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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27

28 **Abbreviations:**
29 PPM - Pine processionary moth
30 *nevadensis* - Pinus sylvestris subspecie nevadensis
31 *iberica* - Pinus sylvestris subspecie 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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Abstract

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

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*

73   *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; Gershenzon & 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   <u>Study site</u>

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.

155

156   <u>Experimental design and sampling of needles</u>

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   <u>Foliar processing for elemental and metabolomic analyses</u>

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 <u>Elemental analysis</u>

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 H_2O_2. 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 <u>Extraction of metabolites for LC-MS analysis</u>

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/H_2O (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 <u>LC-MS analysis</u>

214 Liquid chromatography was performed with a reversed-phase C18 Hypersil gold column (150 × 2.1 mm,

215 3 μm particle size; Thermo Scientific (150 × 2.1 mm, 3μ particle size; Thermo Scientific, Waltham,

Codi de camp canviat

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

Codi de camp canviat

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

Codi de camp canviat

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

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 4ºC. 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 MS^1, 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-gucose, 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   <u>Statistical analyses</u>

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 (χ^2 = 543.44, $P < 0.0001$ for *iberica* and χ^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

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.

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381   <u>Local plant responses to PPM attack</u>

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 H_2O_2 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 & Llusià 2001; Mumm & Hilker 2006; Gershenzon & 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 (Gouinguené *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.

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447 <u>Systemic plant responses to PPM attack</u>

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*.
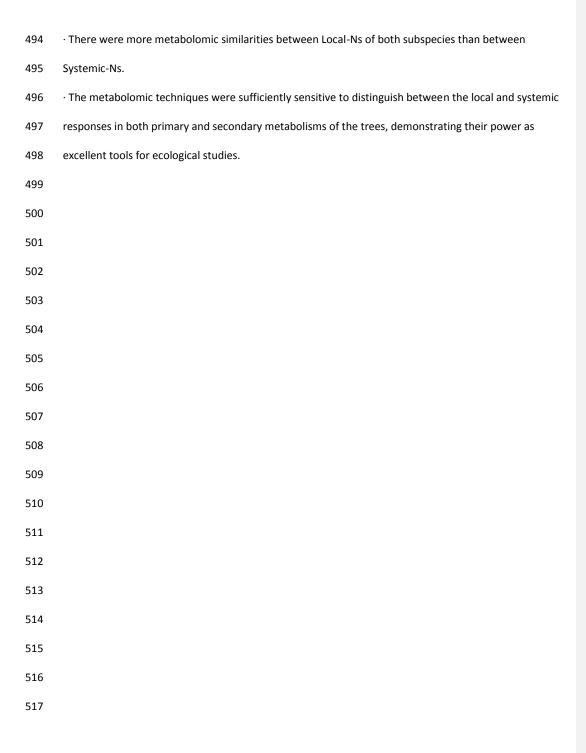
466 Although not statistically significant due its metabolomic proximity to Control-Ns, Systemic-Ns in *iberica*

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					
	Observed for each folivory level			Expected for each folivory level		
	Control-Ns	Systemic-Ns	Local-Ns		χ^2	*P*
iberica	1985 **(26.1%)**	3486 **(45.9%)**	2124 **(28%)**	2530 (33.3%)	543.44	< 0.0001
nevadensis	2326 **(30.6%)**	3259 **(42.9%)**	2010 **(26.5%)**	2530 (33.3%)	332.58	< 0.0001

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