In four species however, *Achillea millefolia*, *Cerastium fontanum*,

330   *Centaurea nigra* and *Leucanthemum vulgare*, sesquiterpenes contributed between 22 and

331   30% of their total VOCs.

332   Snail preferences (AI) were positively correlated with the proportion of monoterpenes

333   (Pearson's product-moment correlation, $r^2 = 0.549$, $t = 2.274$, df = 12, $P = 0.042$) (Fig.

334   2a), and β-ocimene in particular (Pearson's product-moment correlation, $r^2 = 0.568$, $t =$

335   2.394, df = 12, $P = 0.034$), collected from each species (Fig. 2b). We also detected a

336   ('marginally-significant') positive relationship between AI and the proportion of

337   sesquiterpenes in the VOCs profile of each species (Fig 2c) ($r^2 = 0.528$, $t = 2.151$, df =

338   12, $P = 0.053$). Although falling above the $P < 0.05$ convention, there was a negative

339   relationship (Fig. 2d) between AI and total GLVs contribution ($r^2 = -0.490$, $t = -1.948$, df

340   = 12, $P = 0.075$), likely reflecting the influence of the principal GLV, 3-hexen-1-ol acetate

341   ($r^2 = -0.464$, $t = -1.816$, df = 12, $P = 0.094$) (Fig 2e).

342   Based on their anomalously lower post-attack mortality and an assumption that snail

343   damage to *Centuarea nigra* and *Leucanthemum vulgare* elicited a seedling or snail

344   response that prevented further attack (e.g. possible induction of CDSM or VOC

345   defences), we repeated the analysis with the remaining 12 plant species only. In this case,

346   AI remained positively correlated with monoterpenes ($r^2 = 0.587$, t = 2.298, df = 10, $P =$

347   0.044) and β-ocimene ($r^2 = 0.580$, t = 2.251, df = 10, $P = 0.048$) (Fig. 2a,b). While the

348   positive relationship between AI and sesquiterpenes remained tentative (Fig 2c) ($r^2 =$

349   0.497, t = 1.811, df = 10, $P = 0.100$), the negative relationship between AI and total GLVs

350   ($r^2 = -0.701$, t = -3.107, df = 10, $P = 0.011$) and 3-hexen-1-ol acetate ($r^2 = -0.515$, t = -

351   1.900, df = 10, $P = 0.087$) (Fig 2d,e) strengthened. We conclude therefore, that seedling

352   selection by *Cornu* was more closely associated with VOC profiles than CSDMs,

353   specifically that monoterpenes (and potentially sesquiterpenes) have a positive influence

354   on snail selection, while GLVs likely have a negative influence (Hypothesis 1).

355   We performed Kendall's Tau correlation tests between the CSDMs (alkaloids,

356   cyanogenics and phenolics) and the VOC groups (monoterpenes, GLVs and

357   sesquiterpenes) and found that none were correlated. We thus found no evidence to

358   support our second hypothesis that VOCs signal the possession of CSDMs to putative

359   herbivores.

360   **DISCUSSION**

361   Although our results support the assumption (Hulme, 1994; Barlow *et al.,* 2013; Barton

362   and Hanley, 2013) that species-specific variation in seedling herbivory hinges on the

363   expression of chemical defences, surprisingly we found that CSDMs had little or no role

364   in seedling selection by snails. Relative seedling acceptability was instead, most closely

365   associated with the proportions of two major classes of volatile compounds present in

366   seedling VOC profiles; i.e. a positive relationship with monoterpenes and a negative

367   relationship with GLVs. Consequently, not only does this study evidence the likely

368   mechanism by which terrestrial molluscs select and attack seedlings of different grassland

369   plant species, we show that selection is most closely associated with VOCs (olfaction)

370   rather than CSDMs (gustation).

371   *GLVs function as primary seedling defence*

372   To date, and likely by virtue of the fact that GLVs are synthesised in the same oxylipin

373   pathway as jasmonic acids, GLVs have more frequently been associated with the priming

374   or induction of plant defences or so-called SOS signalling, rather than playing a direct

375   role in plant defence (Scala *et al.,* 2013). Recent studies have however, demonstrated a

376   link between GLVs and food plant selection by snails. Hanley *et al.,* (2013) reported that

377   GLV concentrations in *Plantago lanceolata* increased during early ontogeny, while at the

378   same time, snail olfactory selection (of macerated material) declined. Shannon et al.,

379   (2016) also found that seedling acceptability to snails was negatively associated with

