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Effects of experimental warming at the microhabitat scale on oak leaf traits and insect herbivory across abiotically contrasting sites

Running title: **Forest microclimate and herbivory**

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1 ABSTRACT

2 Forest microclimatic variation can result in substantial temperature differences at local
3 scales with concomitant impacts on plant defences and herbivory. Such microclimatic
4 effects, however, may differ across [abiotically contrasting sites depending on](#)
5 [background environmental differences. To test these cross-scale effects shaping species](#)
6 [ecological and evolutionary responses](#), we experimentally tested the effects of
7 aboveground microhabitat warming on insect leaf herbivory and leaf defences
8 (toughness, phenolic compounds) for saplings of sessile oak (*Quercus petraea*) across
9 two abiotically contrasting sites spanning 9° latitude. We found higher levels of
10 herbivory at the low-latitude site, but leaf traits showed mixed patterns across sites.
11 Toughness and condensed tannins were higher at the high-latitude site, whereas
12 hydrolysable tannins and hydroxycinnamic acids were higher at the low-latitude site. At
13 the microhabitat scale, experimental warming increased herbivory, but did not affect
14 any of the measured leaf traits. Condensed tannins were negatively correlated with
15 herbivory, suggesting that they drive variation in leaf damage at both scales. Moreover,
16 the effects of microhabitat warming on herbivory and leaf traits were consistent across
17 sites, i.e., effects at the microhabitat scale play out similarly despite variation in factors
18 acting at broader scales. These findings together suggest that herbivory responds to both
19 microhabitat (warming) and broad-scale environmental factors, whereas leaf traits
20 appear to respond more to environmental factors operating at broad scales (e.g.,
21 macroclimatic factors) than to warming at the microhabitat scale. In turn, leaf secondary
22 chemistry (tannins) appears to [drive](#) both broad-scale and microhabitat-scale variation in
23 herbivory. [Further studies are needed using reciprocal transplants with more populations](#)
24 [across a greater number of sites to tease apart plant plasticity from genetic differences](#)
25 [contributing to leaf trait and associated herbivory responses across scales and, in doing](#)

26 so, better understand the potential for dynamics such as local adaptation and range
27 expansion or contraction under shifting climatic regimes.

28

29 **Keywords:** Latitudinal variation, microclimate, *Quercus petraea*, phenolic compounds,
30 plant-herbivore interactions, saplings

31

32 INTRODUCTION

33 A fundamental goal of global change research is to understand how rising temperatures
34 will affect species interactions which ecological communities and ecosystem
35 functioning depend upon (Parmesan 2006, Kerr et al. 2015). While the threats of climate
36 change are well recognized, previous research has demonstrated that some ecological
37 communities are responding slower than expected to abiotic changes (Bertrand et al.
38 2011, Devictor et al. 2012, Dullinger et al. 2012, De Frenne et al. 2013). Accordingly,
39 the effects of global change drivers operating at broad scales can sometimes be difficult
40 to predict at local scales given biotic and abiotic heterogeneity commonly found at these
41 finer scales (De Frenne et al. 2021). For example, some authors have argued that local,
42 microhabitat-level abiotic heterogeneity can buffer changes in environmental drivers
43 such as radiation, air mixing, evapotranspiration, local topography, and soil properties
44 which would otherwise have more homogeneous effects across the landscape (Chen et
45 al. 2018, Zellweger et al. 2020, De Frenne et al. 2021).

46 Herbivory is strongly shaped by variation in the abiotic environment acting at
47 different spatial scales (Karban 1992, Hunter 2016, Moreira and Abdala-Roberts 2020).
48 For instance, herbivory rates at broad spatial scales often correlate with macroclimatic
49 factors such as mean annual temperature and precipitation, as shown repeatedly by
50 studies on latitudinal gradients in species interactions (Schemske et al. 2009, Anstett et

51 al. 2016). Intervening factors associated with climatic variation which are proposed to
52 explain such patterns include higher productivity, longer growing seasons, and more
53 stable species populations favouring higher herbivore pressure and plant defences at
54 lower latitudes (Rasmann and Agrawal 2011, Salazar and Marquis 2012, Moreira et al.
55 2014, Sanczuk et al. 2021). While predictive of herbivory, these plant traits have also
56 evolved (in some cases perhaps mainly) in response to abiotic forcing (e.g., some types
57 of secondary metabolites, pubescence, etc.), with this in turn driving lower herbivory at
58 high latitudes, i.e., abiotic factors shaping plant traits, and herbivory following the latter
59 (Moreira et al. 2018a). Abiotic variation at small scales, on the other hand, is also highly
60 important in shaping herbivory patterns via multiple pathways. For instance, changes in
61 air temperature at the microhabitat scale have been shown to increase insect metabolic
62 rates, resulting in higher herbivory rates (e.g., Lemoine et al. 2013, Zhang et al. 2020)
63 and increased plant defence induction (Karban, 2011). Likewise, microhabitat warming
64 can also increase the expression of constitutive levels of secondary metabolites such as
65 phenolic compounds (reviewed by Holopainen et al. 2018), in turn leading to reduced
66 herbivory (e.g., Lemoine et al. 2013). Together, these findings highlight different
67 ecological mechanisms jointly affecting plant-herbivore interactions.

68 Abiotic effects acting at different spatial scales can also influence each other and
69 further result in cross-scale dependencies (De Frenne et al. 2021). For example, effects
70 of microclimatic variation on plant traits predictive of herbivory might vary across
71 macro-climatically contrasting sites. One potential mechanism could be that trait
72 plasticity is more limited in warmer compared to colder sites because species in warm
73 climates have evolved under a narrower thermal gradient (e.g., Gugger et al. 2015,
74 Schmid et al. 2017). In another example, herbivore responses to microhabitat-level
75 climatic variation may vary across broad-scale geographical gradients as in the case of

76 insect herbivores from low latitudes being physiologically less tolerant to warming than
77 those found at higher latitudes (Deutsch et al. 2008). Unfortunately, to date, studies
78 testing for local-scale (e.g., microhabitat-scale) abiotic forcing have been usually
79 performed without considering broad spatial extents (e.g., Jamieson et al. 2015, Orians
80 et al. 2019, Moreira et al. 2020a), whereas broader-scale studies (e.g., latitudinal or
81 elevational gradients) have usually ignored microhabitat variability (e.g., Anstett et al.
82 2015, Moreira et al. 2018a). Consequently, our current understanding of commonalities
83 or differences in the mechanisms that shape herbivory at different spatial scales and,
84 especially, cross-scale dependencies, remain quite limited.

