Received: 21 October 2019 | Accepted: 19 February 2020

DOI: 10.1111/1365-2745.13376

RESEARCH ARTICLE

Journal of Ecology BRITISH ECOLOGICAL SOCIETY

Ontogenetic consistency in oak defence syndromes

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Funding information
Regional Government of Galicia Grant, Grant/Award Number: IN607D 2016/001; Ramón y Cajal Research Programme, Grant/Award Number: RYC-2013-13230; Spanish National Research Grant, Grant/Award Number: AGL2015-70748-R

Handling Editor: Tobias Züst

Abstract

1. Plant species allocate resources to multiple defensive traits simultaneously, often leading to so-called defence syndromes (i.e. suites of traits that are co-expressed across several species). While reports of ontogenetic variation in plant defences are commonplace, no study to date has tested for ontogenetic shifts in defence syndromes, and we know little about the ecological and evolutionary drivers of variation in plant defence syndromes across ontogeny.
2. We tested for ontogenetic variation in plant defence syndromes by measuring a suite of defensive and nutritional traits on saplings and adult trees of 29 oak (*Quercus*, Fagaceae) species distributed across Europe, North America, and Asia. In addition, we investigated if these syndromes exhibited a phylogenetic signal to elucidate the nature of their macro-evolutionary variation, whether they were associated with levels of herbivore pressure and climatic conditions, and if any such evolutionary and ecological patterns were contingent on ontogeny.
3. Our analyses revealed three distinct oak defence syndromes: the first included species with high defences, the second species with high defences and low nutrient levels, and the third species with high nutrients and thinner leaves. Interestingly, these defence syndromes remained virtually unchanged across the two ontogenetic stages sampled. In addition, our analyses indicated no evidence for a phylogenetic signal in oak syndromes, a result consistent across ontogenetic stages. Finally, with respect to ecological factors, we found no effect of climatic conditions

on defences for either ontogenetic stage, whereas defence syndromes were associated with differing levels of herbivory in adults but not saplings suggesting an association between herbivore pressure and syndrome type that is contingent on ontogeny.

4. *Synthesis*. Together, these findings indicate that defence syndromes remain remarkably consistent across oak ontogenetic stages, are evolutionarily labile, and while they appear unrelated to climate, they do appear to be associated with herbivory levels in an ontogenetic-dependent manner. Overall, this study builds towards a better understanding of ecological and evolutionary factors underlying multivariate plant defensive phenotypes.

KEYWORDS

adult trees, chemical defences, insect herbivory, nutrients, physical defences, *Quercus*, saplings

1 | INTRODUCTION

Plants are invariably attacked by multiple herbivore species. As a result, plant species have evolved numerous defences against herbivores, including chemical and physical defences, tolerance mechanisms, as well as nutritional traits which determine tissue quality to herbivores (Agrawal, 2007; Núñez-Farfán, Fornoni, & Valverde, 2007). The bulk of research has typically addressed patterns and sources of variation in individual defensive traits, which has often revealed key traits affecting plant–herbivore interactions. However, looking at individual traits in isolation may overlook potentially important phenomena arising from the concurrent contribution of multiple traits to overall defence. For example, a number of studies have reported on patterns of correlated expression in physical and chemical defences (Agrawal & Fishbein, 2006; Kursar & Coley, 2003), and interactive effects among traits (e.g. synergy between chemical compounds; Rasmann & Agrawal, 2009; Richards, Dyer, Smilanich, & Dodson, 2010). These studies have revealed the often complex, multivariate nature of plant defensive phenotypes, thus emphasizing the need for considering the simultaneous expression of multiple traits to fully understand plant defence evolution.

Plant defence syndromes are suites of traits putatively associated with herbivory that are co-expressed, and, by definition, provides a multi-variate view of plant defence (Abdala-Roberts et al., 2018; Agrawal & Fishbein, 2006; Defossez, Pellissier, & Rasmann, 2018; Kariñho-Betancourt, Agrawal, Halitschke, & Núñez-Farfán, 2015; Kursar & Coley, 2003; Moreira, Sampedro, Zas, & Pearse, 2016; Pringle et al., 2011; Walters, 2010). Research has shown that defence syndromes can be driven by shared evolutionary history, whereby phylogenetically related plant species, frequently attacked by the same or similar herbivore species, share similar suites of defensive traits (Berenbaum, 1983; Futuyma & Agrawal, 2009). Alternatively, defence syndromes may be also dictated by evolutionary convergence whereby unrelated plant species growing under similar ecological conditions (i.e. abiotic conditions, herbivore pressure) evolve similar patterns of trait co-expression (Becerra, 2007; Moreira et al., 2016). While these studies have yielded valuable information on plausible evolutionary scenarios underlying plant defence syndromes, research has barely investigated the underlying ecological factors shaping syndrome occurrence and variation. These factors range from plant endogenous processes (e.g. allocation costs, metabolic and developmental constraints) to extrinsic biotic (e.g. herbivory) and abiotic (e.g. climate, soil conditions) pressures. Accordingly, evolutionary, ecological, and plant endogenous factors may concurrently shape plant defence syndromes and thus warrant investigation.

There is good evidence that plant defences vary throughout development (Barton & Boege, 2017; Barton & Koricheva, 2010). For long-lived plants, some authors argue that defence levels build-up from the sapling stage to later stages of adult development due to a greater pool of resources available to fulfil defensive functions (e.g. Barton & Koricheva, 2010; Boege & Marquis, 2005). Consequently, better-defended adults should exhibit lower overall herbivory than saplings. Alternatively, plant defences may vary as a function of the risk of being attacked as described by the 'Plant Apparency Hypothesis' (Feeny, 1976). Because adult plants are larger and more conspicuous than saplings, the former should exhibit higher rates of herbivory (Moreira, Glauser, & Abdala-Roberts, 2017; Smilanich, Fincher, & Dyer, 2016), and, expectedly, invest more in defences. Despite these appealing predictions, few studies have measured both plant defences and herbivory across ontogeny (but see Moreira et al., 2017; Pringle, Dirzo, & Gordon, 2012), thus preventing an assessment of linkages between expression of plant defences and herbivore pressure throughout plant development. More broadly, research has also generally failed to explicitly consider multivariate patterns of defence expression as a function of ontogeny (but see Kariñho-Betancourt et al., 2015). Nonetheless, just as individual traits may exhibit ontogenetic variation, we can expect trait co-expression patterns (i.e. those structuring syndromes) to also vary with plant ontogeny. This could occur due to ontogenetic changes in plant endogenous processes (allocation costs between traits) and exogenous factors (e.g. conspicuousness to attackers, number or composition of attacking herbivores) concurrently affecting multiple correlated traits. To our knowledge, there are no studies available testing for ontogenetic shifts in plant defence syndromes or their underlying drivers or correlates.