380   GLV-dominated volatile profiles and evidenced the mechanism underpinning their

381   deterrent effect when the application of a GLV blend to seedlings of the most acceptable

382   OSR cultivar resulted in reduced olfactory selection. Although we were unable to

383   determine whether one particular GLV compound or blend of compounds influenced

384   snail selection in the present study, we do show that low acceptability species were those

385   expressing GLV-dominated volatile profiles. Moreover, the single most abundant VOC

386   in this study, the GLV 3-hexen-1-ol acetate, was one of the primary constituents of the

387   GLV blend shown to repel snails in Shannon *et al*., (2016) olfactometer trials.

388   *The role of monoterpenes in seedling selection by snails*

389   It is also possible that snails use monoterpenes to locate preferred seedlings. Although

390   relatively little is known about mollusc detection of, and response to, terpenoid

391   compounds, working on six different chemotype populations Linhart and Thompson,

392   (1995) established that snails (*Helix aspersa*) actively selected *Thymus vulgaris* plants

393   containing linalool, but avoided those dominated by monocyclic monoterpenes (carvacrol

394   and thymol). Dodds *et al.,* (1996) also showed that the monocyclic monoterpene carvone,

395   elicited neurophysiological activity in the slug *Deroceras reticulatum* suggestive of a

396   repellent effect on mollusc feeding. We detected relatively few monocyclic monoterpenes

397   in our samples (e.g. β-phellanderene γ-terpinene, and limonene); instead, and particularly

398   for some high acceptability species like *Achillea, Leontodon, Lotus,* and *Plantago*,

399   terpenoid profiles were dominated by acyclic monoterpenes (β-myrcene, and β-ocimene)

400   more similar to linalool, and/or bicyclic compounds (e.g. α- and β-pinene). Interestingly,

401   Shannon *et al*., (2016) identified α-pinene and β-myrcene as putative snail attractants. By

402   comparison, the very low acceptability species in our study (*Jacobaea* and *Holcus*)

403   contained few or no monoterpene compounds at all (0% and 20% respectively).

404   *Possible herbivore induction of volatile defences*

405   The fact that we evidenced a stronger negative relationship between GLVs and seedling

406   acceptability when *Leucantheum vulgare* and *Centaurea nigra* were excluded from the

407   analysis raises two important issues. First, with only around one-third of seedlings killed

408   after the initiation of snail attack, and no apparent exceptional allocation to CDSMs, our

409   results signal that *Centuarea* and *Leucantheum* seedlings may be capable of rapid

410   upregulation of anti-herbivore defences at the cotyledon stage. Induction of anti-

411   herbivore defences in very young seedlings has, however, never been evidenced, nor

412   indeed considered likely, due to the view that plants at such an early ontogenetic stage

413   are unable to synthesise chemical defences *de novo* (Boege and Marquis, 2005; Barton

414   and Hanley, 2013). Secondly, for the remaining 12 species where attack led to complete

415   consumption for at least 75% of all attacked seedlings, our results suggest that high

416   relative concentrations of GLVs in the volatile profile perform a deterrent role, preventing

417   snail attack before any damage occurs.

418   *Implications for our understanding of seedling defence*

419   Given we found no relationship between CSDMs and patterns of seedling selection by

420   snails, our failure to elucidate any association between VOC profile and CSDM

421   expression is perhaps unsurprising. A reliance solely on volatiles means that cotyledon-

422   stage seedlings are unlikely to use VOCs to signal other defences. Indeed, given the

423   usually catastrophic impact herbivory at the cotyledon stage has on seedling fitness and

424   survival (Hanley and Fegan, 2006; Hanley, 2012; Barton, 2013), an ability to deter

425   herbivores before contact is made seems especially adaptive for seedlings with likely

426   limited capacity to allocate resources to anti-herbivore defence. GLVs may be a good

427   candidate for this role; they are C-based, low molecular weight VOCs (Scala *et al*., 2013)

428   and the oxidation and conversion of seed-stored fat reserves to sugars after germination

429   is likely to release GLVs before other secondary defence metabolites accumulate to levels

430   sufficient to defend the seedling.

