RESEARCH ARTICLE

AMERICAN JOURNAL OF BOTANY

Biotic and abiotic factors associated with altitudinal variation in plant traits and herbivory in a dominant oak species[1]

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PREMISE OF THE STUDY: It is generally thought that herbivore pressure is higher at lower elevations where climate is warmer and less seasonal, and that this has led to higher levels of plant defense investment at low elevations. However, the generality of this expectation has been called into question by recent studies.

METHODS: We tested for altitudinal gradients in insect leaf damage, plant defenses (phenolic compounds), and nutritional traits (phosphorus and nitrogen) in leaves of the long-lived tree *Quercus robur*, and further investigated the abiotic factors associated with such gradients. We sampled 20 populations of *Q. robur* distributed along an altitudinal gradient spanning 35–869 m above sea level, which covered most of the altitudinal range of this species and varied substantially in abiotic conditions, plant traits, and herbivory.

KEY RESULTS: Univariate regressions showed that leaf herbivory, phenolics, and phosphorus increased toward higher elevations, whereas leaf nitrogen did not vary with altitude. Multiple regression analyses indicated that temperature was the single most important factor associated with herbivory and appears to be strongly associated with altitudinal variation in damage. Leaf phenolics were also correlated with herbivory, but in a manner that suggests these chemical defenses do not underlie altitudinal variation in damage. In addition, we found that variation in leaf traits (phenolics and nutrients) was in turn associated with both climatic and soil variables.

CONCLUSIONS: Overall, these findings suggest that altitudinal gradients in herbivory and defenses in *Q. robur* are uncoupled and that elevational variation in herbivory and plant traits responds mainly to abiotic factors.

KEY WORDS altitudinal gradient; climate; herbivory; phenolics; plant traits; soil variables; Spain

Geographic variation in species' traits is widespread and frequently follows gradients in biotic (i.e., species interaction intensity) or abiotic (e.g., temperature, soil fertility) conditions experienced by a species throughout its distribution range (Linhart and Grant, 1996; Woods et al., 2012; Pratt and Mooney, 2013). For instance, latitudinal gradients in species interaction strength have traditionally received much attention (Dobzhansky, 1950; Janzen, 1970; Coley and Barone, 1996), and it is often assumed that stronger species interactions toward the equator have led to increasing strength of selection on species traits mediating such interactions (e.g., plant defenses; Schemske et al., 2009; Rasmann and Agrawal, 2011; Marquis et al., 2012). Similarly, altitudinal gradients in biotic or abiotic factors may also drive concomitant variation in species traits (reviewed by Rasmann et al., 2014). Studies have documented that plant species growing at lower elevations experience greater herbivore pressure and therefore invest more in defenses, whereas the opposite is expected for their high-elevation counterparts (Scheidel and Bruelheide, 2001; Rodríguez-Castañeda et al., 2010; Pellissier et al., 2012; but see Rasmann et al., 2014). Although altitudinal clines have received relatively less attention than latitudinal gradients, they are nevertheless characterized by more abrupt changes in biotic and abiotic forcing and may strongly influence species interactions and species traits over short distances (Jonas and Geber, 1999; Rasmann et al., 2014). To date, however, most studies assessing altitudinal clines have been based on interspecific comparisons, whereas much fewer studies have evaluated within-species altitudinal variation. This research gap remains even though knowledge on intraspecific clines is fundamental for uncovering the mechanisms shaping within-species variation along ecological gradients, while also contributing to improve our understanding of how intraspecific

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[1] Manuscript received 19 August 2016; revision accepted 7 November 2016.

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doi:10.3732/ajb.1600310

dynamics scale up to produce macro-ecological patterns (Woods et al., 2012; Pratt and Mooney, 2013; Hahn and Maron, 2016).

Most research on altitudinal gradients in plant defenses and herbivory has implicitly assumed a causative link between amounts of herbivore damage and concomitant plant allocation to defenses (Rasmann et al., 2014). However, it is also well recognized that herbivory is influenced by other plant traits not associated with resistance, such as traits associated with nutritional status (Karban, 1992; Agrawal, 2007). Nutrient concentrations in plant tissues (e.g., nitrogen, phosphorus) are usually low in relation to insect nutritional demands, which frequently makes nutrients a limiting factor for herbivores, and in some cases they are better predictors of herbivory than defenses (Carmona et al., 2011; Loranger et al., 2012). Surprisingly, to date, very few studies have examined altitudinal clines in plant nutritional traits and the extent to which they predict concomitant variation in herbivory. In addition, herbivory may also be influenced by abiotic factors such as climatic variables, which directly or indirectly (e.g., through effects on plant traits) influence herbivores (Masters et al., 1998; Bale et al., 2002). Similarly, climatic conditions have been shown to also influence geographic variation in plant defensive traits, independent of herbivory (Pearse and Hipp, 2012; Abdala-Roberts et al., 2016), and soil nutrient availability may influence plant defense allocation through growth-defense trade-offs whereby slow-growing plants living in nutrient-poor soils invest more in chemical defenses relative to fast-growing plants living in nutrient-rich soils (Coley et al., 1985; Pellissier et al., 2016). Collectively, these findings show that ecological clines in herbivory and plant defenses cannot be fully understood without taking plant nutritional traits and abiotic factors into account (Johnson and Rasmann, 2011; Pellissier et al., 2016).