In this study, we investigated whether patterns of trait co-expression segregated into discernible defence syndromes and whether such syndromes exhibited ontogenetic shifts by measuring multiple leaf traits for naturally occurring saplings and adult trees of 29 oak species (*Quercus*, Fagaceae) distributed across Europe, North America and Asia (Figure 1). In addition, we investigated whether any such syndromes exhibited a phylogenetic signal, whether they were associated with ecological factors (herbivore pressure and climate), and if syndrome phylogenetic patterns and syndrome associations with ecological factors were contingent on oak ontogeny. Specifically, we asked: (a) Do oak species cluster into defence syndromes based on discernible patterns of leaf trait co-expression? (b) Do defence syndromes change with plant ontogeny? and (c) Are oak defence syndromes structured by phylogenetic relatedness and/or associated with climate or differing levels of herbivore pressure, and do these associations depend on oak ontogenetic stage? To this end, for both saplings and adult trees found in naturally occurring populations we quantified physical (specific leaf area and trichomes), chemical (phenolic compounds), and nutritional (phosphorus and nitrogen) traits, as well as leaf damage by chewing insects. By measuring groups of candidate traits structuring defence syndromes across contrasting plant ontogenetic stages, and further assessing ecological and evolutionary features associated with such syndromes, this study advances our understanding of ontogenetic variation in multivariate plant defensive phenotypes.

2 | MATERIALS AND METHODS

2.1 | Natural history

The 29 oak (*Quercus*) species studied cover large forested areas in the Northern Hemisphere (Cavender-Bares, 2019; Manos, Doyle, & Nixon, 1999). Of this total, 16 have a Palearctic distribution range (*Q. acutissima*, *Q. canariensis*, *Q. cerris*, *Q. coccifera*, *Q. faginea*, *Q. frainetto*, *Q. glauca*, *Q. ilex*, *Q. lusitanica*, *Q. petraea*, *Q. phillyraeoides*, *Q. pubescens*, *Q. pyrenaica*, *Quercus robur*, *Q. serrata*, and *Q. suber*), and 13 have a Nearctic distribution (*Q. agrifolia*, *Q. alba*, *Q. bicolor*, *Q. falcata*, *Q. macrocarpa*, *Q. nigra*, *Q. palustris*, *Q. phellos* *Q. rubra*, *Q. shumardii*, *Q. stellata*, *Q. texana*, and *Q. velutina*; Figure 1). Six of these species are evergreen (*Q. agrifolia*, *Q. coccifera*, *Q. glauca*, *Q. ilex*, *Q. phillyraeoides*, and *Q. suber*) and the rest are deciduous. At the sampling sites, leaf burst for deciduous species usually occurs in April and leaves turn brown and are shed in October.

Oak species support a speciose community of specialist and generalist insect herbivores, many of which are leaf chewers (Giffard, Jactel, Corcket, & Barbaro, 2012; Moreira, Castagneyrol, et al., 2018; Moreira et al., 2017; Pearse & Hipp, 2009; Southwood, Wint, Kennedy, & Greenwood, 2005; Tack & Roslin, 2011). Notorious examples of leaf-chewers in the New World include the leaf-tying caterpillar *Psilocorsis quercicella*, the western tussock moth *Orgyia vetusta*, and the gold-spotted oak borer *Agrilus coxalis* (Lill & Marquis, 2001; Marquis et al., 2019; Oswalt, Clatterbuck, & Houston, 2006; Pearse & Hipp, 2009; Sork, Stowe, & Hochwender, 1993), whereas in the Old World common chewing insects are the gypsy moth *Lymantria dispar*, the oak processionary *Thaumetopoea processionea*, the sycamore *Acronicta aceris*, the pale tussock *Elkneria pudibunda*, and the purple hairstreak *Favonius quercus* (Annighöfer, Beckschäfer, Vor, & Ammer, 2015; Giffard et al., 2012; Tack, Ovaskainen, Pulkkinen, & Roslin, 2010).

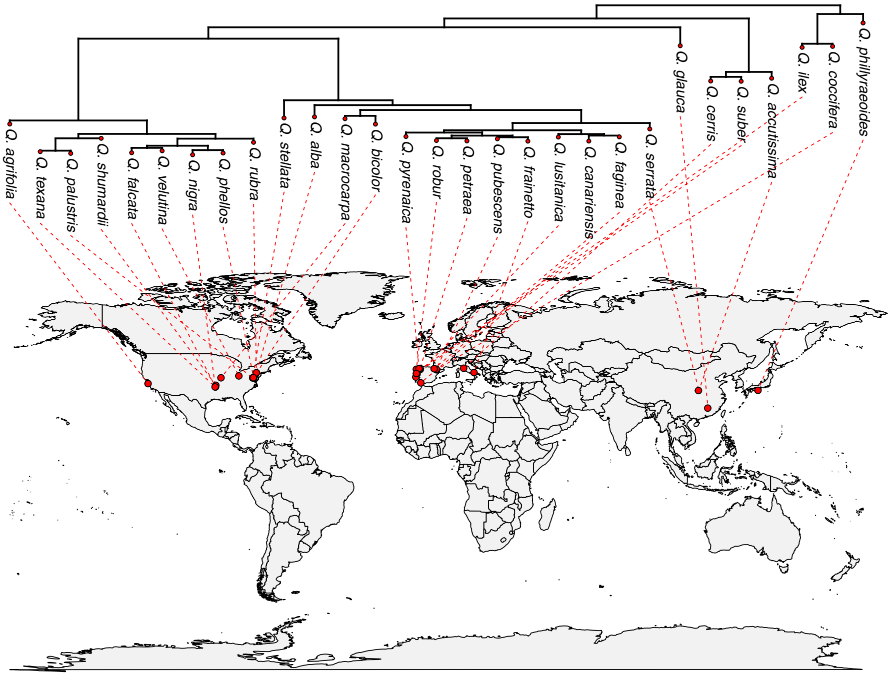


FIGURE 1 Map of the world showing the sampling sites and the phylogenetic tree of the studied oak (*Quercus*) species based on Single Nucleotide Polymorphism matrices by ddRAD sequencing [Colour figure can be viewed at wileyonlinelibrary.com]

In turn, oak species possess several physical (e.g. toughness, trichomes) and chemical (e.g. phenolic compounds) leaf traits which are toxic and deterrent to a broad range of phytophagous insects (Feeny, 1970; Forkner, Marquis, & Lill, 2004; Frost & Hunter, 2008; Galmán, Petry, et al., 2019; Moreira, Castagneyrol, et al., 2018; Moreira & Pearse, 2017; Pearse & Hipp, 2009, 2012; Roslin & Salminen, 2008). In addition, several nutritional traits (e.g. phosphorus, nitrogen) are typically found in low concentrations in oak leaves and can therefore act as limiting resources for herbivore growth and development (Forkner & Hunter, 2000; Moreira, Castagneyrol, et al., 2018).