431   This situation is however, likely to change as the plant ages. Indeed as Barton and Boege,

432   (2017) point out, a failure to establish a clear relationship between trait (CSDM)

433   expression and strength of an interaction (seedling section by herbivores) cannot discount

434   the possibility that these compounds are effective deterrents at later ontogenetic stages

435   and/or have other important functions at the life history stage under investigation. Indeed,

436   for most of our study species, (mono- and sesqui-) terpenes, phenolics, alkaloids and

437   cyanogenic glycosides, are for established plants, important anti-herbivores defences

438   (Grime *et al.,* 2007). It is likely also that their relative roles (in isolation or combination)

439   vary according to the selective impact of different herbivores throughout ontogeny (see

440   Iason *et al.,* 2011 Goodger *et al.,* 2013). Establishing how, why, and when a transition

441   occurs from GLV-dominated defence to one where other metabolites and structural

442   defences assume more importance (with VOCs perhaps playing a more significant role in

443   signalling or induced defence), would go some way to better understanding the evolution

444   and ecology of ontogenetic defence trajectories (Barton and Boege, 2017). Our study

445   suggests however, that some plants species counter the usually fatal consequences of

446   seedling herbivory with GLV-based, volatile defences.

451   **Supporting Information**

452   **Table S1:** Relative (%) composition of total profile of major VOCs

453   **LITERATURE CITED**

454   **Allan E, Crawley MJ. 2011.** Contrasting effects of insect and molluscan herbivores on

455   plant diversity in a long-term field experiment. *Ecology Letters* **14**: 1246–1253.

456   **Barlow SE, Close AJ, Port GR. 2013.** The acceptability of meadow plants to the slug

457   *Deroceras reticulatum* and implications for grassland restoration. *Annals of*

458   *Botany* **112**: 711-720.

459   **Barton KE. 2013.** Ontogenetic patterns in the mechanisms of tolerance to herbivory in

460   *Plantago. Annals of Botany* **112**: 711-720.

461   **Barton KE. 2016.** Low tolerance to simulated herbivory in Hawaiian seedlings despite

462   induced changes in photosynthesis and biomass allocation. *Annals of Botany* **117**:

463   1053–1062.

464   **Barton KE, Boege K. 2017.** Future directions in the ontogeny of plant defence:

465   understanding the evolutionary causes and consequences. *Ecology Letters* **20**:

466   403-411.

467   **Barton KE, Hanley ME. 2013.** Seedling-herbivore interactions: Insights into plant

468   defence and regeneration patterns. *Annals of Botany* **112**: 643-650.

469   **Barton KE, Koricheva J. 2010.** The ontogeny of plant defense and herbivory:

470   characterizing general patterns using meta-analysis. *American Naturalist* **175**:

471   481–493.

472   **Birkett MA, Dodds CJ, Henderson IF, Leake LD, Pickett JA, Selby MJ, Watson P.**

473   **2004.** Antifeedant compounds from three species of Apiaceae active against the

474   field slug, *Deroceras reticulatum* (Muller). *Journal of Chemical Ecology* **30**: 563-

475   576.

476   **Boege K, Marquis RJ. 2005.** Facing herbivory as you grow up: the ontogeny of

477   resistance in plants. *Trends in Ecology and Evolution* **20**: 441-448.

478   **Bradbury JH, Egan SV. 1992.** Rapid screening assay of cyanide content of cassava.

479   *Phytochemical Analysis* **3**: 91-94.

480   **Carroll MJ, Schmelz EA, Meagher RL, Teal PEA. 2006.** Attraction of *Spodoptera*

481   *frugiperda* larvae to volatiles from herbivore-damaged maize seedlings. *Journal*

482   *of Chemical Ecology* **32**: 1911-1924.

483   **Carroll MJ, Schmelz EA, Teal PEA. 2008.** The attraction of *Spodoptera frugiperda*

484   neonates to cowpea seedlings is mediated by volatiles induced by conspecific

485   herbivory and the elicitor inceptin. *Journal of Chemical Ecology* **34**: 291-300.

486   **Clark SJ, Dodds CJ, Henderson IF, Martin AP. 1997.** A bioassay for screening

487   materials influencing feeding in the field slug *Deroceras reticulatum* (Müller)

488   (Mollusca: Pulmonata). *Annals of Applied Biology* **130**: 379-385

489   **van Dam NM, Poppy GM. 2008.** Why plant volatile analysis needs bioinformatics -

490   detecting signal from noise in increasingly complex profiles. *Plant Biology* **18**:

491   29–37.

492   **Dodds CJ, Ford MG, Henderson IF, Leake LD, Martin A, Pickett JA, Wadhams LJ,**

493   **Watson P. 1996.** Slug chemical ecology: electrophysiological and behavioural

494   studies. In: Henderson IF ed. *Slug and Snail pests in Agriculture*, British Crop

495   Protection Council Monograph **66**: 73-81.

496   **Dudareva N, Klempien A, Muhlemann JK, Kaplan I. 2013.** Biosynthesis, function and

497   metabolic engineering of plant volatile organic compounds. *New Phytologist* **198**:

498   16–32.

