Faculty of Science and Engineering

School of Biological and Marine Sciences

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

10.1093/aob/mcy190 Annals of Botany Oxford University Press (OUP)

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.

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

Mick E Hanley^{1*}, Roger WR Shannon^{1,2}, Damien G Lemoine³, Bethan Sandey¹, Philip L Newland², Guy M Poppy²

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

Annals of Botany

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.

Methods We quantified seedling selection by snails (*Cornu aspersum*) for 14
 common, European grassland species. Seedling acceptability was subsequently
 compared with species-specific expression of constitutive secondary defence
 metabolites (CSDMs), and VOCs to determine their relative influence on seedling
 selection.

Results We found no relationship between seedling acceptability and CSDMs, but
seedling selection was strongly associated with VOC profiles. Monoterpenes
(specifically β-ocimene), were identified as likely attractants, while green leaf
volatiles (GLVs) (3-hexen-1-ol acetate) were strongly associated with low seedling
acceptability.

Conclusions By elucidating a relationship between VOCs and seedling acceptability,
 we contradict a long-held, but poorly tested, assumption that seedling selection by
 herbivores in (semi-) natural plant communities centres on CSDMs. Instead, our
 results corroborate recent work evidencing how GLVs, including 3-hexen-1-ol
 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	

Annals of Botany

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

Annals of Botany

Original Article doi.org/10.1093/aob/mcy190

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 Western Australia. This study focussed however, on seedlings exposed to (unknown) 62 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 profiles101 than CSDMs.

102 2: if seedlings possess effective CSDM deterrents, they advertise them with103 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

As the principal seedling herbivore in temperate grasslands (Hulme, 1994; Allan andCrawley, 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°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

Seeds were set to germinate in large plastic trays (350-mm x 215-mm x 70-mm deep) filled with John Innes No2 potting compost and maintained in a controlled plant growth room (mean daily temp = $15.0^{\circ}C \pm 0.2^{\circ}C$; 12-hr day: night). All species germinated within 3-5 days. Immediately following radicle appearance, seedlings were transferred in to 50 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 = $15.0^{\circ}C \pm 0.2^{\circ}C$, 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 x 70 mm deep) filled with commercial potting compost, such that the top of each pot was 159 160 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 (\approx 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 AI per tray = $\frac{\text{Mean number of 'test' seedlings attacked}}{\text{Mean number of 'test' + index seedlings attacked}}$

Average seedling AI for each species was then calculated across all replicate trays for each species. AI ranges between 0 (highly unacceptable) and 1 (highly acceptable) where a value of 0.5 represents equal acceptability to the lettuce control. Acceptability trials were conducted between October 2014 and January 2015 in such a way that the timing of 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 = $15.0^{\circ}C \pm 0.2^{\circ}C$; 12 hr day: night). 178 Fourteen day-old seedlings were cut at ground level and immediately flash frozen in tin foil packets in liquid nitrogen, before storage at -80°C. Samples were subsequently placed 179 180 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 μ l 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 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

Annals of Botany

Original Article doi.org/10.1093/aob/mcy190

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, drying in air and cutting to two cm² pieces. The picrate paper was suspended above the 205 206 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

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 filter and pumped into the bag at a rate of 1000 ml min⁻¹ using a Neuberger KNDC B 231 232 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

Annals of Botany

Original Article doi.org/10.1093/aob/mcy190

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 μ m 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 $5^{\circ}C$
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 step deads (Cience Aldrich I (d. UIZ) and calculating their Research interview in dea
202	with standards (Sigma-Aldrich Ltd., UK) and calculating their Kovats retention index

Annals of Botany

Original Article doi.org/10.1093/aob/mcy190

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

- the VOCs, we used the percentage of each volatile in the total VOCs collected for each
- sample rather than using the Total Ion Count to quantify VOCs, after first removing
- artefacts identified from our controls as arising from the SPME fibres or the GC
- 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,
- 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 Tau correlation tests between the mean values of the three groups of chemicals (phenolics, 276 277 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,

we conducted Kendall's Tau correlation tests between the three groups of CSDMs andthe 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 (Kendall's Tau correlation $r^2 = 0.07$; $\tau = 0.990 P = 0.322$), alkaloid ($r^2 = 0.05$; $\tau = -0.187$, 305 306 P = 0.392), or evanogenic ($r^2 = 0.04$; $\tau = 0.094$, 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 (Pearson's product-moment correlation, $r^2 = 0.549$, t = 2.274, df = 12, P = 0.042) (Fig. 333 2a), and β -ocimene in particular (Pearson's product-moment correlation, $r^2 = 0.568$, t =334 2.394, df = 12, P = 0.034), collected from each species (Fig. 2b). We also detected a 335 336 ('marginally-significant') positive relationship between AI and the proportion of sesquiterpenes in the VOCs profile of each species (Fig 2c) ($r^2 = 0.528$, t = 2.151, df = 337 338 12, P = 0.053). Although falling above the P < 0.05 convention, there was a negative relationship (Fig. 2d) between AI and total GLVs contribution ($r^2 = -0.490$, t = -1.948, df 339 340 = 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, AI remained positively correlated with monoterpenes ($r^2 = 0.587$, t = 2.298, df = 10, P = 346 0.044) and β -ocimene ($r^2 = 0.580$, t = 2.251, df = 10, P = 0.048) (Fig. 2a,b). While the 347 positive relationship between AI and sesquiterpenes remained tentative (Fig 2c) ($r^2 =$ 348 349 0.497, t = 1.811, df = 10, P = 0.100), the negative relationship between AI and total GLVs $(r^2 = -0.701, t = -3.107, df = 10, P = 0.011)$ and 3-hexen-1-ol acetate $(r^2 = -0.515, t = -0.515, t = -0.515)$ 350 351 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 influence354 on snail selection, while GLVs likely have a negative influence (Hypothesis 1).

