

1 **Similar local but different systemic metabolomic responses of closely related pine subspecies**
2 **to folivory by caterpillars of the processionary moth.**

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26 **Key words.** Folivory, plant-insect, stoichiometry, metabolomics, phenolics, systemic responses

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28 **Abbreviations:**

29 PPM - Pine processionary moth

30 *nevadensis* - *Pinus sylvestris* subspecies *nevadensis*

31 *iberica* - *Pinus sylvestris* subspecies *iberica*

32 LC-MS - Liquid chromatography coupled to mass spectrometry

33 Control-Ns – Needles of the not attacked trees.

34 Systemic-Ns – Non-attacked needles of the attacked trees

35 Local-Ns - Attacked needles of the attacked trees

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40 **Abstract**

41 Plants respond locally and systemically to herbivore attack. Most of the research conducted on plant-
42 herbivore relationships at elemental and molecular levels have focused on the elemental composition
43 or/and certain molecular compounds or specific families of defensive metabolites showing that
44 herbivores tend to select plant individuals or species with higher nutrient concentrations and to avoid
45 those with higher levels of defensive compounds. We performed stoichiometric and metabolomics, local
46 and systemic, analyses in two subspecies of *Pinus sylvestris* under the attack by the caterpillars of the
47 pine processionary moth, an important pest in the Mediterranean Basin. Both pine subspecies
48 responded locally to folivory mainly by increasing the relative concentrations of terpenes and some
49 phenolics. Systemic responses differed between subspecies and most of the metabolites presented
50 intermediate concentrations between those of the affected parts and unattacked trees. Our results
51 support the hypothesis that foliar nutrient concentrations are not a key factor of an alleged plant
52 selection by adult female processionary moths for oviposition since folivory was not associated with any
53 of the elements analyzed. Phenolic compounds did not generally increase in the attacked trees
54 questioning thus their commonly proposed induction by folivory attack and their anti-feeding
55 properties. Herbivory attack produced a general systemic shift in pines, including both primary and
56 secondary metabolisms, that was less intense and chemically different from the local responses. Local
57 pine responses were similar between subspecies while systemic responses were more distant between
58 them.

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68 **Introduction**

69 Carbon (C) to nitrogen (N) and C to phosphorus (P) biomass ratios are lower in herbivores than in plants
70 (Fagan *et al.* 2002). Foliar nutrient concentration has been widely reported in recent decades as an
71 important factor in the selection of foliage by insect herbivores, who usually choose plants with the
72 highest nutrient concentrations for maintaining their internal C:N:P stoichiometric homeostasis (Elser *et al.*
73 *et al.* 2000; Sterner & Elser 2002; Ngai & Jefferies 2004; Cosme *et al.* 2011; Sardans *et al.* 2012) and for
74 ensuring larval survival (Hódar *et al.* 2002). The location of oviposition is crucial for most herbivorous
75 insects to ensure larval survival. However, the role of elemental composition on host selection by
76 herbivores remains unclear (Rivas-Ubach *et al.* 2014; Jactel *et al.* 2015); other chemical and physical
77 barriers could play even more significant roles (Tremmel & Müller 2013; Onodera *et al.* 2014). Onodera
78 *et al.* (2014) reported that insects selected organs of a plant species with less defensive compounds than
79 with higher nutrient concentrations, thus demonstrating the importance of defensive metabolites in
80 host selection by herbivores. Plants have developed a wide array of resistance mechanisms against
81 herbivores (Hanley *et al.* 2007; Heil 2009). Secondary plant metabolic compounds are examples of
82 defensive compounds (Herms & Mattson 1992; Kessler & Baldwin 2001) and can be constitutive or
83 induced by a specific stressor. Herbivorous attack induces the synthesis of defensive compounds at a
84 local level but also at a systemic level (Karban & Baldwin 1997; Sticher *et al.* 1997; Heil & Bueno 2007;
85 Heil 2009) through induced internal plant signaling (Howe & Jander 2008; Wu & Baldwin 2009) and the
86 production of reactive oxygen species (ROS) (Orozco-Cardenas & Ryan 1999; Wu & Baldwin 2010).

87 Studies of plant-induced chemical responses to herbivorous attack have generally focused on a
88 single compound or families of metabolites (Sardans *et al.* 2011). The role of volatile organic
89 compounds, such as terpenes, in plant defense has been extensively discussed and reviewed in recent
90 decades (Kessler & Baldwin 2001; Mumm & Hilker 2006; Gershenson & Dudareva 2007; Peñuelas &
91 Staudt 2010). Low foliar terpene concentrations are often directly correlated with higher rates of

92 herbivorous attack, thus showing their role in constitutive defenses (Kessler & Baldwin 2001; Hódar *et*
93 *al.* 2004; Achotegui-Castells *et al.* 2013). Terpene synthesis, though, can also be induced by herbivorous
94 attack (Pare & Tumlinson 1997; Achotegui-Castells *et al.* 2013; Irmisch *et al.* 2014). Phenolics, a diverse
95 group of plant secondary metabolites, are commonly considered one of the most important groups of
96 defensive molecular compounds against folivores (Bennett & Wallsgrove 1994), but evidence of their
97 defensive role in conifers is still very limited and unclear (Mumm & Hilker 2006; Hódar *et al.* 2015).