499   **Elger A, Lemoine DG, Fenner M, Hanley ME. 2009.** Plant ontogeny and chemical

500   defence: older seedlings are better defended. *Oikos* **118**: 767-773.

501   **Fenner M, Hanley ME, Lawrence R. 1999.** Comparison of seedling and adult palatability

502   in annual and perennial plants. *Functional Ecology* **13**: 546-551.

503   **Fritz RS, Hochwender CG, Lewkiewicz DA, Bothwell S, Orians CM. 2001.** Seedling

504   herbivory by slugs in a willow hybrid system: developmental changes in damage,

505   chemical defense, and plant performance. *Oecologia* **129**: 87–97.

506   **Goodger JQD, Heskes AM, Woodrow IE. 2013.** Contrasting ontogenetic trajectories

507   for phenolics and terpenoid defences in *Eucalyptus froggattii*. *Annals of Botany*

508   **112**: 651-660.

509   **Grime JP, Hodgson JG, Hunt R. 2007.** *Comparative Plant Ecology* 2nd edn. Dalbeattie,

510   Scotland, Castlepoint Press.

511   **Hanley ME. 1995.** *The influence of molluscan herbivory on seedling regeneration in*

512   *grassland*. PhD Thesis, University of Southampton, UK.

513   **Hanley ME. 2004.** Seedling herbivory and the influence of plant species richness in

514   seedling neighbourhoods. *Plant Ecology* **170**: 35-42.

515   **Hanley ME. 2012.** Seedling defoliation, plant growth, and flowering potential in native-

516   and invasive-range *Plantago lanceolata* populations. *Weed Research* **52**: 252-259.

517   **Hanley ME, Fegan EL. 2007.** Timing of cotyledon damage affects growth and flowering

518   in mature plants. *Plant, Cell & Environment* **30**: 812-819.

519   **Hanley ME, Lamont BB. 2001.** Herbivory, serotiny and seedling defence in Western

520   Australian Proteaceae. *Oecologia* **126**: 409–417

521   **Hanley ME, Sykes RJ. 2014.** Seedling Herbivory and the Temporal Niche. In: Kelly

522   CK, Bowler MA, Fox GA eds. *Environmental Fluctuation, Temporal Dynamics*

523   *and Ecological Process*. Cambridge, UK: Cambridge University Press, 102-122.

524   **Hanley ME, Bulling M, Fenner M. 2003.** Quantifying individual feeding variability:

525   implications for mollusc feeding experiments. *Functional Ecology* **17**: 673-679

526   **Hanley ME, Collins SA, Swann C. 2011.** Advertising acceptability: is mollusk olfaction

527   important in seedling selection? *Plant Ecology* **212**: 727-731.

528   **Hanley ME, Fenner M, Edwards PJ. 1995.** The effect of seedling age on the likelihood

529   of herbivory by the slug *Deroceras reticulatum*. *Functional Ecology* **9**: 754-759.

530   **Hanley ME, Fenner M, Edwards PJ. 1996.** The effect of mollusc grazing on seedling

531   recruitment in artificially created grassland gaps. *Oecologia* **106**: 240-246.

532   **Hanley, ME, Fenner M, Whibley H, Darvill B. 2004.** Early plant growth: identifying

533   the end point of the seedling phase. *New Phytologist* **163**: 61-66.

534 **Hanley ME, Girling RD, Felix AE, Olliff ED, Newland PL, Poppy GM. 2013.**

535   Olfactory selection of *Plantago lanceolata* declines with seedling age. *Annals of*

536   *Botany* **112**: 671-676.

537 **Hanley ME, Lamont BB, Fairbanks MM, Rafferty CM. 2007.** Plant structural traits

538   and their role in anti-herbivore defence. *Perspectives in Plant Ecology, Evolution*

539   *and Systematics* **8**: 157-178.

540 **Hare JD. 2011.** Ecological role of volatiles produced by plants in response to damage by

541   herbivorous insects. *Annual Review of Entomology* **56**: 161–180.

542 **Horrill JC, Richards AJ. 1986.** Differential grazing by the mollusc *Arion hortensis* Fer.

543   On cyanogenic and acyanogenic seedlings of white clover *Trifolium repens* L

544   *Heredity* **56**: 277-281.

545 **Hulme PE. 1994.** Seedling herbivory in grassland: relative impact of vertebrate and

546   invertebrate herbivores. *Journal of Ecology* **82**: 873-880.