2.2 | Field sampling and leaf herbivory measurements

At the end of the growing season (late September to mid-October), we surveyed 3–5 populations of each oak species separated at least by 5 km. Each population was comprised of at least 15 adult oak trees of which we haphazardly selected five adult (reproductive) trees and five saplings (<1 m tall). In total, we sampled 953 trees corresponding to 29 oak species × 3–5 populations × 2 ontogenetic stages × 5 individuals.

For each adult tree, we randomly selected two low-hanging branches (2–3 m from the ground) and collected 25 leaves per branch. For saplings, we collected as many as 15 leaves distributed throughout the canopy. All sampled leaves were transported to the laboratory, oven-dried for 48 hr at 40°C, and then shipped to Spain where they were scored by the same person (A. Galmán) to avoid biases in leaf damage estimates. Collected leaves were mostly damaged by chewing insects, and to a much lesser extent by leaf miners or other herbivore guilds (<5% of sampled leaves; A. Galmán, personal observation). For each leaf, we visually estimated the percent leaf area removed by chewing insects (‘leaf herbivory’ hereafter) using the following scale: 0 = undamaged; 1 = 1%–5% damaged; 2 = 6%–10% damaged; 3 = 11%–25% damaged; 4 = 26%–50% damaged; 5 = 51%–75% damaged; 6 = >75% damaged; Moreira, Abdala-Roberts, et al., 2019). For adult trees, we averaged values across all leaves to obtain a mean value per branch, and then averaged across branches to obtain a single mean per tree for statistical analyses. For saplings, we averaged values across all leaves to obtain a single mean per plant for statistical analyses. Although this methodology may have underestimated leaf herbivory by mammals (e.g. deer) as removal of entire leaves may have gone unnoticed, direct and indirect evidence suggests that mammals were usually rare or present in low abundances at most of the study sites.

We collected four additional fully expanded leaves per branch of each adult plant and six additional fully expanded leaves near the apical meristem in the case of saplings to quantify leaf traits (see ahead). In the case of evergreen species, we only collected expanded leaves produced during the sampling season (easily identified by their colour and texture). We collected all leaves towards the end of the growing season rather than at the beginning because subsequent mechanical damage and leaf tissue removal due to sampling may affect subsequent defence allocation particularly for saplings of slow-growing species (which averaged merely 10–12 leaves depending on the species). In addition, we selected leaves with little or no evidence of herbivory to reduce variation in defences or nutrients caused by site-specific leaf physiological changes (e.g. induction of defences) due to herbivory (Moreira, Abdala-Roberts, et al., 2019). Still, systemic induction may have occurred and, as a result, our trait measurements likely reflected constitutive levels plus some unknown level of systemic induction. We also took special care to sample only completely expanded leaves of similar age (position on branch, colour and consistency) and location in the canopy (for adults). Similarly, we avoided expanding leaves or those close to senescence to control for variation in leaf age which may influence oak defences and herbivory. Immediately after collection, leaves were oven-dried for 48 hr at 40°C and then shipped to Spain. Once there, leaves were ground with liquid nitrogen, and stored at room temperature before conducting chemical analyses.

2.3 | Quantification of oak physical defences

We estimated leaf trichome density and specific leaf area (SLA), both of which have been identified as oak putative physical defences (Lill et al., 2006). Trichomes can reduce herbivory by physically inhibiting feeding or movement (Fordyce & Agrawal, 2001), whereas low SLA is correlated with high leaf toughness (Hanley, Lamont, Fairbanks, & Rafferty, 2007; Pearse & Hipp, 2009) and can therefore act as a proxy of structural resistance against herbivory. Accordingly, previous work has demonstrated that these traits are associated with plant phylogeny and damage by a diverse array of herbivores (e.g. Pearse, 2011; Pearse & Hipp, 2009). We visually estimated trichome density of each leaf using an index of the percentage of leaf area covered by trichomes: 0 = no trichomes; 1 = 1%–5% of the leaf area covered by trichomes; 2 = 6%–10% of the leaf area covered by trichomes; 3 = 11%–25% of the leaf area covered by trichomes; 4 = 26%–50% of the leaf area covered by trichomes; 5 = 51%–75% of the leaf area covered by trichomes, and 6 = > 75% of the leaf area covered by trichomes. We estimated SLA for each leaf by dividing the surface area of a 20-mm diameter disk by its dry mass. We took measurements of trichomes and SLA from only one leaf per plant as previous trials for some oak species indicated low leaf-to-leaf variation within individual plants for these traits (SLA: mean CV = 6.81; trichome density: mean CV = 5.20).

2.4 | Quantification of oak chemical defences

Phenolic compounds are considered putative defences against insect herbivores in oaks (Feeny, 1970; Moreira, Abdala-Roberts, et al., 2018; Moreira, Castagneyrol, et al., 2018; Roslin & Salminen, 2008), and therefore represent a suitable model for chemical defences for the studied species. Phenolic compounds were extracted from 20 mg of dry leaf tissue with 1 ml of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation (Moreira et al., 2014). We then transferred the extracts to chromatographic vials. For phenolic quantification, we used Ultra-High-Performance Liquid-Chromatograph (UHPLC Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. The compound separation was carried out on a Kinetex™ 2.6 µm C18 82–102 Å, LC Column 100 × 4.6 mm, protected with a C18 guard cartridge. The flow rate was 0.4 ml/min and the 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% B and using a gradient to obtain 30% B at 4 min, 60% B at 10 min, 80% B at 13 min and 100% B at 15 min. The injection volume was 10 µl. For phenolic compound identification, we used an ultra-performance liquid chromatography coupled with electrospray ionization quadrupole (Thermo Dionex Ultimate 3000 LC) time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS; Bruker Compact™). We identified four groups of phenolic compounds: flavonoids, ellagitannins and gallic acid derivatives ('hydrolysable tannins' hereafter), proanthocyanidins ('condensed tannins' hereafter) and hydroxycinnamic acid precursors to lignins ('lignins' hereafter). We quantified flavonoids as rutin equivalents, condensed tannins as catechin equivalents, hydrolysable tannins as gallic acid equivalents, and lignins as ferulic acid equivalents (Galmán, Petry, et al., 2019; Moreira, Abdala-Roberts, et al., 2018). We achieved the quantification of these phenolic compounds by external calibration using calibration curves at 0.25, 0.5, 1, 2 and 5 µg/ml. We expressed phenolic compound concentrations in mg/g tissue on a dry weight basis.

