Drought enhances folivory by shifting foliar metabolomes in Quercus ilex trees

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Summary

• At the molecular level, folivory activity on plants has mainly been related to the foliar concentrations of nitrogen (N) and/or particular metabolites.
• We studied the responses of different nutrients and the whole metabolome of Quercus ilex to seasonal changes and to moderate field experimental conditions of drought, and how this drought may affect folivory activity, using stoichiometric and metabolomic techniques.
• Foliar potassium (K) concentrations increased in summer and consequently led to higher foliar K : phosphorus (P) and lower carbon (C) : K and N : K ratios. Foliar N : P ratios were not lowest in spring as expected by the growth rate hypothesis. Trees exposed to moderate drought presented higher concentrations of total sugars and phenolics and these trees also experienced more severe folivory attack.
• The foliar increases in K, sugars and antioxidant concentrations in summer, the driest Mediterranean season, indicated enhanced osmoprotection under natural drought conditions. Trees under moderate drought also presented higher concentrations of sugars and phenolics; a plant response to avoid water loss. These shifts in metabolism produced an indirect relationship between increased drought and folivory activity.

Introduction

Climate change is expected to lead to longer dry spells, higher evaporative demand and more intense droughts in the coming decades in several regions of the world, including the Mediterranean basin (IPCC, 2007). Increased drought has been demonstrated to enhance tree mortality in forests (Martínez-Vilalta & Pinol, 2002; Allen et al., 2010; Galiano et al., 2012) and may have multiple effects on ecosystem biodiversity and ecosystem structure and function (Anderegg et al., 2013; Peñuelas et al., 2013a). Furthermore, both xeric and mesic forests may be hydraulically vulnerable to drought (Choat et al., 2012).

Several studies showed that herbivores commonly increase their activity in response to increased concentrations of soluble nitrogen (N) in foliage (Larsson, 1989; Larsson & Bjorkman, 1993; Rouault et al., 2006). Plant water status also plays an important role in the resistance of trees against herbivorous attack (Rouault et al., 2006). Drought may affect foliar nutritional quality and indirectly stimulate insect folivory (White, 1984; Rouault et al., 2006). Also, plants are able to respond to folivory attack by producing chemical defenses such as alkaloids, terpenes and phenolics (Bennett & Wallsgrove, 1994; Kessler & Baldwin, 2001; Ali & Agrawal, 2012), and these chemical defenses seem to be more concentrated in those populations of plants historically more frequently in contact with the insects (Raffa et al., 2013). Several other studies have also demonstrated that plant primary metabolism is shifted in response to tissue predation or infection (Ehness et al., 1997; Widarto et al., 2006; Sardans et al., 2013a).

Drought is also a potential driver of changes in the elemental carbon (C) : N : phosphorus (P) : potassium (K) stoichiometries of different plant organs and ecosystems (Rivas-Ubach et al., 2012; Sardans et al., 2012a,b) with consequent effects on ecological processes and ultimately the structure and function of ecosystems (Elser et al., 1996; Sterner & Elser, 2002; Sardans et al., 2011a, 2012b; Peñuelas et al., 2013b). K has been demonstrated to play an important role under low water availability conditions to avoid water losses, especially in summer drought (Ingram & Bartels, 1996; Sardans et al., 2012a,b, Wang et al., 2013), through increases in its foliar concentrations (Sardans et al., 2013b). The study of foliar K concentrations under natural or experimental drought conditions thus requires more attention in the context of ecological stoichiometry (Sardans et al., 2012a,b). The large variation in C : N : P : K biomass stoichiometries in plants, both in time and in space (latitude and altitude), may be a significant factor, among others, in the selection of foliage with high nutritional content by herbivores (Gusewell & Koerselman, 2002; Lindroth et al., 2002; Oleksyn et al., 2002; Raubenheimer & Simpson, 2003; Sardans et al., 2012b). Additionally, the rates of folivory...
have been observed to be directly related to the relative concentrations of foliar structural compounds such as lignin rather than to foliar N concentrations, with high relative concentrations of these structural compounds usually being avoided (Choong et al., 1992; Williams et al., 1998). Thus, C : N ratios have been shown to play a key role in folivory (Ji et al., 2011) through the effects of structural compounds or N concentrations. Furthermore, changes in plant stoichiometry may influence the coevolution of insects by shifting the composition and concentration of defensive chemical compounds of plants (Raubenheimer & Simpson, 2003), as most elements, such as C, N and P, generally do not act in isolation but as components of molecular compounds (Peñuelas & Sardans, 2009a) such as lignin and cellulose in lignified structures or various compounds that defend against herbivorous attack (Bennett & Wallsgrove, 1994; Kessler & Baldwin, 2001).

