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1 **Developing oak buds produce volatile emissions in response to herbivory by freshly**
2 **hatched caterpillars**

3

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10 **Abstract**

11 Plant responses to damage by insectivorous herbivores is well documented in mature leaves. The
12 resulting herbivore-induced plant volatiles (HIPVs) protect the plant by attracting carnivorous
13 arthropods and even some insectivorous vertebrates, to parasitize or consume the plant invaders.
14 However, very little is known about plant production of HIPVs in developing buds, particularly
15 when herbivorous insects are too small to be considered a prey item. It is additionally unclear
16 whether plants respond differently to generalist and specialist chewing insects that overlap in
17 distribution. Therefore, we compared HIPV production of Downy oak (*Quercus pubescens* Willd.)
18 buds infested with freshly hatched caterpillars of *Tortrix viridana* (specialist) and *Operophtera*
19 *brumata* (generalist), against uninfested buds. Of the compounds identified in both years of the
20 experiment, we found that (*Z*)-hex-3-enyl acetate, (*E*)- β -ocimene, acetophenone, linalool, (*E*)-4,8-
21 dimethyl-1,3,7-nonatriene (DMNT), methyl salicylate, α -copaene, α -humulene, (*E*)-caryophyllene,
22 and (*E,E*)- α -farnesene were higher in infested buds compared to controls. We found no difference
23 in HIPV production between the specialist and the generalist herbivores. Production of HIPVs was
24 also associated with leaf damage, with higher HIPV production in more severely attacked buds.
25 Thus, our study shows that oak trees already start responding to insect herbivory before leaves
26 are developed, by producing compounds similar to those found in damaged mature leaves. Future
27 work should focus on how Downy oak may benefit from initiating alarm cues at a time when
28 carnivorous arthropods and insectivorous vertebrates are unable to use herbivorous insects as
29 host or food.

30 **Introduction**

31 Plants have developed multiple methods to reduce damage caused by herbivory. One of
32 these methods is through the release of herbivore-induced plant volatiles (HIPVs). These airborne
33 signals have been shown to attract carnivorous arthropods (Turlings & Benrey 1998; Van Poecke
34 *et al.* 2001) and even some insectivorous vertebrates (Amo *et al.* 2013; Mäntylä *et al.* 2017;
35 Goldberg *et al.* 2019). Using these chemical signals, carnivorous arthropods and insectivorous
36 vertebrates can significantly reduce the number of herbivorous insects, thus preventing further
37 damage to the plant (Kessler & Baldwin 2001).

38 While a wealth of studies have described herbivore-induced volatile emissions from
39 mature foliage, only a few have specifically investigated the emissions from infested buds or very
40 young developing shoots (Röse & Tumlinson 2004; Brilli *et al.* 2009, Rostas & Eggert 2008).
41 Generally, buds are well protected against biotic and abiotic stresses during the dormancy period.
42 The tiny embryo shoots they contain are densely enveloped by tough scales impregnated with
43 gummy or resin type substances, efficiently impeding attacks by insects or pathogens. However,
44 buds become vulnerable during bud break when the scales are spreading or fall off giving access
45 to the inner soft tissues of the developing shoot. Herbivore attacks on these organs can be very
46 detrimental to the plants (Alves-Silva & Del-Claro 2016), because even small damages will cause
47 the complete loss of a new shoot for the coming growing season and not only the fraction of an
48 already existing leaf (Blundell & Peart 2000; Kozlov & Zvereva 2014). Bud herbivory and
49 parasitism particularly threaten deciduous woody plants growing in climates having a very short
50 vegetation period, which limits both the replacement of lost shoots by re-sprouts from dormant
51 buds and the acquisition of resources by the remaining foliage. For example, in the Mediterranean
52 area, the growth of new shoots is restricted to the period of spring due the onset of drought in
53 summer (Montserrat-Marti *et al.* 2009).

54 Although damages inflicted by insect herbivores to buds are presumably more detrimental
55 to the plant than damages inflicted to mature leaves, it is unclear whether HIPV emission is
56 adaptive at this early stage. Buds are mostly present in early spring, at a time when many
57 herbivorous insects like caterpillars only start to hatch from eggs and are therefore still small (van
58 Asch & Visser 2007). At that time, those herbivores might not be valuable enough for predators
59 to consider them as prey items. Considering that the fitness benefits for plants in releasing HIPVs
60 are not always evident, even for mature leaves (Cipollini & Heil 2010; Dicke & Baldwin 2010), the
61 net benefit of emitting HIPVs at the bud stage may be even more questionable. Therefore, to
62 better understand the potential benefits provided by HIPV production, we must first clarify whether
63 developing buds respond to herbivory by releasing HIPVs (Röse & Tumlinson 2004; Brill *et al.*
64 2009).

65 Many herbivorous insects are specialized to feed their larvae on the developing buds and
66 foliage of their host trees (Cízek 2005). Thus, plants are unlikely to be exposed to a single
67 herbivorous insect. For example, the green oak tortrix (*Tortrix viridana*) and the winter moth
68 (*Operophtera brumata*) are serious defoliators of European deciduous tree species (Schroeder &
69 Degen 2008) and specifically attack developing buds and shoots in early spring (Du Merle 1999,
70 Hunter 1990). While the green oak tortrix is a *Quercus* specialist, limited to oak stands (Du Merle
71 1999), winter moths are polyphagous and capable of consuming up to 100 different plant species
72 (Hunter 1990). It is suggested that plants are able to fine-tune the attractiveness of their emissions
73 to carnivores (Dicke & Baldwin 2010) by adjusting their emissions to the type or level of damages
74 they experience (Alborn *et al.* 1997; De Moraes *et al.* 1998; Gaquerel *et al.* 2009; Bricchi *et al.*
75 2010; Girling *et al.* 2011; Zebelo *et al.* 2014). Whether plants differ in HIPV emissions among
76 chewing herbivorous insects has not been extensively studied yet (e.g. De Moraes *et al.* 1998).
77 With newly emerging evidence that polyphagous caterpillar species like *Helicoverpa zea* produce
78 a salivary enzyme that inhibits the emission of HIPVs in host plants (Lin *et al.* 2021), there may

79 be differences in HIPV responses between specialist herbivores that co-evolve with their host,
80 and polyphagous herbivores with a broad diet.