85 We conducted a manipulative field study testing for the effects of experimental
86 warming at the microhabitat-scale on insect leaf herbivory and leaf traits associated with
87 herbivory across two sites with contrasting (e.g., macroclimatic) conditions using sessile
88 oak (*Quercus petraea*) as model species, a common tree species in many Western
89 European broadleaf forests (Supporting Information). Specifically, we established two
90 field sites spanning two thirds of this species' latitudinal range (1.3-fold difference in
91 mean temperature from 11.5°C to 15.1°C, and two-fold seasonality in precipitation from
92 809 mm to 1785 mm), one in Spain (42°N) and one in Belgium (51°N), using three-
93 year-old saplings local to each site. We imposed microhabitat-level warming for half of
94 the saplings at each site using infrared heater arrays. After a full growing season (March
95 to September 2022), we collected leaves to measure insect leaf herbivory and to
96 quantify physical (specific leaf area, correlated with leaf thickness or toughness) and
97 chemical (phenolic compounds) traits known to affect herbivory in oak species.
98 Specifically, we asked: (1) Do leaf defensive traits and leaf herbivory vary in response
99 to warming at the microhabitat scale as well as across sites, the latter pointing at the
100 influence of broad-scale environmental (e.g., macroclimatic) factors? (2) Do the effects

101 of microhabitat warming vary across sites, therefore providing evidence of an
102 interaction or dependency across scales? And (3) are effects of broad-scale
103 environmental [differences](#), microhabitat-level warming, and any such interactive effect
104 across scales on herbivory related to concomitant variation in oak leaf traits? We
105 hypothesize that if herbivory and plant defence patterns are related, then effects on each
106 will be interdependent. For example, microhabitat-level warming and warmer
107 conditions at the southern site could lead to elevated defences which then drive lower
108 herbivory, i.e., a bottom-up mechanism. Alternatively, a top-down mechanism could
109 also take place with warming increasing damage and in turn defence levels. [It is also](#)
110 [possible that](#) broad-scale forcing and microhabitat-level warming lead to independent
111 effects on herbivory and/or defences (responses are uncoupled if traits do not influence
112 damage or vice versa). In addition, we expected interactive effects between micro- and
113 macro-climatic conditions whereby, for example, effects of microhabitat-level warming
114 will be stronger at the most northern site which has the coldest climate because
115 experimental heating would drive the greatest increase in temperature relative to
116 background weather conditions (e.g., reducing within-day temperature fluctuations and
117 buffering against low temperatures at night). Overall, this study provides a unique test
118 of how local warming might interact with regional differences among sites to determine
119 plant traits and patterns of herbivory.

120

121 **MATERIAL AND METHODS**

122 *Experimental design, sampling and measurements*

123 The study sites were nearby the town of Pontevedra (northern Spain, 42.40 N, 8.64 W,
124 39 m a.s.l.) and the other near Ghent (central Belgium, 50.97 N, 3.80 E, 48 m a.s.l.),
125 hereafter low and high-latitude sites, respectively. At each site, we selected an open

126 stand close to an oak-dominated forest. The study sites spanned close to two thirds of
127 the latitudinal range of *Q. petraea*. Specifically, across sites, oak trees and insect
128 herbivores experience a difference of c. 4 °C in mean annual temperature (11.5 °C at the
129 high-latitude site while 15.1 °C at the low-latitude site) and c. 1000 mm in annual
130 precipitation (809 mm at the high-latitude site and 1785 mm at the low-latitude site)
131 based on 2022 climatic data (Supporting Information).

132 In March 2022, before leaf burst, we purchased three-year-old saplings from
133 nurseries sourced from local populations near experimental sites, measured their height
134 (“initial height”) and individually planted them in 2-L pots containing peat (85%) and
135 coconut fibre (15%). Prior to obtaining them, saplings were grown under greenhouse
136 conditions and did not experience herbivory. After transplanting, we transported
137 saplings to the study sites and assigned half of the plants in each stand to one of two
138 warming treatments: (1) microhabitat-level warming or (2) untreated control (i.e.,
139 ambient temperature). In total, there were 125 saplings (35 control saplings and 34
140 warmed saplings at the high-latitude site; 28 control saplings and 28 warmed saplings at
141 the low-latitude site). Saplings were subjected exclusively to natural rainfall throughout
142 the experiment. The warming treatment was imposed by means of infrared heaters of
143 100 W installed at 85 cm height above the saplings (Elstein - Werk M Steinmetz GmbH
144 & Co KG, Northeim, Germany) which remained in place from April to September
145 2022. We grouped saplings in blocks (N = 5 at the high-latitude site and N = 3 at the
146 low-latitude site), and within each block we paired a group of 6-9 control plants and a
147 group of 6-9 saplings under an infrared heater. Distance between potted saplings within
148 each block was 20 cm and the distance between blocks within each site was 1 m. To
149 assess microclimatic changes in air temperature due to the warming treatment, we
150 installed a climatic-data logger (TMS-4, TOMST, Prague, Czech Republic) inside the

151 heated area, next to the saplings. These recordings indicated that, from April to August
152 2022, the warming treatment increased the air temperature, on average, by 1.5 °C
153 compared to plants under ambient temperature at both the low-latitude and high-latitude
154 sites (Supporting Information). While warming of the northern site never reached
155 temperatures of the southern site, thus setting by default a lower temperature upper limit
156 under warming at the colder site, in both cases we achieved realistic increases in
157 temperature predicted under climate change in each region. Finally, we protected all
158 plants from mammalian herbivores (e.g., deer, rabbits, rodents) by installing fences
159 around the experimental sites.

160 In late August and early September 2022 (before leaf senescence), we removed
161 the heaters and measured total height of all saplings (“final height”). Shortly after
162 removing the heaters, we haphazardly collected 10 fully expanded leaves of roughly the
163 same age (based on position along branch, color and consistency) for each sapling to
164 assess herbivory and leaf defensive traits. Leaf collection was performed blindly with
165 respect of treatments (Zvereva and Kozlov 2019). We sampled leaves at the end of the
166 growing season at each site, i.e., in early September at the high-latitude site and in late
167 September at the low-latitude site, to (i) minimize phenological differences in herbivory
168 and leaf defensive traits between sites, and (ii) to provide an assessment of cumulative
169 herbivory over the growing season (given that leaf longevity of *Q. petraea* spans most
170 of the growing season). Most of the damage observed on the collected leaves were due
171 to chewing insects and to a lesser extent by leaf miners or other herbivore guilds (< 2%
172 of sampled leaves). We photographed all leaves with a Samsung Galaxy A30s (25
173 effective megapixels, 4× digital zoom) and estimated the percentage of leaf area
174 consumed by chewing insects (“insect herbivory” hereafter) using BioLeaf - Foliar
175 Analysis™ (Brandoli Machado et al. 2016). We used the average value across leaves

176 per individual sapling for statistical analyses.

