

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

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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

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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* Baumg., *Jacobaeae vulgaris* Gaertn., *Leontodon hispidus* L.,
108 *Lotus corniculatus* L., *Leucanthemum 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 ($15^{\circ}\text{C} \pm$
131 0.2°C , 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 = $15.0^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$; 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 = 15.0°C ± 0.2°C, 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 = 15.0°C ± 0.2°C; 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°C. 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 Butler 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 FeCl₃ 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 Jolicœur (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 MgCl₂) 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 cm² 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 $10^{\circ}\text{C min}^{-1}$ 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 (*Centaurea 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 *Centaurea 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 *Leucanthemum 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 *Centaurea* and *Leucanthemum* 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.

447 **ACKNOWLEDGEMENTS**

448 We thank Jane Akerman, Felicity Thom, Roberto Lo Monaco, & Mike Cotton for
449 technical assistance. This work was supported by the Leverhulme Trust [grant number
450 RPG-083 to MEH, PLN & GMP].

451 **Supporting Information**

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

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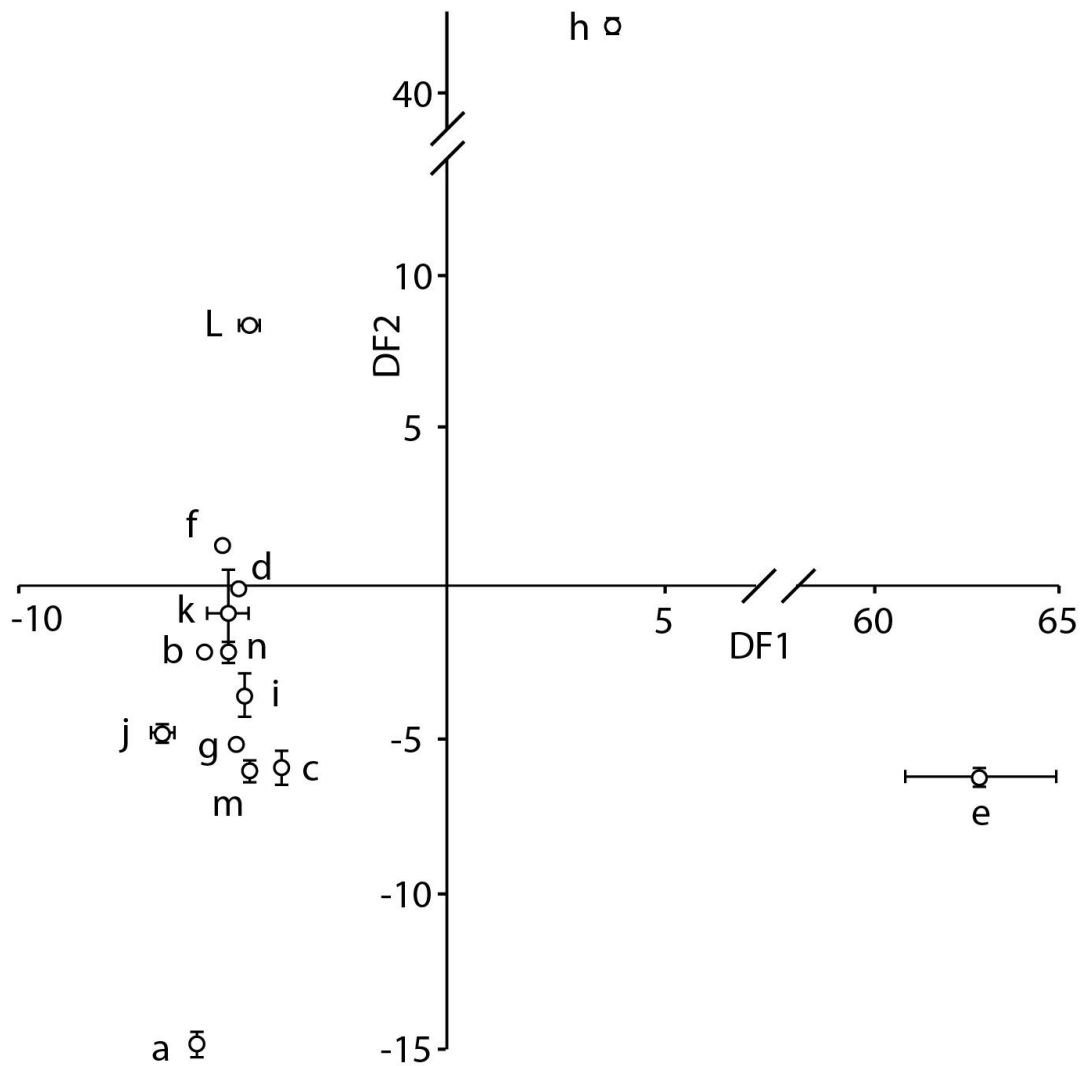
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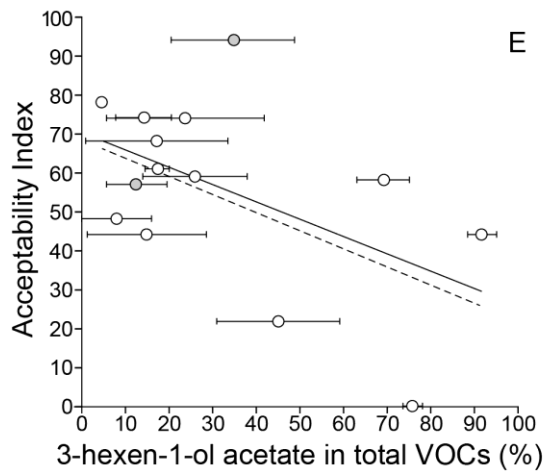
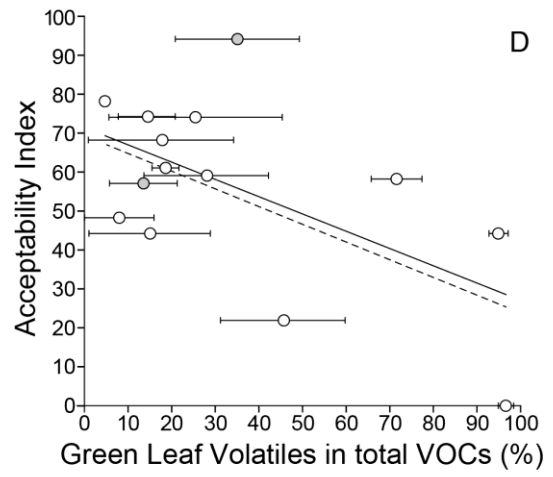
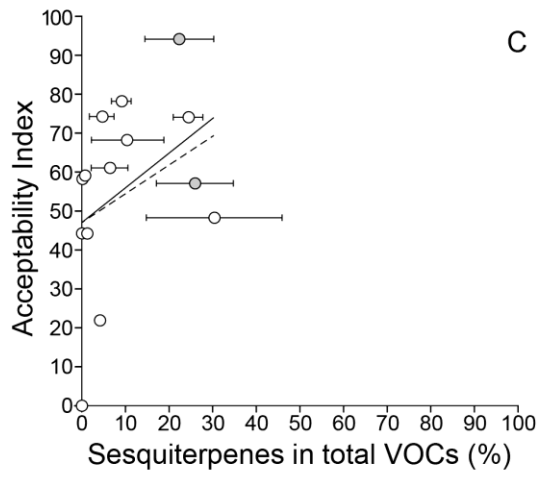
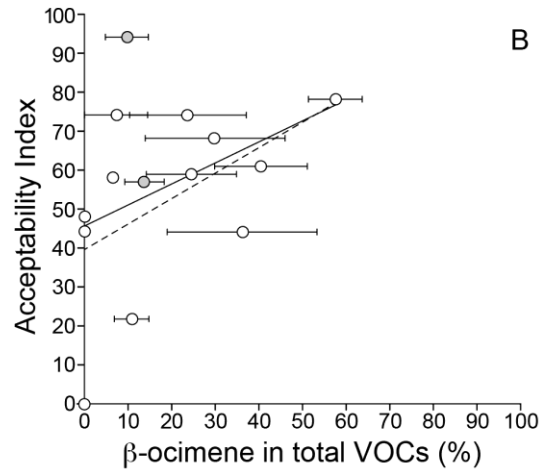
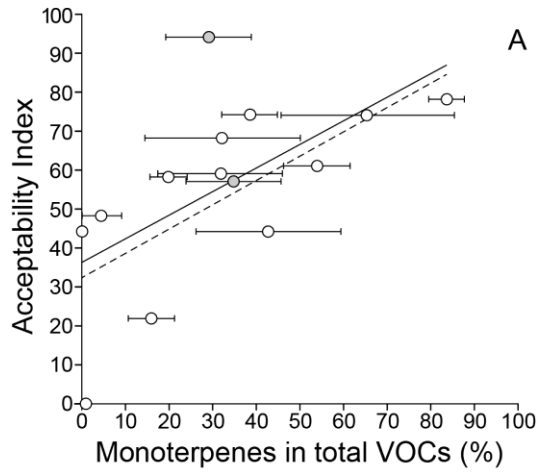
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582 **Figure 1:** Discriminant Factor 1 (DF1) and DF2 from a Canonical Discriminant Analysis
583 (CDA) conducted on the mean (\pm SE) proportion of volatile organic compounds collected
584 from seedlings of 14 grassland species. Key to species: a –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

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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.