81 In the present study, we investigated whether plants release HIPVs in response to insect
82 herbivory during the early stages of leaf development by infesting developing *Quercus pubescens*
83 oak buds with freshly hatched caterpillars and comparing the HIPV emissions of caterpillar
84 infested and uninfested buds. Additionally, we examined if HIPVs released by oak buds early in
85 the season differ between chewing herbivore species by comparing an oak specialist (green oak
86 tortrix) and polyphagous (winter moth) species, and we investigated whether HIPV emissions are
87 proportional to the damages herbivores inflict to the buds.

88

89 **Methods**

90 *Plants*

91 During the spring of 2017 and 2018, 80 Downy oak trees (*Quercus pubescens* Willd.)
92 grown in pots were maintained on the north side of a building ($n = 20$), south side of a building (n
93 $= 20$), or at a higher elevation to delay bud burst ($n = 40$). All trees were treated with copper in a
94 Bordeaux mixture (AMM 9500302) and a rapeseed oil insecticide (Naturen Eradibug, AMM
95 2110150) in the winter preceding each experiment to reduce the chance any fungus and wild
96 insects infested the trees and caused other volatile emissions than the ones induced by our
97 caterpillar infestation.

98 Bud stages were defined using a slightly modified classification from Du Merle & Mazet
99 (1983). Briefly, bud stages ranged from 1 to 9, with 1 corresponding to a winter dormant bud, and
100 9 to a fully developed leaf. Stage 4 corresponds to a bud that elongates and loses its scales, and
101 stage 5 corresponds to a bud that opens up. Once at stage 4, the oak buds were considered
102 ready to use in the experiment as caterpillars are unable to perforate the protective scales to enter
103 the bud prior to that stage.

104

105 *Insects*

106 Winter moth (*Operophtera brumata*) and green oak tortrix (*Tortrix viridana*) adult females
107 lay their eggs next to leaf buds, which hatch in spring contemporary with bud break (Du Merle
108 1999). Winter moth is a more generalist herbivore adapted to cooler temperate climates and is
109 considered as an invasive species in North America, while green oak tortrix is rather adapted to
110 warm temperate climates and feeds mainly on few species overall oaks. The green oak tortrix is
111 widely distributed throughout western Europe, though as a *Quercus* specialist, limited to oak
112 stands (Du Merle 1999). Winter moths are polyphagous, but compete with green oak tortrix for
113 oak hosts in parts of their distribution (Hunter 1990). Green oak tortrix and winter moth eggs were
114 collected the previous summer from Corsica, France and Gelderland, The Netherlands,
115 respectively. Eggs were kept in an unheated building overwinter and moved outdoors in spring,
116 so caterpillars and trees were experiencing the same weather conditions.

117

118 *Volatile Sampling*

119 Push-pull dynamic headspace sampling was used to collect biogenic volatile organic
120 compound (VOC) emissions from infested and uninfested (control) oak buds (Tholl *et al.* 2006).
121 A double-walled glass headspace (170 cm³) enclosed the twig with the focal bud(s). The
122 headspace was closed with a silicone cap pierced with three holes, one holding the twig, and two
123 holding PFA ¼” tubes allowing air entrance and exit from the headspace. Before entering the
124 headspace, ambient air was pumped through a charcoal filter to remove any contaminants. A flow
125 meter (VAF-G2-03M-1-0, Swagelock, Lyon Vannes et Raccords, Genas, France) was used to
126 control the flow of air into the headspace (260 mL min⁻¹). Water from a thermo-stated water bath
127 was circulated through the outside of the headspace to maintain a temperature of 25°C inside the

128 headspace as temperature strongly affects volatile emissions from plants (Niinemets *et al.* 2011;
129 Peñuelas & Staudt 2010; Vallat *et al.* 2005).

130 One day before sampling, the focal trees were moved to the system to allow any volatile
131 emissions produced while moving to dissipate. When the system was initially turned on, clean air
132 was pumped freely through the system for 10 minutes to purge the headspace. The terminal part
133 of an intact twig (holding 1-8 buds, see below) was then placed carefully into the headspace. The
134 system continued to run for at least 30 min to clear any stress-induced compounds released by
135 the plant when placing the buds into the headspace and allow volatile emissions to come to a
136 steady state. A Gilian GilAir air sampler (Sensidyne, LP, St. Petersburg, FL, USA) was attached
137 to the system and pulled air from the headspace at 200 mL min⁻¹ into a thermal-desorption
138 stainless-steel cartridge (Perkin Elmer, Villebon-sur-Yvette, France) containing 280 mg of Tenax[®]
139 TA adsorbent (20/35 mesh, Alltech-Grace Davison, Düren, Germany) to collect volatile emissions.
140 The net influx of clean air kept ambient air from entering the system. Sampling lasted for 2h 30min
141 to collect volatiles from ~30L of air. In addition to sampling buds (with or without caterpillars), a
142 blank sample was collected each day to subtract background compounds from the bud samples
143 collected that day. These samples were collected in the same way as the bud samples, just
144 without anything in the headspace. Cartridges were stored at -20°C until analysis.

145 Thirty-three oak trees were sampled in 2017 ($n = 14$ *tortrix* infested, $n = 10$ winter moth
146 infested, $n = 9$ control) and 2018 ($n = 10$ *tortrix* infested, $n = 12$ winter moth infested, $n = 11$
147 control). A healthy, well-developed terminal twig with 1 – 8 buds was selected for analysis on
148 each tree ($n = 42$ with 1 bud, $n = 11$ with 2 buds, $n = 4$ with 3 buds, $n = 5$ with 4 buds, $n = 1$ with
149 5 buds, $n = 2$ with 6 buds, $n = 1$ with 8 buds). Each twig was repeatedly measured before (T_0) and
150 after caterpillar infestation at 24 h (T_{24}) and 48 h (T_{48}) in 2017, and 48 h (T_{48}) and 96 h (T_{96}) in
151 2018. Bud stage for first measurement ranged between 4 and 7 in both years. After the first
152 measurement, the focal buds were either infested with green oak tortrix caterpillars, winter moth

153 caterpillars, or left as a control. Control twigs were thus repeatedly sampled the exact same way,
154 except that they were never infested with caterpillars.

