Plant-insect interactions patterns in three European paleoforests of the late-Neogene – early-Quaternary

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Since hundred millions of years the interactions between plants and insect are essential in terrestrial ecosystems, as they composed one of the main trophic level in forest communities. Plant-insect interactions are correlated with many different environmental factors (such as the diversity of plants or climatic parameters) but these relations are still poorly understood, especially since human activities may have disturbed these relationships. This study put forward the first comparison between three European fossil leaves assemblages from late Cenozoic (Willershausen, Berga) and early Quaternary (Bernasso). The plant-insect interactions were estimated through insect activity traces identified on the leaf blade. Half of the leaves showed some kind of interaction in Willershausen (Germany) while 25% and 35% of the leaves were affected by insects in Berga (Germany) and Bernasso (35%) respectively. Proportions of specialized damages are significantly higher in Bernasso, which may suggest a more important thermal and hydric seasonality than in Willershausen and Berga. Convergent results reveal that temperature and precipitation seasonality seem to have a larger influence on plant-insect interaction difference than the annual average of these climatic parameters as it traditionally discussed. This is the first time in fossil record that seasonality is highlighted to be a potential factor impacting the plant-insect interaction.

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22 **ABSTRACT**

23 Plant and insect interactions are essential in terrestrial ecosystems and have been so for hundreds

24 of millions of years, as they compose one of the main trophic levels in forest communities. Plant-

25 insect interactions are correlated with many different environmental factors (such as the diversity

26 of plants or climatic parameters) but these relations are still poorly understood, especially since

27 human activities may have disturbed these relationships. This study put forward the first

28 comparison between three European fossil leaves assemblages from late Cenozoic

29 (Willershausen, Berga) and early Quaternary (Bernasso). The plant-insect interactions were

30 estimated through insect activity traces identified on the leaf blade. Half of the leaves showed

31 some kind of interaction in Willershausen (Germany) while 25% and 35% of the leaves were

32 affected by insects in Berga (Germany) and Bernasso (35%) respectively. Proportions of

33 specialized damages are significantly higher in Bernasso, which may suggest a more important

34 thermal and hydric seasonality than in Willershausen and Berga. Convergent results reveal that

35 temperature and precipitation seasonality seem to have a larger influence on plant-insect

36 interaction difference than the annual average of these climatic parameters as it traditionally

37 discussed. This is the first time in fossil record that seasonality is highlighted to be a potential

38 factor impacting the plant-insect interaction.

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40

INTRODUCTION

Climate is a major factor affecting the extension, structure, and composition of terrestrial ecosystems (Taylor et al., 2012; Frank et al., 2015). Hence, past climatic oscillations are of special importance for understanding and interpreting biotic changes in the past (e.g., DeChaine & Martin, 2006) and are of interest in terms of forecasting the biotic response to future global warming (Meehl et al., 2007). Nowadays, it is clear that Human activities have now reached a global impact affecting components of the Earth system as a whole (Turner et al., 1990; Heller & Zavaleta, 2009) while these impacts on terrestrial biotas are still poorly understood (Knight & Harrison, 2012). Nevertheless, previous studies on fossil insect herbivory have provided a variety of ecological and evolutionary information, such as climate (e.g., Wappler, 2010; Wappler et al., 2012), the evolutionary impact of plant radiations (e.g.,Labandeira, 2012; Labandeira & Currano, 2013), food web dynamics (e.g., Wappler & Grímsson, 2016), extinction patterns (e.g., Labandeira, 2002; Labandeira, Johnson & Wilf, 2002; Donovan et al., 2016), and ecosystem recovery after extinction events (e.g., Wappler et al., 2009; Labandeira, Kustatscher & Wappler, 2016). They have also shown that biodiversity loss may greatly impede trophic interactions and change the overall food web structure of ecological systems (Haddad et al., 2009). Moreover, there is increasing concern about the loss of biological diversity from ecosystems (Hooper et al., 2012). The Plio–Pleistocene fossil record offers an exceptional possibility for conducting a meta-analysis for the estimation of the evolution and dynamics of associations between plant species and their dependent insect-herbivore species, as descriptions of Plio–Pleistocene floral changes (Tzedakis, Hooghiemstra & Pälike, 2006; Médail & Diadema, 2009; Postigo Mijarra et al., 2009; Magri, 2010; Migliore et al., 2012). Although a few isolated records of specialized phytophagy categories have been reported from the Pliocene (Straus, 1977; Givulescu, 1984; Titchener, 1999), only a single systematic survey of plant–arthropod interactions has been carried out on an early Pleistocene flora (Adroit et al., 2016).

Thus, an ideal setting for the evaluations of relationships among global climate and biodiversity under conditions warmer than today, but with a similar paleogeographic configuration (Raymo *et al*. 2011; Rohling *et al*. 2009) is possible throughout the famous upper Pliocene fossil Lagerstätten Willershausen (3.2 – 2.6 Ma; MN 16/17; (Hilgen, 1991; Mai, 1995) and the comparisons with Berga (Germany, late Pliocene) and the French Pleistocene locality of Bernasso (Adroit et al., 2016). Willershausen and Berga outcrops are of similar age (Piacenzian)

72 and are located in the surroundings of the Harz Mountains, Germany (Figure 1). The

73 Willershausen paleoforest was dominated by typical taxa of hilly mesophytic woodland

74 (Ferguson & Knobloch, 1998; Knobloch, 1998) such as *Acer, Aesculus, Carpinus, Fagus,*

75 *Quercus, Sassafras, Tilia* (Mai, 1995; Knobloch, 1998) and other taxa such as *Parrotia, Zelkova*

76 and *Liquidambar* were also characteristic elements of Willershausen (Mai, 1995). All of these

77 taxa were also found in Berga (Mai & Walther, 1988). The presence of these taxa indicates

78 relatively warmer conditions in Europe than today during the late Pliocene (Uhl et al., 2007;

79 Thiel, Klotz & Uhl, 2012). Most plant fossil evidence from Central Europe outcrops (Haywood,

80 Sellwood & Valdes, 2000; Uhl et al., 2007; Williams et al., 2009; Thiel, Klotz & Uhl, 2012) and

81 data from marine isotopes, geological evidences (Driscoll & Haug, 1998; Haug, Tiedemann &

82 Keigwin, 2004) are directing in the same way. The decreasing temperatures throughout the

83 Pliocene (Thunell, 1979; Ravelo et al., 2004) lead to the dominant European vegetation changing

84 gradually from highly diverse subtropical and warm-temperate forests to temperate deciduous

85 forests with East Asian and partly North American affinities (Mai, 1995).

86 Through the comparison of three European forest plant communities of the Plio-Pleistocene, the

87 aim of this study was to understand how climatic parameters could have impacted insect

88 interactions of fossil leaves. It has been expected to observe different patterns of plant-insect

89 interactions according to the climatic conditions in each fossil leaf assemblage. Variations of

90 insect herbivory between the three fossil plant communities were statistically correlated plant

91 diversity and climatic parameters to highlight similarities and differences between the 3 contexts.

92 Our study will present a baseline data on the categories of arthropod interactions among plant

93 species at the Pliocene-Pleistocene transition. This will provide a basis for quantitative analyses

94 of individual biotas and improved reconstructions of Plio-/Pleistocene trophic webs in the future.