98 The metabolome is the complete set of metabolites present in an organism at a given time and is
99 considered as the chemical phenotype of an organism (Fiehn 2002). Such metabolites include sugars,
100 amino acids and nucleotides from primary metabolism and terpenes and phenolics from secondary
101 metabolism. The metabolome thus represents a large variety of complex physiological processes for
102 maintaining homeostasis and function under diverse environmental conditions. The initial functional
103 response of an organism to biotic and abiotic stressors produces shifts in metabolomes (Peñuelas &
104 Sardans 2009). The most recently developed metabolomic techniques in the fields of plant physiology
105 and ecology (ecometabolomics) has not only allowed the differentiation of species-specific
106 metabolomes (Deborde & Jacob 2014) or of specific metabolomes under different environmental
107 situations (Robertson 2005; Bundy *et al.* 2009; Sardans *et al.* 2011; Macedo 2012; Rivas-Ubach *et al.*
108 2012; Fester 2015) but has also allowed the understanding of intraspecific metabolic differences
109 between organs (Gargallo-Garriga *et al.* 2014). Herbivores both increase the production of defensive
110 chemical compounds and induce a general shift of the metabolomes of the host plant (Peñuelas &
111 Sardans 2009; Leiss *et al.* 2009; Rivas-Ubach *et al.* 2014). Sardans *et al.* 2014 recently reported a
112 systemic shift of *Quercus ilex* foliar metabolomes after a few hours of simulated wounding. The
113 suitability and sensitivity of ecometabolomics for detecting local and systemic metabolomic shifts in
114 plants under field conditions, however, are not well known but could provide an overview and

115 understanding of how individual plants cope with herbivorous attack both locally and systemically,
116 taking into account the simultaneous primary and secondary metabolisms.

117 The caterpillar of the pine processionary moth *Thaumetopoea pityocampa* (Denis and
118 Schiffermüller) (hereafter PPM) is an important defoliating pest of pines in the Mediterranean region.
119 PPM caterpillars feed on several pine and other coniferous species (Battisti 1988; Hódar *et al.* 2003). The
120 caterpillars develop through various stages from the end of summer to the beginning of spring and
121 present an intense folivore activity that peaks in winter (Battisti *et al.* 2005). The PPM is geographically
122 limited mainly by low winter temperatures (Huchon & Démolin 1971). Scots pine (*Pinus sylvestris*) grows
123 at high altitudes and is exposed to low temperatures and was consequently not usually a host for the
124 PPM (Huchon & Démolin 1971; Hódar *et al.* 2003), but several recent studies have shown that the global
125 increase in temperatures have allowed a geographic and demographic expansion of the PPM, which is
126 thus now able to access Scots pine and other pine species naturally occurring at higher altitudes (Benigni
127 & Battisti 1999; Hódar *et al.* 2003; Battisti *et al.* 2005, 2006). Sierra Nevada Natural Park (alongside
128 Sierra de Baza Natural Park) in Spain is the southernmost limit of distribution of Scots pine in Western
129 Europe (Boratynski 1991). Two sympatric subspecies of *P. sylvestris*, *P. sylvestris* subsp. *nevadensis*
130 (hereafter *nevadensis*) and *P. sylvestris* subsp. *iberica* (hereafter *iberica*), are currently seriously affected
131 by the PPM (Hodar *et al.* 2002) to the point that PPM caterpillars constitute a serious problem for the
132 conservation of pine populations in Sierra Nevada, especially *nevadensis* (Blanca *et al.* 1998). The rising
133 temperatures (IPCC 2013) threaten these pines indirectly by favoring the climatic conditions for the
134 expansion and activity of the PPM.

135 The present study is an initial exploration of the local and systemic shifts in elemental
136 concentrations and metabolomes induced by PPM attack in two wild pine subspecies coexisting in the
137 same environment. This analysis allows understanding which metabolic pathways are altered as a
138 consequence of herbivorous attack. Moreover, the elemental analyses shed light on the the still unclear

139 role of foliar elemental concentrations and C:N:P:K ratios in host selection by herbivores. We sampled
140 needles of both subspecies of Scots pine (*nevadensis* and *iberica*) in winter, when PPM folivorous
141 activity is highest, in Sierra Nevada Natural Park where pine populations are now naturally exposed to
142 PPM attack. The foliar elemental compositions and untargeted metabolomes were analyzed in non-
143 attacked trees, in the attacked branches of attacked trees and in the non-attacked branches of attacked
144 trees of both subspecies.

145

146 **Material and Methods**

147 Study site

148 Samples were collected in March 2011 (late winter) on Collado de Matasverdes in Sierra Nevada
149 National Park (Granada, SE Spain) (37.05°N, 3.27°W; 1900 m a.s.l.), one of the sites where *nevadensis*
150 coexists with *iberica* (Robledo-Arnuncio *et al.* 2009). The climate is Mediterranean, with hot summers,
151 cold winters and usually a severe summer drought. The mean annual temperature is 9.8 °C, and the
152 mean annual precipitation is 945 mm. January is the coldest month, with a mean minimum temperature
153 of -0.1 °C, and July is the warmest, with a mean maximum temperature of 30.1 °C. Rainfall is
154 concentrated mainly in autumn and spring. See Achotegui-Castells *et al.* (2013) for more details.