2.5 | Quantification of oak nutritional traits

Phosphorus and nitrogen in leaves have been shown to be associated with leaf herbivory across a number of plant taxa (Huberty & Denno, 2006; Mattson, 1980), and previous work has shown positive correlations between insect leaf herbivory and these nutrients in oaks (e.g. Eatough Jones, Paine, & Fenn, 2008; Forkner & Hunter, 2000). To quantify these traits, we digested approximately 0.1 g of ground dried leaf material in a mixture of selenous sulphuric acid and hydrogen peroxide (Moreira, Zas, & Sampedro, 2012). Diluted aliquots of the digestion were analysed by colorimetry for quantification of nitrogen (indophenol blue method) and phosphorus (molybdenum blue method) concentration using a Biorad 650 microplate reader (Bio-Rad Laboratories) at 650 and 700 nm, respectively (Walinga, Van Der Lee, & Houba, 1995). We expressed nitrogen and phosphorus concentrations in mg/g tissue on a dry weight basis.

2.6 | Molecular analyses

For each species, we used two leaves from three plants of different origin than those sampled in the field (purchased from Planfor nursery, Uchacq-et-Parentis, France) and grown under greenhouse conditions for molecular analyses. We built a phylogenetic tree of *Quercus* species based on Single Nucleotide Polymorphism matrices by ddRAD sequencing (Figure 1). For this, we extracted DNA following Pandey and Tamta (2015), purified it using the Isolate II Plant DNA kit (Bioline) and quantified it by fluorimetric methods (Qubit, Thermo Fisher Scientific; see Moreira, Abdala-Roberts, et al., 2018) for more details of the molecular analyses).

We used RAxML v8 software for the phylogenetic reconstruction of the *Quercus* phylogeny based on SNP matrices that included variable sites. We enabled the ascertainment bias correction for the GTRGAMMA model (-m ASC GTRGAMMA) and we tested the two types of corrections (–err-corr = lewis and –asc-corr = felsenstein) described in Leaché, Banbury, Felsenstein, Oca, and Stamatakis (2015). We activated the RAxML option for rapid bootstrapping and the number of bootstraps were automatically determined using the option #- autoMRE. Albeit there were some exceptions, relationships for New World oak species in our phylogenetic tree were in general terms similar to those reported by Hipp et al. (2018) (see Figure S1).

2.7 | Climatic variables

To characterize the climatic conditions present at each oak population location, we used the 19 BIOCLIM variables from the WorldClim version 2.0 database (http://worldclim.org/version2/) at the 30-s resolution (Fick & Hijmans, 2017). For statistical analyses, we averaged values for climatic variables across populations of each species.

2.8 | Statistical analyses

For each oak species, we constructed trait distance matrices using Euclidean distances of scaled mean trait values (*dist* function in R, the STATS package; R Core Team, 2019), separately for adults and saplings. We defined patterns of trait similarity among species using hierarchical cluster analysis to create defence phenograms for saplings and adult trees (*hclust* function in R, the STATS package). The clustering was performed based on the Ward's linkage method and Euclidean distances (Ward Jr., 1963) which combines subgroups (initially building from one species) at each iteration so as to minimize the within-cluster ANOVA sum-of-squares (Murtagh & Legendre, 2013). We further described the main clusters for saplings and adult trees using linear discriminant analysis on the scaled trait data matrix (*lda* function in the MASS package in R; Ripley et al., 2019), and we visualised the ordination for each plant defence trait for each ontogenetic stage using LDA loading values (Agrawal & Fishbein, 2006). In addition, we examined the strength with which particular traits contributed to differences among clusters using discriminant function analysis (Agrawal & Fishbein, 2006). To evaluate whether defence syndromes vary between ontogenetic stages, we used a Mantel test to compare the distance matrices based upon defence phenograms of saplings and adult trees (*mantel.test* function in the APE package in R; Paradis, Claude, & Strimmer, 2004). A $p < 0.05$ indicates that the adult and sapling defence phenograms are correlated with each other (i.e. are similar). Additionally, we assessed ontogenetic variation in individual defensive traits which may be masked by our analysis of syndromes, and reported these results as Supporting Information. For these univariate tests, we used phylogenetic generalized least squares (PGLS) analyses based on species trait values to account for phylogenetic non-independence among oak species (pgls function in the CAPER package in R; Orme, 2013). This analysis assumed a Brownian motion model for trait evolution and the λ parameter was estimated by maximum likelihood to avoid inflating Type I errors.

To determine whether any such ontogenetic differences in oak defence syndromes were structured by oak phylogenetic relationships, we used Mantel tests to compare the defensive phenogram and the oak species phylogenetic tree using distance matrices separately for each ontogenetic stage (Moreira et al., 2016). A $p < 0.05$ indicates that the defence phenograms and phylogenetic distance are correlated with each other (Agrawal & Fishbein, 2006). We additionally ran analyses testing for a phylogenetic signal (λ value; Pagel, 1999) of individual traits separately for saplings and adult trees using data at the species level (phyloSignal function in the PHYLOSIGNAL package in R; Keck, Rimet, Bouchez, & Franc, 2016) and report these results as Supporting Information.

To assess whether oak defence syndromes were structured by climatic factors and if any such pattern was contingent on ontogeny, we used Mantel tests to compare the defensive and climatic phenograms separately using distance matrices for each ontogenetic stage. Here again, a $p < 0.05$ indicates that the defensive and climatic phenograms are correlated with each other. As follow-up tests, we performed phylogenetically corrected multiple regressions (pgls function in the CAPER package in R; Orme, 2013) to test for effects of climatic factors on individual defensive traits which may be masked or overlooked by our analysis of syndromes, and these findings are reported in the Supporting Information. For these analyses, we used the *z*-scores of the first principal component (PC) of two independent PCAs, one for temperature-related variables and another for precipitation-related variables.

Finally, to determine whether defence syndromes were associated with leaf herbivory and if any such pattern was contingent on ontogeny, we tested for adults and saplings for differences in leaf herbivory among species clusters from the phenogram analysis based on species trait values by using PGLS analyses to account for phylogenetic non-independence among oak species (pgls function in the CAPER package in R; Orme, 2013).

3 | RESULTS

The hierarchical cluster analysis of leaf defensive and nutritional traits revealed three distinct clusters (i.e. defence syndromes) for both saplings and adults (Table 1; Figure 2). For saplings, the first syndrome (cluster A) was comprised of oak species ($n = 15$) with high nitrogen content and high SLA (i.e. thinner leaves; Table 1a; Figure 2a). The second syndrome (cluster B) contained oak species ($n = 3$) with high lignins and hydrolysable and condensed tannins (Table 1a; Figure 2a). The third syndrome (cluster C) was represented by oak species ($n = 11$) with high flavonoids and low SLA and nitrogen (Table 1a; Figure 2a). Likewise, for adult trees, we observed an equivalent pattern whereby the first syndrome

TABLE 1 Contribution of the leaf defensive and nutritional traits to the clustering of defensive traits across 29 oak species for (a) saplings and (b) adult trees. Trait values (least square mean ± *SE*) for the three defensive clusters (syndromes) and coefficients for linear discriminant analyses (LDA scaling) are shown. Significant ($p < 0.05$) differences between clusters are typed in bold