95

96 **STUDIED AREA**

97 *Willershausen, Lower-Saxony, Germany*

98 Geological studies of Willershausen dated back to the end of the 19th century (e.g., Wegele,

99 1914); see details in (Wegele, 1914; Ferguson & Knobloch, 1998; Meischner, 2000). The

100 absence of bioturbation gave rise to one of the exceptionally well preserved floras and faunas

101 (Briggs et al., 1998). The Willershausen site was a lake which developed in a pond due to the

102 dissolution of underlying Permian evaporites and tumbled of the Triassic and Early Jurassic

103   sediments (Briggs et al., 1998; Meischner, 2000; Kolibáč et al., 2016). Today, Willershausen is

104   an abandoned clay mining operation and it is included in the Geopark Harz, Braunschweiger

105   Land, Ostfalen since 2012. This paleolake was ca. 200m in diameter and approximately 10m

106   deep with a narrow sand beyond which the sides inclined abruptly towards the bottom of the lake

107   (Meischner, 2000). Willershausen geology has been described by (Von Koenen, 1895) and

108   detailed compilations can be found in (Vinken, 1967), (Ferguson & Knobloch, 1998; Meischner,

109   2000).

110   The leaves used in this study are stored in different museum collections in Germany. The

111   majority (6546 leaves) is located at the Geoscience center of the University of Göttingen

112   (GZG.W collection). Additional fossil leaves are stored in the Staatliches Museum für

113   Naturkunde Stuttgart (SMNS.W collection, 957 leaves), in the collections of TU Clausthal of

114   Clausthal-Zellerfeld (320 leaves), in the Naturkundemuseum im Ottoneum of Kassel (NMOK.W,

115   236 leaves) and in the Senckenberg Natural History Collections Dresden (14 leaves). Some of

116   the best well-preserved fossil specimens are presented in Figure 2.A. The flora from

117   Willershausen comprised a rich vegetation community including the presence of *Acer, Alnus,*

118   *Betula, Carpinus, Carya, Fagus, Pterocarya*, *Populus, Quercus, Tilia, Ulmus, Zelkova* (Straus,

119   1977; Ferguson & Knobloch, 1998; Knobloch, 1998). The vertebrates *Anancus (Mastodon)*

120   *arvernensis* and *Tapirus* were found in Willershausen and seems to indicated a Piacenzian age

121   (late Pliocene, ca. 3.2 – 2.4 Ma; MN 16/17; (Mai, 1995), which is corroborate by the presence of

122   *Parrotia persica* and *Liquidambar europaeum* (Mai, 1995).

123   Most plant fossil evidence from Willershausen (*vide infra*) indicates warmer conditions than

124   today (Ferguson & Knobloch, 1998). The mean annual temperature (MAT) in Willershausen was

125   estimated between 10.6°C and 15.6°C on the base of the leaf morphology and of diversity of

126   plant species niches (Uhl et al., 2007); these different approaches explain the wide range of

127   temperature estimated. The mean temperature of the coldest month (CMMT) is estimated

128   between 0.6°C and 3.2°C and the mean annual precipitation (MAP) between 897 and 1151 mm

129   per year (Uhl et al., 2007; Thiel, Klotz & Uhl, 2012).

130

131   *Berga, Thüringen, Germany*

132   Berga was a lake in which compressions and impressions (some with the cuticles preserved) of

133   leaves were found in silty sediments (Mai & Walther, 1988). It is 70km far from the

134 Willershausen outcrop. The stratigraphic age of the Berga sediments is estimated on the basis of

135 sedimentological correlations referring to the Piacenzian (ca. 3 Ma – 2.6 Ma) (Bachmann et al.,

136 2008).

137 This leaf collection (534 specimens) is housed in the collection of the Senckenberg Natural

138 History Collections Dresden, Germany. It contained many fossils of different origins (Mai &

139 Walther, 1988), including 30 angiosperms leaf taxa (Figure 2.B). They represent different

140 environments: a freshwater plant community, a swamp and riparian associations and a zonal

141 mixed broadleaved conifer forest (which dominates the taphocoenosis). The temperatures were

142 estimated with the same approaches than Willershausen; MAT is comprised between 7.4°C and

143 16.6°C, the CMMT is between -4.3°C to +0.6°C and the MAP is between 897 and 1297 mm per

144 year (Uhl et al., 2007; Thiel, Klotz & Uhl, 2012).

145

146 *Bernasso, France*

147 Bernasso was a lake developed when a basaltic flows shut on a canyon valley (Leroy & Roiron,

148 1996). Diatomites were formed and fossil leaves, often with rest of cuticle, were preserved. It is

149 located close to Lunas (Hérault, southern France) (Leroy & Roiron, 1996; Adroit et al., 2016).

150 The fossil deposit is dated from the early Pleistocene on the basis of K/Ar analysis (Ildefonse et

151 al., 1972) on a basaltic dyke that crosses the diatomite layers. A complementary analysis on

152 cyclostratigraphy (Suc & Popescu, 2005) and paleomagnetism (Ambert et al., 1990) corroborated

153 these results and estimated an age around 2.16 to 1.96 Ma.

154 The collection included 800 fossil leaves and 535 specimens well-preserved were described in

155 (Adroit et al., 2016). These same specimens were also used for comparisons in the present study.

156 The fossil leaves were conserved the Institut des Sciences de l'Evolution de Montpellier in

157 France. Different preparation of fossil leaves were done by (Leroy & Roiron, 1996) and their

158 impact on interpretation were discussed in (Adroit et al., 2016). The flora represents a

159 mesothermic forest, mixing Mediterranean and Caspian elements (Suc, 1978; Leroy & Roiron,

160 1996). The MAT in Bernasso was estimated about 14-15°C and the MAP is around 1500 mm/y

161 (Leroy & Roiron, 1996). It is important to note that CLAMP results in Bernasso suggest a

162 probably lower temperature (Girard et al., in review).

163

164 **DATA ANALYSES**

165 *Plant-insect interaction identifications*

166 The plant-insect interactions were identified following the Damage Type Guide (Labandeira et

167 al., 2007). The damages type (DTs) are divided in 7 Functional Feeding Groups (FFG): hole

168 feeding, margin feeding, skeletonization, surface feeding, piercing & sucking, mining and

169 galling. Leaves without damage were also categorized in an eighth FFG called the undamaged

170 leaves. The leaves were examined under a binocular Leica MZ95 and all photographed with a

171 Canon EOS 350D camera fitted with a Canon EF-S 60-mm f/2.8 macro lens. A Nikon Coolpix

172 E4500 was used sometimes for precise pictures through the binocular. All pictures were

173 developed using Abode Lightroom CC v.2015 especially in order to improve contrast of the leaf.

174 The insect interactions on leaves were scored according to the richness, frequency and

175 distribution on the different plant species for each outcrop. For each DT, a host specificity value

176 has been attributed by (Labandeira et al., 2007) that allowed to classify our DTs into generalist

177 interactions (made by polyphagous organisms) and specialized interactions (made by

178 monophagous organisms) (Labandeira, 2002). Detailed plates of fossil leaves from

179 Willershausen are available in Table S1 including the original descriptions of the plant-insect

180 interactions made by (Straus, 1977) and our actual updates with the guide of insect (and other)

181 damage types on compressed plant fossils (Labandeira et al., 2007).

182 The results obtained for Willershausen and Berga were compared to those recently published for

183 the outcrop of Bernasso (Adroit et al., 2016). For some comparisons with Bernasso, new values

184 were calculated based on raw data.