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156 Experimental design and sampling of needles

157 Twenty-four adult *iberica* and *nevadensis* trees, >45 years old and >5 m in height, were randomly
158 selected as study cases (total n = 48), 12 with no signs of caterpillar attack and 12 with caterpillars in the
159 canopy, easily located by their winter tents (2-4 per tree). A small branch exposed to the sun was
160 removed from the not-attacked trees, from the not-attacked area of the attacked trees and from the
161 attacked area of the attacked trees with a pole (see Fig. S1). The needles of not-attacked trees thus

162 served as controls (hereafter; Control-Ns), the not-attacked needles of the attacked trees and the
163 attacked needles of the attacked trees were used for determining the systemic and local responses to
164 folivory (hereafter; Systemic-Ns and Local-Ns respectively), referred to as folivory levels (FLs) throughout
165 this article. We acknowledge that metabolomes of plants can shift due environment conditions, for this
166 reason, in order to get robust comparative metabolomic data, needle samples were collected in a
167 narrow window of time (from 10:30 to 14:30 local time) under sunny, non-windy and with insignificant
168 temperature variation. A bunch of the youngest well-developed needles (over 100) from each sampled
169 branch were collected, packed in plastic bags and quickly frozen and stored in liquid nitrogen. It took
170 often less than 1 minute from branch sampling until needle freezing.

171 Our selection of trees in the wild was based on the presence/absence of natural defoliation, so the
172 pines were not assigned to the different levels of this factor completely randomly. However, this
173 problem should not affect the reliability of our results. While many studies analyzed between-species
174 host selection by PPM, none of them established a clear pattern of individual tree selection within
175 species based on nutritional and/or chemical cues yet (see Jactel *et al.* 2015 for a recent review). Rather,
176 it is usually admitted that moths in monospecific stands, as in our case, base their selection on visual
177 cues to focus on isolated or taller trees that are more likely to provide optimal microclimatic conditions
178 (high solar radiation) for egg survival and successful development of larvae, rather than on chemical
179 differences between individuals (Jactel *et al.* 2015). The assignment of attacked/unattacked levels by
180 female moths when ovipositing can thus be reliably considered as a random selection of the prior
181 chemistry of the trees.

182

183 Foliar processing for elemental and metabolomic analyses

184 The foliar processing is described in detail in Rivas-Ubach *et al.* (2013). Briefly, pine needles frozen in
185 liquid nitrogen were lyophilized and stored in plastic cans at -20 °C. The samples were ground with a ball

186 mill at 1600 rpm for 8 min (Mikrodismembrator-U, B. Braun Biotech International, Melsungen,
187 Germany). The fine homogeneous powder produced was stored at -80 °C until the extraction of the
188 metabolites for analyses by liquid chromatography-mass spectrometry (LC-MS).

189

190 Elemental analysis

191 C and N concentrations were determined for 1.4 mg of sample powder by elemental analysis using
192 combustion coupled to gas chromatography with a CHNS-O Elemental Analyser (EuroVector, Milan,
193 Italy). P and K were extracted by acid digestion in a MARSXpress microwave reaction system (CEM,
194 Mattheus, USA) under high temperature and pressure (Sardans *et al.* 2010). Briefly, 250 mg of sample
195 powder were placed in a Teflon tube with 5 mL of nitric acid and 2 mL of H₂O₂. The digested material
196 was transferred to 50-mL flasks and resuspended in Milli-Q water to a final volume of 50 mL. After
197 digestion, the P and K concentrations were determined by ICP-OES (Optic Emission Spectrometry with
198 Inductively Coupled Plasma) (Perkin-Elmer Corporation, Norwalk, USA). See Elemental Analyses section
199 of the supporting information for more details.

200

201 Extraction of metabolites for LC-MS analysis

202 Polar and semi-polar metabolites were extracted as described by t'Kindt *et al.* (2008) with some
203 modifications. Briefly, two sets of 2-mL centrifuge tubes were labeled: set A for the metabolite
204 extractions and set B for the extracts from set A. One hundred milligrams of needle powder for each
205 sample were weighed into each tube of set A, and 1 mL of extractant (MeOH/H₂O (80:20)) was added.
206 All tubes were vortexed for 15 min, sonicated for 5 min at 24 °C and then centrifuged at 23 000 × *g* for 5
207 min. After centrifugation, 0.6 mL of the supernatant from each tube of set A was transferred to the
208 corresponding 2-mL centrifuge tube of set B. This procedure was repeated to perform two extractions of

209 each sample. The tubes of set B were centrifuged at 23000 × *g* for 5 min, and the extracts were collected
210 by crystal syringes, filtered through 0.22-μm microfilters and transferred to a labeled set of high
211 performance liquid chromatography (HPLC) vials. Extracts were stored at -80 °C until the LC-MS analysis.