Most studies examining the relationships between plant metabolites and herbivory predation at the molecular level have generally focused on the identification of single compounds or families of metabolites (Sardans et al., 2013a). The application of the new metabolomic techniques in ecology and plant physiology (ecometabolomics) allows the study of the complete metabolome of an organism, the total set of metabolites in an organism at a specific moment (Fiehn, 2002), and its response to abiotic and biotic environmental shifts (Peñuelas & Sardans, 2009b; Sardans et al., 2011b; Sardans & Peñuelas, 2012; Peñuelas et al., 2013a; Rivas-Ubach et al., 2013). Recent ecometabolomic studies have reported metabolomic changes in plants exposed to a biotic stress such as herbivorous predation (Jansen et al., 2009; Leiss et al., 2009) or an abiotic stress such as drought (Charlton et al., 2008; Lugan et al., 2009; Sardans et al., 2011b; Rivas-Ubach et al., 2012). The link between shifts in foliar stoichiometries and shifts in foliar metabolomes throughout the seasons and under experimental drought in the field has been recently demonstrated (Rivas-Ubach et al., 2012), so the nutritional quality of food for herbivores may thus also change under stress conditions (Rouault et al., 2006).

The relationship between plant stoichiometry and metabolism and herbivorous attack under drought conditions, however, is still unclear and warrants further study (Raubenheimer & Simpson, 2003). The rates of folivory can thus be influenced directly by food quality and indirectly by plant water status in what could be a cascade effect of drought. As a broader and better understanding of the metabolomic shifts in plants grown under single and interacting stress factors such as drought and herbivory is greatly needed, we performed this study where we hypothesized that shifts in both foliar elemental stoichiometry and metabolomics produced by drought would influence folivory activity. By affecting the quality of the food available to herbivores, drought-induced shifts in foliar chemical composition may lead to long-term cascade effects that could alter trophic webs.

Once per season, we sampled leaves of Quercus ilex trees from a mature forest in Catalonia (northeastern Iberian Peninsula) exposed to conditions of moderate experimental drought similar to those projected for the coming decades. The metabolomes and elemental compositions of all samples were analyzed. The rates of folivory were also determined for each sampled tree. Our goal was to understand: (1) the stoichiometric responses of Q. ilex, the most dominant tree in the forests of the Mediterranean basin, to seasonal changes and to experimental conditions of moderate drought; (2) the foliar metabolomic responses of Q. ilex to different seasons and moderate drought; (3) how the foliar stoichiometric and metabolic shifts of Q. ilex may affect the selection of food by herbivores, and (4) how Q. ilex is able to shift its metabolome to respond to herbivore attack. For this purpose, the Q. ilex forest was used to test our hypothesis that drought, simulated in experimental plots, could alter folivorous activity by altering the foliar metabolome. Our results could thus help to predict the impact of climate change on trophic webs (Peñuelas & Sardans, 2009a).

Materials and Methods

Study site

This study was performed in a natural Quercus ilex L. forest in the Prades Mountains in southern Catalonia (see Ogaya & Peñuelas, 2007 for details; 41°21′N, 1°2′E). All sampled plots faced south-southeast on a 25% slope at 930 m above sea level. The climate of the region is mesic-Mediterranean with a marked summer drought for 3 months (Supporting Information Fig. S1a). The vegetation consists of a forest dominated by Q. ilex, followed by Phillyrea latifolia and Arbutus unedo, among others.

