

RESEARCH PAPER

Leaf habit does not determine the investment in both physical and chemical defences and pair-wise correlations between these defensive traits

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Deciduous; evergreen; herbivory; phenolic compounds; proteins; *Quercus*; Resource availability hypothesis; trichomes.

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ABSTRACT

- Plant life-history strategies associated with resource acquisition and economics (e.g. leaf habit) are thought to be fundamental determinants of the traits and mechanisms that drive herbivore pressure, resource allocation to plant defensive traits, and the simultaneous expression (positive correlations) or trade-offs (negative correlations) between these defensive traits. In particular, it is expected that evergreen species – which usually grow slower and support constant herbivore pressure in comparison with deciduous species – will exhibit higher levels of both physical and chemical defences and a higher predisposition to the simultaneous expression of physical and chemical defensive traits.
- Here, by using a dataset which included 56 oak species (*Quercus* genus), we investigated whether leaf habit of plant species governs the investment in both physical and chemical defences and pair-wise correlations between these defensive traits.
- Our results showed that leaf habit does not determine the production of most leaf physical and chemical defences. Although evergreen oak species had higher levels of leaf toughness and specific leaf mass (physical defences) than deciduous oak species, both traits are essentially prerequisites for evergreenness. Similarly, our results also showed that leaf habit does not determine pair-wise correlations between defensive traits because most physical and chemical defensive traits were simultaneously expressed in both evergreen and deciduous oak species.
- Our findings indicate that leaf habit does not substantially contribute to oak species differences in plant defence investment.

INTRODUCTION

Herbivores consume approximately 15% of annual net primary productivity in most terrestrial ecosystems (Cyr & Pace 1993), and to resist them, plants have evolved potent anti-herbivore defences (Agrawal 2011; Johnson 2011). This plant defensive arsenal includes a range of both physical structures (e.g. spines, trichomes) and chemical compounds (e.g. phenolics, alkaloids) that reduce plant nutritional quality and are toxic or deterrent to herbivores (Agrawal 2007, 2011; Núñez-Farfán *et al.* 2007). These physical and chemical traits exhibit tremendous variation among plant species and even among individual plants within species (Agrawal 2007; Núñez-Farfán *et al.* 2007). Accordingly, over the past half century much of the ecological and evolutionary research on plant defences has focused on trying to understand the origin and maintenance of such diversity of defensive traits (Stamp 2003; Agrawal 2007).

The basic assumption underlying most previous research on plant defence variation is that plant defences are costly to produce and maintain (Stamp 2003; Mason *et al.* 2016). On the one hand, because plants possess a finite pool of resources and some defensive traits are redundant in their actions against

herbivores, trade-offs between different defensive traits (e.g. physical and chemical defences; Koricheva *et al.* 2004; Eichenberg *et al.* 2015) should be common and underlie variation in plant defence allocation (Stamp 2003). However, previous studies that have evaluated the emergence of trade-offs between defensive traits have found mixed support for univariate trade-offs; furthermore some of these studies found positive relationships (Koricheva *et al.* 2004; Moles *et al.* 2013). As a single defensive trait or strategy frequently does not provide effective resistance against multiple herbivore species (Agrawal & Fishbein 2006), plants might need to simultaneously express two defensive traits including both plant chemical and physical defences (Moles *et al.* 2013; Johnson *et al.* 2014; Moreira *et al.* 2016). On the other hand, variation in plant defences might occur as a result of the costs and benefits of investing in defences as a function of inherent plant growth rate, which is basically determined by resource availability [Resource availability hypothesis (RAH); Coley *et al.* 1985; Endara & Coley 2011]. Negative fitness impact of losing tissue area through herbivory is higher for slow-growing plant species adapted to resource-poor environments than for fast-growing species adapted to resource-rich environments, as the former cannot

quickly produce new tissues after herbivory damage (Coley *et al.* 1985; Endara & Coley 2011). Accordingly, slow-growing species tend to allocate more resources to defences as resource limitation makes the replacement of tissues consumed by herbivores more difficult (Coley *et al.* 1985; Endara & Coley 2011).