185

186 *Leaf Mass per Area*

187 Leaf mass per area (LMA) was estimated by using the robust relationship between petiole width

188 squared and leaf mass standardized by leaf area (Royer et al., 2007). Generally, plants with a low

189 LMA have thinner leaves with a short lifespan and should also have high nutrient concentrations,

190 thus being more palatable for insects (Coley & Barone, 1996; Royer et al., 2007). Because of the

191 difficulties in having specific measurements needed to obtain the LMA, we considered plant

192 species statistically represented, i.e. when at least 10 specimens were measured (as it was done in

193 (Currano et al., 2016)

194

195 *Statistical analyses*

196   For each outcrop, the statistical analyses were performed on two different databases as described

197   in (Knor et al., 2012). The first one is the whole assemblage of plant-insect interactions. The

198   second one considers only the interactions of the species that are significantly represented (more

199   than 20 leaves). The quantitative analyses were done in R version 3.1.2 (R Development Core

200   Team, 2014). The differences among the proportions of occurrences from all FFG were tested

201   with Chi^2-test. The remaining information needed for this test was obtained by using the

202   generalized linear model of binominal distribution. Sample-based rarefaction curves were done

203   to compare the different damage richness between the different outcrops (Gotelli & Colwell,

204   2001). At last, in order to observe the different distributions of plant-insect interactions among

205   the paleoforests, principal component analysis (PCA) were performed with the software Past3

206   (v3.14) (Hammer, Harper & Ryan, 2001) in a biplot way. The data matrix used for it comprises

207   the diversity of each eight FFGs on every plant species for each outcrop.

208

209   **RESULTS**

210   *Comparisons of plant-insect interactions*

211   In Willershausen 50.4% of the leaves are damaged, and only 25.1% in Berga. This percentage

212   was 34.6% in Bernasso (Adroit et al., 2016). These differences are statistically significant

213   ($p<0.001$) (Figure 3, Table S2).

214   The frequencies of generalist interactions are 42.8% for Willershausen, 17.8% for Berga and

215   19.8% for Bernasso (Adroit et al., 2016). Only Willershausen frequency is significantly different

216   from the others ($p<0.001$). Willershausen leaves have especially much more hole feedings

217   (26.9%) and margin feedings (9.9%) than Berga (respectively 12.7% and 1.9%) and Bernasso

218   leaves (respectively 9.8% and 7%) (Figure 3).

219   The frequencies of specialized interactions are 11.2% for Willershausen, 8.4% for Berga and

220   17.9% for Bernasso (Adroit et al., 2016). Only the Bernasso frequency is significantly different

221   from the others ($p<0.001$). This difference is mainly due to the important quantity of galling in

222   Bernasso (12%) which is significantly higher than in Willershausen and Berga, respectively 7%

223   and 6% ($p<0.01$) (Figure 3).

224   Rarefaction tests on plant species diversity highlight that Willershausen has more plant species

225   (>100) than Berga (33) and Bernasso (20) (Figure 4). However, the DT richness in

226   Willershausen (36 DTs) and Berga (25 DTs) are lower than in Bernasso (40 DTs) (Figure 4).

227

228 *LMA per species at Willershausen*

229 The LMA calculations in Bernasso and Berga were not possible due to the low quantity of leaves

230 per species and the bad preservation of many fossil leaves. The LMA was calculated only on

231 leaves in Willershausen. For the 10 species on which LMA was determinable, values in

232 Willershausen vary from 46.18 g/m^2 to 79.26 g/m^2 (Figure 5, Table S3). For the same 10 species,

233 the percentage of damaged leaves varies between 38.8 and 71.2 % (Figure 5, Table S3). Lastly,

234 on the figure 5, we clearly observed (with also standard deviation in Table S3) that there is no

235 significant difference of herbivory according to the different values of LMA.

236

237 *Structure of the paleoforests with the damage distribution on plant species*

238 Figure 6 presents the different PCA realized for the 3 outcrops with the data of plant and DT

239 diversities. For each outcrop, only the first two axes are presented as for Willershausen they

240 represent 77% (Figure 6A), for Berga 93% (Figure 6B) and for Bernasso 91% (Figure 6C) of the

241 whole distribution.

242 For Willershausen (Figure 6A) the FFGs hole feeding and skeletonization are positively

243 correlated with PCA-axis 1 (respectively 0.76 and 0.61) and undamaged is negatively correlated

244 with this axis (-0.97) (Data S1). Skeletonization and galling are positively correlated with PCA-

245 axis 2 (respectively 0.62 and 0.63) while hole feeding is negatively correlated with this axis (-

246 0.73) (Data S1). Concerning the species, three pools of plant species can be distinguished. The

247 *Tilia* (*T. saportae, T.* cf. *saviana*), the *Ulmus* (*U. caprinifolia, U. campestris*), the *Fagus* (*F.*

248 *grandifolia, F. pliocenica*), *Acer integerrimum* and *Quercus roburoides* are all along the positive

249 part of the PCA-axis 1. The leaves of these species have the highest DT diversity of hole feeding

250 and skeletonization. A second set of taxa is composed, for the most evident species, by *Acer*

251 *cappadocicum, A. laetum, Carya minor*, cf. *Magnolia* sp1 and 2, *Populus willershausensis*,

252 *Quercus praeerucifolia* and *Zelkova ungeri*. They are along the negative part of the PCA-axis 1

253 and along the positive part of the PCA-axis 2. They are mainly affected by the FFG galling

254 (specialized interaction) or have no damage. At last, the third set of species is composed of

255 Fagales (*Fagus sylvatica*, all the *Quercus*, *Alnus* and *Betula* species) and is in the negative part of

256 the PCA-axis 2. These leaves are mainly undamaged or only impacted by hole feeding

257 (generalist interaction).

258   For Berga (Figure 6B) the FFGs hole feeding, skeletonization and undamaged are positively

259   associated with the PCA-axis 1, respectively with a correlation of 0.56, 0.73 and 0.99 (Data S1).

260   Hole feeding and skeletonization are also correlated with the PCA-axis 2, negatively for hole

261   feeding (-0.79) and positively for skeletonization (0.66) (Data S1). Concerning the species, we

262   can note that *Taxodium dubium, Zelkova ungeri, Cercidiphylum crenatum* and *Acer*

263   *integerrimum* are correlated with this undamaged category. *Fagus attenuata, Acer tricuspidatum*

264   and *Quercus* sp. are mainly correlated with hole feeding.

265   For Bernasso (Figure 6C; (Adroit et al., 2016), the skeletonization and galling are positively

266   correlated with the PCA-axis 1 (0.82 and 0.92) while undamaged is negatively correlated with

267   this axis (-0.94) (Data S1). Hole feeding and skeletonization are positively correlated with PCA-

268   axis 2 (0.92 and 0.25) while undamaged and galling are negatively correlated with this axis (-

269   0.25 and -0.37) (Data S1). *Acer monspessulanum* and *Sorbus domestica* are in the positive part of

270   the PCA-Axis 1 while the other are in the negative one (to note that *Parrotia persica* is close to

271   zero). Concerning the PCA-axis 2, *Acer monspessulanum, Carpinus orinetlais* and *Carya minor*

272   are in the positive part of the PCA-axis 2 while the others are in the negative part (to note that

273   *Zelkova ungeri* is close to zero).

274   Furthermore, *Zelkova ungeri* is a species found in the 3 outcrops (Figure 6) and comparing to its

275   position in the different PCAs, we can note that *Z. ungeri* is mostly associated with the FFG

276   undamaged. However, for other common plant species, their relative position on the PCAs could

277   be different. *Acer integerrimum* in Berga (Figure 6B) is mostly associated with the FFG

278   undamaged while in Willershausen is opposite to this FFG as it is mainly associated to the FFG

279   hole feeding and more weekly with skeletonization, margin and galling (Figure 6A).