Experimental design

Four plots (15 × 10 m) of mature Q. ilex forest were established 15 m apart in March 1999 (Ogaya et al., 2003). Two randomly assigned plots received a drought treatment, and the other two served as control plots. The drought treatment consisted of covering c. 30% of the soil surface with 1 × 14 m PVC strips oriented down the slope and 0.5–0.8 m above the soil surface to prevent irrigation by rainwater. PVC strips do not produce an increase in soil temperature (Fig. S1d). A ditch 0.8–1 m in depth was dug along the entire top edge of the treatment plots to intercept up-slope runoff. All water intercepted by the strips was channeled to the bottom edge of the drought plots. Soil moisture, air humidity, precipitation and air and soil temperatures were monitored every 30 min. The drought treatment resulted in an average annual reduction of 18% in relative soil moisture (Fig. S1b,c; see Barbeta et al., 2013 for details). This reduction in soil moisture has increased tree mortality and reduced growth during the last 10 yr (Fig. S2).

Sampling of leaves

Five adult Q. ilex individuals 25–50 cm in diameter were randomly selected from each plot as study cases (total n = 20). Leaves were sampled once each season: in February (winter), May (spring), August (summer) and November (autumn). A small branch exposed to the sun was removed from each tree with a pole, and a sample of the youngest well-developed leaves was frozen in liquid nitrogen for the stoichiometric and metabolomic analyses. The remaining leaves were stored in bags at 6–8°C for
determination of water content and for photographic analysis of the extent of consumption by herbivores.

Calculation of the proportion of leaves consumed by herbivores

 Fifteen randomly selected leaves stored at 6–8°C from each tree were placed on a flat white surface and photographed with a Nikon D80 camera with a Nikkor AF-S 18–135/3.5–5.6 G DX lens to calculate the percentage of folivory. The area of the leaves consumed was calculated using Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA, USA) by following the method of Peñuelas et al. (2013c). The assigned value of consumed area for each tree was the mean of the 15 leaves analyzed (see Fig. S3 for details). The values of folivory for each tree were then standardized by the total foliar biomass of *Q. ilex* in its plot. All values were thereafter transformed for normality (arcsin (square root(percentage))). Additionally, the fresh leaves were also used for calculation of fresh weight, dry weight, foliar water content, foliar size and leaf mass per unit area (LMA).

Foliar processing for elemental and metabolomic analyses

The processing of the leaves is described in detail in Rivas-Ubach et al. (2013). Briefly, leaves frozen in liquid nitrogen were lyophilized and stored in plastic cans at ~20°C. Samples were ground with a ball mill at 1600 rpm for 6 min (Mikrodismembrator-U; B. Braun Biotech International, Melsungen, Germany), producing a fine powder that was then stored at ~80°C until the extraction of the metabolites.

Elemental analysis

For the C and N analyses, 1.4 mg of the powder from each sample was analyzed. The C and N concentrations were determined by elemental analysis using combustion coupled to gas chromatography with a CHNS-O Elemental Analyser (EuroVector, Milan, Italy).

Macroelements (P and K) were extracted by acid digestion in a microwave reaction system under high pressure and temperature (Sardans et al., 2010). Briefly, 250 mg of leaf powder was placed in a Teflon tube with 5 ml of nitric acid and 2 ml of H2O2. A MARSXpress microwave reaction system (CEM, Mattheus, NC, USA) was used for the acid digestions (see the ‘Chemical analyses’ section of Methods S1 for details). The digested material was transferred to 50-ml flasks and resuspended in Milli-Q water (EMD Millipore, Darmstadt, Germany) to a final volume of 50 ml. After digestion, the P and K concentrations were determined by optic emission spectrometry with inductively coupled plasma (ICP-OES) (Optima 4300; Perkin-Elmer Corporation, Norwalk, CT, USA; see Methods S1 for details).