Here we tested for altitudinal gradients in insect leaf herbivory, plant chemical defenses (phenolic compounds), and plant nutritional traits (phosphorus and nitrogen) in leaves of the long-lived tree *Quercus robur* L. (Fagaceae), and further investigated the abiotic factors associated with these altitudinal clines. To address this, we sampled 20 populations of *Q. robur* distributed along an altitudinal gradient in northwestern Spain spanning 35–869 m above sea level (Fig. 1). This gradient covered 85% of the altitudinal range of this oak species and varied substantially in abiotic factors (e.g., precipitation, temperature). Specifically, we asked the following questions: (1) Are there altitudinal clines in herbivore damage, plant chemical defenses, and nutritional traits? (2) Is altitudinal variation in herbivory associated with concomitant variation in plant defenses and nutritional traits? (3) Are abiotic factors (climate and soil variables) associated with altitudinal gradients in herbivory and plant traits? We tested for the concurrent effects of multiple abiotic factors associated with altitudinal variation in herbivory and plant traits by running separate multiple regressions including climatic and soil variables as predictors of herbivore damage, leaf defenses, and leaf nutrients. Overall, this study builds toward a better understanding of altitudinal clines in plant-herbivore interactions and the joint influence of biotic and abiotic factors shaping such patterns.

MATERIALS AND METHODS

Natural history—The pedunculate or English oak, *Q. robur*, is a long-lived deciduous tree that grows 20–40 m tall, and is widely distributed from the Iberian Peninsula to southern Scandinavia (Petit et al., 2002). This species tolerates a wide range of climatic conditions, but is frequently associated with nutrient-rich and well-drained soils and is dominant in most deciduous forests in northwestern Spain (Jones, 1959). The altitudinal range of this species spans from close to sea level up to 1000 m above sea level, although it is rarely found at elevations greater than 900 m in northwestern Spain. Leaf-burst occurs in early spring (April) and leaf drop takes place during early October. Leaves are approximately 8–12 cm long with 4–7 pairs of lobes on each side and have a very short petiole. Throughout its native range, this oak species is fed upon by a number of dietary specialist and generalist insect herbivores, mainly leaf chewers, miners, and gall formers (Southwood et al., 2005; Tack et al., 2010; Castagneyrol et al., 2012; Giffard et al., 2012).

Field sampling and leaf herbivory measurements—From early to late September (towards the end of the growing season) we surveyed 20 *Q. robur* populations distributed throughout northwestern Spain and spanning 35–869 m above sea level (Fig. 1; Appendix S1, see Supplemental Data with the online version of this article). The sampled populations spanned most of the altitudinal gradient experienced by this species, and they varied substantially in climatic conditions including a 1.7-fold gradient in mean annual precipitation (897–1563 mm) and close to a 4°C variation in mean annual temperature (10.7–14.5°C) (Fig. 1). All the populations were sampled within a three-week period and exhibited no detectable differences in leafing phenology, which could influence herbivory and plant defensive traits. None of the sites had a recent history of human impacts (e.g., cattle raising) or natural disturbances (e.g., fire).

Each population included at least 10 mature, reproductive individuals. We randomly selected five of these individuals per population. For each tree, we visually inspected leaf damage by insect herbivores on two randomly chosen low-hanging branches. Herbivory was estimated as the proportion of herbivore-damaged leaves by randomly choosing 25 leaves per branch and counting the number of damaged leaves, including consumption by chewers, miners, and gallers, but without differentiating among them (sucking insects were rare). For each of the two branches, we collected two fully expanded and undamaged leaves, which were then oven-dried for 48 h at 40°C, ground, and stored for subsequent quantification of phenolic compounds and nutrients. Careful inspection suggested relatively low leaf-to-leaf variation in herbivore damage within- and among-individual plants (X. Moreira, personal observation). Although young leaves typically exhibit higher levels of insect damage than mature leaves (Coley and Barone, 1996), we sampled mature leaves at the end of the growing season because this allowed us to assess cumulative leaf herbivory occurring over the entire growing season. This is possible given that leaf longevity of *Q. robur* is 5–6 months and that we sampled several weeks before the initiation of leaf drop. In addition, we note that although measuring the frequency of damaged leaves is less accurate than measuring the percentage of area consumed per leaf, we opted for the former approach because leaf miners and gallers were relatively common in some populations (recorded in approximately 10% of the leaves, X. Moreira, personal observation) and damage by these insects is difficult to assess in the field. By sampling undamaged leaves we reduced variation in defense levels caused by site-specific induction (Abdala-Roberts et al., 2016). However, some systemic