280   Comparing Willershausen and Bernasso (Figure 6A & 6C), *A. monspessulanum* is mainly

281   associated to skeletonization and galling in Willershausen (Figure 6.A) and in Bernasso it is with

282   skeletonization, galling too but also with hole feeding (Figure 6C).

283   *Sorbus domestica* and *Carpinus orientalis* are both associated to the FFG undamaged in

284   Willershausen (Figure 6A). In Bernasso, *S. domestica* is associated with galling and

285   skeletonization and *C. orientalis* is associated with hole feeding and undamaged (Figure 6C).

286   *Carya minor* is associated to skeletonization and galling in Willershausen (Figure 6A) while in

287   Bernasso it is associated to undamaged and hole feeding (Figure 6C). *Parrotia persica* is

288   associated to galling and undamaged in Bernasso (Figure 6C) while in Willershausen, it is

289   associated with the FFGs skeletonization and galling (Figure 6A).

290

291   **DISCUSSION**

292   *Floristic richness and herbivory representativeness*

293   All genera and many plant species from Berga are also present in Willershausen (Table

294   S4) that can be expected because they have similar age and geographic proximity. Bernasso had

295   nearly the same composition of plant genera found in Willershausen (except *Ilex* only found in

296   Bernasso) and also the majority of plant species (Table S4) despite its geographical situation and

297   its younger age. There is quite a difference of plant richness between Bernasso and Berga, but

298   the genera are the same (Table S4). This may suggest a difference in specific richness between

299   those paleoforests. However, it could also be due to the fossil collections themselves. The great

300   difference of collection size between the three outcrops could have led to artificial difference of

301   plant species richness (Table S2). However, rarefaction data tests indicate that there is in fact

302   highest richness of plant species in the Willershausen outcrop compared to Berga and Bernasso

303   outcrops when the collections are similarly re-sized (Figure 4).

304   Sampling effort tests indicate that enough specimens were taken into account to have a

305   representative overview of the interactions on plant species found into the different outcrops

306   (Figure 4). The large standard deviation observable on the Willershausen rarefaction curves on

307   Figure 4 is due to this size of the fossil collection that includes around 8.000 specimens while the

308   others are only 534 for Berga and 535 for Bernasso.

309

310   *Absence of correlation between LMA and plant-insect interaction*

311   In Willershausen, no significant correlation between the LMA of plant species used and

312   the herbivory was observed (Figure 5) while previous studies (Currano et al., 2008; Currano,

313   Labandeira & Wilf, 2009; Wappler et al., 2009) showed a negative correlation between the LMA

314   and the damage on the leaves. These correlations were measured on leaf assemblages including

315   both deciduous and evergreen plant species. In the case of Willershausen, only deciduous plant

316   species have been measured. Finally, the relationship between LMA and leaf damage by insect

317   feeding is not evident (Poorter et al., 2009). For example, a small increase of secondary defense

318   compounds is not really affecting LMA but could significantly reduce the herbivory rate (Coley,

319 1988). Moreover, under different resource availabilities for plants, (Züst & Agrawal, 2017) have

320 shown that LMA values could be similar between leaves while their nutrient concentrations were

321 totally different.

322

323 *Parameters involved in insect damages differences*

324   Climatic conditions seem to be in relation with variations in richness and frequency of

325 plant-insect interactions (Currano et al., 2016). If an increase of temperature seems to stimulate

326 insect herbivory (Zvereva & Kozlov, 2006), it is still complex to understand the complete role of

327 temperature in the modulation of herbivory (DeLucia et al., 2012).

328 (Thiel, Klotz & Uhl, 2012) indicated, through leaf morphological analyses, that temperatures

329 estimated for Willershausen were approximately 3°C higher than those for Berga. These

330 paleoforests were geographically very close to each other (less than 70 km) and at a similar

331 latitude (51°N) (Figure 1). Today the nearest meteorological stations of these locations

332 (Willershausen: Göttingen, Lower-Saxony; Berga: Nordhausen, Thüringen) indicates the same

333 mean annual temperature also for the coldest and warmest months over the last years

334 (www.worldweatheronline.com). Such current similarities make the argument for similar

335 paleoclimates of the two fossil localities if they were strictly of the same age. However, between

336 3 Ma and 2.5 Ma, CO_2 concentration progressively decreased (Kürschner et al., 1996; van de

337 Wal et al., 2011) implicating a continuous decrease of mean annual temperatures (Willeit et al.,

338 2015). Consequently, as Willershausen was warmer than Berga, the paleoforest of Willershausen

339 grew under higher atmospheric CO_2 concentration than the Berga paleoforest. It seems to be

340 corroborate by the higher damage frequency observed in Willershausen that can have been

341 favored by an increase of C/N ratios and an increase of photosynthesis rates (due to the high CO_2

342 concentration) (Bezemer & Jones, 1998; Stiling & Cornelissen, 2007; DeLucia et al., 2012).

343 However, Willershausen and Berga had different sedimentological contexts and the preservation

344 of the fossil leaves did not follow the same taphonomical constrains in the two outcrops. This

345 could have influenced interpretation of the climate trough morphological analyses.

346 For this reason, (Thiel, Klotz & Uhl, 2012) were in favor of the Co-existence approach for

347 climate interpretation which estimated similar temperature for Berga and Willershausen. It has

348 been highlighted that the diversity of insects is often correlated to diversity of plant species

349 (Siemann, Tilman & Haarstad, 1996; Wright & Samways, 1998; Knops et al., 1999; Mulder et

350   al., 1999) and should be expected to have higher damage richness in the more diverse paleoforest

351   (Price, 1991, 2002). Thus, the higher richness and frequency of damage in Willershausen than in

352   Berga could also be due to a higher insect diversity. Nevertheless, despite its higher plant

353   diversity Willershausen had less DT richness than Bernasso (Figures 3, 4). Bernasso had also

354   more damage richness and frequency than Berga (Figures 3, 4). Thus, these observations make

355   this assumption unsustainable for our case study.

356   For Bernasso, the latitudinal position is different from Berga and Willershausen, as it located

357   1,000 km to the South. It has been highlighted that the insect diversity increases getting closer to

358   the tropics (Hutchinson, 1959; Klopfer, 1959; Klopfer & MacArthur, 1960; MacArthur, 1972;

359   Coley & Barone, 1996; Fraser, 2017). The southern position of Bernasso could partly explain the

360   measured damage type richness. Nevertheless, the quantity of damage is not exclusively link to

361   the insect diversity (Currano, Labandeira & Wilf, 2010). Latitudinal differences could led to a

362   difference of thermal seasonality (Saikkonen et al., 2012) which is the key to the latitudinal

363   gradient of insect diversity (Archibald et al., 2010). (Leroy & Roiron, 1996) indicated that

364   Bernasso paleoforest grew under temperatures of 14–17°C and precipitations of 1300-1500

365   mm/a. Recently, Girard et al. (in review) re-estimated Bernasso climate with different

366   approaches and some results, based on leaf morphological traits, estimated temperatures in

367   Bernasso to be cooler than estimations of (Leroy & Roiron, 1996), while the pollen analysis from

368   the same study tend to corroborate previous estimations done by (Leroy & Roiron, 1996).