Extraction of metabolites for analysis by nuclear magnetic resonance (NMR)

The extraction of foliar metabolites for NMR analysis is described in detail in Rivas-Ubach et al. (2013). First, two sets of 50-ml centrifuge tubes were labeled: set A for the extraction of metabolites and set B for lyophilization. A set of crystal jars for the organic fractions were also labeled. The tubes of set A received 200 mg of powdered leaf material of each sample. Six milliliters of water/methanol (1 : 1) and 6 ml of chloroform were added to each tube. The samples were vortexed for 15 s and then sonicated for 2 min at room temperature. All tubes were centrifuged at 1100 g for 15 min. Four milliliters of each fraction (aqueous and organic) was collected independently; aqueous fractions were transferred to the centrifuge tubes of set B, and organic fractions were transferred to the crystal jars. This procedure was repeated twice for two extractions of the same sample. The aqueous samples, previously resuspended in water to reduce the proportion of methanol (< 15% methanol), were lyophilized, and then 4 ml of water was added to each tube, which was vortexed and centrifuged at 23 000 g for 3 min. The samples were frozen at ~80°C and lyophilized again. The organic fractions were transferred to round-bottomed evaporation flasks and dried in a rotary vacuum evaporator. Finally, 1 ml of KH2PO4-NaOD-buffered D2O + 0.01% trimethylsilyl propionic acid sodium salt (TSP; pH 6.0) was added to the dried aqueous fractions, and 1 ml of chloroform-D + 0.01% tetramethylsilyl (TMS) was added to the dried organic fractions. TSP and TMS were used as internal standards. All solutions were resuspended with a micro-pipette, transferred to 2-ml centrifuge tubes and centrifuged at 23 000 g for 3 min. The supernatants were transferred to NMR sample tubes.

Extraction of metabolites for liquid chromatography–mass spectrometry (LC-MS) analysis

The extraction of metabolites followed the protocol of t’Kindt et al. (2008) with minor modifications. Two sets of Eppendorf tubes were labeled: set A for extractions and set B for the extracts from set A.

The tubes of set A received 100 mg of sample powder from each sample, and then 1 ml of MeOH/H2O (80 : 20) was added to each tube. The tubes were vortexed for 15 min, sonicated for 5 min at room temperature and then centrifuged at 23 000 g for 5 min. After centrifugation, 0.6 ml of the supernatant from each tube was transferred to the corresponding Eppendorf tubes of set B. This procedure was repeated for two extractions of the same sample. The tubes of set B were centrifuged at 23 000 g for 5 min. The supernatants were collected using crystal syringes, filtered through 0.22-μm pore microfilters and transferred to a labeled set of high-performance liquid chromatography (HPLC) vials. The vials were stored at ~80°C until the LC-MS analysis.

LC-MS analysis

LC-MS chromatograms were obtained using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific/Dionex RSLC, Waltham, MA, USA) coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionization (HESI II) source.
Chromatography was performed on a reversed-phase C18 Hypersil gold column (150 × 2.1 mm, 3 μm particle size; Thermo Fisher Scientific) at 30°C. The mobile phases consisted of acetonitrile (A) and water (0.1% acetic acid) (B). Both mobile phases were filtered and degassed for 10 min in an ultrasonic bath before use. At a flow rate of 0.3 ml min⁻¹, the elution gradient began at 10% A (90% B) and was maintained for 5 min, and then was changed to 10% B (90% A) for the next 20 min. The initial proportions (10% A; 90% B) were gradually recovered over the next 5 min, and the column was then washed and stabilized for 5 min before injecting the next sample. The injection volume of the samples was 5 μl. All samples were injected twice, once with the HESI operating in negative ionization mode (−H) and once in positive ionization mode (+H). The Orbitrap mass spectrometer was operated in Fourier transform mass spectrometry (FTMS) full-scan mode with a mass range of 50–1000 m/z and high-resolution (60 000). The resolution and sensitivity of the spectrometer were monitored by injecting a standard of caffeine after every 10 samples, and the resolution was further monitored with lock masses (phthalates). Blank samples were also analyzed during the sequence.