Plant life-history strategies associated with resource acquisition and economics are likely important determinants of the traits and mechanisms that drive herbivory patterns, resource allocation to different defensive traits and pair-wise correlations between these defensive traits (Wright *et al.* 2004). For example, leaf habit of plant species has been considered a fundamental driver of plant-level resource partitioning and storage, as well as levels of herbivore damage (Givnish 2002; Piper & Fajardo 2014; Wyka *et al.* 2016). Evergreen species exhibit a conservative resource-use strategy (relative to deciduous species), with lower photosynthetic and respiration rates and reduced resource allocation to growth (Lohbeck *et al.* 2015). In contrast, deciduous species exhibit an exploitative resource-use strategy (relative to evergreen species), with higher photosynthetic and respiration rates, traits commonly associated with a rapid resource capture and high growth rates (Reich *et al.* 1998; Poorter & Garnier 2007). According to the RAH, we might thus expect that slower-growing evergreen species invest more in both physical and chemical defences (Moreira *et al.* 2014). Moreover, because evergreen species have leaves with a longer lifespan leaf tissue is more consistently available to herbivores compared with deciduous species that have a period without leaves (Orians & Solbrig 1977; Karban 2007; Pringle *et al.* 2011; Pearse & Karban 2013). This pattern can hold for short-lived plants or plants with shorter leaf longevity because these plants can avoid some herbivores in time and do not need to invest in traits that impart defence or tolerance (Krimmel & Pearse 2016). Finally, we might also expect that evergreen species, which are subjected to longer and more persistent episodes of herbivore damage, will tend to simultaneously produce physical and chemical defences, whereas deciduous species, which support shorter herbivore events (Orians & Solbrig 1977; Pringle *et al.* 2011; Pearse & Karban 2013), should be inherently more predisposed to trade-offs between different defensive traits. Unfortunately, how the simultaneous expression or trade-offs between both physical and chemical defences differ among plant species with contrasting leaf habit has been addressed in only a handful of studies (Pringle *et al.* 2011).

By using a dataset that included 56 oak species (*Quercus* genus) we previously evidenced that leaf habit is phylogenetically conserved and markedly influences herbivory patterns by leaf chewers and miners (Pearse & Hipp 2009). Here we go a step further and investigate whether leaf habit of plant species governs the investment in both physical and chemical defences and pair-wise correlations between these defensive traits. Based on the predictions of the RAH and the expected patterns of herbivore pressure, we hypothesised that evergreen species – which usually grow slower and support constant herbivore pressure – should (i) exhibit higher levels of both physical and chemical defences, and (ii) exhibit a higher predisposition to the simultaneous expression of physical and chemical defensive traits than deciduous species. By addressing these goals our study builds towards a better understanding of how life-history traits contribute to tree species differences in plant defence investment.

MATERIAL AND METHODS

Study system and field site

To test our objectives, we used the dataset of Pearse & Hipp (2009, 2012), which included 56 oak species with contrasting leaf habit (24 deciduous and 32 evergreen species; Table S1). These species represents each of the five major oak clades (*Quercus* with 32 species, *Cerris* with 12 species, *Cyclobalanopsis* with one species, *Lobatae* with eight species and *Protobalanus* with three species) and includes species from eastern North America, western North America and Mexico (Nearctic region), Europe and Asia (Palearctic region; Table S1). This sample represents 15% of the species that comprise the entire genus and covers a substantial portion of the distribution range, as well as phylogenetic and ecological diversity of the genus (Pearse & Hipp 2009). We studied these oak species in a 40-year-old stand of oaks at the University of California-Davis Arboretum, where each species was represented by three mature (acorn-bearing) individuals, except in cases where fewer were present in the arboretum (see more details of the experimental design in Pearse & Hipp 2009, 2012). Leaf traits in oaks show strong differences among species (Pearse & Hipp 2009), and affect the survival of a generalist herbivore (Pearse 2011). Most defensive traits of oak leaves are not phylogenetically conserved (Pearse & Hipp 2009) and show strong heritability within a species (Pearse *et al.* 2015) and in hybrid crosses (Pearse & Baty 2012), suggesting that leaf defences are easily evolvable characteristics of oaks. To some degree, leaf defences may be the product of gene \times environment interactions, which could complicate the interpretation of measurements of traits in a common garden. However, predictors of herbivory to oaks planted at sites across North America were similar among sites (Pearse & Hipp 2014), suggesting consistency of oak phenotypes across environments.