Ontogeny	Variables	LDA scaling	Cluster A	Cluster B	Cluster C	*p*-value
(a) Saplings	Flavonoids	0.10	−0.56 ± 0.59	0.12 ± 0.56	0.73 ± 1.10	**0.017**
	Lignins	−3.13	−0.36 ± 0.52	2.50 ± 0.74	−0.19 ± 0.44	**<0.001**
	Condensed tannins	0.62	−0.60 ± 0.53	1.11 ± 1.17	0.51 ± 0.97	**0.006**
	Hydrolysable tannins	−0.72	−0.70 ± 0.49	1.85 ± 0.81	0.45 ± 0.61	**<0.001**
	Trichomes	0.66	−0.40 ± 0.70	−0.49 ± 0.31	0.68 ± 1.12	0.085
	Specific leaf area (SLA)	−0.30	0.62 ± 0.84	0.30 ± 0.48	−0.93 ± 0.44	**<0.001**
	Phosphorus	−0.41	0.29 ± 1.01	0.30 ± 0.65	−0.48 ± 0.93	1
	Nitrogen	0.16	0.48 ± 0.94	0.60 ± 0.37	−0.82 ± 0.6	**0.006**
(b) Adults	Flavonoids	0.46	−0.36 ± 0.75	0.10 ± 0.54	1.22 ± 0.98	**0.007**
	Lignins	−1.54	−0.18 ± 0.82	2.50 ± 0.54	−0.20 ± 0.39	**<0.001**
	Condensed tannins	−1.40	−0.31 ± 0.79	2.13 ± 0.37	0.37 ± 0.82	**0.006**
	Hydrolysable tannins	−0.67	−0.31 ± 0.71	2.38 ± 1.11	0.29 ± 0.77	**<0.001**
	Trichomes	0.87	−0.05 ± 0.94	−0.91 ± 0.32	0.47 ± 1.21	1
	SLA	1.38	0.29 ± 0.86	0.60 ± 0.00	−1.23 ± 0.55	**0.007**
	Phosphorus	−0.58	0.22 ± 1.02	0.12 ± 0.46	−0.82 ± 0.63	0.577
	Nitrogen	0.04	0.25 ± 0.83	1.16 ± 0.44	−1.26 ± 0.26	**<0.001**

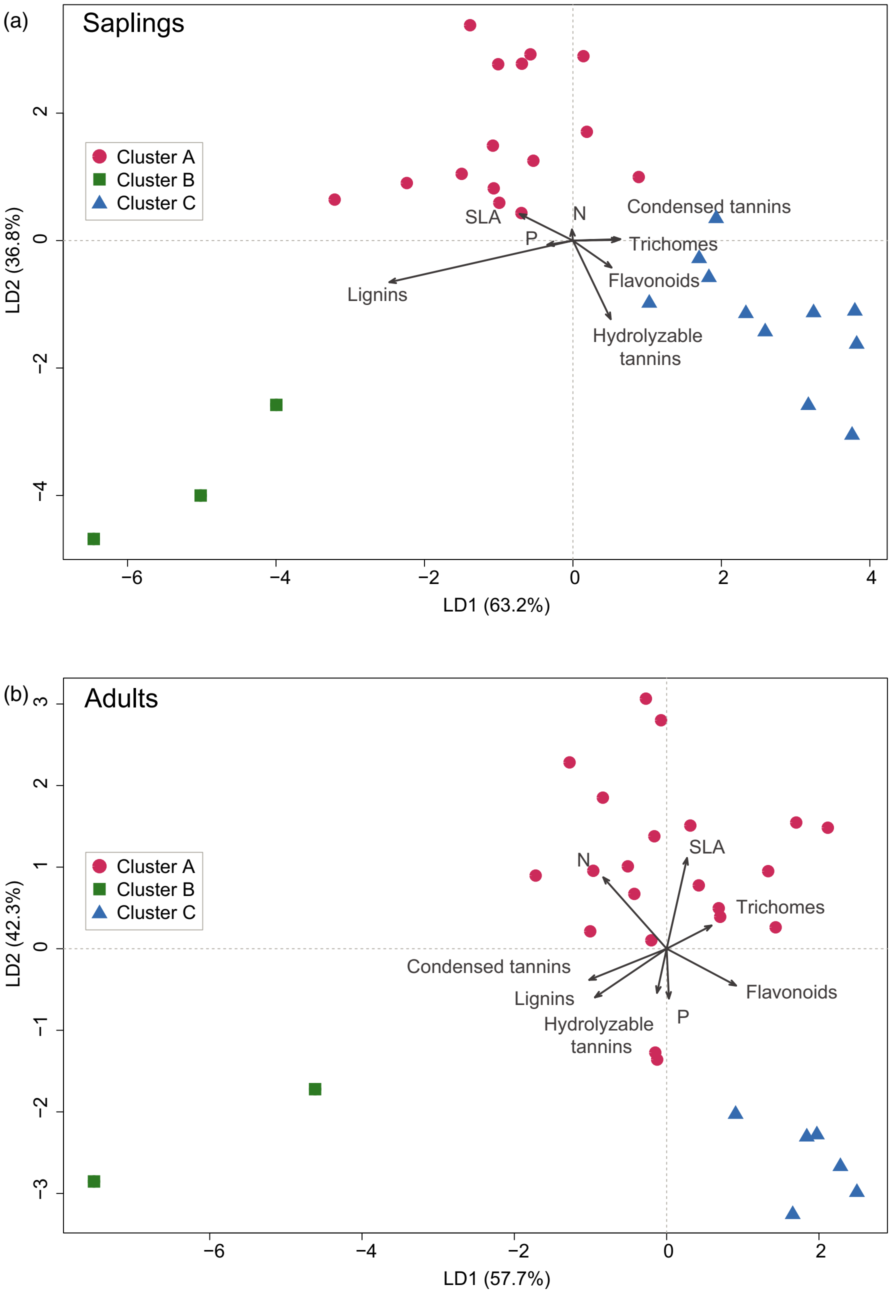


FIGURE 2 Ordination of oak defensive and nutritional traits. Shown are the linear discriminant analyses loading values for eight defensive and nutritional measured in (a) saplings and (b) adult trees. Species are colour-coded based on their defence syndromes (cluster A in magenta circles, cluster B in green squares, and cluster C in blue triangles)

(cluster A) had oak species (*n* = 21) with high nitrogen content and high SLA, the second syndrome (cluster B) had species (*n* = 2) with high lignins and hydrolysable tannins, and the third syndrome (cluster C) had species (*n* = 6) with high flavonoid levels and low levels of SLA and nitrogen (Table 1b; Figure 2b). The Mantel test showed that defence phenograms for saplings and adult trees were correlated (Mantel test, $p < 0.001$; Figure 3), indicating that oak defence syndromes did not significantly differ across ontogenetic stages. Analyses at the level of individual traits similarly indicated non-significant ontogenetic differences across species for all studied traits except SLA and phosphorus (Figure S2).