NMR analysis

NMR experiments were performed on a Bruker AVANCE 600 spectrometer working at a magnetic field of 14.1 T (1H and 13C NMR frequencies of 600.13 and 150.13 MHz, respectively) and equipped with an automatic sample changer, a multinuclear triple resonance TBI probe and a temperature control unit (Bruker Biospin, Rheinstetten, Germany). The temperature into the probehead was previously calibrated and maintained constant for all the experiments at 298.0 K; for this purpose a temperature equilibration delay of 2 min is left for each sample before the shimming process. All NMR sample handling, automation, acquisition and processing was controlled using TOPSPIN 3.1 software (Bruker Biospin). Spectra were referenced to TSP (1H and 13C at δ 0.00 ppm) in the case of polar samples and to the signal of the residual solvent, CHCl₃ (1H at δ 7.26 ppm and 13C at δ 77.16 ppm), in the case of nonpolar samples.

1H NMR fingerprinting

All extract samples were analyzed by high-resolution one-dimensional (1D) 1H NMR spectroscopy following the conditions described in Rivas-Ubach et al. (2013) and using standard pulse-acquisition 1D 1H-NMR experiments. In the case of the water/methanol extract samples, proton spectra were acquired with suppression of the residual water resonance. The water resonance signal was presaturated at a power level of 55 dB, corresponding to an effective field of 30 Hz during a relaxation delay of 2 s. Each experiment was acquired as a set of 32 000 data points, over a spectral width of 16 ppm, as the sum of 128 transients and with an acquisition time of 1.7 s. The resulting free induction decays (FIDs) were Fourier-transformed and the spectra obtained were phased and baseline-corrected. The FIDs of polar samples were multiplied by an exponential apodization function equivalent to 0.2-Hz line broadening before the Fourier transform. The experimental time was c. 8 min per sample.

NMR metabolite identification

Standard 2D NMR experiments (1H–1H correlated spectroscopy (COSY), 1H–13C total correlation spectroscopy (T2QC), 1H–13C heteronuclear single quantum correlation (HSQC) and 1H–13C heteronuclear multiple bond correlation (HMBC)), and 1D selective 1H TOCSY experiments were performed on representative polar and nonpolar samples for the identification of metabolites. For the water–methanol extract samples, 2D experiments were carried out with standard presaturation of the residual water peak during the relaxation delay. Experiments were acquired using standard Bruker pulse sequences and routine conditions (see Methods S2 for details). The procedure followed was that described by Rivas-Ubach et al. (2013). All elucidated metabolites were further confirmed using data reported in the literature (see Tables S1 and S2 for references).

Processing of LC-MS chromatograms

The raw data files from the spectrometer were processed using MZmine 2.10 (Pluskal et al., 2010). Chromatograms were baseline-corrected, deconvoluted, aligned and filtered before the numerical database was exported in CSV format (Table S3 for details). Metabolites were assigned by exact mass and retention time from the measurements of the standards in the MS and MSⁿ modes of the spectrometer (Table S4 for details). Different assigned variables corresponding to the same molecular compounds were summed. The numerical values of the variables extracted from the LC-MC chromatograms correspond to the absolute peak areas of the chromatograms detected by the spectrometer. The area is directly proportional to the concentration of the variable, so we use the term ‘concentration’ in this article when referring to changes in the amount of metabolites between the studied factors (control vs drought or between seasons).

NMR bucketing

The processing of 1H NMR spectra is detailed in Rivas-Ubach et al. (2013). Briefly, before the exportation of the 1H NMR numerical databases, all spectra were phased, baseline-corrected and referenced to the resonance of the internal standard (TSP for polar and TMS for nonpolar samples) at δ 0.00 ppm with TOPSPIN 3.1. A variable-size bucketing was thus applied to all 1H NMR spectra using AXMY software (Bruker Biospin), scaling the buckets relative to the internal standard (TMS or TSP). The output was a data set containing the integral values for each assigned 1H NMR spectral peak in the described pattern. The buckets corresponding to the same molecular compound were summed.