Estimation of physical and chemical plant defences

Constitutive (*i.e.* always expressed in the plants) investment in four physical (specific leaf mass, toughness, trichome density and water content) and four chemical [total phenolics, condensed tannins, proteins and peroxidase activity (POX)] defensive traits in the leaves were measured (Pearse & Hipp 2009, 2012). These defensive traits have been demonstrated to provide oak resistance against a diverse array of herbivores (*e.g.* Feeny 1970; Forkner *et al.* 2004; Roslin & Salminen 2008; Pearse & Hipp 2009; Pearse 2011; Abdala-Roberts *et al.* 2016a). In particular, lower water and protein content and higher specific leaf mass, toughness, trichome density, total phenolics, condensed tannins and peroxidase activity are associated with lower levels of herbivory in oaks (Feeny 1970; Roslin & Salminen 2008; Pearse 2011). By estimating only constitutive defences, our goal was to reduce variation in defences caused by species-specific induction of plant defences (Abdala-Roberts *et al.* 2016b). Sampling undamaged leaves does not completely eliminate induction, as induced responses may be systemic. Therefore, levels of physical and chemical defences measured likely represent a mixture between constitutive levels of defence plus some unknown level of induction, where our sampling scheme was aimed at reducing some of the variation due to induction.

As physical defensive traits, the specific leaf mass on five undamaged leaves per individual tree (as $\text{g}\cdot\text{cm}^{-2}$ dry weight) was measured. Leaf toughness on three undamaged leaves per tree was recorded with a penetrometer (Chatillon & Sons, New York, USA; as $\text{g}\ 3\ \text{mm}\ \varnothing\ \text{needle}^{-1}$). The proportion of water content on five undamaged leaves per individual tree was recorded as (fresh weight – dry weight)/fresh weight. Trichome density on the leaves was recorded as the average of trichome density on the upper and lower surface of three undamaged leaves per individual tree using a transparent grid (number of trichomes per 25-mm^2 grid). To estimate chemical defensive traits, fresh leaf material from at least five undamaged leaves per oak individual was collected and ground in liquid nitrogen. The chemical defensive traits quantified were: (i) concentration of total phenolics using a colorimetric and Prussian Blue assay (Graham 1992), (ii) concentration of condensed tannins using a colorimetric acid butanol assay (Porter *et al.* 1986), (iii) concentration of proteins with the Bradford reagent method (Bradford 1976), and (iv) peroxidase (POX) activity ($\Delta[\text{Absorbance}_{470\text{nm}}]\ \text{min}^{-1}$) with the fluorimetric guaiacol method (Harauchi & Yoshizaki 1982). Details of protocols for defensive trait quantification are listed in Pearse & Hipp (2009, 2012). It is important to note that in addition to their roles in deterring herbivores some of these plant traits (*e.g.* specific leaf mass, water and protein content) are key ecophysiological traits related to resource economics and might also arise due to selection on water- and nutrient-use dynamics.

Statistical analyses

Leaf habit effect on physical and chemical defensive traits

In order to determine whether there was an effect of leaf habit (deciduous *versus* evergreen species) on levels of physical and chemical defences, we used phylogenetic generalised least-squares models (PGLS; Martins & Hansen 1997). We entered defensive traits as a response variable (using species means) and the leaf habit as the main factor. We log-transformed the proportion of water content data to achieve normality.