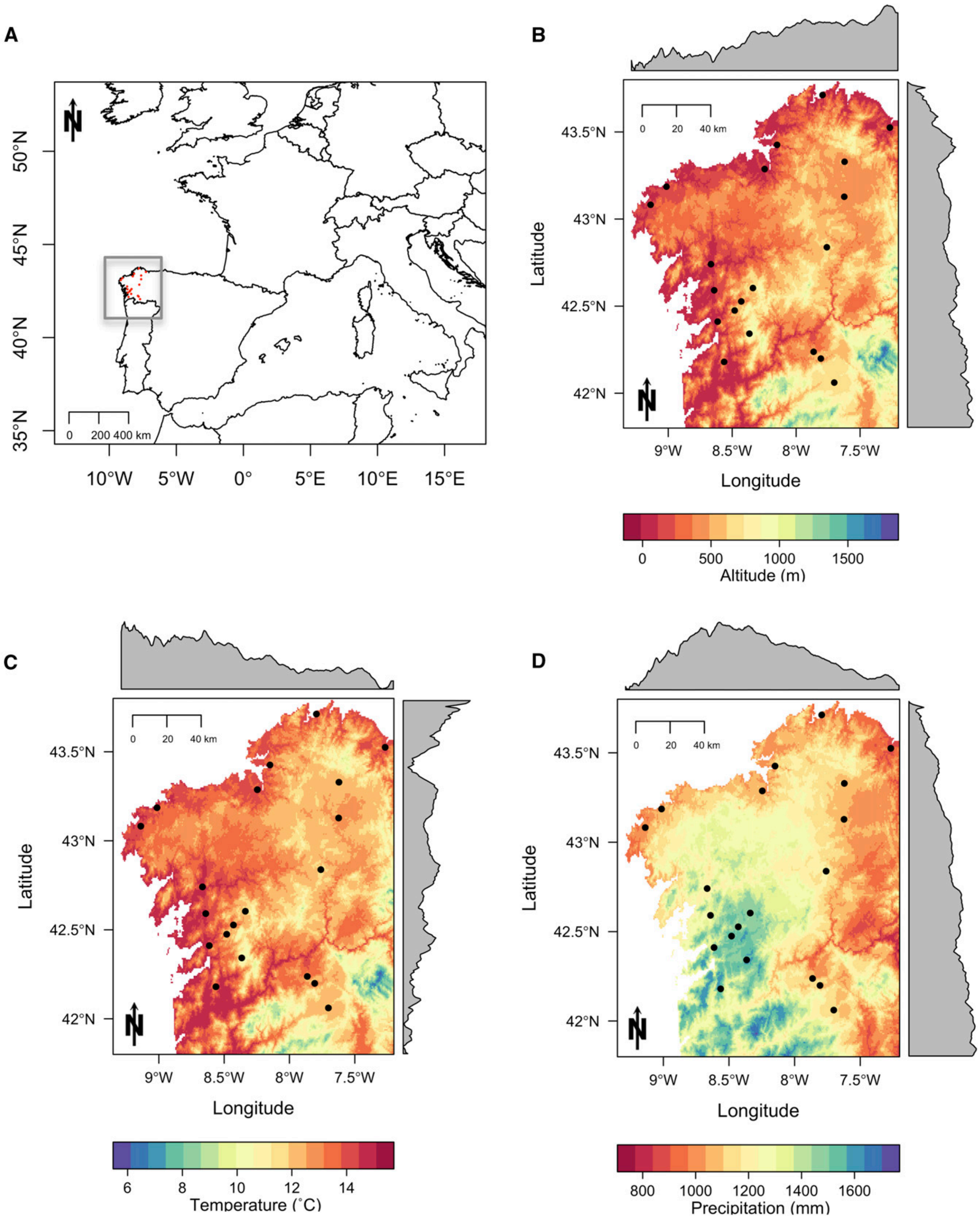

FIGURE 1 Overview of Western Europe indicating the study region in northwestern Spain (A), and a close-up of the study region showing (B) altitude (m above sea level), (C) mean annual temperature (°C), and (D) annual precipitation (mm). Black dots indicate the location of the sampled *Quercus robur* populations ($N = 20$).

induction may still occur, and therefore, the observed concentrations of defenses should be conservatively interpreted as a combination of induced and constitutive levels.