212

213 LC-MS analysis

214 Liquid chromatography was performed with a reversed-phase C18 Hypersil gold column (150 × 2.1 mm,
215 3 μm particle size; Thermo Scientific) and a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific/Dionex RSLC,
216 Massachusetts, USA) and a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific/Dionex RSLC,
217 Dionex, Waltham, USA) at a constant temperature of 30 °C and a flow rate of 0.3 mL min⁻¹. Five
218 microliters of each sample were injected. We used water (0.1% acetic acid) (A) and acetonitrile (B) as
219 mobile phases. Both A and B were previously filtered and degassed for 10 min in an ultrasonic bath. The
220 elution gradient was initiated at 90% A (10% B) and held for 5 min, then the solvent was linearly
221 changed from 90% A (10% B) to 10% A (90% B) from 5 to 25 minutes. The gradient then returned linearly
222 to the starting conditions from 25 to 30 minutes. The gradient was then held at these conditions for 5
223 minutes to re-equilibrate the chromatographic system prior to the analysis of the next sample.

224 HPLC was coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher
225 Scientific, Waltham, USA) equipped with an HESI II (heated electrospray ionization) source for mass
226 spectrometric analyses. All samples were injected twice, once with the HESI operating in positive
227 ionization mode (+H) and once in negative ionization mode (-H). The mass spectrometer was operated in
228 FTMS (Fourier Transform Mass Spectrometry) full-scan mode with high-mass resolution (60000) and a
229 mass range of 50-1000 *m/z*. For both ionization modes, capillary temperature was set at 275 °C, sheath
230 and auxiliary gas flow rates were operated at 35 and 5 respectively (arbitrary units). Heater temperature
231 was 250°C for +H and 150°C for -H. Capillary voltage operated at 4 and 10 V for +H and -H respectively.
232 Tube lens operated at 100 and -125 V for +H and -H ionization modes respectively. A caffeine standard

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233 was injected every 10 samples to monitor the resolution and sensitivity of the spectrometer. The
234 resolution was further monitored with lock masses (phthalates). Blank samples were also analyzed
235 during the sequence. Auto sampler temperature was set at 4°C. See LC-MS analyses section of the
236 supporting information for more details.

237

238 Processing of LC-MS chromatograms

239 The raw data files obtained from the spectrometer were processed with MZmine 2.17 (Pluskal *et al.*
240 2010). The chromatograms of the positive and negative modes were always treated separately. All
241 chromatograms were first baseline corrected and posteriorly ion chromatogram lists were extracted.
242 Those ions chromatogram were thus deconvoluted, retention time normalized, aligned and
243 automatically assigned (see Table S1 for parameter details). Metabolite assignation is putative since it
244 was based on total exact mass of the metabolite, exact mass of the fragments and retention time using
245 the measurements of standards in the LC-MS Orbitrap system (See table S2 for assigned metabolites).
246 However, high resolution and RT allow reducing the number of false positives considerably. The
247 numerical data sets were then exported to CSV format and posteriorly filtered. Due chromatogram
248 builder and deconvolution, diverse ions with the same mass may present slightly different retention
249 times among them, for this reason all those identified variables assigned to a same molecular compound
250 were summed to obtain only one variable per metabolite. With the used chromatographic method,
251 certain groups of carbohydrates with the same molecular mass co-elute at the same retention time
252 making thus impossible to differentiate them at MS¹, for this reason, different carbohydrates were
253 classified in groups according their mass and retention time (Hexoses: glucose, fructose, mannose and
254 galactose, Pentoses: arabinose, ribose and xylose, Disaccharides: Saccharose and maltose, Group of
255 sugars 1 (S1): deoxi-glucose, deoxi-galactose and D-fucose, Group of Sugars 2 (S2): sorbitol and mannitol,
256 and Group of sugars 3 (S3): xylitol and arabitol). Outliers and variables present in fewer than 8

257 individuals were removed from the data set. Outlier variables were defined as measurements 3-fold
258 higher than the 3rd quartile or 3-fold lower than the 1st quartile of each cell factor. The numerical values
259 of the variables of the data sets correspond to the absolute peak areas of the chromatograms detected
260 by the spectrometer. The area value of the deconvoluted chromatograms is directly proportional to the
261 concentration of the variable, so it is a suitable value for comparative analyses as demonstrated in
262 several metabolomics studies (Rivas-Ubach *et al.* 2012, 2013, 2014; Lee & Fiehn 2013; Mari *et al.* 2013;
263 Leiss *et al.* 2013; Gargallo-Garriga *et al.* 2014) although it does not reflect the real concentration in
264 terms of weight of metabolite per weight of the sample. For this reason, we use the term *relative*
265 *concentration* when referring to differences in the amount of metabolites among the studied factors
266 (season, subspecies and FL).

267

268 Statistical analyses

269 We first performed Shapiro and Levene's tests on all variables to assess the normality and homogeneity
270 of the variances, respectively. All identified metabolites were normally distributed, and any unidentified
271 metabolomic variable that was not normally distributed was removed from the data set to comply with
272 the assumptions of the statistical tests. After the processing and filtering of chromatograms, 43 (0.57%)
273 unidentified variables were not normally distributed in the dataset. The main dataset of this study is
274 composed by two categorical independent variables, subspecies (*iberica* and *nevadensis*) and FLs
275 (Control-Ns, Systemic-Ns and Local-Ns), and 7595 dependent continuous variables, nine of which were
276 elemental concentrations and stoichiometric variables (C, N, P, K, C:N, N:P, C:P, N:K and K:P) and 7586 of
277 which were metabolomic variables, including 64 identified by our plant metabolite library.