177 For each sapling, we selected four leaves (out of the 10 originally sampled) with
178 little or no herbivore damage and oven-dried them at 40 °C for 48 h to quantify physical
179 and chemical defences. We used undamaged leaves to partially control for variation due
180 to induced defences, namely local induction (Abdala-Roberts et al. 2016). However,
181 variation due to systemic induction is also possible and was not prevented by this
182 sampling approach. Accordingly, measured levels of leaf traits, particularly secondary
183 metabolites, represent constitutive levels, plus variation due to systemic induction
184 (Abdala-Roberts et al. 2016).

185

186 ***Quantification of physical defences***

187 We estimated specific leaf area (SLA) as a proxy for physical defence (Lill *et al.* 2006;
188 Moreira *et al.* 2020b). Previous work has shown that low SLA correlates negatively
189 with high leaf toughness (Hanley et al. 2007, Pearse and Hipp 2009) and can therefore
190 be used as a proxy for structural resistance against herbivory. We measured SLA (cm²
191 g⁻¹) by dividing the surface area of the four selected leaves by its dry mass and then
192 averaged these values across leaves to obtain a single (mean) value for statistical
193 analyses.

194

195 ***Quantification of chemical defences***

196 Phenolic compounds have been shown to defend oaks against insect herbivores (Feeny
197 1970, Roslin and Salminen 2008. Moreira et al. 2018a,b. Moreira et al. 2020b), and
198 therefore represent a suitable class of compounds to assess chemical defences in species
199 of this group. For these analyses, we used the same four leaves used to quantify SLA
200 but ground them all together using liquid nitrogen to obtain a single pooled sample. We

201 then extracted phenolic compounds from 20 mg of dry pulverized leaf tissue with 1 mL
202 of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation (Moreira
203 et al. 2014). We then transferred the extracts to chromatographic vials and performed
204 ultra-high-performance liquid chromatography analyses (Supporting Information). We
205 identified four groups of phenolic compounds: (i) flavonoids; (ii) ellagitannins and
206 gallic acid derivatives (“hydrolysable tannins” hereafter); (iii) proanthocyanidins
207 (“condensed tannins” hereafter); and (iv) hydroxycinnamic acids, based on the
208 comparison of their parent ion mass and fragmentation pattern, UV spectra and
209 retention time with commercial standards and literature data. We quantified flavonoids
210 as rutin equivalents, condensed tannins as catechin equivalents, hydrolysable tannins as
211 gallic acid equivalents, and hydroxycinnamic acids as ferulic acid equivalents (Moreira
212 et al. 2018b, Galmán et al. 2019). We achieved the quantification of these phenolic
213 compounds by external calibration using calibration curves at 0.16, 0.8, 4, 20, 100 and
214 500 $\mu\text{g mL}^{-1}$. We expressed phenolic compound concentrations in mg g^{-1} dry tissue.

215

216 *Statistical analysis*

217 We ran general linear mixed models (GLMMs) testing for the effect of site (two levels:
218 high- vs. low-latitude), microhabitat-level warming (two levels: control or ambient
219 temperature vs. experimental warming) and their interaction on four different response
220 variables: plant relative growth rate (estimated as: $[\text{final height} - \text{initial height}] / \text{initial}$
221 height), phenolic compounds (separately for each group of compounds), SLA and insect
222 leaf herbivory. The site by warming interaction tested whether the effects of
223 microhabitat-level warming varied across sites, i.e., a cross-scale dependency. In all
224 models, we also included the three-way interaction between block, site and warming
225 treatment as a random effect in order to analyse the main factors (i.e., site and warming)

226 with the appropriate error terms.

227 To assess which leaf traits were associated with herbivory, we firstly ran a
228 stepwise multiple regression with herbivory as a response and all leaf traits as
229 predictors. We used a backward model selection based on the difference in the values of
230 the Akaike information criterion (AIC) between models (AIC; delta AIC values
231 between two competing models being higher than 2) to identify the most important
232 traits predictive of herbivory (Supporting Information). We tested for leaf trait effects
233 on herbivory rather than the reverse as preliminary analyses indicated that traits, namely
234 phenolic compounds, significantly negatively correlated with leaf damage [suggesting](#)
235 [that these compounds predict leaf damage, i.e., confer plant biotic resistance. While this](#)
236 [interpretation is not definitive as other factors correlated with phenolics could underlie](#)
237 [this correlation \(see *Results*\), it is the best evidence we have suggestive of causality](#)
238 [based on available data](#)). Second, to assess whether environmental conditions (broad-
239 scale vs. microhabitat scale due to warming) influenced herbivory via effects on leaf
240 trait, namely broad-scale (e.g., abiotically-driven) [differences](#) vs. plasticity responses to
241 local warming, respectively, traits retained in the above stepwise regression were
242 included as predictors (covariates) of herbivory in the initial leaf damage model testing
243 for main effects (site, microhabitat-level warming) and their interaction (see above). For
244 this regression analysis, we standardized herbivory data (mean=0, SE=1) by site-by-
245 warming treatment combination. We expected that if a given trait explains site or
246 microhabitat-level warming effects, then any such effects on leaf damage in the initial
247 model without covariates should turn non-significant after its inclusion (Abdala-Roberts
248 et al. 2016, Moreira et al. 2018a). By the same token, if effects remain significant this
249 would suggest that site or microhabitat-scale warming effects on herbivory act via other
250 (unmeasured) leaf traits or through some other mechanism, e.g., direct effect of

251 warming on insects influencing leaf damage (e.g., mobility, physiological state)
252 independently of leaf defensive traits.