369   Berga has a low temperature of the coldest months (from -6.4°C to 2°C) compared to

370   Willershausen which had the highest temperatures (from -0.5°C to 5.1°C) (Uhl et al., 2007;

371   Thiel, Klotz & Uhl, 2012). These lower temperatures during the cold period could explain the

372   lowest damage frequency observed in Berga. Indeed, insects are poikilotherms, meaning that

373   their body temperature is extremely dependent to the environment temperature (Meglitsch,

374   1972). Cooler temperatures decrease the insect metabolism (leading to diapause of insects) and

375   the quantity of generations per year (Archibald et al., 2010), consequently it could also reduce

376   the herbivory rates during the year (Bale & Hayward, 2010). Concerning Bernasso, the different

377   estimations of temperatures, included the CMMT, are lower than those of Willershausen (Uhl et

378   al., 2007; Thiel, Klotz & Uhl, 2012), thus the lowest frequency of damage could also be due to a

379   lower insect metabolism in Bernasso than in Willershausen. The lowest frequency of damage in

380   Berga than in Bernasso could also be due to insect diapause in the case of coolest temperatures

381 are lower in Berga. However, the estimated temperatures of Bernasso overlap the ones of Berga

382 (especially for the coolest temperatures) and therefore complicate any interpretations about the

383 damage frequency between these two paleoforests.

384 Moreover, it is important to note that no data about insect richness of these different paleoforests

385 are available. Although as it could be assumed that insect richness between Willershausen and

386 Berga could be similar because outcrops are geographically and temporally similar, the insect

387 richness of Bernasso could be quite different. Consequently, in case of differences in the insect

388 faunas, the previous relation could be disturbed as some insects, such as larvae of *Thaumetopoea*

389 *pityocampa,* feed plant during winter season (Battisti et al., 2005; Buffo et al., 2007), when

390 others insects have no or lower activity (Hahn & Denlinger, 2007).

391

392 *More precisions provided by proportion of generalized/specialized damages*

393   The comparison of plant-insect interaction between different locations or through

394 different time periods could still be upset by local disturbances (fires, floodings, etc.) or other

395 constraints (such as different soils) that are not perceptible in fossil record and could impacted

396 damage pattern in general (Currano et al., 2011; García, Castellanos & Pausas, 2016). Moreover,

397 taphonomic biases, especially fossil preservation and different excavation histories, could also

398 interfere with our analyses. For example, the damage frequency observed in fossil record could

399 be partly distorted because the damaged leaves had less chance to be preserved in the fossil

400 record than the complete and undamaged leaves (Ferguson, 2005). For all these reasons, we

401 suggested complementing analyses by comparison of the proportions of generalized and

402 specialized damage patterns.

403 (Leckey et al., 2014) indicate that the proportion of generalist and specialist herbivores may

404 change between different forests because the difference of abiotic parameters (such as climate).

405 There are the lowest proportions of specialist interactions (mainly based on galling) in

406 Willershausen and Berga, and conversely the highest proportion is in Bernasso (Figure 3), this

407 may due to climatic factors (e.g., Leckey et al., 2014). Indeed, precipitation in Bernasso was

408 higher than in Willershausen and Berga (Leroy & Roiron, 1996; Uhl et al., 2007) and hydric

409 seasonality was probably more important in Bernasso (Girard et al., in review). This is in

410 agreement with the proposed Mediterranean climate for Bernasso that provided heavy constrain

411 to plants here due to less water availability during the dry season (Bagnouls & Gaussen, 1957;

412   Daget, 1977, 1984). The higher seasonality conditions in Bernasso compared to conditions
413   proposed for Berga and Willershausen could also be supported the idea that regional conditions
414   of Northern Atlantic realm were more marked by higher seasonlity during Pleistocene than the
415   Pliocene (Williams et al., 2009; Hennissen et al., 2015; Utescher et al., 2017). Water stress
416   should have a positive impact on galling diversity (especially the frequency), as many studies
417   already mentioned that galling is an adaptation of stressful environment (Fernandes & Martins,
418   1985; Fernandes & Price, 1988, 1992; Price et al., 1998; Lara, Fernandes & Gonçalves-Alvim,
419   2002). In addition, (Cuevas-Reyes et al., 2003), who studied the development of galling, showed
420   that it exists a negative correlation between gall-forming insect species richness and plant species
421   richness. It could also partly explain the highest proportion of specialized interactions in
422   Bernasso. Additionally, a forest in its late successional stage, as it has been proposed for
423   Bernasso (Leroy & Roiron, 1996; Adroit et al., 2016), tend to favor the richness of gall-inducing
424   insects (that increase the proportion of specialized interaction) (Fernandes, Almada & Carneiro,
425   2010; Adroit et al., 2016).
426   This global comparison of specialized and generalized damages between the fossil leaf
427   assemblage of Bernasso, Willershausen and Berga are also observable precisely on the common
428   plant species statistically represented on each outcrop. However, the FFGs and especially the
429   undamaged feature on some plant taxa are similar or could be slightly different between the
430   fossil leaf assemblage (Figure 6). It tends to confirm that the abiotic parameters are important
431   determinant factors involving significant variation of herbivory between different
432   paleoenvironments (Cuevas-Reyes et al., 2004, 2003; Leckey et al., 2014). Biotic parameters can
433   also be involved in the difference of interaction structures. For example, a decrease in food
434   quality caused by higher concentration of carbon in plants could also have a negative impact on
435   herbivory (Stiling & Cornelissen, 2007), but in general, it is compensated by an increase of
436   insect feeding (Bezemer & Jones, 1998). The impact of biotic factors seems to be further
437   confirmed as in Willershausen we can note that most Fagales (Betulaceae: *Alnus, Betulus,*
438   *Carpinus*; Fagaceae: *Fagus, Quercus*; Juglandaceae: *Carya, Juglans*) are all associated to hole
439   feeding and to undamaged feature (Figure 6A). This measurement cannot be due to hazard but it
440   probably reflects an effect of some biotic parameters (such as genetic background, plant
441   competition, host specificity, etc.).
442

443 **CONCLUSION**

444   In the actual context where plant-insect interactions are essential to understand the food

445 web of the paleo/-forests (May, 1988; Wilf, 2008; Cornelissen, 2011), this study allows the first

446 comparison of the plant-insect interaction structure of three Plio-Pleistocene leaf assemblages.

447 Such a comparison highlighted that relationships between climate parameters (such as

448 temperature and precipitation) and plant-insect interactions are not evident in European

449 paleoforests around the Neogene – Quaternary transition. Temperature and precipitation

450 undoubtedly have major impacts on herbivory rate and our results suggest that the annual

451 variation of those climatic parameters (i.e. seasonality) could have a greater influence on plant-

452 insect interactions than the average annual of these parameters. This makes sense as the insects'

453 response to climatic variation is very sensitive (Bale & Hayward, 2010) and could be predicted

454 with their feeding behavior on leaves (Bale et al., 2002).

455 These statements about the impacts of seasonality on plant-insect interactions should to be

456 considered for further studies in the fossil record.