547 **Iason GR, O'Reilly-Wapstra JM, Brewer MJ, Summers RW, Moore BD. 2011.** Do

548   multiple herbivores maintain chemical diversity of Scots pine monoterpenes?

549   *Philosophical Transactions of the Royal Society B* **366**: 1337–1345.

550 **Kiss T. 2017.** Do terrestrial gastropods use olfactory cues to locate and select food

551   actively? *Invertebrate Neurophysiology* **17**: 9. doi.org/10.1007/s10158-017-0202-

552   2

553 **Linhart YB, Thompson JD. 1995.** Terpene-based selective herbivory by *Helix aspersa*

554   (Mollusca) on *Thymus vulgaris* (Labiatae). *Oecologia* **102**: 126-132.

555   **Pothier J, Lenoble M, Pousset J-L. 1983.** Dosage rapide des différents alcaloïdes de

556   *Lupinus albus* L. et de *Lupinus mutabilis* Sweet pour la sélection. *Agronomie* **3**:

557   391-393.

558   **Rohloff J, Bones AM. 2005.** Volatile profiling of *Arabidopsis thaliana* – Putative

559   olfactory compounds in plant communication. *Phytochemistry* **66**: 1941-1955.

560   **Scala A, Allmann S, Mirabella R, Haring MA, Schuurink RC. 2013.** Green Leaf

561   Volatiles: A plant's multifunctional weapon against herbivores and pathogens.

562   *International Journal of Molecular Sciences* **14**: 17781–17811.

563   **Shannon RWR, Felix A-E, Poppy GM, Newland PL, van Dam NM, Hanley ME.**

564   **2016.** Something in the air? The impact of volatiles on mollusc attack of oilseed

565   rape seedlings. *Annals of Botany* **116**: 1073-1082.

566   **Smolders AJP, Vergeer LHT, van Der Velde G, Roelofs JGM. 2000.** Phenolic

567   contents of submerged, emergent and floating leaves of aquatic and semiaquatic

568   macrophyte species: why do they differ? *Oikos* **91**: 307-310.

569   **Speiser B, Harmatha J, Rowell-Rahier M. 1992.** Effects of pyrrolizidine alkaloids and

570   sesquiterpenes on snail feeding. *Oecologia* **92**: 257-265.

571   **Stewart-Jones A, Poppy GM. 2006.** Comparison of glass vessels and plastic bags for

572   enclosing living plant parts for headspace analysis. *Journal of Chemical Ecology*

573   **32**: 845–864.

574   **Tikhomiroff C, Jolicoeur M. 2002.** Screening of *Catharanthus roseus* secondary

575   metabolites by high-performance liquid chromatography. *Journal of*

576   *Chromatography* **955**: 87-93.

577   **Unsicker S, Kunert G, Gershenzon J. 2009.** Protective perfumes: the role of vegetative

578   volatiles in plant defense against herbivores. *Current Opinion in Plant Biology*

579   **12**: 479-485.