253 We performed all analyses with PROC MIXED in SAS ver. 9.4 (SAS Institute,
254 Cary, NC) (Littell et al. 2006). For standardisation of herbivory data, we used PROC
255 STANDARD in SAS 9.4. We log-transformed herbivory, condensed and hydrolysable
256 tannins, flavonoids, and plant relative growth rate to achieve normality of the residuals,
257 and report model least-squares means and standard errors as descriptive statistics. We
258 calculated the post hoc statistical power of our analyses by using the online tool at
259 <https://clincalc.com/stats/Power.aspx>, using the experimental unit (group of control or
260 heated plants) as the level of replication (consistent with the statistical models above).
261 The statistical power always exceeded 65%, indicating that it was sufficient to detect an
262 effect of site and warming treatment on herbivory and plant defences if that effect did
263 exist (Kozlov et al. 2022).

264

265 RESULTS

266 *Effects of site and microhabitat-level warming on oak traits*

267 There were significant effects of site on most groups of phenolic compounds, except
268 flavonoids (Table 1, Fig. 1A-D), though the latitudinal direction was contingent on the
269 type of compound. For example, the concentration of condensed tannins was, on
270 average, 1.2-fold higher for saplings growing at the high-latitude site (**mean \pm SE of**
271 **back-transformed log data: $5.11 \pm 1.05 \text{ mg g}^{-1} \text{ d.w.}$**) than for those growing at the low-
272 latitude site (**$4.32 \pm 1.06 \text{ mg g}^{-1} \text{ d.w.}$**) (Fig. 1A). In contrast, hydrolysable tannins and
273 hydroxycinnamic acids were, on average, 2.0-fold and 1.2-fold higher for saplings
274 growing at the low-latitude site than for those growing at the high-latitude site (**mean \pm**
275 **SE of back-transformed log data for hydrolysable tannins: 4.56 ± 1.04 vs. 2.32 ± 1.03**

276 mg g⁻¹ d.w.; **mean ± SE for** hydroxycinnamic acids: 15.81 ± 0.57 vs. 13.52 ± 0.51 mg g⁻¹
277 d.w.) (Fig. 1B, 1D). Microhabitat warming and the site by warming interaction were
278 not significant in any case (Table 1).

279 We also found a significant effect of site on SLA (Table 1), whereby the mean
280 value was 1.3-fold higher for saplings growing at the low-latitude site (**mean ± SE:**
281 136.59 ± 3.38 cm² g⁻¹) relative to those growing at the high latitude site (104.78 ± 2.90
282 cm² g⁻¹) (Fig. 2). We found no significant effects of microhabitat warming and the site
283 by warming interaction on SLA (Table 1).

284 There were no significant effects of site, microhabitat warming, or their
285 interaction on sapling relative growth rate (Table 1, Supporting Information).

286

287 *Effects of site and microhabitat warming on herbivory*

288 **Levels** of insect leaf damage were somewhat low (mean ± SE: 4.35 ± 0.32%, range: 0-
289 15.93%, **data based on individual plants**). There was a significant effect of site (Table
290 1), whereby herbivory was, on average, 1.4-fold higher for saplings growing at the low-
291 latitude site (**mean ± SE of** back-transformed log data: 5.05 ± 1.11%) relative to those
292 growing at the high-latitude site (3.65 ± 1.10%) (Fig. 3). At the microhabitat scale, there
293 was a significant effect of warming on herbivory (Table 1), leaf damage being, on
294 average, 1.4-fold higher for saplings subjected to warming (**mean ± SE of** back-
295 transformed log data: 5.03 ± 1.10%) than for control saplings (3.66 ± 1.10%) (Fig. 3).

296 We found no significant interaction site by warming interaction (Table 1), i.e.,
297 microhabitat warming effects were similar across sites (Fig. 3).

298

299 *Leaf traits underlying site and microhabitat-level warming effects on herbivory*

300 We found that condensed tannins and hydrolysable tannins were the only traits retained

301 after multiple regression backward elimination, but only the former had a significant
302 negative effect (condensed tannins: $\text{mean} \pm \text{SE of slope estimator } [\beta] = -0.067 \pm 0.031$,
303 $P = 0.033$; hydrolysable tannins: slope estimator $[\beta] = 0.075 \pm 0.040$, $P = 0.066$).
304 Following from this, we ran again the herbivory model with main effects but now
305 including also condensed and hydrolysable tannins as covariates. Results from this
306 model indicated that both the site and warming effects turned non-significant (Table 2
307 vs. 1).

308

309 DISCUSSION

310 We found significant effects of broad-scale site **differences** and microhabitat-scale
311 warming on leaf defensive traits and insect herbivory on *Q. petraea* saplings. Across
312 sites, herbivory was highest on saplings growing at the low-latitude site (Spain),
313 whereas leaf traits showed mixed responses, i.e., both increases and decreases at low
314 latitude depending on the focal trait. At the microhabitat scale, warming increased
315 herbivory but did not affect plant traits. Condensed tannin concentrations, the only trait
316 significantly predicting leaf damage (negative effect), were lower at the low latitude site
317 and seemingly accounted for site and warming differences in herbivory. Together, these
318 findings indicate different responses acting at each scale, namely: leaf defences were
319 more responsive to broad-scale environmental **differences** than to microhabitat warming
320 whereas herbivory was affected at both scales. Furthermore, our findings also indicated
321 no evidence of cross-scale dependencies, namely that warming effects at the
322 microhabitat scale played out similarly across latitudes.

323 Insect leaf damage was highest at the low-latitude site, supporting predictions of
324 latitudinal variation in species interactions (reviewed by Schemske et al. 2009),
325 including herbivory (Coley and Barone 1996, Rasmann and Agrawal 2011, Pearse and

326 Hipp 2012, Salazar and Marquis 2012, Moreira et al. 2014). This result is also
327 consistent with our previous work on pedunculate oak *Q. robur*, another common
328 European oak, showing higher insect leaf herbivory at lower latitudes (Moreira et al.
329 2018a). In addition, we found that some defensive traits such as condensed tannins and
330 SLA (proxy for leaf thickness or toughness) were higher at the high-latitude site,
331 whereas hydrolysable tannins and hydroxycinnamic acids were higher at the low-
332 latitude site. Further analyses indicated that herbivory significantly correlated
333 (negatively) only with condensed tannins and that these compounds accounted for site
334 differences in herbivory. These results are also consistent with our previous work on *Q.*
335 *robur* suggesting condensed tannins drive latitudinal variation in herbivory (Moreira et
336 al. 2018b). We argue that for this species, and possibly also for sessile oak, higher
337 temperature and precipitation drive lower tannin concentrations at lower latitudes and
338 this in turn results in higher herbivory, i.e., a bottom-up causality that differs from
339 classical theory which postulates that higher herbivore pressure drives higher defences
340 at low latitude, a top-down mechanism (Moreira et al. 2018a). It is also plausible that
341 higher herbivory at the low-latitude site is shaped by direct effects of climate on insect
342 performance and resulting impacts on population growth or life cycle dynamics (e.g.,
343 voltinism) leading to greater leaf damage. For example, previous studies have reported
344 that higher temperatures (as at our low-latitude site) increase physiological activity and
345 metabolic rates of insect herbivores which in turn consume more plant tissues and grow
346 faster (Bale et al. 2002).