278 The whole dataset, including the assigned and non-assigned metabolomic variables, (7595 variables
279 in total) of the *P. sylvestris* needles were subjected to PERMANOVA analysis using the *Bray curtis*
280 distance to test the overall stoichiometric and metabolomic differences between subspecies and FLs.

281 The number of permutations was set at 10000. One-way ANOVAs between subspecies and FL were also
282 performed for each individual stoichiometric or metabolite variable. ANOVAs of known metabolites are
283 shown in table S3 and retention time and m/z for the 200 unknown metabolomic variables (ions)
284 presenting the largest significant differences of means between SystemicNs and Control-Ns and
285 between Local-Ns and Control-Ns are represented in the table S4. Benjamini-Hochberg correction
286 algorithm was applied to the entire list of one-way ANOVAs (7595) for a rigorous false positive control. A
287 heat map with the FL means of all identified variables was constructed for each subspecies. All means of
288 each variable for each FL were scaled to the same range of values for producing a good graphical
289 representation of the heat maps.

290 We counted the following for each subspecies: i) the number of metabolomic variables the Control-
291 Ns had intermediate values between those of the Systemic-Ns and Local-Ns, ii) the number of
292 metabolomic variables the Systemic-Ns had intermediate values between those of the Control-Ns and
293 Local-Ns and iii) the number of metabolomic variables the Local-Ns had intermediate values between
294 those of the Control-Ns and Systemic-Ns. The data was subsequently analyzed by chi-square tests to
295 detect if any of the FLs (Control-Ns, Systemic-Ns or Local-Ns) presented overall intermediate
296 metabolomes between those of the other two. The expected probability under the assumption of equal
297 probability of intermediate values for each of the three FLs should thus be 1/3 of the total studied
298 variables.

299 The whole datasets of each one of the subspecies, including both assigned and unassigned
300 metabolomic variables, were subjected to principal component analysis (PCA) to identify the shifts in
301 foliar stoichiometry and metabolome between FLs for *nevadensis* and *iberica* separately. The score
302 coordinates of the variables of the PCAs were subjected to one-way ANOVAs to identify statistical
303 differences among the groups (see Supporting Information Rivas-Ubach *et al.* 2013).

304 All statistical analyses were performed with R (R Core Team 2013). Benjamini-Hochberg *P* value
305 corrections and Shapiro and chi-square tests were performed with the *p.adjust*, *shapiro.test* and
306 *Chisq.test* functions, respectively, in the “R stats” package (R Core Team 2013). Levene’s test was
307 performed with the *leveneTest* function in the “car” package (Fox & Weisberg 2011). The PERMANOVA
308 analysis was conducted with the *adonis* function in the “vegan” package (Oksanen *et al.* 2013). Heat
309 maps were constructed with the *heatmap.2* function in the “gplots” package (Gregory 2015). The PCAs
310 were performed by the *pca* function of the R “*mixOmics*” package (Dejean *et al.* 2013). The matrix data
311 included in the PCAs was scaled by setting the parameter *SCALE = T* of the *pca* function in R.

312
313

314 **Results**

315 The PERMANOVA of the entire dataset identified significant differences in the overall stoichiometric and
316 metabolomes among all the different levels of the studied factors (subspecies and FLs) and their
317 interactions (Table 1).

318 One way ANOVAs of all 73 known variables identified several significant differences ($p < 0.05$) after
319 Benjamini-Hochberg correction between Control-Ns, Systemic-Ns and Local-Ns in both subspecies; 32
320 (43.84%) for *iberica* and 31 (42.5%) for *nevadensis* (Fig. 1; Table S3). The heat map with the relative
321 concentrations between FLs of the 73 known variables showed that Systemic-Ns of both subspecies
322 were stoichiometrically and metabolically closer to Control-Ns than to Local-Ns (Fig. 1). Chi-square tests
323 on the number of intermediate relative concentrations of each variable of each FL within each
324 subspecies and season showed that Systemic-Ns had intermediate relative metabolite concentrations
325 between those of Control-Ns and Local-Ns in 3486 of 7595 (45.9%) metabolomic variables in *iberica* and
326 in 3259 of 7595 (42.9%) metabolomic variables in *nevadensis*, indicating that the overall intermediate

327 response was not a random effect ($\chi^2 = 543.44$, $P < 0.0001$ for *iberica* and $\chi^2 = 332.58$, $P < 0.0001$ for
328 *nevadensis*) (Table 2).

329 The PCAs of each subspecies clearly separated the FLs (Fig. 2). The first four PCs of the PCA for
330 *iberica* explained a 31.7% of the total variance, 14.4% by PC1 and 7.3% by PC2 (Fig. 2). For *nevadensis*,
331 the first four PCs of the PCA explained a 30.6% of the total variance, 12.3% by PC1 and 7.8% by PC2 (Fig.
332 2). Case plot of PCAs in both subspecies represented Systemic-Ns in an intermediate position between
333 Control-Ns and Local-Ns, but closer to Control-Ns (Fig. 2A, C).