155 A paintbrush was used to gently place newly hatched caterpillars on the buds where they
156 remained until the end of sampling. Though caterpillars were observed entering the buds in 2017,
157 several buds had no caterpillars remaining at the end of sampling ($n = 2$ *tortrix* infested, $n = 3$
158 winter moth infested). Thus, in 2018, wire cages with mesh cloth bags were attached to the branch
159 with the focal bud to prevent caterpillars from dispersing. Cages were attached the same way to
160 the sampled branches of control trees.

161

162 *Gas Chromatography Mass Spectrometry (GC-MS)*

163 Analyses were carried out at the PACE (Plateforme d'Analyses Chimiques en Ecologie),
164 with the support of LabEx CeMEB, an ANR "Investissements d'avenir" program (ANR-10-LABX-
165 04-01). Cartridges were placed on a TD-20 two-stage thermal desorption system coupled to a
166 QP-2010-SE gas chromatography mass spectrometry system (GC-MS; Shimadzu Corporation,
167 Kyoto, Japan). VOCs were first thermally desorbed by flushing the cartridge heated at 250°C
168 during 10 minutes with a 30mL min⁻¹ flow of helium, and absorbed for the second stage on a -
169 10°C low-dead volume cold trap containing Tenax[®] TA adsorbent (80/100 mesh, Shimadzu
170 Corporation, Kyoto, Japan). The cold trap was subsequently heated to 250°C for 5 minutes. VOCs
171 were then injected into a 250°C heated transfer line in the GC-MS column (Optima-5MS, 30 m x
172 0.25 mm x 0.25 µm, Macherey-Nagel, Düren, Germany). The oven temperature program was as
173 follow: 40°C held for 2 minutes, then increased at a rate of 5°C min⁻¹ until reaching 200°C, and
174 finally increased at a rate of 10°C min⁻¹ up to 270°C held for 6 minutes. Helium was used as carrier
175 gas at 36.1cm s⁻¹. The temperature of the transfer line and ion source were 250 and 200°C,
176 respectively. MS was then used with electron impact at 70eV in the scan mode, from 35 to 350
177 m/z.

178 Peaks were identified by comparing their retention indices calculated with alkanes (Sigma
179 Aldrich®, Darmstadt, Germany) and mass spectra with those of commercial databases (Adams,
180 2007 and NIST 2005, respectively). Pure standards dissolved in methanol were used to i) confirm
181 identification and ii) make three-level calibration curves of four specific standards, representing
182 classes of VOCs, in order to estimate emissions (α -pinene, (*E*)-caryophyllene, methyl-salicylate
183 and (*Z*)-hex-3-enyl acetate for monoterpenoids, sesquiterpenoids, shikimic acid and fatty acids
184 derivatives, respectively). The class mean calibration factor (ng unity⁻¹ of peak area) were applied
185 to quantify the peaks identified in the corresponding classes, and the mean calibration factor of
186 all the standards was applied to peaks to unidentified peaks.

187 Area under the curve (ng) divided by volume of air sampled (L) was used to calculate the
188 concentration for each compound (ng.L⁻¹). Since the air flow in the headspace was always kept
189 constant, changes in the net HIPV concentrations must reflect changes in the emission rate per
190 enclosed shoot.

191

192 *Bud Damage*

193 After sampling was complete in 2018, infested buds ($n = 21$) were dissected to assess
194 the level of damage to the bud after being infested for 96 h. Buds were dissected and damaged
195 leaves were photographed against graph paper and analyzed using ImageJ software (Schneider
196 *et al.* 2012). Leaf damage area (mm²) was measured for all 21 samples from infested trees. Buds
197 sampled later in the season ($n = 13$) have photographs of both the damaged and undamaged
198 leaves that allowed us to calculate damaged leaf area divided by total leaf area (i.e., proportion
199 of total bud damage). However, using this measure reduced sample size. We found a significant
200 correlation between damaged area (mm²) and proportion of total bud damage in the 13 samples
201 with both measures ($F_{1,11} = 35.48$, $p < 0.001$, $r^2 = 0.76$). Thus, damaged area (mm²) is used in
202 analyses reported here.

203

204 *Statistical Analyses*

205 All analyses were conducted in R version 3.5.1 (R Core Team 2020). Initially, 68 and 52
206 volatile organic compounds were found in at least 1 sample in 2017 and 2018, respectively. We
207 reduced our analyses to the following compounds that were identified in both years: (Z)-hex-3-
208 enyl acetate, (E)- β -ocimene, acetophenone, linalool, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT),
209 methyl salicylate, α -copaene, (E)-caryophyllene, (E,E)- α -farnesene, (Z)-hex-3-enyl benzoate, (E)-
210 nerolidol, and dendrolasin. In addition, we included aromadendrene and α -humulene that were
211 only regularly found in 2017 samples, but that have been associated with larval feeding (Martins
212 & Zarbin 2013; Pinto-Zevallos *et al.* 2013). Two samples contained values greater than nine
213 standard deviations from the mean and were thus, excluded from analyses.

214 To identify the experimentally controlled factors influencing VOC variation, data was
215 analyzed using redundancy analysis (RDA) (Hervé *et al.* 2018). Prior to this analysis, a small
216 constant was added to the data to remove zeroes. Data was pre-transformed using centered log
217 ratio (package: *Hotelling*, Curran 2018) and then auto-scaled (Hervé *et al.* 2018). An RDA model
218 was fit with treatment (winter moth infested, tortrix infested, or control), time (T₀, T₂₄, and T₄₈), and
219 the interaction of treatment and time using the package *vegan* (Oksanen *et al.* 2019). We
220 excluded T₉₆ samples, which were only collected in 2018, from the analysis because the non-
221 detection of aromadendrene and α -humulene in 2018 required entering missing values as zeros.
222 We did not want to include a sampling time that would increase the likelihood of type I error for
223 these compounds by having a mean of 0 ng/L at T₉₆ compared to positive, non-zero values in all
224 other sampling times. The condition function was used to control for repeated measures in
225 individual trees and across years.