The analyses correlating defence syndromes and the oak phylogenetic tree indicated that the similarity among oak species in leaf traits was incongruent with the molecular phylogeny of oak species for both saplings (Mantel test, $p = 0.58$; Figure S3a) and adult trees (Mantel test, $p = 0.69$; Figure S3b). This means that there was no phylogenetic signal in oak defence syndromes and this finding was consistent across ontogenetic stages. Subsidiary tests for individual traits in saplings and adult trees indicated that there was no detectable phylogenetic signal for all studied traits except, again, SLA and phosphorus (Table S1).

Analyses further indicated that similarity among oak species in leaf traits was incongruent with the climatic phenogram for both saplings (Mantel test, $p = 0.615$; Figure S4a) and adult trees (Mantel test, $p = 0.853$; Figure S4b). Multiple regression analyses using individual traits indicated that saplings and adult trees of oak species growing at warmer sites exhibited higher defensive levels (increased trichomes and decreased SLA, phosphorus and nitrogen; Table S2). There were no significant effects of precipitation on individual

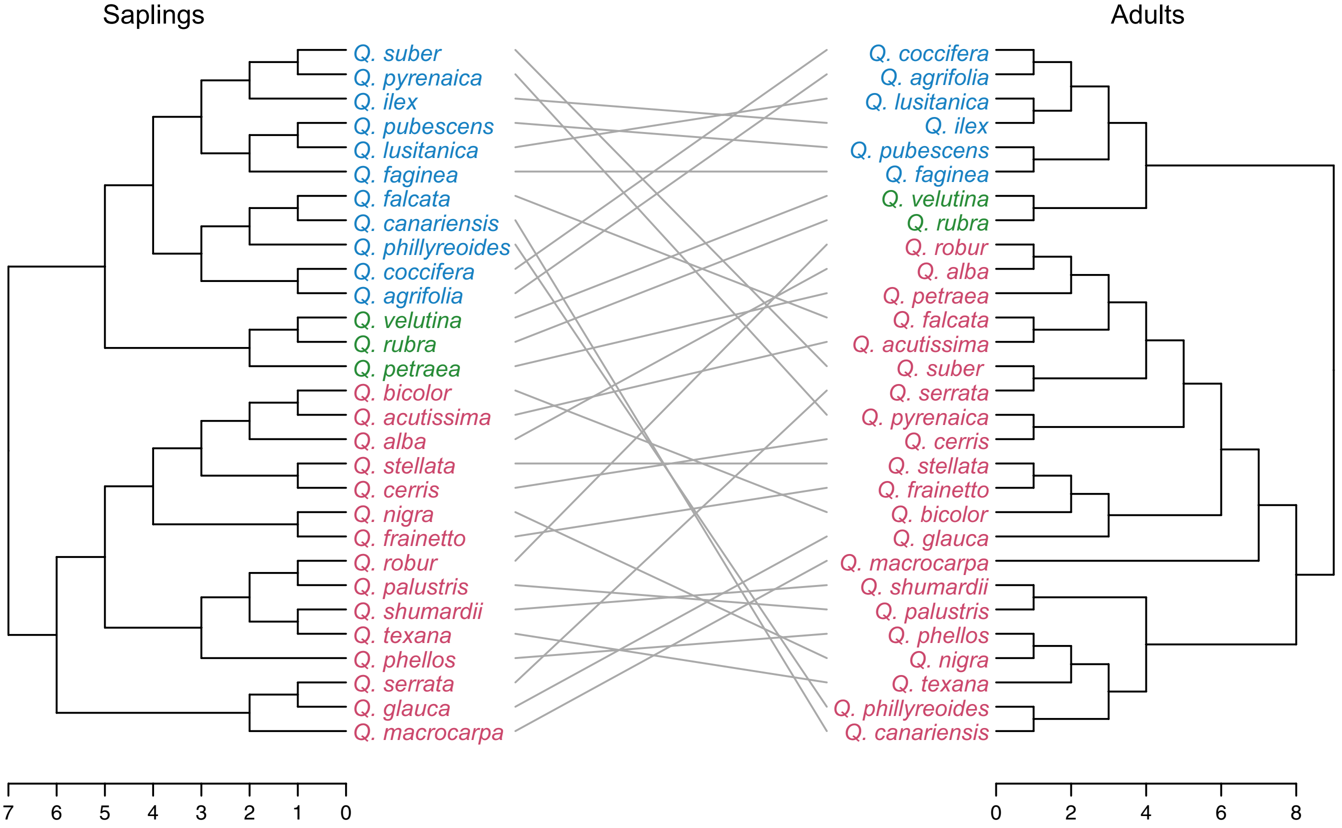
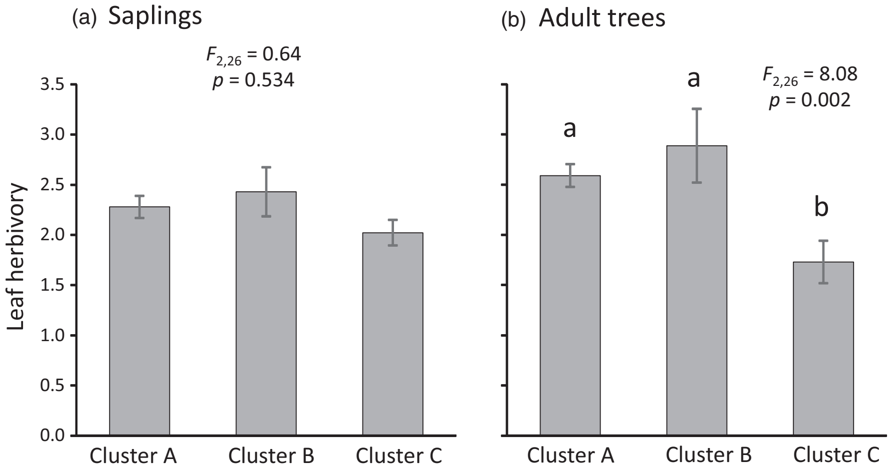


FIGURE 3 Defensive phenograms showing similarity among 29 oak species generated by hierarchical cluster analysis of leaf defensive and nutritional traits for saplings and adult trees. Closely clustered species show similar integrated defensive phenotypes and form three defence syndromes (cluster A in magenta font, cluster B in green font and cluster C in blue font)

FIGURE 4 Differences in mean leaf herbivory score (see *Methods*) by insect leaf chewers among defensive clusters (syndromes) comprised of 29 oak (*Quercus*) species. *F*- and *p*-values for the effect of cluster after controlling for phylogeny using phylogenetic generalized least squares are shown. Bars are least square mean ± *SEM*. Different letters indicate significant differences between clusters



defensive traits, except a positive association between precipitation and leaf nitrogen in saplings (Table S2).