We performed Kendall's Tau correlation tests between the CSDMs (alkaloids, cyanogenics and phenolics) and the VOC groups (monoterpenes, GLVs and sesquiterpenes) and found that none were correlated. We thus found no evidence to support our second hypothesis that VOCs signal the possession of CSDMs to putative 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

To date, and likely by virtue of the fact that GLVs are synthesised in the same oxylipin
pathway as jasmonic acids, GLVs have more frequently been associated with the priming
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 in our samples (e.g. β -phellanderene γ -terpinene, and limonene); instead, and particularly 397

Annals of Botany

Original Article doi.org/10.1093/aob/mcy190

for some high acceptability species like *Achillea, Leontodon, Lotus,* and *Plantago,* terpenoid profiles were dominated by acyclic monoterpenes (β -myrcene, and β -ocimene) more similar to linalool, and/or bicyclic compounds (e.g. α - and β -pinene). Interestingly, Shannon *et al.*, (2016) identified α -pinene and β -myrcene as putative snail attractants. By comparison, the very low acceptability species in our study (*Jacobaea* and *Holcus*) 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

Given we found no relationship between CSDMs and patterns of seedling selection bysnails, 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 cyanogenic glycosides, are for established plants, important anti-herbivores defences 437 438 (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 signalling or induced defence), would go some way to better understanding the evolution 443 444 and ecology of ontogenetic defence trajectories (Barton and Boege, 2017). Our study

suggests however, that some plants species counter the usually fatal consequences ofseedling herbivory with GLV-based, volatile defences.

447 ACKNOWLEDGEMENTS

- 448 We thank Jane Akerman, Felicity Thom, Roberto Lo Monaco, & Mike Cotton for
- 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

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: 563475 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
- *frugiperda* larvae to volatiles from herbivore-damaged maize seedlings. *Journal 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

- van Dam NM, Poppy GM. 2008. Why plant volatile analysis needs bioinformatics detecting signal from noise in increasingly complex profiles. *Plant Biology* 18:
 29–37.
 Dodds CJ, Ford MG, Henderson IF, Leake LD, Martin A, Pickett JA, Wadhams LJ,
- Watson P. 1996. Slug chemical ecology: electrophysiological and behavioural
 studies. In: Henderson IF ed. *Slug and Snail pests in Agriculture*, British Crop
 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.
- Elger A, Lemoine DG, Fenner M, Hanley ME. 2009. Plant ontogeny and chemical
 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
- herbivory by slugs in a willow hybrid system: developmental changes in damage,
 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-
- 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
- Hanley ME, Sykes RJ. 2014. Seedling Herbivory and the Temporal Niche. In: Kelly
 CK, Bowler MA, Fox GA eds. *Environmental Fluctuation, Temporal Dynamics*
- *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.
- Hanley, ME, Fenner M, Whibley H, Darvill B. 2004. Early plant growth: identifying
 the end point of the seedling phase. *New Phytologist* 163: 61-66.

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

Original Article doi.org/10.1093/aob/mcy190

535	Olfactory selection of Plantago lanceolata declines with seedling age. Annals of
536	<i>Botany</i> 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	<i>Heredity</i> 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.
- **Rohloff J, Bones AM. 2005.** Volatile profiling of *Arabidopsis thaliana* Putative
 olfactory compounds in plant communication. *Phytochemistry* 66: 1941-1955.
- Scala A, Allmann S, Mirabella R, Haring MA, Schuurink RC. 2013. Green Leaf
 Volatiles: A plant's multifunctional weapon against herbivores and pathogens. *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.





581 582 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 officianal; m – 588 *Trifolium pratensis*; n – *Trifolium repens*.





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

Species	Acceptability (Al)		N	Phenolics (mg g DM ⁻¹)		Alkaloids (mmol g DM ⁻¹)		Cyanide (mmol g DM-1)	
openie	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	

Original Article

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.

	Monoterpenes (%)		GLV	s (%)	Socquitornonos	Other	Unknown	
Species	β–ocimene	Total	3-hexen-1-ol Total acetate		(Total %)	Compounds (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	

Original Article

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).