580
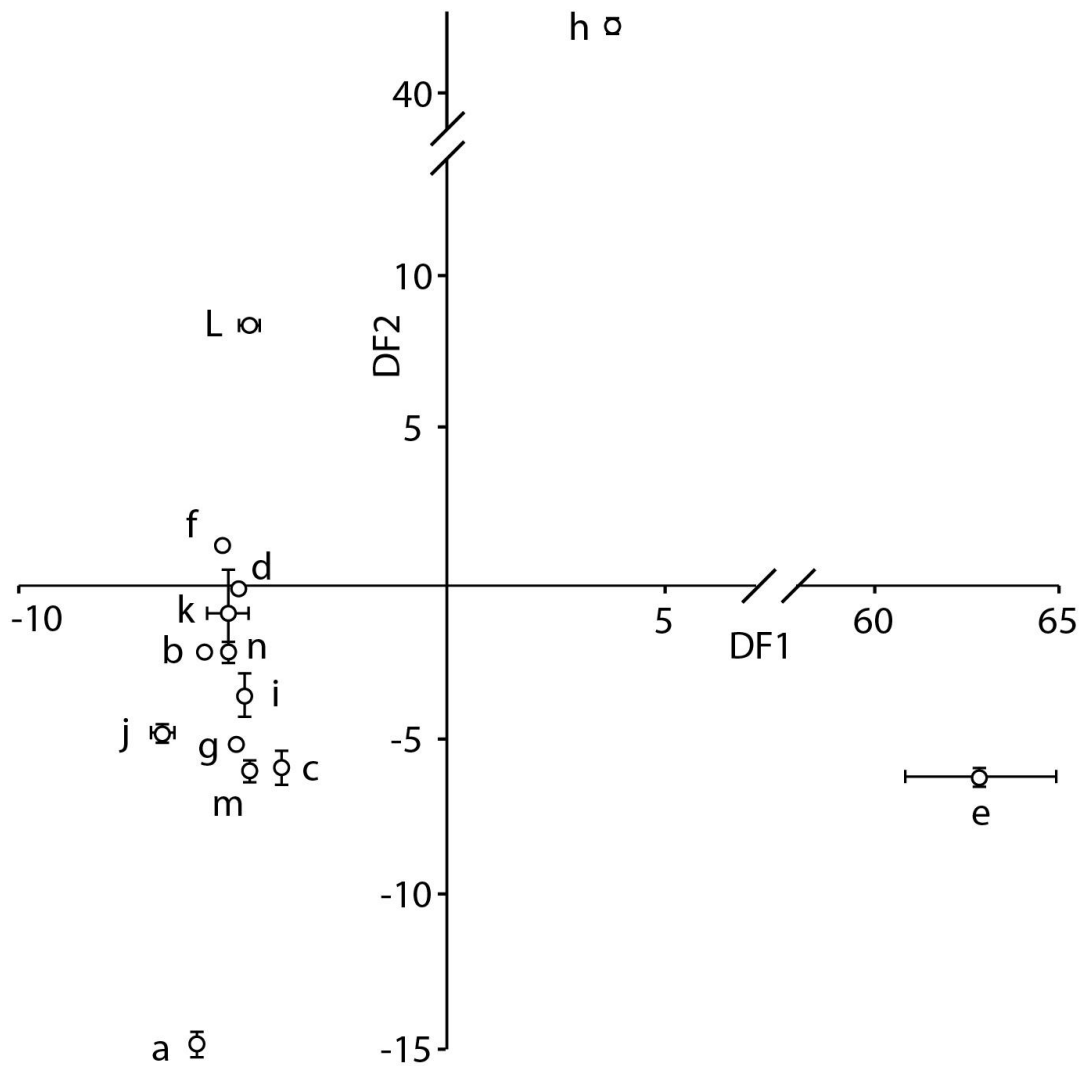
Figure 1: Discriminant Factor 1 (DF1) and DF2 from a Canonical Discriminant Analysis (CDA) conducted on the mean (± SE) proportion of volatile organic compounds collected from seedlings of 14 grassland species. Key to species: a – *Achillea millefolia*; b – *Cerastium fontanum*; c – *Centaurea nigra*; d – *Dactyllis glomerata*; e – *Festuca rubra*; f – *Holcus lanatus*; g – *Leontodon hispidus*; h – *Lotus corniculatus*; i – *Leucanthemum vulgare*; j – *Plantago lanceolata*; k – *Jacobaea vulgaris*; L – *Taraxacum officianal*; m – *Trifolium pratensis*; n – *Trifolium repens*.
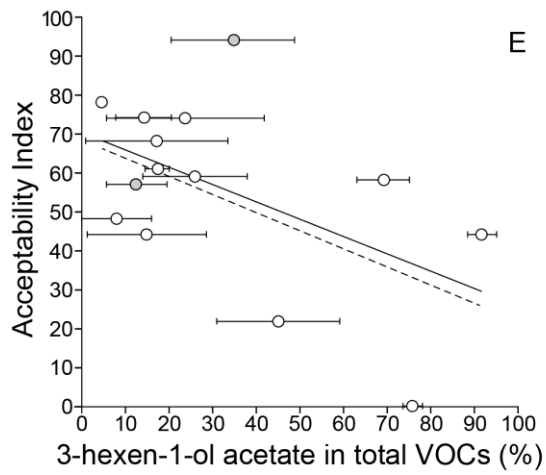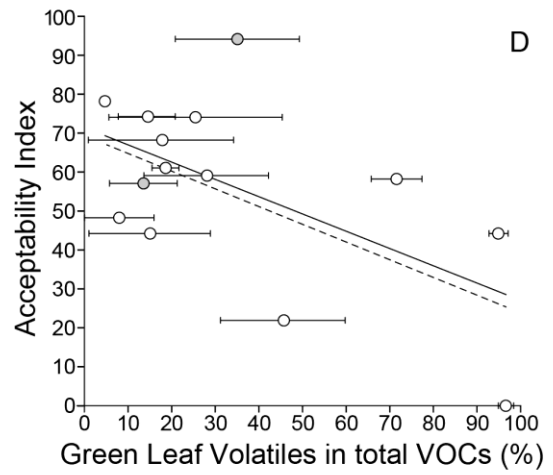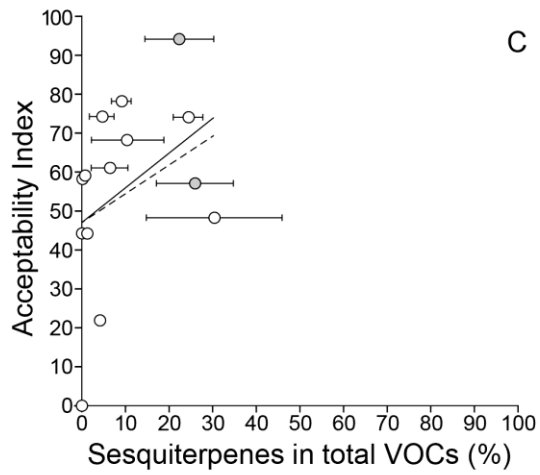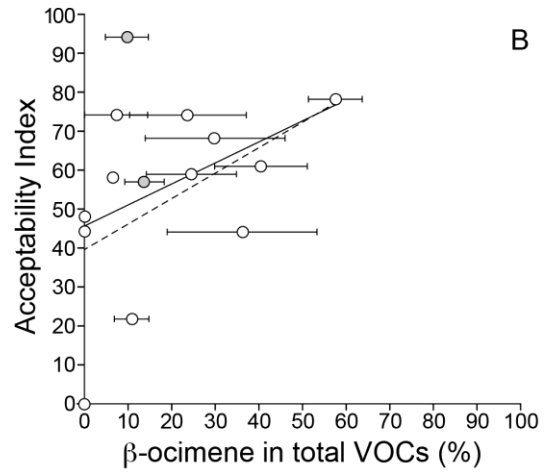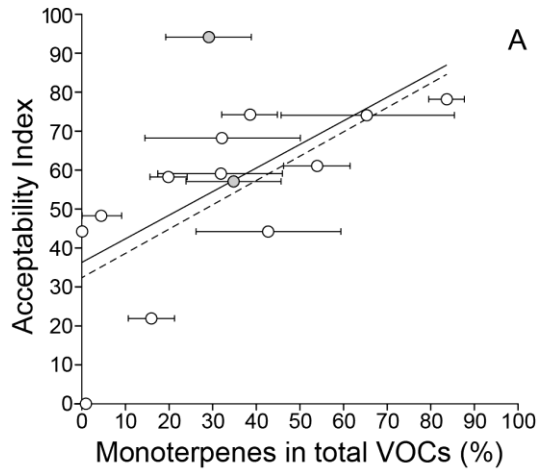
591