Quantification of plant chemical traits—As a proxy of leaf defenses, we chose phenolic compounds because they are widely recognized as herbivore feeding deterrents across many plant taxa (Salminen and Karonen, 2011; Mithöfer and Boland, 2012) and have been shown to confer resistance against insect herbivores in *Q. robur* (Feeny, 1970; Roslin and Salminen, 2008). Phenolic compounds were extracted using 20 mg of dry plant tissue with 1 mL of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation (Moreira et al., 2014). We diluted these methanolic extracts (1:5 vol:vol) and transferred them to chromatographic vials. We performed phenolic profiling according to Moreira et al. (2015a) with some modifications. Briefly, we used ultrahigh-pressure liquid chromatography-quadrupole-time-of-flight mass spectrometry (UHPLC-QTOF-MS) to detect, identify, and quantify phenolic compounds. The separation was carried out on a 50 × 2.1 mm Acquity UPLC BEH C18 column (Waters, Milford, CT, USA) thermostated at 25°C. Solvents were A= water + 0.05% vol. formic acid; B = acetonitrile + 0.05% vol. formic acid. The gradient program was performed at a flow rate of 0.4 mL/min under the following conditions: 5–30% B in 6 min, 30–100% B in 2 min, holding at 100% B for 2 min followed by re-equilibration at 5% B for 2 min. The injection volume was 2 µL. The QTOF-MS was operated in MS^E negative mode over an m/z range of 85–1200 Da with the following parameters: capillary voltage at −2.5 kV, cone voltage −25 V, source temperature 120°C, desolvation gas temperature 350°C, desolvation gas flow 800 L/hr. Internal calibration of the instrument was obtained by infusing a solution of leucine-enkephaline at 400 ng/mL at a flow rate of 15 µL/min through the Lock Spray probe (Waters Corporation, Milford, Massachusetts, USA). We identified phenolic compounds on the basis of their molecular formula (as determined from exact mass measurements), fragment ions, and comparison with available databases such as the Dictionary of Natural Products (Chapman & Hall, CRC Informa, London; version 20.2) or ReSpect for Phytochemicals (Sawada et al., 2012). We quantified flavonoids as rutin equivalents, condensed tannins as catechin equivalents, hydrolysable tannins as gallic acid equivalents, and lignins as ferulic acid equivalents. The gallic acid standard was obtained from Sigma-Aldrich. We achieved the quantification of these phenolic compounds by external calibration using calibration curves at 0.2, 0.8, 2, 5, and 20 µg/mL. For statistical analyses, we used the concentration of phenolic compounds measured as milligram per gram of tissue on a dry weight basis for each compound group, and also calculated total phenolics by summing the concentrations across these groups of phenolics.

For leaf nutrients, we chose phosphorus and nitrogen concentration because both of these variables are strongly (positively) correlated with levels of leaf herbivory across many plant taxa (Karban, 1992; Huberty and Denno, 2006), and previous work has reported such correlations for other oak species (e.g., Forkner et al., 2004; Eatough Jones et al., 2008). We digested approximately 0.1 g of grounded leaf material in a mixture of selenous sulphuric acid and hydrogen peroxide (Moreira et al., 2012). Diluted aliquots of the digestion were analyzed by colorimetry for quantification of nitrogen (indophenol blue method) and phosphorus (molybdenum blue method) concentration using a Biorad 650 microplate reader (Bio-Rad Laboratories, Philadelphia, Pennsylvania, USA) at 650 nm and 700 nm, respectively (Walinga et al., 1995). For statistical analyses, we used the concentration of nitrogen or phosphorus as milligram per gram of tissue on a dry weight basis.

Geographic and climatic and soil variables—We obtained the geographic coordinates of each *Q. robur* population using a Global Positioning System device (Garmin, Olathe, Kansas, USA). To characterize the climatic and soil conditions present at each population site, we used a subset of the bioclimatic variables of the WorldClim database (http://www.worldclim.org/) at the 30 s resolution, and soil variables from the SoilGrids database (http://www.soilgrids1km.isric.org) at 1 km grid resolution. Specifically, we used BIO1 (annual mean temperature, °C), BIO4 (temperature seasonality, expressed as the standard deviation of temperature among months × 100), BIO5 (maximum temperature of the warmest month, °C), BIO6 (minimum temperature of the coldest month, °C), BIO12 (annual precipitation, mm), BIO13 (precipitation of the wettest month, mm), BIO14 (precipitation of the driest month, mm), BIO15 (precipitation seasonality, expressed as standard deviation of precipitation across months) as climatic variables. For the soil variables, we used bulk density (kg m^{-3}), cation exchange capacity (cmolc kg^{-1}), coarse fragments (cm^{-3}), organic carbon stock (tonnes per ha), organic carbon content (parts per thousand), PH, percentage of clay, percentage of silt and percentage of sand in the topsoil (2.5 cm) and subsoil (150 cm). The procedures used to calculate these climatic and soil variables are fully described in Hijmans et al. (2005) and Hengl et al. (2014), respectively.

Statistical analyses—*Population variation in leaf herbivory and plant traits*—Prior to testing for altitudinal gradients, we determined whether there was variation among *Q. robur* populations in leaf herbivory, and in the concentration of leaf chemical defenses and nutrients. In each case, we ran general linear models testing for an effect of population.

Altitudinal gradients in herbivory and plant traits—We first assessed whether there were altitudinal clines in leaf herbivory, leaf chemical defenses, and nutrients by performing simple regressions between elevation and each response variable using population means. Subsequently, we proceeded to test for the effects of underlying factors associated with altitudinal variation in herbivory and plant traits by running separate multiple regressions for herbivory, leaf defenses, and leaf nutritional traits. To make use of the information from all climatic variables without inflating Type I error caused by multiple tests for each variable, prior to running these multiple regressions we summarized the eight climatic variables into two principal components (PC) by conducting principal component analyses (PCA) (Moreira et al., 2014; 2015b). Two axes explained 78% of the variance in the eight climatic variables across populations. The first PC (hereafter "PC precipitation") was positively associated with annual precipitation, precipitation of the wettest month, and precipitation seasonality. The second PC (hereafter "PC temperature") was positively associated with mean annual temperature and minimum temperature of the coldest month. Elevation was significantly (negatively) correlated with PC temperature ($r = -0.92$, $P < 0.0001$), but not with PC precipitation ($r = 0.30$, $P = 0.194$). Similarly, two axes explained 64% of the variance in 18 soil variables across populations. The first PC ("PC1 soil" hereafter) was positively associated with coarse texture in the topsoil and subsoil and negatively associated with percentage of sand in the topsoil and subsoil. The second