457

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465
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The location of Willershausen and Berga outcrops

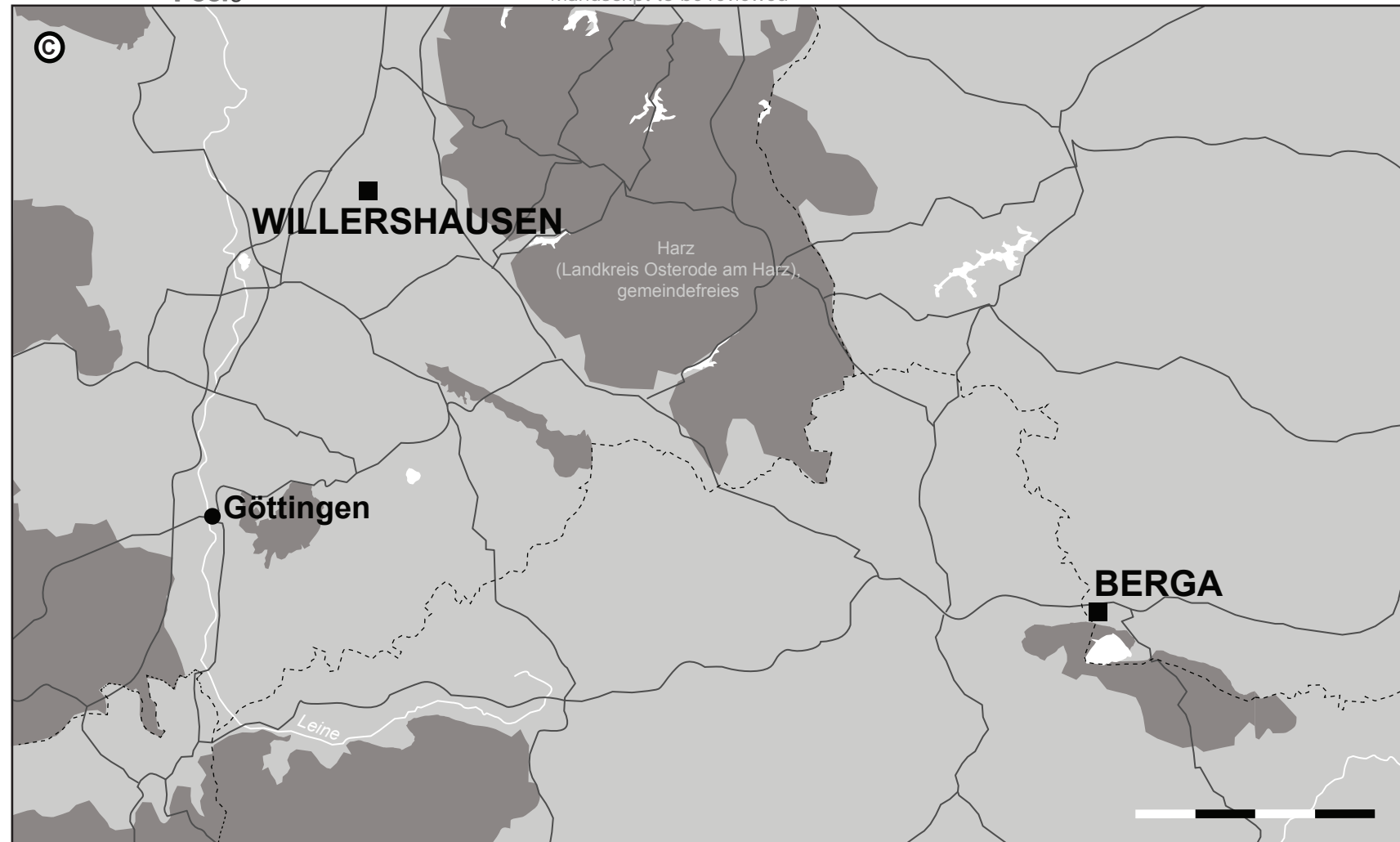
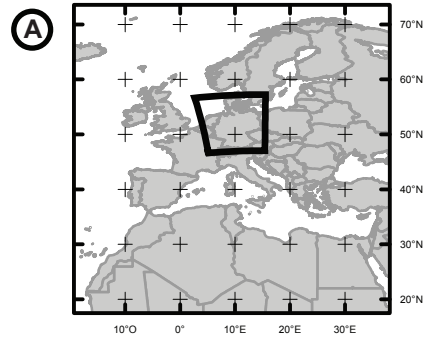
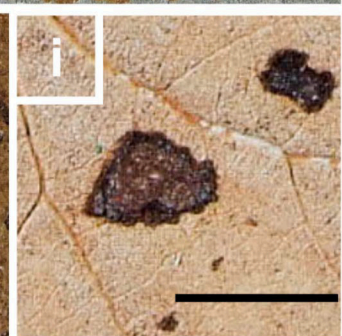
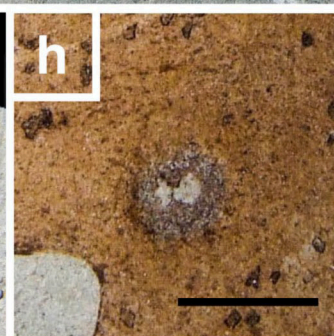
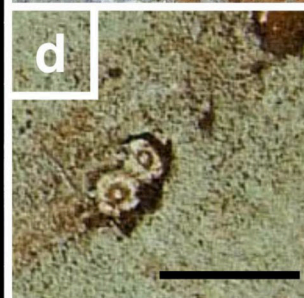
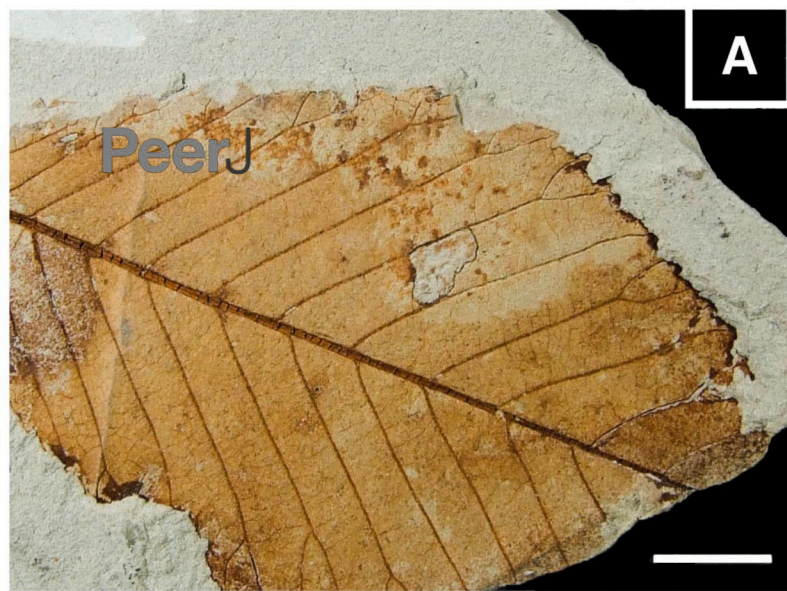
A- The location of Germany in Europe. B- The locations of both outcrops in Germany. C- Zoom on the area near Göttingen. On the scale, each dash (black or white) represents 5km. The data from the maps A and B come from Natural Earth database (http://www.naturalearthdata.com)

Samples of fossil leaves from the German outcrops

Plate 1. Fossil leaves from Willershausen (Göttingen coll.). A- *Ulmus caprinifolia* with Hole feeding (DT05). B- *Alnus spaethii* with Margin feeding (DT14). C- *Fagus* sp with Piercing & Sucking (DT168). enlarged in 'd'. F- *Ulmus campestris* with Mining (DT109) enlarged in 'e'. G- *Quercus praeerucifolia* with Galling (DT145) enlarged in 'h'. J- *Ulmus caprinifolia* with Skeletonization (DT17). K- *Populus tremula* with Surface feeding (DT30) enlarged in 'i'.