592

593   **Figure 2:** Relationship between seedling acceptability (AI) and the relative proportion

594   (% total profile) of major volatile organic compounds quantified from cotyledon-stage

595   seedlings of 14 grassland plant species. Panels show a) monoterpenes, b) β-ocimene, c)

596   sesquiterpenes, d) green leaf volatiles, e) 3-hexen-1-ol acetate, and include results

597   obtained when two species (*Centaurea nigra* and *Leucanthemum vulgare* – denoted by

598   light grey circles) were excluded from analysis (see text for explanation). The solid line

599   represents the regression when all 14 spp. are included, dashed line when 12 spp. are

600   included.

601

602

Species	Acceptability (AI)		N	Phenolics (mg g DM⁻¹)		Alkaloids (mmol g DM⁻¹)		Cyanide (mmol g DM-1)	
	Mean	SE		**Mean**	SE	**Mean**	SE	**Mean**	SE
Asteraceae									
Achillea millefolium	**0.74**	0.07	5	**14.3**	1.1	ND		ND	
Centaurea nigra	**0.94**	0.08	4	**15.3**	1.7	ND		ND	
Jacobaea vulgaris	**0**	0	4	**12.6**	1.1	**3.0**	0.4	**0.9**	0.1
Leontodon hispidus	**0.74**	0.10	5	**15.4**	1.1	ND		ND	
Leucanthemum vulgare	**0.57**	0.06	7	**16.6**	1.1	**6.7**	1.3	ND	
Taraxacum officinale	**0.44**	0.10	4	**20.3**	0.9	**6.0**	1.6	ND	
Caryophyllaceae									
Cerastium fontanum	**0.48**	0.10	3	**10.4**	0.5	**6.1**	0.5	ND	
Fabaceae									
Lotus corniculatus	**0.78**	0.09	4	**11.9**	0.8	**0.4**	0.1	**12.5**	1.6
Trifolium pratense	**0.61**	0.03	5	**11.1**	0.6	ND		**13.3**	1.5
Trifolium repens	**0.59**	0.12	5	**12.0**	0.4	ND		**10.8**	1.6
Plantaginaceae									
Plantago lanceolata	**0.68**	0.09	4	**16.9**	0.9	**5.7**	1.3	ND	
Poaceae									
Dactylis glomerata	**0.44**	0.13	4	**6.9**	0.9	ND		ND	
Festuca rubra	**0.58**	0.16	4	**9.8**	0.5	ND		ND	
Holcus lanatus	**0.22**	0.07	4	**9.8**	0.9	ND		ND	