334 The foliar stoichiometry of both subspecies did not differ among the FLs, except Control-Ns of
335 *nevadensis* which had the highest K:P ratio. The Control-Ns and Systemic-Ns of both subspecies
336 generally had higher relative foliar concentrations of amino acids. Adenine and guanine also tended to
337 be higher in Control-Ns and Systemic-Ns of both subspecies, but adenosine was highest only in Control-
338 Ns of *nevadensis*. Relative concentrations of the various sugars also differed as a function of FL and
339 subspecies, but Control-Ns and Systemic-Ns of both subspecies generally had higher relative
340 concentrations of hexoses and Xylitol/Arabitol (the latter two categorized as group 3 sugars), and Local-
341 Ns had higher relative concentrations of disaccharides. The identified organic acids typically related with
342 tricarboxylic acid cycle did not show major shifts among FLs of both subspecies, but especially in
343 *nevadensis* which any of them changed significantly after Benjamini-Hochberg correction. Succinic acid
344 increased significantly in Local-Ns (Figures 1 and 2). Control-Ns and Systemic-Ns of both subspecies had
345 higher relative concentrations of most phenolics, but Local-Ns of *iberica* had higher relative
346 concentrations of catechin, epicatechin, epigallocatechin and vitexin while Local-Ns of *nevadensis* only
347 had higher relative concentrations of vitexin. Local-Ns also had higher relative concentrations of d-
348 tocopherol and eugenol in both subspecies. Caryophyllene and carvone (terpenes) were also at higher
349 relative concentrations in the Local-Ns of both subspecies. Control-Ns and Systemic-Ns in both
350 subspecies had the highest relative concentrations of growth factors such as abscisic acid.

351

352 **Discussion**

353 Our results show clearly that pine subspecies and folivory levels presented different metabolome
354 structure (Table 1, Figures 1 and 2). Commonly, responses of plants to folivory have been focused on
355 changes in concentrations of defensive compounds (Karban & Baldwin 1997; Sticher *et al.* 1997; Heil &
356 Bueno 2007; Heil 2009); however, our results showed that those shifts are also produced in the whole
357 metabolome at both local and systemic levels of the plant.

358 Elemental composition of needles

359 PPM caterpillars only feed in the trees they hatch. The concentrations of N, P and K in pine needles were
360 not related with PPM oviposition since the FLs did not differ significantly in either pine subspecies
361 (Figures 1 and 2). Some studies have reported herbivore preference for plants with higher
362 concentrations of N (Cosme *et al.* 2011; Loaiza *et al.* 2011) or P (Cosme *et al.* 2011), even within the
363 same plant species. If elemental composition of needles were a key factor for stand selection, we would
364 expect to find differences between needles of attacked and non-attacked trees. PPM is able to feed on
365 different species of conifers (Battisti 1988; Hódar *et al.* 2003) which may differ in foliar elemental
366 concentrations, however, our study was performed in a monospecific forest with two subspecies of
367 Scots pine. The lack of significance in our elemental and stoichiometric results could be thus mainly
368 interpreted by two different hypotheses: 1) PPM females were not able to discriminate foliar
369 concentrations of C, N, P and K between individuals of either subspecies for oviposition, in agreement
370 with other studies performed with this Lepidoptera species (Hódar *et al.* 2002; Jactel *et al.* 2015). This
371 could be due the very short reproductive life of adult female moths, often mating and ovipositing within
372 the first 24 hours after pupal emergence (Hódar *et al.* 2003). 2) Although adult female moths of PPM
373 could discern the elemental differences among individuals, other factors may play more important roles

374 for stand selection since there were no differences in foliar concentrations of C, N, P and K, as other
375 studies had also reported (Tremmel & Müller 2013; Onodera *et al.* 2014). However, although further
376 research is still necessary regarding the role of elements in the plant selection by folivores, our results of
377 wild pine populations and recent literature of PPM suggest that the concentrations of C, N, P and K are
378 not a key factor in stand selection for female PPM moths, at least in selection of stands within the same
379 plant-host species.

380

381 Local plant responses to PPM attack

382 Local PPM attack induced several different metabolomic responses in both subspecies (Table. 1).
383 Phenolic compounds have usually been associated as important defensive molecular compounds in
384 response to herbivorous attack, especially in conifers (Swain 1977; Franceschi *et al.* 2005). However in
385 this study, Control-Ns and Systemic-Ns of both subspecies were the groups presenting the highest
386 relative concentrations of most of the 18 phenolic compounds identified by our metabolomic analyses
387 (Figures 1). Vitexin, epicatechin, catechin, luteolin, robinetin, quercetin, epigallocatechin and, myricetin
388 are examples of phenolics that changed significantly amongst the different FLs in one or both subspecies
389 (Figures 1). Local-Ns of both subspecies had the highest relative concentrations of vitexin and Local-Ns
390 of *iberica* had also higher relative concentrations of catechin, epicatechin and epigallocatechin in Local-
391 Ns (Figures 1 and 2; Table S3). All those compounds have been described as flavonoids with strong
392 antioxidant properties that protect lipid membranes and other cellular structures from peroxidation.
393 They decrease the oxidative stress produced by the accumulation of cellular H₂O₂ and other ROS (Rice-
394 Evans *et al.* 1996; Kim *et al.* 2005) and may be directly induced by folivory (Orozco-Cardenas & Ryan
395 1999; Wu & Baldwin 2010). The fact that most phenolics did not increase in Local-Ns suggests that these
396 compounds are not necessarily induced by the attack of PPM and supports the premise that phenolics
397 have multiple and diverse, even more significant, functions in plants rather than only defensive