In contrast to results for phylogenetic and climatic analyses, we found that oak defensive syndromes differed significantly in their leaf herbivory scores in the case of adult trees (Figure 4b), but not for saplings (Figure 4a). Mean leaf herbivory score for adult trees of oak species grouped in cluster C was 33% and 40% lower than mean leaf herbivory for oak species belonging to clusters A and B, respectively (Figure 4b). Thus, despite no overall change in oak defence syndromes with ontogeny (see above), clusters of species belonging to different defence syndromes appeared to vary in their level of herbivory but in an ontogenetic-dependent manner.

4 | DISCUSSION

Defence syndromes arise when two or more defence traits are co-expressed by multiple plant species, but other traits are not. We found evidence for three defence syndromes across the 29 oak species studied: The first syndrome (Cluster A in Figure 2) was composed of species with high leaf nitrogen and high SLA. This combination of traits resembles a strategy of tolerance to or escape from herbivores (sensu Agrawal & Fishbein, 2006) achieved through fast growth in high-resource environments. In contrast, the other two defence syndromes (clusters B and C in Figure 2) captured traits that lower plant nutritional quality for herbivores; cluster B was associated with high lignin and tannin levels, whereas cluster C was similarly characterized by high chemical defences (flavonoids), but coupled with low SLA and low nutrients. The observed clusters of leaf traits resemble different extremes along the leaf economic spectrum and defence investment, with species in cluster A presumably associated with fast carbon turnover (i.e. acquisitive strategy) combined with low defence. In contrast, species in clusters B and C exhibit slower carbon turnover (i.e. conservative strategy) and higher defence investment (Abdala-Roberts et al., 2018; Defossez et al., 2018; Díaz et al., 2016; Mason et al., 2016; Wright et al., 2004).

Our findings share similarities but also point to differences found for other comparisons of species within other genera. Agrawal and Fishbein (2006) reported a pattern of syndromes in milkweed (*Asclepias*) species that suggests weak constraints among traits involved in resource acquisition and defence. Specifically, they found two syndromes associated with low nutritional quality (high physical and chemical defences) and a third depicting both high resource quality and high defences, a syndrome type that we did not find for the studied oaks. A study of sunflowers (*Helianthus*) by Mason et al. (2016) found no evidence that species cluster into syndromes but they did find a pattern suggesting constraints between resource-use traits and defensive traits whereby conservative resource use was associated with higher defences. Taken together, available studies suggest that very different evolutionary scenarios have played out among the studied plant taxa. Specifically, species can cluster into syndromes (oaks and milkweeds) or not (sunflowers). Further, constraints between resource-use traits and defensive traits can be important (oaks and sunflowers) or not (milkweeds). Thus, even this limited number of studies of closely related plant species suggests that the role of defence constraints and their impact the evolution of defence syndromes may vary depending on the plant taxon sampled. More systems need to be studied. The focus should be on particularly speciose taxa, as it is unclear how the likelihood of finding syndromes (clusters) depends on the number of species sampled. By increasing species sampling effort we can start to answer key questions such as which ecological and evolutionary factors favour the emergence of syndromes, when the syndromes are more or less likely associated with constraints between resource use/acquisition and defence traits, and what are the implications of contrasting patterns for the correlated evolution of plant traits and syndrome features. In addressing these points, it is important to consider indirect defences (e.g. Defossez et al., 2018), inducibility of defensive traits (e.g. Moreira et al., 2016) or plant tolerance (e.g. Stowe, Marquis, Hochwender, & Simms, 2000). These additional components of the plant defensive arsenal should be addressed to fully characterize plant defence syndromes.

We found no overall difference in oak defence syndromes across ontogenetic stages, which counters previous theoretical and empirical work arguing for marked ontogenetic changes in the expression of plant defences (reviewed by Barton & Boege, 2017; Boege & Marquis, 2005). In one of the few studies addressing multiple traits, Mason and Donovan (2015) found marked ontogenetic changes in the expression of structural and chemical defences for sunflower species (*Helianthus*) which presumably then shaped ontogenetic changes in traits related to the leaf economic spectrum. However, studies such as these have not explicitly assessed patterns of correlated trait expression (e.g. Elger, Lemoine, Fenner, & Hanley, 2009; Moreira et al., 2017). Having said this, it is also important to note that in our study several species included in cluster C as saplings (e.g. *Q. canariensis*, *Q. pyrenaica*, *Q. suber*) moved to cluster A as adults, suggesting that while syndromes remain consistent across ontogenetic stages, there are underlying shifts in trait expression at the intra-specific level which deserve attention (Figure 3). Accordingly, this finding also implies changes in the magnitude of expression of individual traits for which we have found evidence in our previous work with oaks (Galmán, Abdala-Roberts, Covelo, Rasmann, & Moreira, 2019; Moreira et al., 2017) and, to a lesser extent, in the present study. Therefore, parallel assessments of ontogenetic changes in individual traits (i.e. magnitude of variation) as well as their co-variation (e.g. in the form of syndromes) can yield a better understanding of developmental variation in plant defence allocation. Additionally, further ontogenetic assessments that include the seedling stage as well as several intermediate ontogenetic stages are also needed to more robustly test for ontogenetic effects (e.g. non-linear ontogenetic trajectories; see Quintero, Barton, & Boege, 2013) in oak defence syndromes and underlying changes in defensive and resource-use traits. In addition, it is also important to note that the generality of our interpretations could be limited by the fact that traits were measured late in the growing season and using mature leaves. While this methodology has clear advantages (e.g. assessing cumulative herbivory over the season), it could also overlook patterns of early-season defence expression and herbivory (e.g. Forkner et al., 2004) as well as resource use and acquisition. Accordingly, this calls for including multiple sampling points during the growing season, particularly early-season measurements of growing leaf tissues for which defence levels can be especially pronounced and herbivory levels acute.

Our results also showed that defence syndromes were incongruent with the oak molecular phylogeny, suggesting that syndromes are evolutionary labile and presumably shaped mainly by evolutionary convergence of leaf trait co-expression patterns. Similarly, previous studies have also reported that defence syndromes do not track phylogenetic history, including work with milkweeds (*Asclepias* spp.; Agrawal & Fishbein, 2006), wild tomatoes (Haak, Ballenger, & Moyle, 2014), neotropical *Piper* (Salazar, Jaramillo, & Marquis, 2016), *Bursera* spp. (Becerra, 2007), *Inga* spp. (Kursar & Coley, 2003), and pines (*Pinus* spp.; Moreira et al., 2016), suggesting convergence is a common macro-evolutionary pattern in plant defence syndromes. In addition, our results further showed that the absence of phylogenetic signal in oak defence syndromes was consistent across ontogenetic stages. Still, further tests including a greater number of species are necessary to increase inference. The question remains open for other plant taxa whether developmental changes in defence syndromes affect the way in which syndromes map onto phylogenies.