Table 1 Seedling acceptability (AI) and the concentrations of major constitutive secondary metabolites quantified from 14 d-old seedlings of 14 grassland plant species. ND (Not Detected) denotes failure to detect any quantity above the detection limit. All acceptability trials were conducted on a minimum 10 replicate assays.

Species	Monoterpenes (%)		GLVs (%)		Sesquiterpenes (Total %)	Other Compounds (Total %)	Unknown (%)
	β–ocimene	Total	3-hexen-1-ol acetate	Total			
Asteraceae							
Achillea millefolium	7.3 ± 7.3	38.5 ± 6.3	14.2 ± 6.6	14.3 ± 6.7	24.3 ± 3.4	9.6 ± 9.1	13.2 ± 7.3
Centaurea nigra	9.6 ± 5.0	29.0 ± 9.8	34.6 ± 14.1	35.1 ± 14.3	22.3 ± 7.9	1.3 ± 1.3	12.3 ± 4.9
Jacobaea vulgaris	ND	1.0 ± 1.0	75.7 ± 2.2	96.6 ± 1.7	ND	0.4 ± 0.4	2.1 ± 0.9
Leontodon hispidus	23.6 ± 13.3	65.4 ± 19.7	23.7 ± 18.1	25.4 ± 19.8	4.7 ± 2.8	ND	4.5 ± 0.6
Leucanthemum vulgare	13.6 ± 4.6	34.8 ± 10.8	12.5 ± 6.9	13.4 ± 7.8	25.8 ± 8.9	4.2 ± 1.3	21.8 ± 5.2
Taraxacum officinale	ND	ND	91.6 ± 3.5	94.8 ± 2.1	ND	1.9 ± 0.4	3.3 ± 1.7
Caryophyllaceae							
Cerastium fontanum	ND	4.6 ± 4.6	7.9 ± 7.9	7.9 ± 7.9	30.2 ± 15.6	9.4 ± 9.4	47.9 ± 19.7
Fabaceae							
Lotus corniculatus	57.4 ± 6.2	83.5 ± 4.0	4.6 ± 0.9	4.6 ± 0.9	9.0 ± 2.4	1.9 ± 1.2	0.9 ± 0.5
Trifolium pratense	40.3 ± 10.6	53.8 ± 7.6	17.3 ± 2.8	18.7 ± 2.9	6.3 ± 4.4	9.7 ± 7.3	11.4 ± 4.3
Trifolium repens	24.4 ± 10.3	31.7 ± 14.3	25.9 ± 12.0	27.8 ± 13.7	0.6 ± 0.6	28.8 ± 23.8	11.1 ± 6.4
Plantaginaceae							
Plantago lanceolata	29.8 ± 10.3	32.2 ± 17.8	17.1 ± 16.3	17.5 ± 16.7	10.4 ± 8.4	ND	40.0 ± 19.8
Poaceae							
Dactylis glomerata	36.1 ± 17.3	42.8 ± 16.5	14.8 ± 13.7	14.9 ± 13.8	1.2 ± 0.9	23.3 ± 12.9	17.7 ± 8.4
Festuca rubra	6.6 ± 1.2	19.8 ± 4.3	69.1 ± 6.0	71.5 ± 6.0	ND	5.1 ± 0.8	3.6 ± 1.2
Holcus lanatus	10.8 ± 3.9	15.9 ± 5.4	45.0 ± 14.1	45.5 ± 14.1	4.0 ± 1.1	9.8 ± 9.8	24.7 ± 3.6

Table 2. Relative composition (% of total profile) of major groups of volatile organic compounds (most common individual component of each shown) quantified in 14 d-old seedlings of 14 grassland plant species. ND (Not Detected) denotes failure to detect any quantity above the detection limit. $N = 4$ for all samples except for *Dactylis glomerata* ($N = 3$).