226 Using the package *RVAideMemoire* (Hervé 2019), a constrained and unconstrained
227 principle component analysis (PCA) was run on the RDA model. The constrained PCA determined
228 the proportion of the variance that was explained by the controlled variables in the experimental
229 design (treatment, time, and the interaction of treatment and time) and the unconstrained PCA to

230 determine the proportion of the variance explained by unknown factors that were not included as
231 controlled variables. An analysis of variance (ANOVA) *F*-test with 999 permutations tested
232 whether the proportion of variance explained by the controlled variables was significant. In the
233 case of significant models, a multivariate ANOVA was used to determine which controlled
234 variables from the model (treatment, time, and the interaction between treatment and time)
235 contributed significantly to the proportion of variance explained by the experimental design.
236 Pairwise tests were conducted on significant variables with more than two factors to determine
237 which groups were significantly different from each other. In groups that were significantly
238 different, a biplot was produced to visualize which compounds appeared to be driving the
239 observed differences.

240 When analyzing leaf area damage, all analyses were restricted to the 96hr sampling point
241 from infested trees only ($n = 21$ samples). Controls were excluded because we assumed no
242 damage was done to the buds. We first ran a linear model to determine if one species of caterpillar
243 caused more damage to the buds than the other. To further identify if caterpillar species or
244 damaged leaf area influenced HIPV production, we ran a second linear model to measure the
245 effects of damage area, species, and the interaction of damage area and species on changes in
246 overall HIPV emissions from the plants. For each sample, the emissions for all 12 HIPVs identified
247 in the 2018 samples were summed to avoid any issues arising from repeated analyses and
248 collinearity between HIPV emissions. Mean values are reported \pm SEM. We set $\alpha = 0.05$.

249

250 **Results**

251 Table 1 provides details of VOC concentrations from control, green oak tortrix infested, and winter
252 moth infested buds.

253

254 **Table 1.** Mean concentrations (ng/L \pm SEM) of the 14 HIPVs identified for analysis from the three
 255 sampling times for control, green oak tortrix (*Tortrix*) infested, and winter moth infested buds.
 256 GC-MS linear retention time (RI) and type of volatile organic compound are also included. GLV
 257 = green leaf volatile; HMTP = homoterpene; MNTP = monoterpene; SAD = shikimic acid
 258 derivative; SSQP = sesquiterpene.

VOC	Type	RI	Treatment	T0	T24	T48
(Z)-Hex-3-enyl acetate	GLV	1006	Control	1.545 \pm 0.678	1.252 \pm 0.825	0.168 \pm 0.143
			<i>Tortrix</i>	1.179 \pm 0.739	2.485 \pm 1.011	1.081 \pm 0.557
			Winter Moth	6.664 \pm 3.921	3.595 \pm 1.556	1.326 \pm 0.861
(E)- β -Ocimene	MNTP	1050	Control	0.016 \pm 0.007	0.076 \pm 0.043	0.060 \pm 0.030
			<i>Tortrix</i>	0.100 \pm 0.038	1.457 \pm 0.744	2.926 \pm 1.950
			Winter Moth	0.129 \pm 0.060	0.166 \pm 0.072	0.461 \pm 0.223
Acetophenone	SAD	1061	Control	0.399 \pm 0.219	1.359 \pm 0.545	0.618 \pm 0.306
			<i>Tortrix</i>	2.335 \pm 1.058	5.680 \pm 2.973	3.286 \pm 1.192
			Winter Moth	0.407 \pm 0.205	2.603 \pm 1.521	0.789 \pm 0.457
Linalool	MNTP	1100	Control	0.030 \pm 0.018	0.034 \pm 0.024	0.014 \pm 0.010
			<i>Tortrix</i>	0.098 \pm 0.034	0.221 \pm 0.194	0.337 \pm 0.273
			Winter Moth	0.252 \pm 0.156	0.033 \pm 0.033	0.382 \pm 0.350
(E)-4,8-dimethyl-1,3,7-Nonatriene (DMNT)	HMTP	1118	Control	0.076 \pm 0.045	0.033 \pm 0.017	0.027 \pm 0.011
			<i>Tortrix</i>	0.079 \pm 0.033	0.378 \pm 0.112	0.698 \pm 0.308
			Winter Moth	0.276 \pm 0.138	0.152 \pm 0.060	0.636 \pm 0.296
Methyl salicylate	SAD	1194	Control	0.024 \pm 0.024	0.068 \pm 0.039	0.038 \pm 0.016
			<i>Tortrix</i>	0.030 \pm 0.018	0.336 \pm 0.117	0.359 \pm 0.119
			Winter Moth	0.018 \pm 0.011	0.173 \pm 0.074	0.087 \pm 0.035
α -Copaene	SSQP	1384	Control	0.001 \pm 0.001	0.000 \pm 0.000	0.001 \pm 0.001
			<i>Tortrix</i>	0.002 \pm 0.001	0.004 \pm 0.003	0.007 \pm 0.003
			Winter Moth	0.004 \pm 0.002	0.003 \pm 0.003	0.003 \pm 0.002
(E)-Caryophyllene	SSQP	1429	Control	0.009 \pm 0.004	0.049 \pm 0.044	0.033 \pm 0.019
			<i>Tortrix</i>	0.034 \pm 0.016	0.217 \pm 0.107	0.570 \pm 0.349
			Winter Moth	0.027 \pm 0.009	0.053 \pm 0.035	0.267 \pm 0.170
Aromadendrene	SSQP	1457	Control	0.054 \pm 0.032	0.024 \pm 0.012	0.019 \pm 0.008
			<i>Tortrix</i>	0.056 \pm 0.024	0.272 \pm 0.080	0.362 \pm 0.227
			Winter Moth	0.195 \pm 0.100	0.109 \pm 0.043	0.345 \pm 0.214
α -Humulene	SSQP	1452	Control	0.000 \pm 0.000	0.045 \pm 0.025	0.025 \pm 0.010
			<i>Tortrix</i>	0.014 \pm 0.011	0.244 \pm 0.091	0.262 \pm 0.125
			Winter Moth	0.009 \pm 0.007	0.122 \pm 0.047	0.109 \pm 0.049
(E,E)- α -Farnesene	SSQP	1511	Control	0.023 \pm 0.008	0.120 \pm 0.059	0.100 \pm 0.036
			<i>Tortrix</i>	0.136 \pm 0.037	1.358 \pm 0.615	2.657 \pm 1.754
			Winter Moth	0.195 \pm 0.076	0.206 \pm 0.083	0.521 \pm 0.327
(E)-Nerolidol	SSQP	1566	Control	0.020 \pm 0.011	0.066 \pm 0.039	0.056 \pm 0.025
			<i>Tortrix</i>	0.075 \pm 0.030	1.122 \pm 0.541	2.071 \pm 1.408
			Winter Moth	0.154 \pm 0.076	0.154 \pm 0.056	0.273 \pm 0.176
	SAD	1573	Control	0.060 \pm 0.032	0.329 \pm 0.113	0.166 \pm 0.071