347 Microhabitat-level warming, on the other hand, increased leaf herbivory,
348 consistent with previous studies pointing at temperature-driven increases in insect
349 metabolic rates or changes in feeding behaviour as potential mechanisms (Bale et al.
350 2002, Hamann et al. 2021). For instance, Nakamura et al. (2022) recently reported that

351 microhabitat-scale warming of 5°C above ambient temperature using heat resistance
352 cables (in the soil and branches) markedly increased herbivory by chewing insects on
353 birch trees. Similarly, Lemoine et al. (2013) found that consumption rates by the
354 generalist herbivore *Popillia japonica* on several plant species increased under
355 microhabitat warming. Contrarily to leaf damage, microhabitat warming did not affect
356 SLA and the concentration of any of the analysed phenolic compounds. Such lack of
357 warming effects does not agree with results from other studies reporting increases in
358 chemical defences with experimental warming in *Q. rubra* (Top et al. 2017), *Betula*
359 *nana* (Graglia et al. 2001) and *Populus tremuloides* (Li et al. 2021). Differences
360 between studies in the timing of leaf collection could explain these differences in
361 results, provided seasonal changes in plasticity responses to warming in phenolic
362 compound concentrations (Hunter and Price 1992, Forkner and Hunter 2000) as well as
363 changes in secondary chemistry with leaf ontogeny or between leaf flushes early vs.
364 later in the growing season which overrun warming effects (Matsuki et al. 2004, van
365 Asch and Visser 2007, Gaytán et al. 2022).

366 The fact that most leaf traits were not affected by microhabitat warming would
367 have precluded trait plasticity-mediated effects on herbivory. Despite this, once having
368 accounted for hydrolysable tannins and condensed tannins (retained traits after stepwise
369 procedure), the warming effect on herbivory turned non-significant, suggesting that the
370 effects of microhabitat temperature manipulation on damage were underlain by
371 warming-induced changes in these compounds. However, the fact that warming did not
372 significantly influence these compounds casts doubt on this trait-mediated
373 interpretation. Further work assessing warming-induced changes in additional leaf traits
374 such as terpenoids and fiber content is needed, including diurnal and nocturnal plant
375 secondary chemistry-related metabolism as well as insect behavioural changes under

376 warming. In addition, while the achieved temperature increase is within the range of
377 predicted increases due to climate change, our experiment did not test for temporal
378 variability in temperature increases, including peaks in temperature which have been
379 shown to have especially strong impacts on plant physiology and defensive responses
380 (e.g., heat waves; Tian et al. 2022).

381 The warming effect on herbivory and leaf traits was consistently non-significant
382 across the studied distant sites despite substantial differences in abiotic (e.g., climatic)
383 conditions. Few studies have experimentally tested for microhabitat-level warming
384 effects across abiotically-contrasting sites (e.g., Birkemoe et al. 2016, Pepi and Karban
385 2021, Zvereva et al. 2022). Consistent with our findings, Pepi and Karban (2021)
386 reported that microhabitat warming drove an increase in herbivory by woolly bear
387 caterpillars (*Arctia virginalis*) on pacific silverweed (*Argentina anserina* ssp. *pacifica*),
388 with such effect being similar in magnitude across two sites located at the two extremes
389 of a precipitation gradient in California. In contrast, Birkemoe et al. (2016) found
390 contrasting effects of microhabitat warming on herbivory for two plant species growing
391 at two sites differing in elevation. Namely, warming increased herbivory by
392 leaf-chewing insects on the mountain avens *Dryas octopetala* but only at low elevation,
393 whereas for the alpine bistort *Bistorta vivipara* it increased herbivory only at high
394 elevation (Birkemoe et al. 2016). These authors argued that different herbivores appear
395 to be responsible for damage on the studied plant species, pointing at herbivore-specific
396 responses to warming (Birkemoe et al. 2016). At our field sites, *Q. petraea* is mainly
397 attacked by lepidopteran species such as *Tortrix viridana*, *Lymantria dispar*, and
398 *Malacosoma neustria* (Moreira et al. 2017, 2018a, 2020b). Unfortunately, were unable
399 to record the identity of the herbivores based on observed leaf damage patterns to
400 inform site [differences](#) in herbivore composition to assess whether the same or different

401 herbivores responded similarly to warming across sites.

402

403 *Study limitations and future work*

404 The first limitation of our study is that by using only one source of saplings at each site
405 we cannot disentangle plant genetically based from plasticity effects on species traits
406 which is essential for predicting species responses to climatic variation and its
407 consequences for species interactions. To this end, follow-up studies should make use
408 of plants from multiple source sites located at each latitude and sampling at multiple
409 points along the studied gradient (including expanding beyond the latitudinal range
410 used). Another limitation is that by not including a detailed inspection of the biotic and
411 abiotic drivers of broad-scale effects we cannot identify mechanisms operating at this
412 scale, tease apart their relative effects, as well as their potential to interact locally with
413 microclimatic effects. To this end, further studies should include direct measurements of
414 target factors across sites (e.g., using meteorological stations, soil samples, herbivore
415 community sampling, etc.) or using available databases (e.g., climate). An additional
416 limitation worth emphasizing is that by including a single time point of leaf collection
417 over the same growing season we cannot provide a fine-tuned picture of within- or
418 among-season variation in leaf chemistry, and a better understanding of its driving
419 factors and cause-effect linkages to herbivory. To this end, further studies should
420 sample leaves across different moments of the growing season and during multiple
421 growing seasons. Broadly, research aiming to address one or several of these features
422 will lead to a better understanding of the drivers of plant-herbivore interactions and how
423 abiotic factors acting at different spatial scales shape species traits and interactions
424 under climate change.