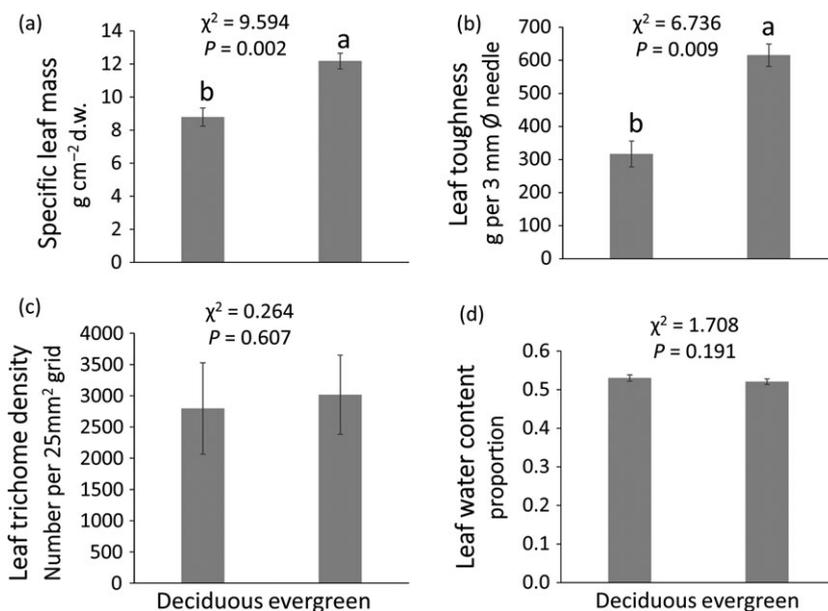


Fig. 1. Effect of leaf habit on leaf physical defences. Levels of (a) specific leaf mass, (b) leaf toughness, (c) leaf trichome density and (d) proportion of leaf water content in 56 oaks species with contrasting leaf habit (24 deciduous and 32 evergreen species) from Palearctic and Nearctic regions. Bars are least square means \pm SE ($N = 3$ trees per species). Chi-square values (χ^2) and associated significance levels (P) of the effect of leaf habit after controlling for phylogeny using phylogenetic generalised least-squares models (PGLS) are shown. Different letters indicate significant ($P < 0.05$) differences between leaf habits.

Pair-wise correlations between physical and chemical defences

To test for pair-wise correlations between physical and chemical defences, we performed species-level correlations among these traits separately for deciduous and evergreen species, accounting for phylogenetic non-independence in a maximum likelihood PGLS framework (Pagel 1999). We used the phylogenetic tree of *Quercus* genus developed by Pearse & Hipp (2009). We estimated phylogeny of these oak species using Bayesian Markov chain Monte Carlo and minimum evolution methods on an amplified fragment length polymorphism (AFLP) dataset (Pearse & Hipp 2009).

RESULTS

Physical defences

Two physical defensive traits in the leaves significantly differed between deciduous and evergreen oak species (Fig. 1). Specifically, specific leaf mass and toughness were 38% and 94% higher, respectively, in evergreen species than in deciduous species (Fig. 1a and b). However, trichome density and proportion of water content in leaves did not significantly differ between deciduous and evergreen oak species (Fig. 1c and d).

Chemical defences

Leaf habit did not significantly affect the production of chemical defences in leaves (Fig. 2). Specifically, concentration of leaf phenolics (Fig. 2a), condensed tannins (Fig. 2b) and proteins (Fig. 2c) and POX activity (Fig. 2d) in the leaves did not significantly differ between deciduous and evergreen oak species.

Pair-wise correlations between physical and chemical defences

We found 22 significant pair-wise correlations (18 of them considered as simultaneous expression of two defensive traits) across evergreen oak species between levels of physical and chemical defences in leaves (Table 1). In particular, for

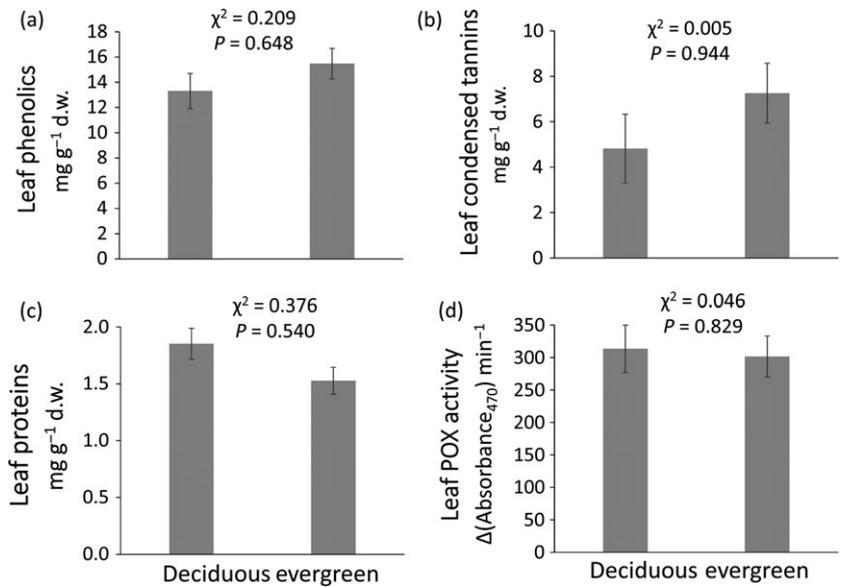


Fig. 2. Effect of leaf habit on leaf chemical defences. Levels of (a) phenolics, (b) condensed tannins, (c) proteins and (d) peroxidase (POX) activity in the leaves of 56 oak species with contrasting leaf habit (24 deciduous and 32 evergreen species) from Palearctic and Nearctic regions. Bars are least square means \pm SE ($N = 3$ trees per species). Chi-square values (χ^2) and associated significance levels (P) of the effect of leaf habit after controlling for phylogeny using phylogenetic generalised least-squares models (PGLS) are shown.

evergreen species we found that species that had higher levels of specific leaf mass had higher levels of toughness and lower levels of water content, and invested more in phenolics (Table 1). Evergreen species that had higher levels of toughness invested more in phenolics (Table 1). Evergreen species that had higher levels of trichomes invested more in condensed tannins (Table 1). Evergreen species that had lower levels of proteins invested more in phenolics and condensed tannins (Table 1).