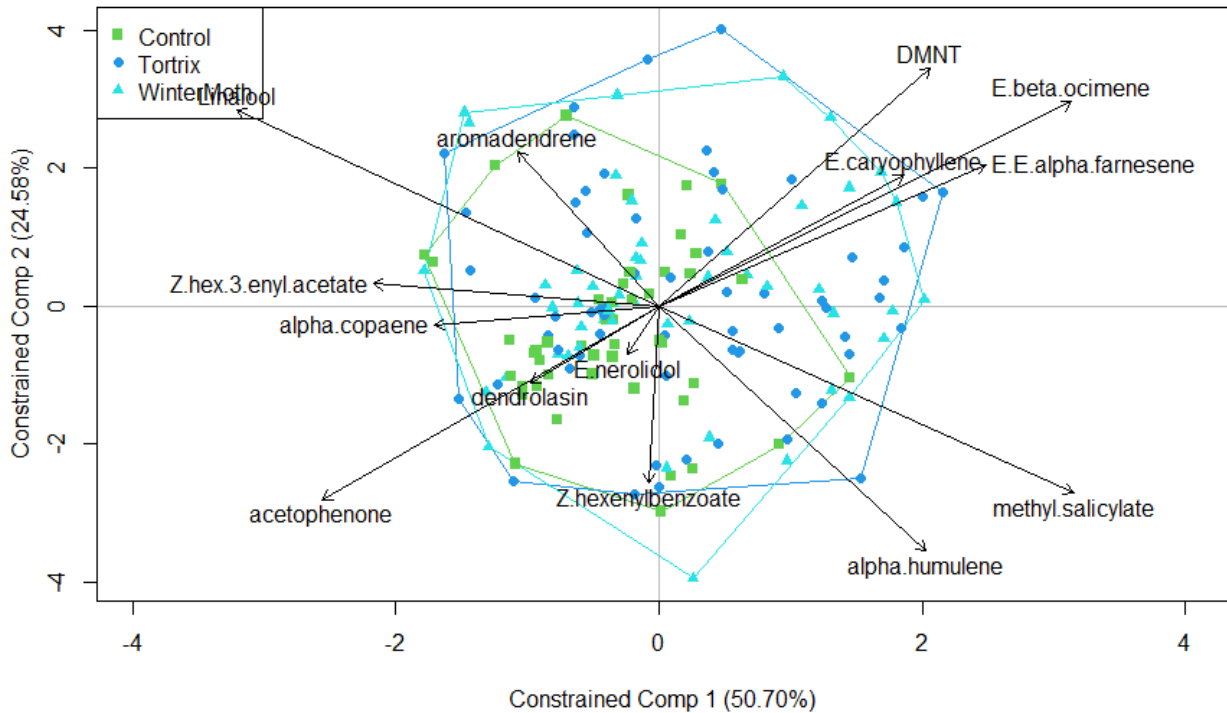
(Z)-Hex-3-enyl benzoate				<i>Tortrix</i>	0.238 ± 0.118	0.806 ± 0.325	1.001 ± 0.443
				Winter Moth	0.105 ± 0.043	0.492 ± 0.165	0.443 ± 0.211
Dendrolasin	SSQP	1581	Control	0.162 ± 0.056	0.260 ± 0.086	0.155 ± 0.063	
			<i>Tortrix</i>	0.264 ± 0.099	0.763 ± 0.240	0.982 ± 0.402	
			Winter Moth	0.340 ± 0.170	0.339 ± 0.077	0.641 ± 0.368	