425

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576 **Table 1.** Effects of the site (two levels: low-latitude site in Spain, and high-latitude site
 577 in Belgium), microhabitat-level warming treatment (two levels: control or ambient
 578 temperature vs. experimental warming), and their interaction on the concentration of
 579 leaf chemical defences (condensed and hydrolysable tannins, flavonoids and
 580 hydroxycinnamic acids), specific leaf area, leaf herbivory by chewing insects and plant
 581 relative growth rate in *Quercus petraea* saplings. We also included the three-way
 582 interaction between block, site and warming treatment as a random effect. *F*-values with
 583 the degrees of freedom (numerator, denominator) and associated significance levels (*P*-
 584 values) are shown. Significant *P*-values ($P < 0.05$) are in bold.

	Site (S)		Warming (W)		S × W	
	<i>F</i> _{1,12}	<i>P</i>	<i>F</i> _{1,12}	<i>P</i>	<i>F</i> _{1,12}	<i>P</i>
Condensed tannins	4.64	0.033	0.23	0.634	0.03	0.873
Hydrolysable tannins	194.06	<0.001	1.20	0.294	0.80	0.388
Flavonoids	0.51	0.489	0.07	0.803	0.01	0.929
Hydroxycinnamic acids	9.08	0.011	0.05	0.829	0.81	0.386
Specific leaf area	51.07	<0.001	2.85	0.117	4.37	0.059
Insect leaf herbivory	5.40	0.039	5.23	0.041	1.20	0.296
Relative growth rate	0.84	0.377	0.32	0.583	0.37	0.555

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595 **Table 2.** Effects of the site (two levels: low-latitude site in Spain, and high-latitude site
 596 in Belgium), microhabitat-level warming treatment (two levels: control or ambient
 597 temperature vs. experimental warming), and their interaction on leaf herbivory by
 598 chewing insects in *Quercus petraea* saplings. In this model, we included selected leaf
 599 defensive traits (see statistical analysis in *Methods*) as covariates to test whether the
 600 effects of the site and microhabitat-level warming treatment on insect leaf herbivory
 601 were explained by changes in leaf traits. We also included the three-way interaction
 602 between block, site and warming treatment as a random effect. For these analyses, we
 603 standardized herbivory data (mean=0, SE=1) by site-by-warming treatment
 604 combination. Degrees of freedom (numerator, denominator), *F*-values and associated
 605 significance levels (*P*-values) are shown.

	Insect leaf herbivory		
	DF _{num,den}	F	<i>P</i>
Site	1, 12	1.42	0.256
Warming	1, 12	0.00	0.996
Site × Warming	1, 12	0.02	0.887
Condensed tannins	1, 107	3.79	0.054
Hydrolysable tannins	1, 107	1.51	0.221

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615 **FIGURE LEGENDS**

616

617 **Figure 1.** Concentration of leaf (A) condensed tannins, (B) hydrolysable tannins, (C)
618 flavonoids, and (D) hydroxycinnamic acids in *Quercus petraea* saplings subjected to
619 experimental warming (gray bars) and ambient temperature (white bars) at two macro-
620 climatically contrasted sites (low-latitude site in Spain, and high-latitude site in
621 Belgium). Bars are back-transformed least square means \pm standard error from the
622 linear mixed models (except for hydroxycinnamic acids). Statistical results are shown in
623 Table 1. Different letters above the bars indicate significant differences (at $P < 0.05$)
624 between treatments.

625

626 **Figure 2.** Specific leaf area in *Quercus petraea* saplings subjected to experimental
627 warming (gray bars) and control or ambient temperature (white bars) at two macro-
628 climatically contrasted sites (low-latitude site in Spain, and high-latitude site in
629 Belgium). Bars are least square means \pm standard error. Statistical results are shown in
630 Table 1. Different letters above the bars indicate significant differences (at $P < 0.05$)
631 between treatments.

632

633 **Figure 3.** Percent leaf herbivory by insect chewers on *Quercus petraea* saplings
634 subjected to experimental warming (gray bars) and control or ambient temperature
635 (white bars) at two macro-climatically contrasted sites (low-latitude site in Spain, and
636 high-latitude site in Belgium). Bars are back-transformed least square means \pm standard
637 error from the linear mixed model. Statistical results are shown in Table 1. Different
638 letters above the bars indicate significant differences (at $P < 0.05$) between treatments.

639

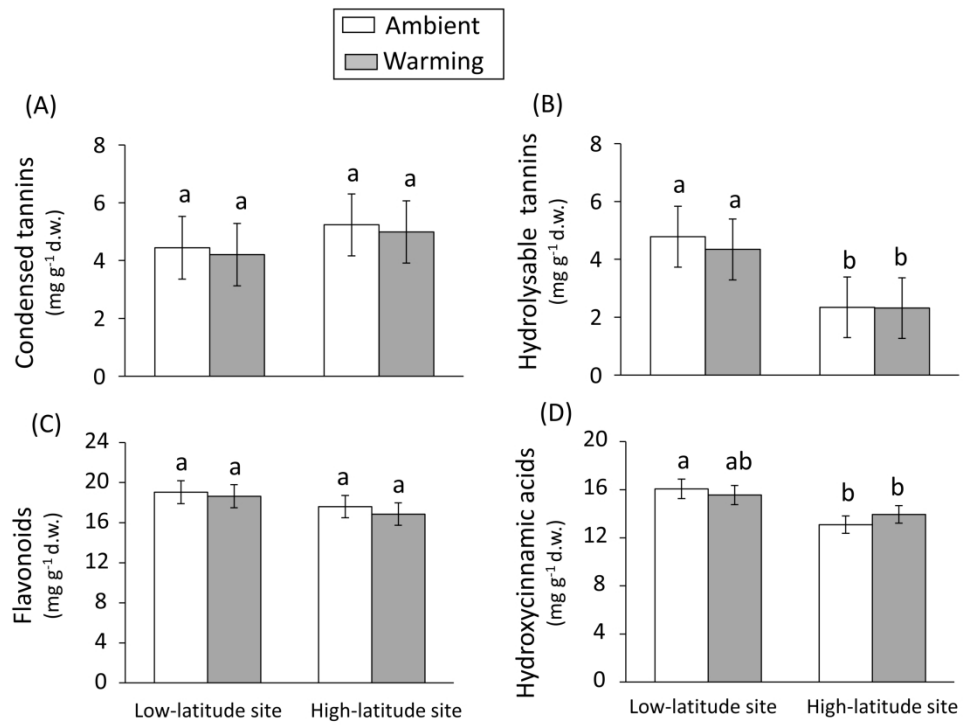


Figure 1

258x190mm (300 x 300 DPI)