Statistical analyses

To test for differences between seasons and drought treatments in foliar elemental stoichiometry and metabolome, the LC-MS and
NMR metabolomic fingerprints of the *Q. ilex* leaves were subjected to PERMANOVA analysis (Anderson *et al.*, 2008) using the Euclidean distance, with season (spring, summer, autumn and winter) and treatment (control and drought) as fixed factors and folivory as a covariable. The number of permutations was set at 999. The PERMANOVA analysis was conducted with PERMANOVA+ for the PRIMER v.6 software (Anderson *et al.*, 2008). One-way ANOVAs were performed for each individual stoichiometric or metabolite variable (Tables S5, S6). Statistically significant results of those ANOVAs are indicated by asterisks in Figs 1 and 2.

Additionally, to understand how the foliar stoichiometry and metabolome of *Q. ilex* shifted with the factors studied (season and climatic treatment), the foliar stoichiometric and metabolomic fingerprints were also subjected to principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). The seasonal PCA and PLS-DA included the fingerprints of all seasons combined (Figs 1, S4). Trees in summer season present the accumulated foliar signals of folivory in the Mediterranean basin; for this reason, in order to study the effects of drought on folivory, the fingerprints from summer leaves were additionally submitted to separate PCAs and PLS-DA (Figs 2, S5). The PCAs and PLS-DA were performed using the *pca* and *pls.da* functions, respectively, of the *mixOmics* package of R (R Development Core Team, 2011). The score coordinates of the variables of the PCAs were subjected to one-way ANOVAs to detect statistical differences among groups (see supporting information of Rivas-Ubach *et al.*, 2013). A Kolmogorov–Smirnov (KS) test was performed on each variable to test for normality. All assigned and identified metabolites were normally distributed, and any unidentified metabolomic variable that was
not normally distributed was removed from the data set. The KS tests were performed with the `ks.test` function of the `tuncogof` package of R (R Development Core Team, 2011).

Folivory begins in spring and early summer, and trees do not produce new leaves in summer, so the sampled leaves had accumulated signs of folivory by the middle of the summer. An additional PCA was thus conducted with summer data excluding the proportions of herbivorous consumption (Fig. S6). The PC1 scores of this PCA, including the entire elemental, stoichiometric and metabolomic variation of the case of trees in summer, were plotted against the degree of folivory (Fig. 3). Folivory was analyzed with a general linear model (GLM) as a function of the climatic treatment and the metabolomic variation (PC1 scores). Another GLM analyzed the total foliar sugar and phenolic concentrations as functions of climatic treatment and season. **STATISTICA v8.0** (StatSoft, Tulsa, OK, USA) was used for the one-way ANOVAs and the post hoc tests of the score coordinates of the PCAs. GLM analyses were performed with the `lme` function of the `nlme` package of R (R Development Core Team, 2011). All statistical analyses were performed at the individual level.

**Results**

The PERMANOVA analysis performed with all elemental, stoichiometric and metabolomic data (assigned and unassigned metabolites) indicated that leaves in different seasons, under drought treatment and with different degrees of folivory had different foliar chemistries and metabolisms (folivory: pseudo-$F = 2.4832$; $P < 0.001$; season: pseudo-$F = 2.4749$; $P < 0.001$; and treatment: pseudo-$F = 3.1031$; $P < 0.001$).

**Elemental, stoichiometric and metabolomic shifts across seasons and experimental drought treatments**

The PCA conducted with all seasons, including all the elemental, stoichiometric and metabolomic data, indicated that more than 50% of the total variance was already gathered by the first four PCs ($PC_1 = 15.2\%$, $PC_2 = 14.7\%$, $PC_3 = 14.1\%$ and $PC_4 = 12.7\%$; Fig. S4). Differences between seasons were explained by PC1 ($P < 0.001$), PC3 ($P < 0.05$) and PC4 ($P < 0.05$) in one-way ANOVAs (Supporting Information Table S7).