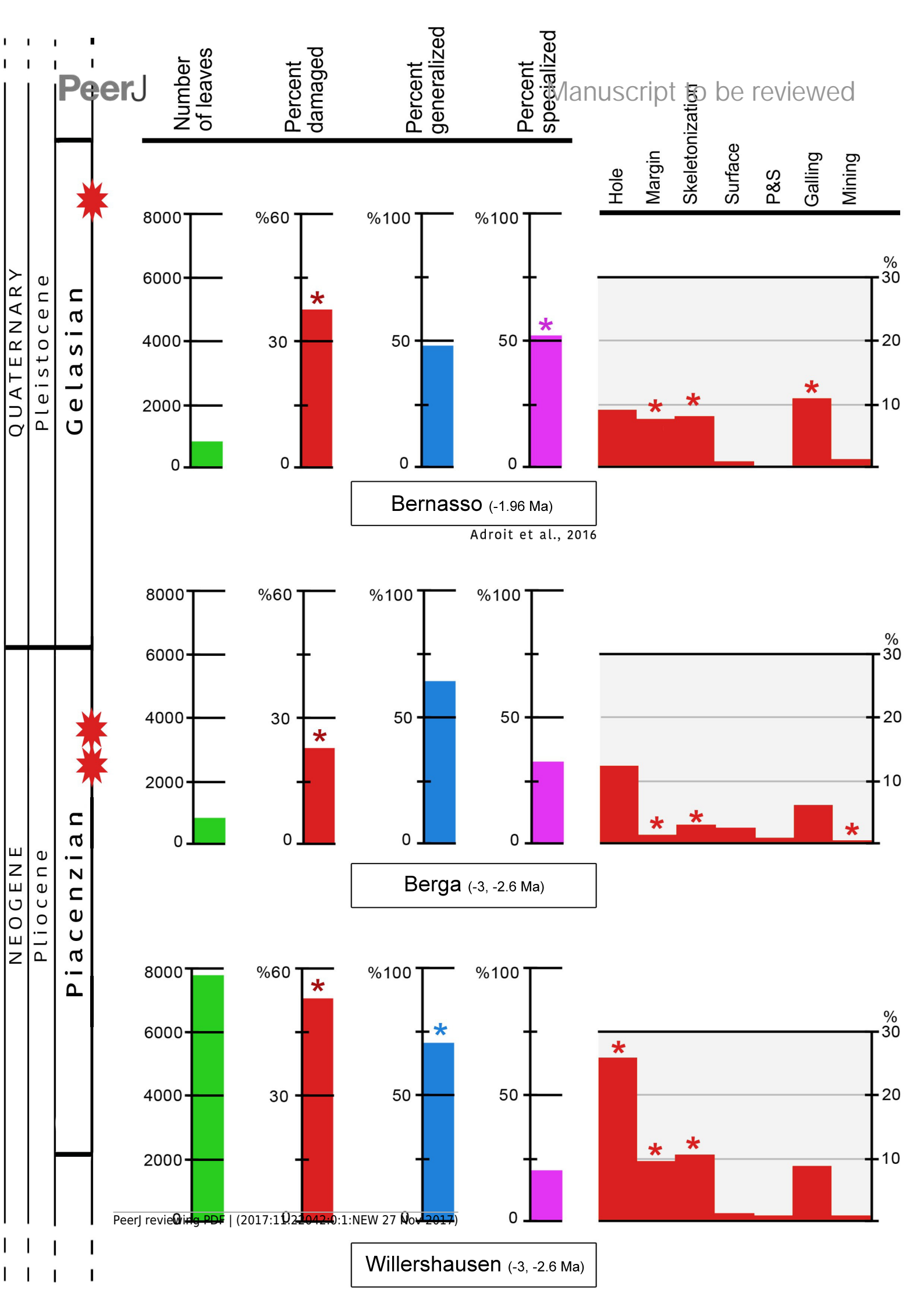
Plate 2. Fossil leaves from Berga (Dresden coll.). L- *Cercidiphyllum crenatum*. M- *Fagus attenuata* with Hole feeding (DT01). N- *Juglans* sp. with Galls (DT34) . O- *Pterocarya paradisiaca*. P- *Quercus pseudocastanea* with Galling (DT116). Q- *Quercus castaneifolia.*

White scale bar represents 1cm, black scale bar represents 0.5cm.

Quantitative distribution of plant-insect interactions from Willershausen, Berga outcrops and the fossil deposit of Bernasso (Adroit et al., 2016).