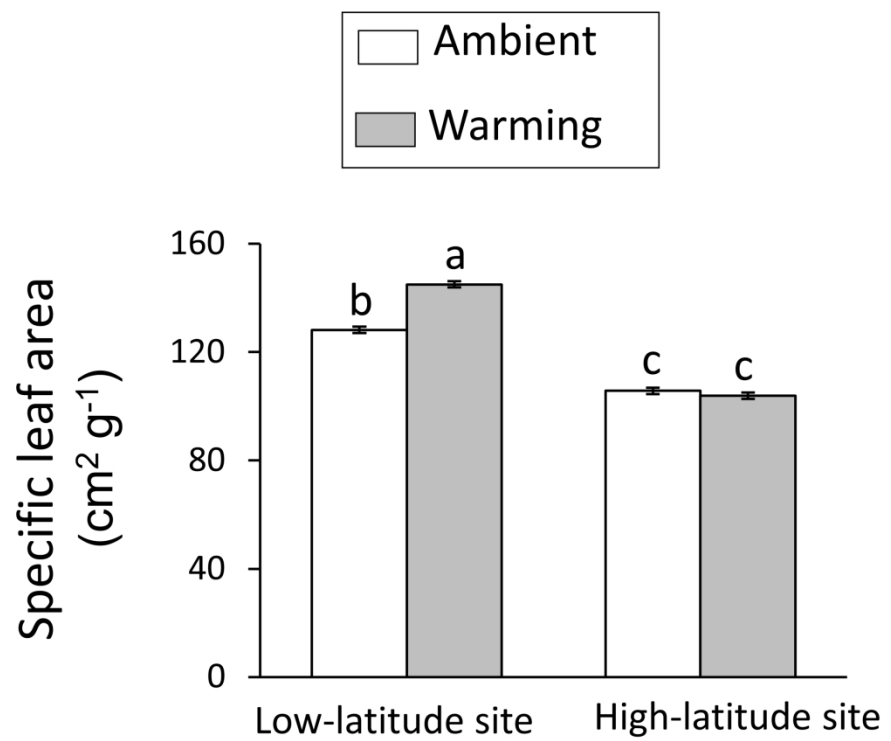


Figure 2

238x190mm (300 x 300 DPI)

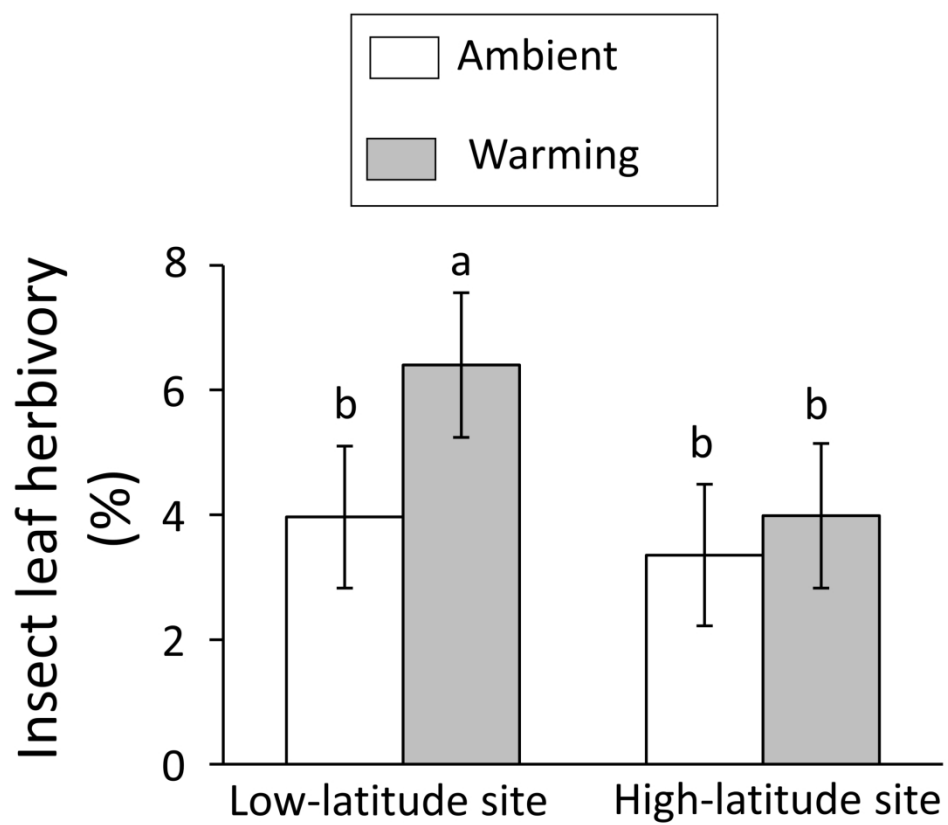


Figure 3

208x180mm (300 x 300 DPI)

TABLE OF CONTENTS

Appendix S1. Natural history of *Quercus petraea* and its herbivores.

Appendix S2. Detailed description of the methods to quantify and identify phenolic compounds.

Table S1. Climatic data of the two studied sites (Spain, Belgium) in 2022.

Table S2. Air temperature above the ground surface in experimental warming and ambient temperature conditions at the two studied sites (Spain, Belgium).

Table S3. Akaike information criterion of stepwise multiple regression models including herbivory as a response and all leaf traits as predictors.

Figure S1. Effects of site and experimental warming treatment on plant relative growth rate.

For Review Only

Appendix S1. Natural history

Sessile oak *Quercus petraea* (Matt.) Liebl. is a long-lived, deciduous tree species native to most of Europe. It is distributed across an extremely wide range of climatic conditions from the south of Scandinavia to the north of the Iberian Peninsula, and from Ireland to Iran (Eaton et al. 2016). Leaf burst in this species usually occurs in April for southern Europe and in May for northern Europe, whereas leaf senescence and leaf drop typically starts in September for northern Europe and in October for southern Europe. Within its native range, this species supports a diverse community of insect herbivores such as leaf chewers, miners, and galls (Galmán et al. 2019, Moreira et al. 2020). In addition, as other oak species, it possesses both physical (e.g., toughness) and chemical (e.g., phenolic compounds) leaf traits which have been shown to be negatively associated with herbivory and potentially act against a broad range of phytophagous insects (Moreira et al. 2018, Galmán et al. 2019, Moreira et al. 2020).