259

260

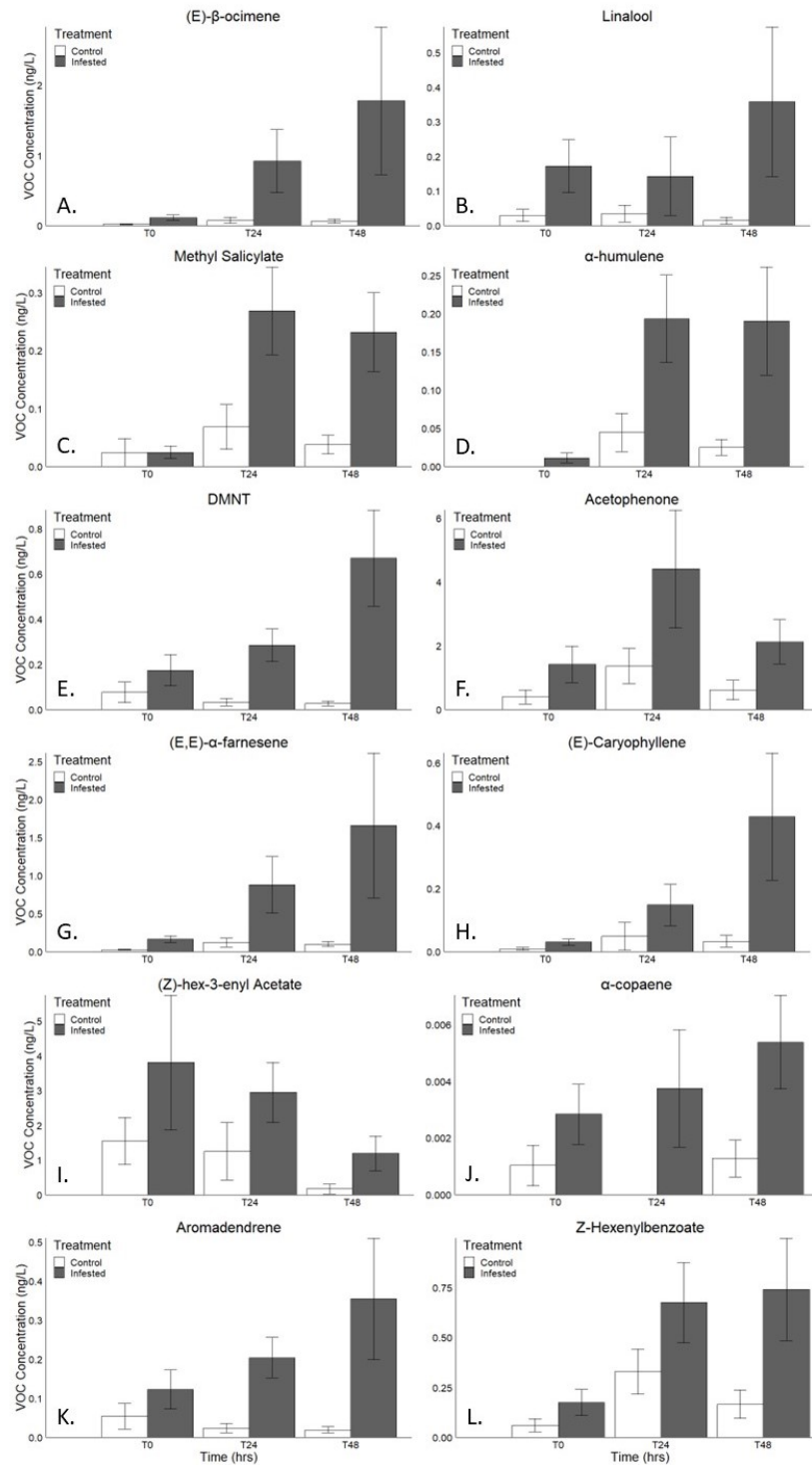
261 *HIPV Emissions*

262 The variance explained by the experimental design was significant (constrained variance
263 = 7.81%, $F_{1,8} = 1.97$, $p = 0.001$). Treatment ($F_{1,2} = 1.84$, $p = 0.014$) and time ($F_{1,2} = 4.06$, $p = 0.001$)
264 were both significant, but the interaction between treatment and time was not ($F_{1,4} = 0.99$, $p =$
265 0.46). Pairwise comparisons of treatment found that the controls differed significantly from both
266 *tortrix* ($p = 0.015$) and winter moths ($p = 0.015$), but *tortrix* and winter moths were not significantly
267 different from each other ($p = 0.825$). Pairwise comparisons of time showed T_0 significantly
268 differed from T_{24} ($p = 0.003$) and T_{48} ($p = 0.003$). T_{24} significantly differed from T_{48} ($p = 0.011$).
269 While there was overlap between VOCs produced in caterpillar infested and control buds, the
270 following compounds appeared to significantly differ in caterpillar infested buds compared to
271 uninfested buds (figure 1): (*E*)- β -ocimene, linalool, methyl salicylate, α -humulene, DMNT,
272 acetophenone, (*E,E*)- α -farnesene, (*E*)-caryophyllene, (*Z*)-hex-3-enyl acetate, and α -copaene.
273 Compounds that appear to be borderline significant between controls and caterpillar infested buds
274 were aromadendrene and (*Z*)-hex-3-enyl benzoate. Non-significant VOCs included dendrolasin
275 and (*E*)-nerolidol. Therefore, we consider all VOCs except dendrolasin and (*E*)-nerolidol as
276 HIPVs.



277
 278 **Figure 1.** Biplot of VOC emissions shows predicted overlap in compounds between infested (green oak
 279 tortrix = blue, winter moth = light blue) and control trees (green), with infested trees extending
 280 beyond the area of the controls. VOCs extending clearly beyond the boundaries of the infested
 281 trees were considered as HIPVs. Aromadendrene and (Z)-hex-3-enyl benzoate were considered
 282 borderline significant, still being within the boundaries of the control trees, but near the border.
 283 The compounds dendrolasin and (E)-nerolidol were clearly within the boundary of the control trees
 284 and therefore were not considered as specific HIPVs in our study.
 285

286
 287 To visualize the patterns in HIPV emissions, the means (\pm SEM) for the compounds found to be
 288 significant and nearly significant in the previous analysis were graphed (figure 2). There was a
 289 general pattern for concentrations in the infested buds to increase compared to the control buds.
 290 The exception to this pattern was (Z)-hex-3-enyl acetate, which appeared to decrease over time
 291 (figure 2I).