We found no detectable influence of climate on oak defence syndromes, and this pattern was consistent across ontogenetic stages. This result is somewhat surprising given that our previous work has shown that climatic conditions influence patterns of trait co-expression in oak saplings (e.g. species distributed in warmer and drier climates had less leaf nitrogen, lower SLA, and more chemical defences; Abdala-Roberts et al., 2018). Having said this, such study was performed with a much lower number of species (n = 11) and involved seedlings grown in controlled greenhouse conditions, which limits direct comparisons with the present work. Although our findings are unsupportive of climatic controls over oak defence syndromes, previous work has emphasized the importance of accounting for abiotic factors in testing for intra- and inter-specific patterns of plant defence (Johnson, Ives, Ahern, & Salminen, 2014; Moreira et al., 2014). Further, it is important to note that abiotic forces may interact or correlate with biotic factors (e.g. herbivory, competition) to the extent that species interactions are dictated by abiotic conditions (which often is the case; e.g. Moreira, Castagneyrol, et al., 2018). The few studies addressing the linkages between abiotic context and plant defence syndromes showed that abiotic (climate) and biotic (herbivory) factors concurrently shape defence syndromes in *Cardamine* species along elevational gradients (Defossez et al., 2018; Pellissier et al., 2016). Adding the ontogenetic component to this type of study and an assessment of plant endogenous processes (e.g. genetic correlations and allocation costs) would provide a fuller understanding of how developmental variation, the abiotic context, and biotic pressures interact to shape plant defence syndromes, particularly in long-lived species.

Our analysis of insect herbivory levels indicated significant differences between groups of oak species associated with each defensive syndrome for adult plants, namely: clusters A (high nutritional content) and B (high chemical defences) had a higher level of herbivory than cluster C (high chemical defences coupled with low SLA and nutrients). This therefore suggests that high defences combined with low nutrients, rather than exclusively high defences (or low nutrients), is associated with lower herbivory in adult trees. In light of these findings, recent work of ours with oak seedlings (Moreira, Vázquez-González, et al., 2019) and that by authors studying other plant taxa (see Carmona, Lajeunesse, & Johnson, 2011; Wetzel, Kharouba, Robinson, Holyoak, & Karban, 2016) have reported an influence of leaf nutrients (in addition to putative defences) on herbivory. Interestingly, this syndrome-dependent pattern in herbivory was not found for saplings, suggesting ontogenetic variation in how herbivore pressure and defence syndromes relate to each other. That said, the fact that adults and saplings exhibited essentially the same defensive syndromes suggests that other unaccounted defensive traits (e.g. volatile organic compounds) or strategies (e.g. induced defences, tolerance) varied across clusters for adults but not saplings to produce differences in herbivory. For example, theory predicts an ontogenetic shift from induced (a cost-saving strategy) to constitutive (a high-cost strategy) chemical defences from early to later stages of plant development (reviewed by Barton & Koricheva, 2010). This may reflect a greater importance of resource limitation and allocation constraints early in ontogeny, when plants prioritize establishment and fast growth over defences to outcompete their neighbouring plants (Barton & Boege, 2017). Distinguishing between constitutive and induced levels of the measured traits would not only add another dimension in characterizing oak defence syndromes but could also explain the observed herbivory patterns (e.g. determine whether constitutive defences have a stronger impact on herbivory and play a more important role in shaping defence syndromes in adults than in saplings).

4.1 | Closing points and outlook

Our study highlights important aspects pertaining to the relationship between plant defence syndromes, plant development, ecological factors and evolutionary features using oaks as a model system. Crucially, results indicate that oak syndromes can remain unchanged across plant ontogenetic stages despite considerable variation in expression level of individual traits. This finding can be seen as consistent with previous studies on ontogenetic variation in plant defences in that these have shown quantitative developmental changes in the magnitude of expression of individual defensive traits but not qualitative changes in the types of defences that are expressed. Still, there are good reasons to expect selection to favour ontogenetic shifts in patterns of multivariate plant defence, making these types of inquiry a worthy endeavour to advance the field. In this sense, a closer mechanistic understanding of ecological factors ranging from life-history traits (e.g. leaf habit, growth rate) and dominant herbivore species or guilds to abiotic factors—at both micro (e.g light availability at different canopy heights; Castagneyrol, Giffard, Valdés-Correcher, & Hampe, 2019) to meso- and macro-scale (e.g. climatic variability; Pearse et al., 2009)—and plant endogenous processes (genetic linkages, allocation constraints) under phylogenetically controlled frameworks will pave the road for explaining the presence, absence or variability in plant defensive syndromes and their generative processes. In doing so, it will also be of key importance to consider methodological efforts such as conducting comprehensive measurements of defensive trait types (ranging from induced direct and induced and constitutive indirect defences to tolerance) which account for phenological variation in herbivory and leaf development, as well as the inclusion of multiple ontogenetic stages to fully characterize developmental variation.

ACKNOWLEDGEMENTS

We thank Patricia Toledo, Yolanda Arnaiz, Teresa Quijano-Medina, Nele Florina Ziehm, Clementina Villaverde, Manuel Malvar, Yuri Kanno, Zhangqi Ding and Shealyn Marino for their help in the field and laboratory. Comments and suggestions by the Associate Editor (Tobias Züst), Kasey Barton, and an anonymous reviewer helped to improve the manuscript. Washington University provided access to the Tyson Research Center, the Missouri Department of Natural Resources to Cuivre River State Park, and the Missouri Botanical Garden to Shaw Nature Reserve, all in Missouri. This research was financially supported by a Spanish National Research Grant (AGL2015-70748-R), a Regional Government of Galicia Grant (IN607D 2016/001) and the Ramón y Cajal Research Programme (RYC-2013-13230) to X.M.

AUTHORS' CONTRIBUTIONS

X.M. formulated the idea of the manuscript, wrote the first draft of the manuscript and designed the experiment; X.M., A.G., A.W.B., E.C., S.F., N.M.F., Y.H., S.R.L., R.J.M., M.N., C.S.N., M.B.P., M.A.S., C.V.-G. and S.Z. performed the experiment; X.M., F.C. and A.G. performed the chemical analyses; M.d.l.F. performed the molecular analyses; X.M. and S.R. contributed reagents/materials/analysis tools; X.M., S.R. and J.C.B.-M.y.T. analysed the data; L.A.-R., S.R. and R.J.M. contributed critically to the writing.

DATA AVAILABILITY STATEMENT

The data used in this study are archived at the Dryad Digital Repository: https://doi.org/10.5061/dryad.c59zw3r42 (Moreira et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Moreira X, Abdala-Roberts L, Galmán A, et al. Ontogenetic consistency in oak defence syndromes. *J Ecol*. 2020;108:1822–1834. https://doi.org/10.1111/1365-2745.13376