398 properties against biotic stressors (Treutter 2006). In agreement with our results, some studies with
399 lepidopteran folivores neither detected direct relationships between folivory rate and phenolic
400 allocation (Zou & Cates 1997; Hódar *et al.* 2004; 2015). Other plant-herbivore studies have reviewed a
401 wide variety of phenolic functions diverging from defensive roles (Close & McArthur 2002; Treutter
402 2006; Rivas-Ubach *et al.* 2014). Our metabolomic results, though, indicated that local-Ns of both
403 subspecies activate metabolic pathways related with oxidative stress. PMM attack induced increases in
404 tocopherol (vitamin E) relative concentrations in needles, with the highest values in the needles of Local-
405 Ns of both subspecies (Figures 1 and 2). Tocopherols are among the most important antioxidants,
406 protecting the stability of biomembranes from the effects of ROS (Munné-Bosch & Peñuelas 2004; Falk
407 & Munné-Bosch 2010) by reacting with them and forming a tocopheryl radical that is then reduced by
408 hydrogen donors (Traber & Stevens 2011). The higher concentration of tocopherols and some end-
409 product flavonoids (epicatechin, catechin, vitexin) in Local-Ns of both subspecies support the idea of
410 antioxidant requirement of the attacked needles. However, other end-products such as luteolin,
411 robinetin, quercetin, and myricetin showed lowest concentrations in Local-Ns in one or both subspecies
412 which thus questions their induction by herbivore attack and consequently, the anti-feeding role of
413 those flavonoids (Figure 1). Even though the common association of phenolics with deterrent function
414 against herbivores (Swain 1977; Franceschi *et al.* 2005), our results suggest that phenolics should not be
415 considered only as a group of compounds with defensive properties. Further research more focused in
416 the anti-feeding properties of phenolic is still required (Close and McArthur 2002), especially in conifers
417 (Mumm & Hilker 2006).

418 Metabolomic analyses also suggested certain non-phenolic compounds related to herbivore attack.
419 Eugenol was found in higher relative concentrations in needles of Local-Ns of both subspecies (Figures 1
420 and 2). Eugenol is a secondary metabolite described as an essential oil with toxic properties against
421 nematodes and insects (Sangwan *et al.* 1990; Isman 2000) and acting as an inhibitor of acetylcholine

422 esterase (Maffei *et al.* 2011). On the other hand, Local-Ns had the highest relative concentrations of the
423 two identified terpenes; carvone and caryophyllene (Figures 1 and 2), thus suggesting that their
424 presence was induced by local attack. Terpenes are a varied class of organic secondary metabolites
425 produced by diverse plants and are typically associated with direct and indirect defenses to insect attack
426 (Peñuelas & Llusà 2001; Mumm & Hilker 2006; Gershenson & Dudareva 2007; Achotegui-Castells *et al.*
427 2013). Terpene production is a principal constitutive and induced defensive chemical mechanism,
428 together with the production of phenolics, against insect folivory, especially in pines and other conifers
429 (Mumm & Hilker 2006). Carvone is an oxygenated monoterpene with certain repellent and antifeedant
430 properties in conifers against coleopterans and lepidopterans (Klepzig & Schlyter 1999; Schlyter *et al.*
431 2004). Increases in caryophyllene, a volatile sesquiterpene, though, have been reported in wild plants in
432 response to herbivorous damage (Gouinguéné *et al.* 2001). Caryophyllene has been described to attract
433 parasitoids or predators and thus act as indirect defensive compound in both above and belowground
434 parts of the plant in response to the injuries of folivores (Rasmann *et al.* 2005; Köllner *et al.* 2008).

435 Some studies have reported increases in glucose (hexose) in wounded plants (Widarto *et al.* 2006;
436 Lafta & Fugate 2011; Peñuelas *et al.* 2013) that may be involved in the increases in the assimilation and
437 efficiency of photosynthetic C (Seco *et al.* 2011; Sardans *et al.* 2014) and the changes in carbohydrate
438 metabolism produced by the defensive responses against wounding (Ehness *et al.* 1997; Seco *et al.*
439 2011). Hexoses did not increase in the needles of the Local-Ns in our study, but the relative
440 concentrations of disaccharides were highest in Local-Ns in both subspecies (Figures 1 and 2). Ness (Ness
441 2003) reported a stimulation of the rates of sucrose excretion in leaves damaged by folivores that
442 attracted insect predators, indicating an indirect defensive mechanism. The attraction of other insect
443 visitors due to the increase in disaccharides in our study could indicate an indirect defensive strategy in
444 Scots pine, but the roles of the various sugars released under herbivorous attack still remain unclear and
445 warrant further research.

446

447 Systemic plant responses to PPM attack

448 The heat maps (Fig. 1) and PCAs (Fig. 2) identified significant differences in several metabolites among
449 folivory levels in both subspecies, demonstrating the general systemic response induced by PPM attack.
450 Interestingly, chi-square tests on the number of intermediate metabolite relative concentrations in both
451 subspecies (Table 2) indicated that Systemic-Ns tended to have intermediate metabolomes between
452 those of Control-Ns and Local-Ns. This results is also corroborated by the dendograms of FLs in the heat-
453 map analyses (Fig. 1) and case plots of PCAs, which Systemic-Ns are represented between Control-Ns
454 and Local-Ns (Fig. 2). Furthermore, Systemic-Ns in both subspecies clustered closer to Control-Ns than
455 to Local-Ns in the case plot of the PCAs showing thus major induced metabolomic shifts in Local-Ns than
456 in Systemic-Ns. Even so, metabolomic shifts between Systemic-Ns and Control-Ns were still significantly
457 different (Fig. 2A, C). These results supported the premise that local PPM attack is able to trigger
458 significant responses in Scots pine systemically by shifting a large proportion of the overall pine
459 metabolomes (Sticher *et al.* 1997; Heil & Bueno 2007; Heil 2009).