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Appendix S2. Methods to quantify and identify phenolic compounds

For phenolic quantification, we used ultra-high-performance liquid chromatography system (UHPLC; Nexera LC-30AD, Shimadzu) equipped with a UV/VIS photodiode array detector (PDA; SPD-M20A Shimadzu) The compound separation was carried out on a Kinetex Core-Shell C18 column (2.6 μm , 100 \times 4.6 mm; Phenomenex), protected with a C18 guard cartridge. The flow rate was 0.4 mL min⁻¹ and the column oven temperature was set at 25 °C. The mobile phase consisted of two solvents: water-formic acid (0.05%) (A) and acetonitrile-formic acid (0.05%) (B), starting with 5% of B and using a gradient to obtain 30% of B at 4 min, 60% of B at 10 min, 80% of B at 13 min and 100% of B at 15 min. The injection volume was 15 μL . Phenolic compound were measured at 280 and 330 nm. For phenolic compound identification, we used an UHPLC-PDA system (Thermo Dionex Ultimate 3000) coupled with an electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-Q-TOF-MS/MS) (Bruker Compact™). We used the same chromatographic conditions as in quantification analysis, but injection volume was 5 μL . Mass spectra (MS) and MS² were acquired in full scan and in negative ionization mode in a range starting from 100 to 1200 m/z¹.

Table S1. Monthly mean temperature (in °C) and total precipitation (in mm) in 2022 at two macro-climatically contrasted sites (low-latitude site in Spain, and high-latitude site in Belgium). Data were collected from nearby weather stations (Hoeilaart, Belgium, 50.766N, 4.449E; Pontevedra, Spain, 42.406N, 8.643W).

Month	Belgium		Spain	
	Temperature	Precipitation	Temperature	Precipitation
January	4.1	82	8.2	68
February	6.4	107	10.1	67
March	7.6	3	10.9	113
April	9.6	45	12.4	115
May	14.3	55	17.6	59
June	16.8	112	17.8	73
July	18.5	10	22.3	3
August	20.0	11	21.5	7
September	14.0	143	19.1	108
October	13.4	55	18.0	255
November	8.8	78	11.8	413
December	3.9	108	11.6	503

Table S2. Mean values and standard errors (N = 2975) of air temperature (°C) at 5 cm height above the ground surface from May to August in experimental warming and ambient temperature conditions at two macro-climatically contrasted sites (low-latitude site in Spain, and high-latitude site in Belgium).

		Belgium	Spain
May	Control	16.87 ± 0.09	19.77 ± 0.06
	Warming	18.21 ± 0.11	21.13 ± 0.11
June	Control	19.05 ± 0.12	21.61 ± 0.07
	Warming	20.22 ± 0.07	23.20 ± 0.12
July	Control	21.51 ± 0.14	24.58 ± 0.07
	Warming	23.17 ± 0.14	26.34 ± 0.14
August	Control	22.83 ± 0.11	25.93 ± 0.06
	Warming	24.48 ± 0.11	27.26 ± 0.14

Table S3. Akaike information criterion (AIC) of stepwise multiple regression models including herbivory as a response and all leaf traits as predictors.

Model	AIC	Predictors
1	-83.1226	Condensed tannins, hydrolysable tannins
2	-82.2612	Condensed tannins, hydrolysable tannins, SLA
3	-81.9713	Hydroxycinnamic acids, condensed tannins
4	-81.6432	Condensed tannins
5	-81.2933	Hydroxycinnamic acids, condensed tannins, hydrolysable tannins
6	-81.2172	Flavonoids, condensed tannins hydrolysable tannins
7	-80.4736	Hydrolysable tannins
8	-80.2949	Hydroxycinnamic acids, condensed tannins, hydrolysable tannins, SLA
9	-80.2907	Flavonoids, condensed tannins hydrolysable tannins, SLA
10	-80.1536	Hydroxycinnamic acids, condensed tannins, SLA

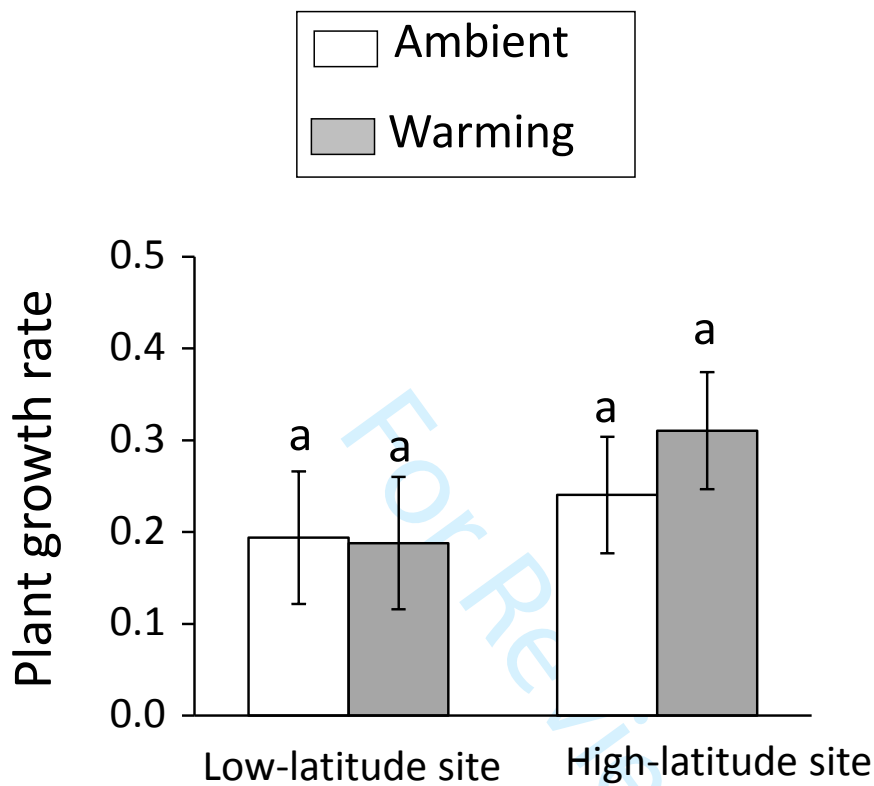


Figure S1. Relative growth rate (estimated as: [final height – initial height] / initial height) of *Quercus petraea* saplings subjected to experimental warming (gray bars) and control or ambient temperature (white bars) at two macro-climatically contrasted sites (low-latitude site in Spain, and high-latitude site in Belgium). Bars are least square means \pm standard error (statistical results shown in Table 1).