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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									
<i>Achillea millefolium</i>	0.74	0.07	5	14.3	1.1	ND		ND	
<i>Centaurea nigra</i>	0.94	0.08	4	15.3	1.7	ND		ND	
<i>Jacobaea vulgaris</i>	0	0	4	12.6	1.1	3.0	0.4	0.9	0.1
<i>Leontodon hispidus</i>	0.74	0.10	5	15.4	1.1	ND		ND	
<i>Leucanthemum vulgare</i>	0.57	0.06	7	16.6	1.1	6.7	1.3	ND	
<i>Taraxacum officinale</i>	0.44	0.10	4	20.3	0.9	6.0	1.6	ND	
Caryophyllaceae									
<i>Cerastium fontanum</i>	0.48	0.10	3	10.4	0.5	6.1	0.5	ND	
Fabaceae									
<i>Lotus corniculatus</i>	0.78	0.09	4	11.9	0.8	0.4	0.1	12.5	1.6
<i>Trifolium pratense</i>	0.61	0.03	5	11.1	0.6	ND		13.3	1.5
<i>Trifolium repens</i>	0.59	0.12	5	12.0	0.4	ND		10.8	1.6
Plantaginaceae									
<i>Plantago lanceolata</i>	0.68	0.09	4	16.9	0.9	5.7	1.3	ND	
Poaceae									
<i>Dactylis glomerata</i>	0.44	0.13	4	6.9	0.9	ND		ND	
<i>Festuca rubra</i>	0.58	0.16	4	9.8	0.5	ND		ND	
<i>Holcus lanatus</i>	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							
<i>Achillea millefolium</i>	7.3 \pm 7.3	38.5 \pm 6.3	14.2 \pm 6.6	14.3 \pm 6.7	24.3 \pm 3.4	9.6 \pm 9.1	13.2 \pm 7.3
<i>Centaurea nigra</i>	9.6 \pm 5.0	29.0 \pm 9.8	34.6 \pm 14.1	35.1 \pm 14.3	22.3 \pm 7.9	1.3 \pm 1.3	12.3 \pm 4.9
<i>Jacobaea vulgaris</i>	ND	1.0 \pm 1.0	75.7 \pm 2.2	96.6 \pm 1.7	ND	0.4 \pm 0.4	2.1 \pm 0.9
<i>Leontodon hispidus</i>	23.6 \pm 13.3	65.4 \pm 19.7	23.7 \pm 18.1	25.4 \pm 19.8	4.7 \pm 2.8	ND	4.5 \pm 0.6
<i>Leucanthemum vulgare</i>	13.6 \pm 4.6	34.8 \pm 10.8	12.5 \pm 6.9	13.4 \pm 7.8	25.8 \pm 8.9	4.2 \pm 1.3	21.8 \pm 5.2
<i>Taraxacum officinale</i>	ND	ND	91.6 \pm 3.5	94.8 \pm 2.1	ND	1.9 \pm 0.4	3.3 \pm 1.7
Caryophyllaceae							
<i>Cerastium fontanum</i>	ND	4.6 \pm 4.6	7.9 \pm 7.9	7.9 \pm 7.9	30.2 \pm 15.6	9.4 \pm 9.4	47.9 \pm 19.7
Fabaceae							
<i>Lotus corniculatus</i>	57.4 \pm 6.2	83.5 \pm 4.0	4.6 \pm 0.9	4.6 \pm 0.9	9.0 \pm 2.4	1.9 \pm 1.2	0.9 \pm 0.5
<i>Trifolium pratense</i>	40.3 \pm 10.6	53.8 \pm 7.6	17.3 \pm 2.8	18.7 \pm 2.9	6.3 \pm 4.4	9.7 \pm 7.3	11.4 \pm 4.3
<i>Trifolium repens</i>	24.4 \pm 10.3	31.7 \pm 14.3	25.9 \pm 12.0	27.8 \pm 13.7	0.6 \pm 0.6	28.8 \pm 23.8	11.1 \pm 6.4
Plantaginaceae							
<i>Plantago lanceolata</i>	29.8 \pm 10.3	32.2 \pm 17.8	17.1 \pm 16.3	17.5 \pm 16.7	10.4 \pm 8.4	ND	40.0 \pm 19.8
Poaceae							
<i>Dactylis glomerata</i>	36.1 \pm 17.3	42.8 \pm 16.5	14.8 \pm 13.7	14.9 \pm 13.8	1.2 \pm 0.9	23.3 \pm 12.9	17.7 \pm 8.4
<i>Festuca rubra</i>	6.6 \pm 1.2	19.8 \pm 4.3	69.1 \pm 6.0	71.5 \pm 6.0	ND	5.1 \pm 0.8	3.6 \pm 1.2
<i>Holcus lanatus</i>	10.8 \pm 3.9	15.9 \pm 5.4	45.0 \pm 14.1	45.5 \pm 14.1	4.0 \pm 1.1	9.8 \pm 9.8	24.7 \pm 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* ($N = 3$).