For deciduous species we found that species that had higher levels of specific leaf mass had higher levels of toughness and trichome density, lower levels of water content and invested more in phenolics (Table 1). Deciduous species that had lower levels of water content invested more in phenolics and had lower levels of proteins (Table 1). Deciduous species that had more trichomes invested more in condensed tannins and had higher levels of leaf toughness and lower levels of proteins (Table 1). Finally, deciduous species that invested more in condensed tannins and phenolics had lower levels of proteins (Table 1).

Finally, we also found four significant pair-wise correlations that are considered as defensive trade-offs. Specifically, for evergreen species we found that species with higher levels of leaf trichomes had lower levels of leaf phenolics, and species with higher levels of leaf POX had also higher levels of leaf proteins (Table 1). For deciduous species we found that species with higher levels of leaf POX had lower levels of leaf phenolics and higher levels of leaf proteins (Table 1).

DISCUSSION

Contrary to our initial hypotheses, we found that leaf habit does not determine the production of leaf physical and chemical defences in oak species. Although evergreen oak species had higher levels of leaf toughness and specific leaf mass than deciduous oak species, both traits are fundamental requirements for evergreenness. Similarly, our results also showed that leaf habit does not determine pair-wise correlations between defensive traits because most physical and chemical defensive

traits were simultaneously expressed in both evergreen and deciduous oak species. Together, these findings indicate that leaf habit makes a weak contribution to oak species differences in plant defence investment.

Although there is substantial theory to suggest that leaf habit should affect investment in leaf defences (e.g. Pringle *et al.* 2011), in our case there were no overall differences in defence investment between evergreen and deciduous species other than the physical traits that are required for evergreenness (*i.e.* toughness and specific leaf mass). These findings suggest that while leaf habit somewhat overlaps with the leaf economic spectrum (as described by specific leaf mass and related physical defences), other leaf physical defences (e.g. trichomes and water content) and all chemical defences can apparently evolve independently (lack of differences with leaf habit, no pair-wise correlations between specific leaf mass and defences like POX and tannins, and inconsistent/context dependent pair-wise correlations between specific leaf mass and trichomes and total phenolics). Our findings do not support the predictions of the RAH (Coley *et al.* 1985; Endara & Coley 2011) because evergreen oaks with usually lower growth rates and higher costs of tissue production and replacement should have allocated more resources to defence. It is important to note that confounding effects of phenotypic plasticity in response to short-term changes in resources, in combination with selection for different strategies in different habitats may make it difficult to capture physical and chemical defence responses to leaf habit. The fact that the species are growing in a 40-year-old stand, and not in their natural habitats, could explain the absence of effect of leaf habit on these defences.

Results of previous studies demonstrating the existence of trade-offs between physical and chemical defences or the simultaneous expression of these defensive traits have been mixed (e.g. Koricheva *et al.* 2004; Moles *et al.* 2013; Eichenberg *et al.* 2015). For instance, using a dataset that included four chemical and six physical defensive traits on 261 plant species, Moles *et al.* (2013) observed that only five of the 45 pair-wise correlations between physical and chemical defensive traits were significant, three of them were trade-offs and two of them

Table 1. Pearson correlation coefficients (and *P*-values within brackets) from pair-wise correlations between several leaf physical [toughness, proportion of water content, specific leaf mass (SLM) and trichome density] and chemical [concentration of phenolics, condensed tannins, peroxidase (POX) and proteins] defensive traits in deciduous (above diagonal, *N* = 24 species) and evergreen (below diagonal, *N* = 32 species) oak species. The *P*-values are from a log-likelihood test between a model (pGLS) with the second trait in question as the predictor of the first *versus* an uninformed model. Significant coefficients (*P* < 0.05) are in bold.