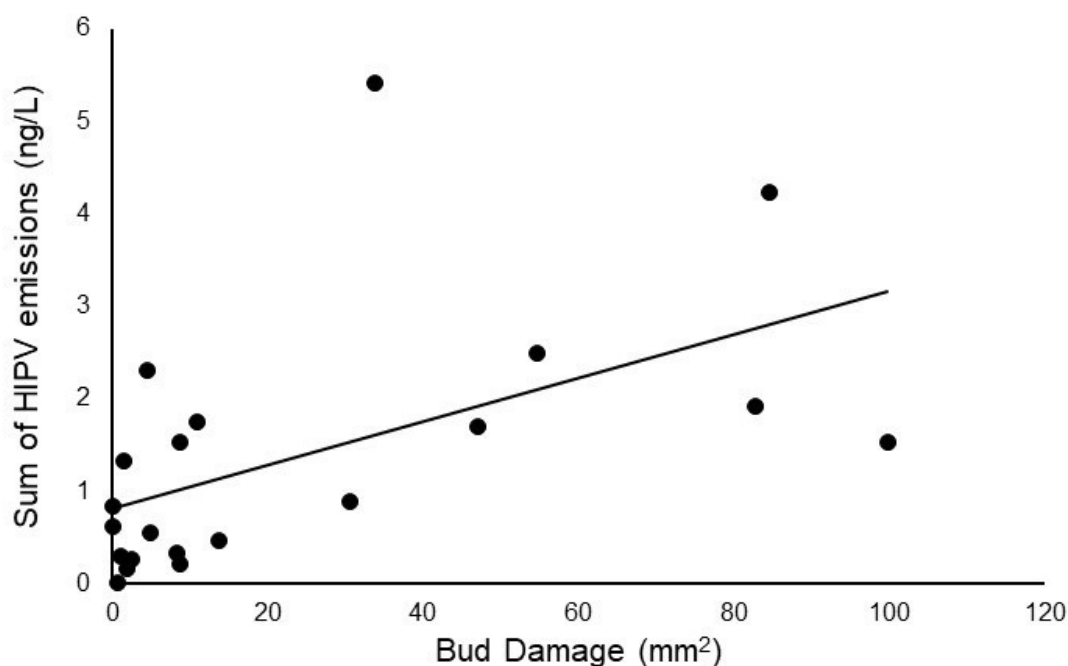
292

293 **Figure 2.** Comparisons of the mean concentrations \pm SEM of 12 VOCs identified as significant or near
 294 significant from the analysis. While the interaction between time and treatment was not significant,
 295 a significant difference between time ($p = 0.001$) and between treatments ($p = 0.014$) was
 296 detected. Visualization of the data shows an increase over time within the infested trees (gray
 297 bars), with the exception of (Z)-hex-3-enyl acetate (panel I), and higher concentrations in the
 298 infested trees compared to the uninfested trees (white bars).

299 2018 Caterpillar Damages

300 After 96hr of infestation, green oak tortrix caused damage to an average of 23.58 ± 10.48
301 mm^2 of leaf area while winter moth caterpillars caused damage to an average of 24.07 ± 9.55
302 mm^2 corresponding to relative damages of 4.10 ± 2.02 and 3.83 ± 1.13 %, respectively. Relative
303 damages ranged from 0.12 % to 14.6 %. There were no differences in the amount of leaf damages
304 done by each species ($F_{1,19} = 0.001$, $p = 0.97$). When analyzing the effect of species and quantity
305 of damage on the sum of HIPV emissions at 96h post-infestation, there is a significant, positive
306 relationship with damaged leaf area ($F_{1,17} = 8.62$, $p = 0.009$, $r = 0.54$, figure 3). Species ($F_{1,17} =$
307 3.25 , $p = 0.09$) and the interaction between species and damaged leaf area ($F_{1,17} = 0.76$, $p = 0.39$)
308 were not significantly correlated with HIPV emissions.

309



310

311 **Figure 3.** As the area of leaf damage increased, the sum of the VOC concentrations identified in 2018
312 samples also increased ($r = 0.54$). Species of caterpillar did not affect the quantity of damaged
313 leaf area or the sum of HIPV emissions, suggesting the buds were responding to caterpillar
314 herbivory in the same way, regardless of species.

315

316 Discussion

317 Downy oak is known to be a strong constitutive emitter of isoprene (Rodríguez-Calcerrada
318 *et al.* 2013), a compound we did not observe in our study on bud emissions. In fact, isoprene is
319 produced only in mature leaves after induction by high temperatures (Monson *et al.* 1994) and
320 therefore is irrelevant as a semiochemical in trophic relationships at earlier stages (see also for
321 example Müller *et al.* 2015). Except for dendrolasin and (*E*)-nerolidol, all HIPVs released from
322 Downy oak during bud break were significantly enhanced by caterpillar feeding, with a positive
323 correlation between quantity of bud damage and HIPV production. This strongly suggests that
324 these emissions were essentially non-constitutive. Indeed, DMNT, ocimenes, linalool,
325 farnesenes, methyl-salicylate and hexenyl-acetate are among the most commonly observed
326 HIPVs (for overviews, see e.g. McCormick *et al.* 2012; Dudareva *et al.* 2013; Turlings & Erb 2018).
327 The minor emissions of these VOCs observed in control buds can be explained by some
328 unavoidable mechanical stress they experienced during chamber installation and/or the presence
329 of endo or epiphytial microorganisms (Junker & Tholl 2013). (*E*)-nerolidol is also a commonly
330 reported HIPV. The non-significant link to caterpillar feeding of this VOC as well as of dendrolasin
331 observed in the present study is likely due to their low volatility, making it difficult to measure
332 emissions quantitatively. Both are oxygenated sesquiterpenes known to be chemically unstable
333 and to adhere and dissolve in surfaces including those of the plant tissues (Helmig *et al.* 2004;
334 Niinemets *et al.* 2011). Moreover, (*E*)-nerolidol is the natural precursor of DMNT (Tholl *et al.* 2011)
335 and therefore its emissions might evolve differently in response to biotic stresses. Generally, the
336 induction kinetics of HIPV emissions can largely differ among individual compounds. Green leaf
337 volatiles such as hexenyl-acetate and other volatile derivatives of fatty acid breakdown are almost
338 instantaneously formed upon injury and rapidly disappear when stress ceases (see e.g. Staudt *et*
339 *al.* 2010). By contrast, the induction of HIPV terpenoids usually involves gene activation following
340 a signaling cascade, which takes hours to days (Bonaventure *et al.* 2011; Joo *et al.* 2018). In line
341 with this, our results show that most of the emitted terpenoids steadily increased during 96 h

342 enclosure period while hexenyl acetate emissions rather decreased. This decrease over time,
343 which was also observed in the control buds, suggests that the hexenyl acetate emissions were
344 induced in part already during the installation of the branches in the headspace and that many of
345 the caterpillars reduced their feeding activities during the confinement, as indicated by their
346 absence in some buds at the end of the experiment.