PC ("PC2 soil" hereafter) was positively associated with carbon content and stock in the topsoil and subsoil. Elevation was not correlated with either PC1 soil ($r = 0.03$, $P = 0.885$) or PC2 soil ($r = 0.17$, $P = 0.478$). The standardized z-scores of these climatic and soil PCs were used as predictors in the multiple regressions. First, we modeled herbivory as dependent upon leaf phosphorus concentration, nitrogen concentration, total phenolics, climatic variables (PC precipitation and PC temperature), and soil variables (PC1 soil and PC2 soil). Soil effects on herbivory are expected to be indirect and mediated by changes in plant traits such as leaf nutrients and defense compounds (already included in this model), but could also influence herbivory through effects on other unaccounted plant traits. Second, we ran separate multiple regressions where we modeled total leaf phenolics, leaf phosphorus or nitrogen as dependent upon climatic (PC precipitation and PC temperature) and soil (PC1 soil and PC2 soil) variables. We also performed separate multiple regressions for each group of phenolic compounds (Appendix S2).

General considerations—All GLM models were run in PROC GLM, the PCA analysis was conducted in PROC FACTOR (rotation = varimax), and simple and multiple regression models were run with PROC REG (SAS 9.4, SAS Institute, Cary, North Carolina, USA). In all cases, residuals were normally distributed.

RESULTS

Population variation in leaf herbivory and plant traits—There was substantial variation among *Q. robur* populations in leaf herbivory and in the concentration of total leaf phenolics. The frequency of leaf damage varied up to 3.9-fold among populations (0.22 ± 0.04 to 0.85 ± 0.05; $F_{19,80} = 4.87$, $P < 0.001$), whereas total leaf phenolics varied up to 2.5-fold (7.04 ± 0.66 to 17.16 ± 1.15 mg g^{-1} d.w.; $F_{19,80} = 4.01$, $P < 0.0001$). When broken down by group of phenolic compound we also found population variation in leaf flavonoids (2.4-fold, $F_{19,80} = 2.21$, $P = 0.0001$), lignins (5.3-fold, $F_{19,80} = 5.79$, $P < 0.0001$), condensed tannins (6.7-fold, $F_{19,80} = 3.17$, $P = 0.0002$), and hydrolysable tannins (4.8-fold, $F_{19,80} = 8.59$, $P < 0.0001$). Likewise, we also found population variation in leaf phosphorus concentration (1.7-fold, $F_{19,80} = 4.68$, $P < 0.001$) and nitrogen (1.4-fold, $F_{19,80} = 2.18$, $P = 0.008$) concentration.

Altitudinal gradients in herbivory and plant traits—Univariate regressions revealed a significant positive association between leaf herbivory and elevation, where plants located at higher altitudes exhibited a greater proportion of damaged leaves (Fig. 2A). Similarly, we also found a significant positive association between the concentration of total leaf phenolics and elevation (Fig. 2B). Individual analyses for each class of phenolic compounds indicated a significant altitudinal gradient only for flavonoids (Appendix S3) suggesting these compounds drove the relationship for total phenolics. In addition, we found a significant positive association between leaf phosphorus concentration and elevation (Fig. 2C), but no association between leaf nitrogen concentration and elevation (Fig. 2D).

Correlates of altitudinal gradients in herbivory and plant traits—The multiple regression model for leaf herbivory indicated that PC temperature and total phenolics were significantly negatively associated with herbivory (Table 1), where plants at warmer sites and with higher concentrations of phenolics exhibited lower amounts of leaf damage. In contrast, leaf phosphorus, nitrogen, PC precipitation, PC1 soil, and PC2 soil were not significantly associated with leaf herbivory (Table 1). We again ran this model including all four groups of phenolic compounds instead of total phenolics and found that hydrolysable tannins were the only defensive group significantly associated with herbivory (suggesting that these compounds drove the effect of total phenolics on damage). All the other results remained qualitatively unchanged relative to the multiple regression with total phenolics (Appendix S4).

Multiple regression also showed that PC temperature and PC2 soil were significantly associated with total leaf phenolics (Table 2), where plants at colder sites and growing in soils with higher amounts of carbon exhibited higher levels of leaf phenolics (Table 2). The PC precipitation and PC2 soil were not significantly associated with leaf phenolics (Table 2). Multiple regressions performed separately for each class of phenolic compounds indicated significant effects of temperature and soil variables only on flavonoids (Appendix S2), suggesting these compounds drove the association between temperature and total phenolics. On the other hand, multiple regressions indicated that PC temperature was the only significant predictor of leaf phosphorus concentration (Table 2), where populations at colder sites had higher amounts of phosphorus in leaves. The PC precipitation was significantly associated with leaf nitrogen concentration (Table 2), where populations at wetter sites had lower amounts of nitrogen in leaves, and marginally significantly associated with PC1 soil (i.e., coarser, less sandy soils) (Table 1).