	toughness	water	SLM	trichomes	phenolics	tannins	POX	proteins
toughness	×	−0.11 (0.583)	0.71 (<0.001)	0.41 (0.022)	0.27 (0.225)	−0.02 (0.918)	−0.31 (0.133)	−0.18 (0.428)
water	−0.07 (0.590)	×	−0.61 (0.005)	−0.10 (0.606)	−0.71 (<0.001)	−0.03 (0.870)	0.17 (0.394)	0.48 (0.023)
SLM	0.43 (0.009)	−0.67 (<0.001)	×	0.35 (0.020)	0.46 (0.010)	0.21 (0.189)	−0.27 (0.112)	−0.28 (0.140)
trichomes	−0.12 (0.484)	0.04 (0.852)	0.05 (0.795)	×	0.09 (0.723)	0.76 (<0.001)	−0.38 (0.079)	−0.50 (0.030)
phenolics	0.44 (0.009)	−0.16 (0.481)	0.41 (0.018)	−0.52 (0.022)	×	−0.07 (0.663)	−0.43 (0.015)	−0.55 (0.004)
tannins	−0.12 (0.488)	0.13 (0.569)	0.13 (0.484)	0.39 (0.023)	−0.30 (0.083)	×	−0.14 (0.523)	−0.49 (0.035)
POX	−0.23 (0.100)	0.21 (0.240)	−0.27 (0.052)	0.10 (0.499)	−0.21 (0.131)	−0.32 (0.072)	×	0.68 (0.001)
proteins	−0.25 (0.124)	−0.01 (0.958)	−0.20 (0.223)	0.27 (0.095)	−0.42 (0.006)	−0.32 (0.041)	0.60 (0.002)	×

were considered as simultaneous expression of two traits. Similarly, a meta-analysis by Koricheva *et al.* (2004), which included 31 studies on 22 different plant species, found that pair-wise correlations between physical and chemical defensive traits varied from positive to negative. In this sense, our initial hypotheses in the current study aimed at providing a new potential explanation for these contrasting findings, *i.e.* plant life-history strategies such as leaf habit might markedly determine the strength and direction of associations between different defensive traits. Compared with deciduous species, leaves of evergreen species are available for a longer time (Orians & Solbrig 1977; Karban 2007; Pearse & Karban 2013), which would, in turn, increase herbivore chance of locating and completing their development on them. Under a scenario of constant herbivore pressure, we hypothesised that evergreen species (relative to deciduous species) should be inherently more predisposed to the simultaneous expression of two defence traits, which would provide higher levels of resistance than independent defensive traits. However, across a geographically broad sample of oak species, our results showed that in most cases physical and chemical defensive traits were simultaneously expressed in both evergreen and deciduous species. In one of the few available studies, Pringle *et al.* (2011) observed that contrary to our results, correlations between leaf physical and chemical traits largely varied between deciduous and evergreen tropical trees. Although the assumption of constant herbivore pressure in evergreen species has been demonstrated in a few systems (Karbon 2007, 2008; Pearse & Karban 2013; but see Pringle *et al.* 2011), this pattern has been poorly investigated in plant systems that support a large population of herbivores with members of different feeding guild and diet breadth. In this sense, a previous study in our common garden showed that evergreen oaks had marginally higher levels of leaf chewing damage, but lower levels of leaf mining damage in comparison with deciduous oak species (Pearse & Hipp 2009). This result suggests that the coexistence between herbivore species that

share the same resource on species with contrasting leaf habit might be compromised (Denno *et al.* 1995). Therefore, it is likely possible that certain groups of herbivores will be dominant in deciduous species. This fact might support the need for simultaneous expression of multiple defences in both evergreen and deciduous oak species. Alternatively, it is also possible that co-evolutionary dynamics between evergreen and deciduous species and herbivores determine the sign (direction) of the relationship between herbivore damage and plant defences to vary from a positive (*i.e.* herbivory drives defences) to a negative (*i.e.* defence drives herbivory) relationship, depending on which stage of the co-evolutionary arms race the plant–herbivore interaction is found at (Abdala-Roberts *et al.* 2016b). Accordingly, two fruitful avenues of future research will be (i) to examine how leaf habit in plants drives herbivory across multiple herbivore species varying in traits such as feeding guild and dietary specialisation, and (ii) to identify the current stage of the co-evolutionary arms race between oaks and herbivores.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. List of the 56 oak species (*Quercus* genus) used in this study. Information about leaf habit (deciduous *versus* evergreen) and biogeography (Palearctic *versus* Nearctic) is shown.

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