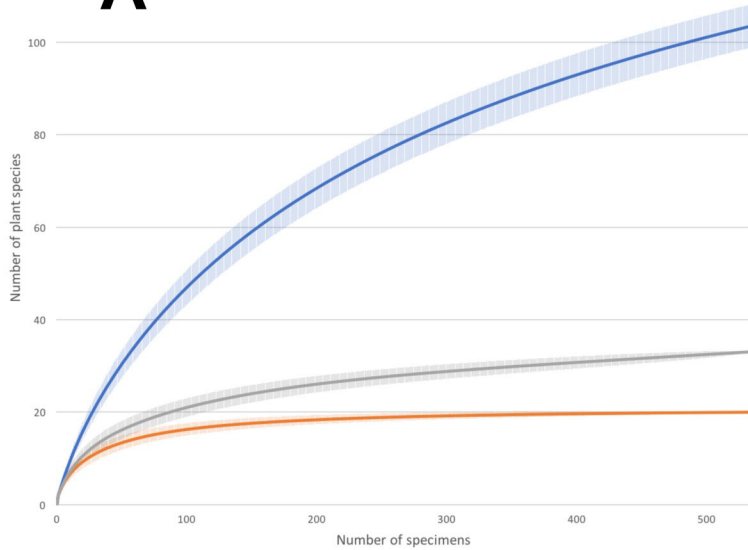
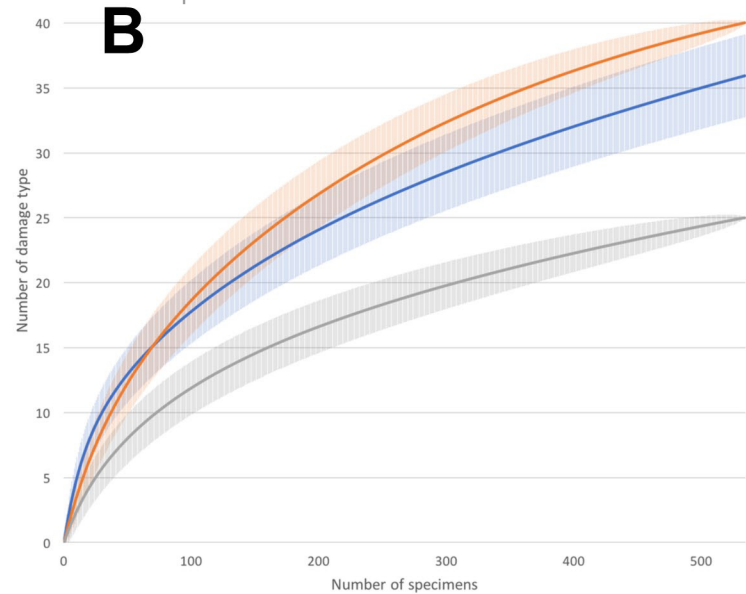
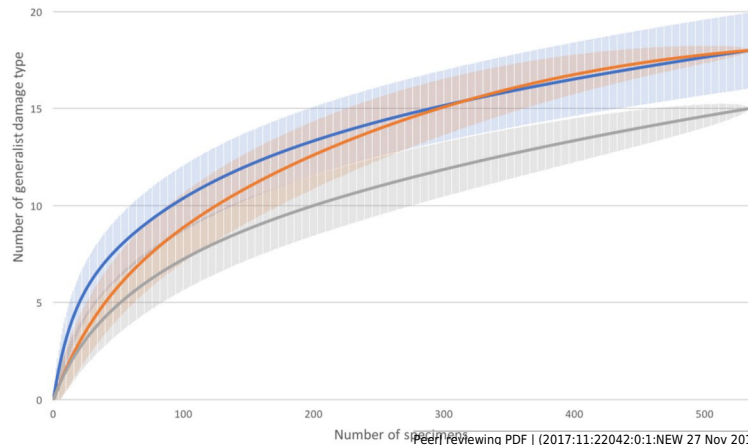
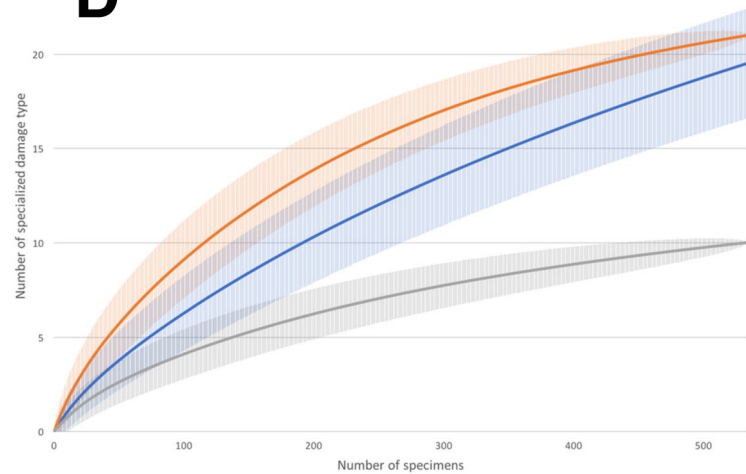
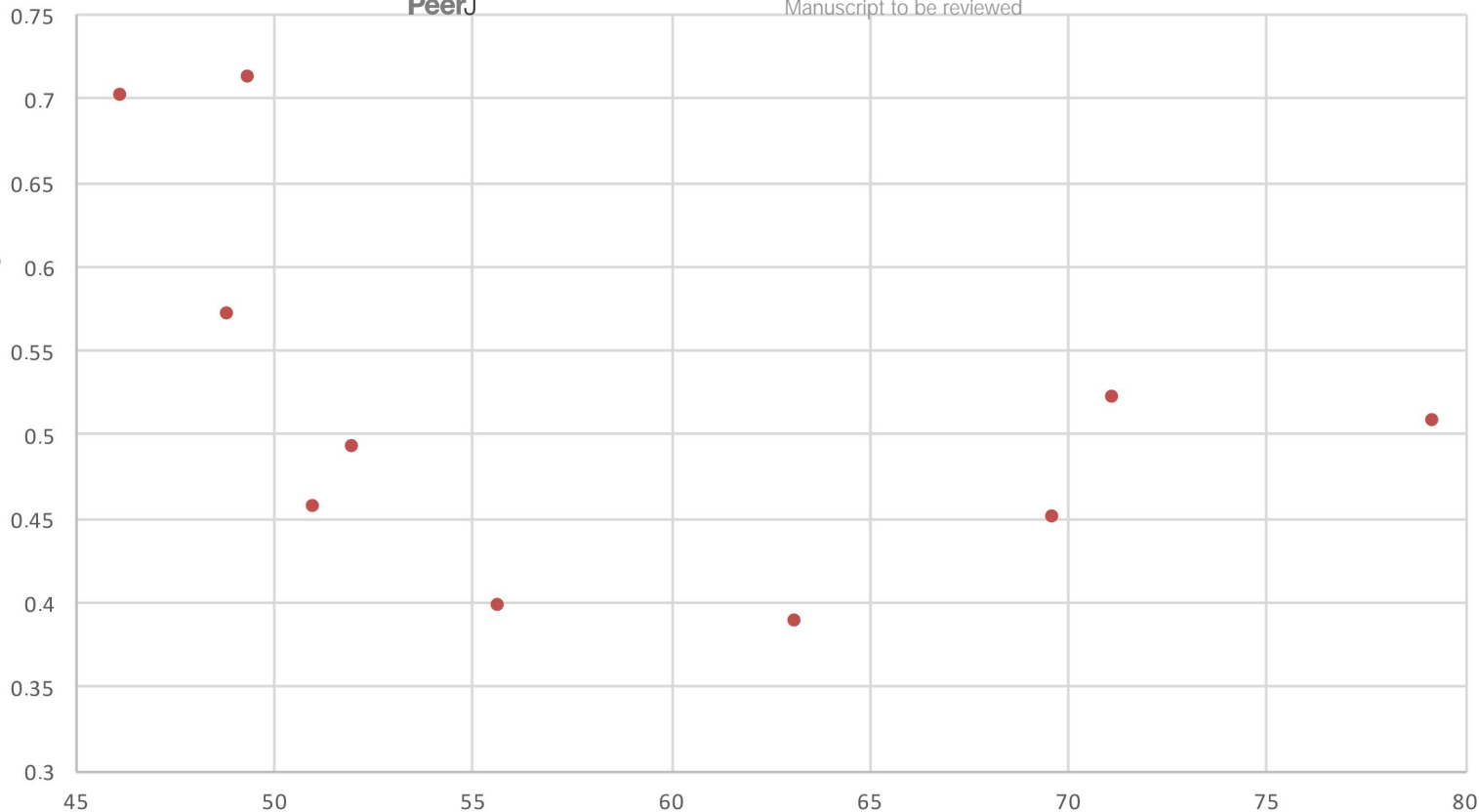
Significant difference (alpha <0.05) from an outcrop to another one is marked by an asterisk. The percent of generalized and specialized damages are computed with the damaged leaves, thus their sum is 100%.

Bernasso (-1.96 Ma)

Adroit et al., 2016

Berga (-3, -2.6 Ma)

Willershausen (-3, -2.6 Ma)

Rarefaction curves on the leaves from Willershausen, Berga and Bernasso (Adroit et al. 2016).

The grey curves represent Berga, the blue curves represent Willershausen outcrop and the orange curves represent Bernasso. The shaded area represents the standard deviation below and above the average of the resamples, with the method from Heck et al. (1975). Rarefaction curves represent the number of specimens by: A- Richness of plant species; B- Richness of damage type (DT); C- Richness of generalized damage; D- Richness of specialized damage

Percent of leaves damaged according to the leaf mass per area (LMA).

The LMA was estimated using the method of Royer et al. (2007). Each dot represents a plant species. Each species was considered only if at least 10 leaf from the species were measurable.

Principal components analysis (PCA) of plant species according to the diversity of damage type (DT).

A- Willershausen, B- Berga, C- Bernasso. Due to large amount of plant species names on A-Willershausen, the species names near the axes intersection were replace by alphabetic letter for visibility concerns: a- *Malus pulcherrima*, b- *Prunus mahaleb*, c- *Fagus sylvatica*, d- *Sorbus gabbrensis*, e- *Populus tremula*, f- *Populus willershausensis*, g- *Zelkova caprinifolia*, h- *Betula pubescens*, i- *Betula_sp1*, j- cf_*Toona*.