347
348 It has been hypothesized that specialist herbivores induce different defensive responses
349 than generalist herbivores, because specialists gained the ability to tolerate direct plant defenses
350 by sequestering phytotoxins (Ali & Agrawal 2012; McCormick *et al.* 2012; Rowen & Kaplan 2016).
351 They may also manipulate the host metabolism to their benefit (Lin *et al.* 2022) and even use
352 HIPVs as cues to choose the right host plant. For example *Tortrix viridana* females are more
353 attracted by the VOC profiles emitted by susceptible pedunculate oak ecotypes than by the profile
354 of resistant ecotypes, whose leaves contain secondary metabolites reducing larval growth and
355 survival (Ghirardo *et al.* 2012; Bertić *et al.* 2021). Owing to their adaptation, specialists are
356 expected to inflict more feeding damage on host trees than generalists, which in turn could induce
357 more HIPVs, possibly favoring the plant's indirect defenses against the herbivore. However, meta
358 analyses on literature data provide only limited support for this hypothesis, and revealed that
359 comparative studies sometimes confounded specialization (diet breadth) and feeding guild (i.e.,
360 chewing insects vs sap feeders; (Ali & Agrawal 2012; Rowen & Kaplan 2016). Sap feeders such
361 as aphids indeed induce less and different HIPV patterns than chewing insects, because they
362 inflict little to no tissue damage but are potential vectors of pathogens (e.g., Staudt *et al.* 2010).
363 Here, we found no detectable differences in the VOCs produced in response to herbivory by the
364 generalist *Operophtera brumata* and the oak specialist *Tortrix viridana*. HIPV production was
365 correlated with damage, but it was similar for both caterpillar species. This suggests that the
366 induction of HIPVs depended predominantly on damage derived factors rather than on insect

367 specific elicitors (Arce *et al.* 2021). Indeed, Mithöfer *et al.* (2005) showed that mechanical
368 wounding simulating caterpillar foraging induces VOC emission patterns similar to real herbivory.

369
370 One result we consider having a significant ecological relevance is that total HIPV
371 concentrations were related to the amount of damages inflicted to the buds. Positive correlations
372 between biotic, stress-induced VOC emission and the degree of damage has been previously
373 reported (Niinemets *et al.* 2013). Since alarm signals emitted by plants are considered cry-for-
374 help (Dicke 2009), releasing more HIPVs when plants are more severely attacked (Girling *et al.*
375 2011), may seem adaptive as studies have shown that parasitic wasps were more attracted to
376 plants emitting more HIPVs (Guerrieri *et al.* 1999). Most studies were however conducted with
377 plants that were already in full leaf, and which were attacked by mature insects (but see for
378 example Hare 2010). In the present study, we show that emissions proportional to infestation
379 already occur at a much earlier stage, when oaks are still in their bud stages and are attacked by
380 newly hatched caterpillars. Those caterpillars are only about 1 mm long, hide inside buds
381 (DuMerle & Mazet 1983), and are still too small to be considered preys for predators like birds
382 that need large biomass of insects to feed their chicks (Blondel *et al.* 1999). Furthermore,
383 predation of caterpillars by birds at this early stage would likely entail the complete loss of the
384 infested buds (Betts 1955), more so than a caterpillar attack by specific parasitoid wasps. There
385 is thus a potential conflict between plants that need their precious buds to be protected from
386 irreversible damages, and insect predators that may not be very interested by newly hatched
387 caterpillars, or cause even more severe damages than caterpillars. From the plants' perspective,
388 the net benefits of HIPV emission from buds via tri-trophic interactions thus remains to be clarified
389 (Dicke & Baldwin 2010). One potential explanation comes from recent studies suggesting that
390 those early emissions already elicit physiological and behavioural reactions from insectivorous
391 songbirds that may use these odours as signals to modulate their reproductive timing and effort
392 (Graham *et al.* 2021, Caro *et al.* 2023). Birds could for example modulate their clutch size, i.e. the

393 number of chicks they intend to raise, based on the amount of HIPVs they detect at this early
394 stage. In this scenario, bud HIPVs would be used as a cue to infer the future available biomass
395 of caterpillars. Therefore, even if the immediate benefits for plants of emitting HIPVs while they
396 are still in buds is not clear yet, they might at least guarantee that there will be enough manpower
397 around to rid them of their parasites at a later stage.

398

399 In the present study, we did not investigate whether HIPVs were only emitted from the
400 damaged buds or also from other non-attacked plant parts. Many studies have shown that HIPVs
401 can be systemically emitted upon stress (e.g. Röse & Tumlinson 2004) possibly increasing its
402 signaling range for receivers (McCormick *et al.* 2012). However, HIPVs released into the air
403 masses are subject to rapid turbulent transport, mixing, and chemical reactions with other trace
404 gases (Conchou *et al.* 2019). Therefore, the distances odor plumes can travel and be perceived
405 by other organisms are highly variable, independent of the shape and the strength of the emission
406 source (Douma *et al.* 2019; Pannunzi & Nowotny 2019). Furthermore, few studies have quantified
407 local and systemic emission rates. In a study of holm oaks infested with gypsy moth, Staudt &
408 Lhoutellier (2007) reported that HIPV emission rates from uninfested leaves were about an order
409 of magnitude lower than rates from infested leaves, indicating that the source of HIPVs is
410 essentially, although not exclusively, local. From the perspective of a caterpillar predator using
411 HIPVs for foraging, locally emitted HIPVs should be much more efficient for spotting their prey in
412 the close range (Volf *et al.* 2021), unless cues other than olfactory ones play a role.

413

414 In conclusion, few studies exist that measure the HIPVs of tree buds in response to
415 herbivore attacks. Our study suggests that, even when herbivorous insects are too small to be
416 considered food or host for insect predators, Downy oak are upregulating the production of HIPVs.
417 The purpose of this upregulation remains unclear. Thus, future studies should investigate whether

418 and how herbivorous insect predators use these cues, and whether there are reciprocal benefits
419 to the tree itself.

420

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590 **Statements and Declarations**

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