DISCUSSION

We found evidence for altitudinal gradients in insect leaf damage and leaf chemistry in *Q. robur*, whereby the proportion of damaged leaves as well as the concentration of leaf phenolics and phosphorus increased toward higher elevations. In contrast, leaf nitrogen concentration was not associated with elevation. In addition, there was a significant negative association between insect herbivory and leaf phenolics, suggesting that leaf chemical defenses decrease leaf damage in *Q. robur*. However, our results indicate that altitudinal variation in these secondary metabolites was not associated with concomitant variation in insect leaf damage across the studied populations. If this had been the case, we would have expected the positive association between phenolics and elevation to result in a decrease in herbivory with increasing elevation, rather than the observed increase. Temperature was the most important factor associated with the altitudinal increase in herbivory, indicating that altitudinal gradients in herbivory are associated with abiotic factors independently of altitudinal clines in plant defenses.

A long-held view in ecology is that warmer and less seasonal climatic conditions lead to stronger plant-herbivore interactions, which in turn select for higher defenses in plants (Schemske et al., 2009). It is therefore expected that plants growing closer to sea level (where climate is warmer and more stable) experience higher amounts of herbivore damage and should invest more in defenses than their high-elevation counterparts (which experience colder, more seasonal conditions and lower herbivory) (Rasmann et al., 2014). Although a number of studies have found support for this prediction (e.g., Scheidel and Bruelheide, 2001; Rodríguez-Castañeda