460 Ecometabolomics has been an excellent tool for the simultaneous detection of general shifts of
461 metabolomes induced by herbivorous attack, including primary and secondary metabolisms rather than
462 only molecular compounds directly linked to the systemically acquired resistance (Gorlach 1996; Sticher
463 *et al.* 1997; Heil & Bueno 2007; Erb *et al.* 2011). From the assigned metabolites, we did not detect
464 several significant shifts of Systemic-Ns compared to Control-Ns in *iberica* (Table S3). An increase in the
465 relative concentrations of flavones (Kim *et al.* 2005) was one clear systemic response in *iberica*.
466 Although not statistically significant due its metabolomic proximity to Control-Ns, Systemic-Ns in *iberica*
467 showed increases in the relative concentrations of eugenol, catechin, vitexin, epigallocatechin (Rice-
468 Evans *et al.* 1996), and terpenes (Rasmann *et al.* 2005) (Figures 1 and 2, Table S3), compounds that
469 increased significantly in Local-Ns in *iberica*, supporting again the presence of a systemically acquired

470 resistance. The systemic response in *nevadensis* nevertheless differed respect to *iberica* and consisted of
471 significant higher relative concentrations of choline, robinetin and flavones relative to Control-Ns
472 (Figures 1 and 2, Table S3). Choline has proven to act as an osmolite after membrane injury (McNeil *et*
473 *al.* 2001), and robinetin is a flavonol with strong antioxidant properties (Sroka 2005). Similarly to
474 Systemic-Ns of *iberica*, Systemic-Ns of *nevadensis* also showed slight increases in terpenes and eugenol
475 respect to Control-Ns although still not significant (Fig. 1). Interestingly, the relative concentrations of
476 several amino acids were higher in the Systemic-Ns of *nevadensis*, such as proline, a multifunctional
477 amino acid with important antioxidant properties (Szabados & Savouré 2010). This overall amino acid
478 shift did not occur in Systemic-Ns of *iberica* (Figures 1 and 2, Table S3).

479

480 **Conclusions**

481 · None of the concentrations of the elements analyzed (N, P or K) differed between attacked and non-
482 attacked trees. Although, there is no evidence of within-species selection in adult female processionary
483 moths of PPM for oviposition, our results support the hypothesis that foliar concentrations of N, P or K
484 are probably not key components of an alleged within-species selection by PPM moths.

485 · Each folivory level (Control-Ns, Systemic-Ns, Local-Ns) showed increases of different phenolic
486 compounds which questions their induction produced by folivory attack and the role of phenolics as a
487 general group with deterrent properties .

488 · Local-Ns had higher relative concentrations of terpenes such as carvone and caryophyllene, which were
489 likely more directly involved as a defensive function against folivores.

490 · The non-attacked branches of the attacked trees (Systemic-Ns) had metabolomes intermediate
491 between those of the non-attacked trees (Control-Ns) and the attacked branches of the attacked trees
492 (Local-Ns), demonstrating an induced gradual response of metabolomes of the entire plant (systemic
493 plant response) front herbivore attack.

494 · There were more metabolomic similarities between Local-Ns of both subspecies than between
495 Systemic-Ns.

496 · The metabolomic techniques were sufficiently sensitive to distinguish between the local and systemic
497 responses in both primary and secondary metabolisms of the trees, demonstrating their power as
498 excellent tools for ecological studies.

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773 **Table 1.** Full factorial PERMANOVA model with all stoichiometric and metabolomic variables:
774 subspecies, folivory level (FL) and subspecies*FL.

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	Df	F.Model	Pr(>F)
Subspecies	1	10.0	<0.0001
Folivory level (FL)	2	5.98	<0.0001
Subspecies*FL	2	3.37	<0.0001
Residuals	66		
Total	71		

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794 **Table 2.** Chi-square analyses comparing the expected and observed number of variables with
 795 intermediate means for each folivory level (FL), using an expected value of 33.3% of the total observed
 796 compounds. This expected value was based on the neutral supposition that each of the three levels of
 797 folivory should have the same probability of having intermediate concentrations of each variable (2530
 798 variables for each FL). The proportion of metabolites for each group with intermediate means respect to
 799 the total is represented in bold.

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	Number of variables with intermediate relative concentrations			Expected for each folivory level	χ^2	<i>P</i>
	Observed for each folivory level					
	Control-Ns	Systemic-Ns	Local-Ns			
<i>iberica</i>	1985 (26.1%)	3486 (45.9%)	2124 (28%)	2530 (33.3%)	543.44	< 0.0001
<i>nevadensis</i>	2326 (30.6%)	3259 (42.9%)	2010 (26.5%)	2530 (33.3%)	332.58	< 0.0001

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