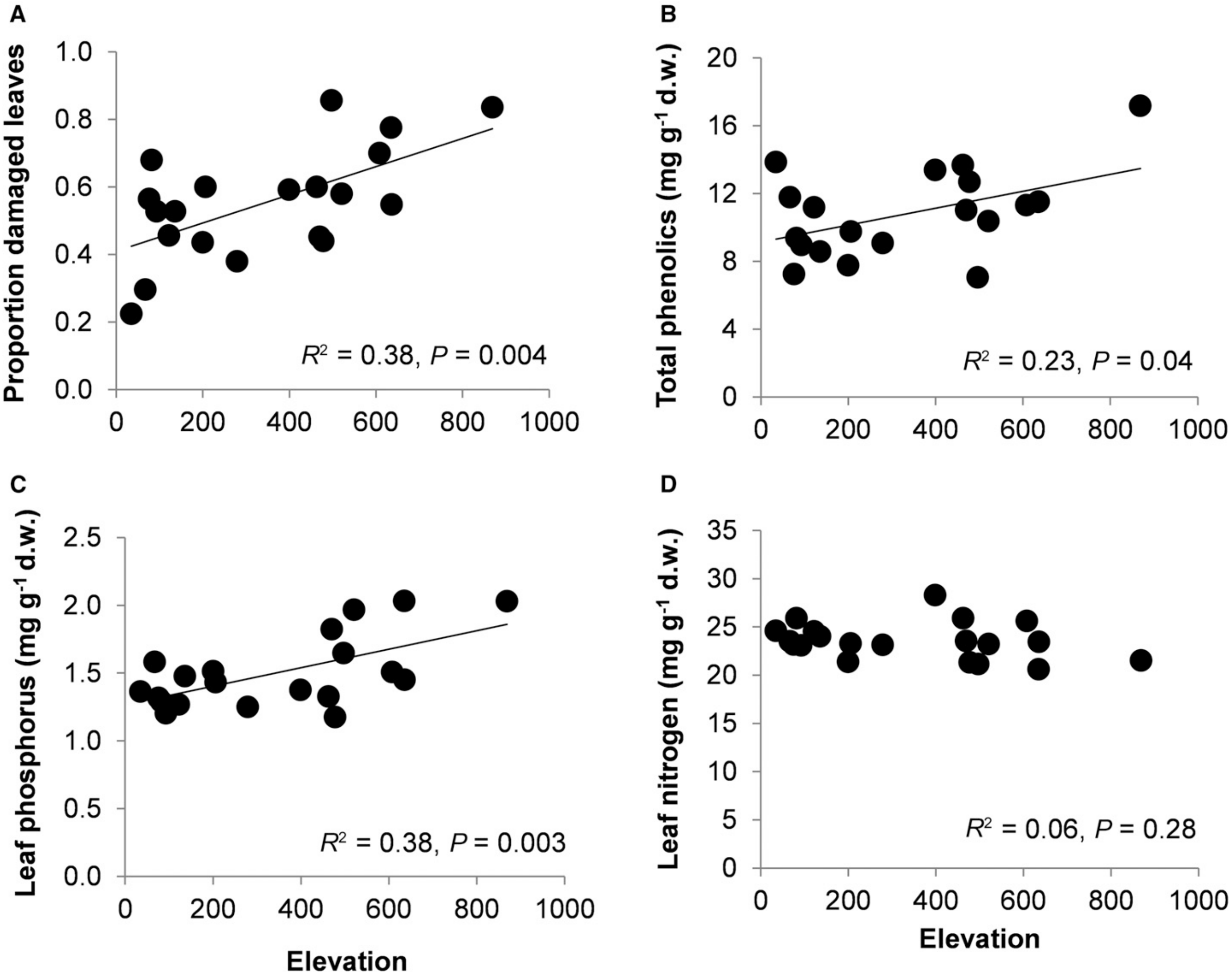

FIGURE 2 Altitudinal clines for (A) leaf herbivory (proportion of damaged leaves), and (B) the concentration of total phenolics, (C) phosphorus, and (D) nitrogen in leaves of *Quercus robur* sampled across 20 populations spanning an altitudinal gradient (35–869 m above sea level) in northwestern Spain. Circles represent population means. Predicted relationships, R^2 and P-values come from simple linear regressions.

et al., 2010), other studies have found no evidence for altitudinal gradients in plant defense or herbivory, and in some cases have even found opposite trends, i.e., greater herbivory and/or higher plant defenses with increasing elevation (reviewed by Rasmann et al., 2014). Our study is in line with the latter group of studies, because we found that *both* herbivory and plant phenolics in *Q. robur* increased with decreasing temperature toward higher altitudes. These results could be explained by the fact that the upper limit of our altitudinal gradient was relatively low (900 m a.s.l.), whereby *Q. robur* (and its associated insect fauna) found at high-elevation sites experience more moderate abiotic conditions (e.g., maximum summer temperatures decrease with increasing elevation) than their low-elevation counterparts that are exposed to high temperatures and water stress, which are characteristic of summers in the study region. This observation reconciles our results with the general expectation that plants growing in less stressful and climatically more stable environments (presumably at higher elevations in this case) experience more herbivory and invest more in defenses. Nonetheless, despite there being a significant negative association between damage and total leaf phenolics suggesting that, as for other oak species (Feeny, 1970; Roslin and Salminen, 2008), these compounds confer resistance against insect herbivores, our results indicate that altitudinal clines in leaf defenses and herbivory are presumably unrelated in *Q. robur*. Indeed, the negative association between phenolics and herbivory would have presumably led to decreasing herbivory with altitude, but we instead found increasing damage with altitude. Based on these findings, we argue that altitudinal clines in herbivory and chemical defenses in *Q. robur* are uncoupled.

Recent work has underscored the importance of considering abiotic factors in testing for altitudinal gradients in plant traits and herbivory (Pellissier et al., 2016). Our study is one of the few to test for and disentangle the concurrent effects of multiple abiotic factors associated with altitudinal gradients in herbivory and plant traits. We found that temperature was the single most important

TABLE 1. Results from a multiple regression model testing for the effects of leaf phosphorus concentration, leaf nitrogen concentration, total concentration of phenolics in leaves, climatic factors (z-score values from a principal components analysis summarizing a suite of variables associated with precipitation or temperature), and soil variables (z-score values from a principal components analysis summarizing a suite of variables associated with soil properties) on insect leaf herbivory in *Quercus robur* trees sampled from 20 populations spanning an altitudinal gradient (35–869 m a.s.l.) in northwestern Spain. Values used in the regression were population means, β = slope estimator, r^2 = partial correlation coefficient. Significant effects ($P < 0.05$) are bolded.

	Leaf herbivory		
Predictor	β	r^2	*P*-value
Phosphorus	0.140	0.078	0.333
Nitrogen	−0.0008	0.0001	0.971
Total phenolics	−0.043	0.395	**0.016**
PC temperature	−0.140	0.517	**0.003**
PC precipitation	−0.039	0.105	0.258
PC1 soil	−0.014	0.016	0.664
PC2 soil	−0.051	0.171	0.142

factor associated with leaf herbivory in *Q. robur*, whereby damage increased with decreasing temperature toward higher elevations. The fact that temperature significantly influenced herbivory after accounting for leaf traits in the multiple regression suggests that temperature directly influences herbivores (and thus damage) by, for example, altering insect physiological tolerance, behavior, or reproduction (Chown and Nicolson, 2004). Given the relatively low upper limit of the altitudinal range sampled, high-altitude sites likely enjoyed a more moderate climate relative to hotter and drier low-elevation sites imposing greater abiotic stress on insect herbivores (and plants). Nonetheless, we cannot discard the possibility that temperature indirectly influenced herbivory through changes in some other unmeasured plant traits along the elevational gradient. Further work measuring a larger number of plant traits is needed to robustly evaluate and separate direct and indirect effects of temperature on herbivory in *Q. robur*.

Interestingly, we found that phosphorus concentration in leaves of *Q. robur* increased with elevation, a pattern that may have resulted from a concomitant gradient in soil phosphorus availability or higher rates of phosphorus uptake by *Q. robur* with increasing elevation (phosphorus deposition from human-related activities is negligible because the study sites have no recent history of farming). There was no detectable effect of phosphorus on herbivory in *Q. robur*, but a relatively strong negative correlation between phosphorus and PC temperature ($r = -0.53$, $P = 0.01$) could have made it difficult to separate the effects of these two factors in this study. Further work involving a larger sample size and experimental manipulations (e.g., greenhouse experiments, or reciprocal transplants along the altitudinal gradient) is necessary to demonstrate whether leaf phosphorus content (or other nutritional traits) is associated with altitudinal gradients in herbivory for *Q. robur*. More broadly, we argue that future studies are needed to test for the joint effects of plant nutritional traits and defenses on altitudinal gradients in herbivory (Cuevas-Reyes et al., 2004; Carmona et al., 2011).

In addition to the association of temperature with herbivory, abiotic factors were also independently correlated with the concentration of phenolics and nutritional traits in leaves of *Q. robur*. Our finding that the concentration of leaf phenolics increased with decreasing temperature toward higher elevations is in line with previous work (e.g., De Long et al., 2015). In addition, we also found that populations growing in soils with greater amounts of carbon exhibited higher levels of leaf phenolics presumably because decreasing soil carbon availability limits the ability of plants to invest in carbon-based defenses such as phenolics. There was no evidence that abiotic factors influenced defense allocation through growth-defense trade-offs, whereby we would have expected populations found in wetter sites (presumably higher in resource availability) or in soils associated with greater resource availability to invest more in growth and less in defense (Abdala-Roberts et al., 2016; Pellissier et al., 2016). Furthermore, precipitation-related variables and soil characteristics did not covary with altitude, suggesting that the altitudinal gradient in leaf phenolics was not influenced by these abiotic factors. It is also worth noting that some groups of phenolic compounds (e.g., flavonoids) are involved in photo-protection (Ballaré, 2014) and may be induced by higher solar radiation at higher elevations. However, there was no association between solar radiation and altitude ($R^2 = 0.008$, $P = 0.78$), suggesting that this abiotic factor was not responsible for the observed altitudinal trends in leaf phenolics. Collectively, our findings suggest that temperature, which was associated with phenolics and covaried with altitude, is the primary factor associated with (and potentially drive) altitudinal clines in *Q. robur* defensive chemistry. Likewise, temperature was the only factor significantly associated with leaf phosphorus concentration suggesting that this climatic factor is also associated with altitudinal variation in this nutrient.

Research on altitudinal gradients in plant-herbivore interactions and concomitant variation in species traits has gained momentum in recent years (Rasmann et al., 2014). Further work should ideally combine observational data with manipulative experiments to assess the relative importance of genetic vs. environmental sources of trait variation and rigorously demonstrate clinal adaptation while controlling for confounding factors (e.g., Pennings et al., 2009; Woods et al., 2012). There is also an increasing recognition of the

TABLE 2. Results from multiple regression models testing for the effects of climatic variables (z-score values from a principal components analysis summarizing a suite of variables associated with precipitation or temperature) and soil variables (z-score values from a principal components analysis summarizing a suite of variables associated with soil properties) on leaf chemical defenses (total phenolics, mg g^{-1}) and nutrients (phosphorus and nitrogen, mg g^{-1}) in *Quercus robur* trees from 20 populations spanning an altitudinal gradient in northwestern Spain. Values used in the regression are population means, β = slope estimator, r^2 = partial correlation coefficient. Significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.10$) effects are bolded and italicized, respectively.

	Total phenolics			Phosphorus			Nitrogen		
Predictor	β	r^2	*P*-value	β	r^2	*P*-value	β	r^2	*P*-value
PC temperature	−1.247	0.312	**0.019**	−0.160	0.38	**0.008**	0.270	0.03	0.464
PC precipitation	−0.355	0.036	0.465	0.056	0.07	0.294	−0.970	0.33	**0.015**
PC1 soil	−0.220	0.013	0.651	−0.078	0.13	0.159	0.755	0.23	*0.054*
PC2 soil	1.105	0.267	**0.034**	−0.047	0.05	0.379	0.345	0.056	0.347

importance of measuring damage by multiple herbivore species or guilds (e.g., Moreira et al., 2015b), screening for a large number of plant defensive and nutritional traits, and differentiating between plant resistance and tolerance (Więski and Pennings, 2014). These are examples of aspects to consider if we are to advance current understanding of the ecological drivers and evolutionary consequences of environmental clines in plant-insect interactions.

ACKNOWLEDGEMENTS

The authors thank Silvia Portela and Joaquín Moreira for their assistance during fieldwork; and Helena Pazó, Silvana Poceiro, and Patricia Toledo for their help with chemical analyses. We also thank Prof. Jean-Luc Wolfender (University of Geneva) for kindly providing rutin and ferulic acid standards. The authors thank Nina Sletvold (the subject editor), Carina Baskett, and an anonymous reviewer for comments on a previous version of the paper. Research was financially supported by a grant from the Regional Government of Galicia (IN607D 2016/001), a Spanish National Research Grant (AGL2015-70748-R), and the Ramón y Cajal Research Program (RYC-2013-13230) to XM. LAR was supported by the CONACyT Repatriation Program (#250934).

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