![Component 1 of the partial least squares discriminant analysis (PLS-DA) of the metabolomic and stoichiometric variables for the summer data. (a) Variable plot and (b) case plot. Carbon (C) : nitrogen (N) : phosphorus (P) : potassium (K) ratios are shown in red and herbivory is shown in dark red. Different metabolomic families are indicated by different colors: blue, sugars; green, amino acids; cyan, nucleotides; violet, phenolics; yellow, related compounds of amino acid and sugar metabolism (RCAA); brown, terpenes; dark blue, overlapped nuclear magnetic resonance (NMR) signals; light orange, nonpolar metabolites. Unassigned metabolites are not represented in the graph. Details of the variables are given in Fig. 1. Control trees, asterisks; droughted trees, triangles. Variables marked with asterisks showed statistical significance ($P < 0.05$) or marginal statistical significance ($P < 0.1$) in one-way ANOVAs (Supporting Information Table S7).](image)

![Principal component 1 (PC1) scores of a principal component analysis (PCA), excluding the degree of folivory, for summer metabolomic and stoichiometric data (Supporting Information Fig. S1) vs the proportion of foliar consumption (arcsin(square root(percentage of consumption))). Gray circles, control trees; gray crosses, droughted trees. Black circle, mean of control trees ± SE; black cross, mean of droughted trees ± SE.](image)
The C, N, P and K foliar concentrations and the ratios C : N, C : P, C : K, N : K and K : P, but not N : P, changed with the seasons (Fig. 1, Table S5). The highest K : P and K : N concentration ratios occurred in summer, coinciding with the highest foliar K concentrations. Foliar N and P concentrations were highest in autumn, thus resulting in the lowest C : N and C : P ratios (see Table S5 and Sardans et al., 2013b for more details).

Most assigned and identified metabolites in the leaves of Q. ilex shifted across the seasons (Fig. 1, Table S5). The main changes were between spring and summer, two of the most critical seasons in the Mediterranean basin, because spring is the period of growth and summer is the driest season. Summer leaves generally had higher concentrations of polyphenolics and sucrose, whereas spring leaves had higher concentrations of amino acids, some related compounds of amino acid and sugar metabolism (RCAAS) and some sugars such as pentoses and disaccharides, products directly related to growth. The effects of experimental drought were also detected on the seasonal PLS-DA and PCA plots (Figs 1, S4, Tables S6, S7). In the case plot for both analyses, PLS-DA and PCA, droughted trees tended to be distributed in the same direction as the summer trees; summer is the driest season in Mediterranean climates (Fig. S1a).

Total concentrations of amino acids, sugars and polyphenolics

The concentrations of the assigned and identified variables of the different metabolite families (amino acids, sugars and phenolics) were summed (Fig. 4). The integral values of $^1$H NMR spectra relative to the internal standard were standardized to sum with the peak area of the LC-MS chromatograms. Factorial ANOVAs indicated that trees presented higher concentrations of foliar amino acids in spring and winter (Fig. 4a). The concentrations of sugars were higher in winter than in the other seasons (Fig. 4b). Trees had higher concentrations of total phenolics in summer, and the drought treatments increased the phenolic concentrations significantly in summer and winter (Fig. 4c). The drought treatment affected the trees in all seasons, increasing the concentrations of total sugars, but the concentrations in summer were not significantly different from those of the control trees (Fig. 4b).

The GLM analysis with total sugars as a dependent variable, season and climatic treatment as fixed independent variables and individuals as a random variable showed statistical significance for both climatic treatment ($P<0.001$) and season ($P<0.05$). The GLM analysis with the same independent variables but with total phenolics as a dependent variable also showed statistical significance for both climatic treatment ($P<0.005$) and season ($P<0.05$).

We did not detect significant differences between leaves of control trees and droughted trees in foliar fresh weight ($F=0.74; P=0.398$), foliar dry weight ($F=0.64; P=0.43$), foliar water content ($F=1.45; P=0.25$) and foliar size ($F=0.33; P=0.57$). Mean values of LMA was 13.6% lower in droughted plants (19 mg DW cm$^{-2}$) than in control plants (22 mg DW cm$^{-2}$; $P<0.05$) in summer, the analyzed season for folivory. The PLS-DA and PCA conducted to investigate the relationships of folivory with drought summer also indicated a difference between control and droughted trees in the multidimensional space (Figs 2, S5). PC1 of the PCA of summer explained 18.7% of the total variance and separated significantly control from droughted trees ($P<0.001$; Fig. S5).

The GLM conducted with the summer data, including the accumulated proportions of folivory and with folivory as a
dependent variable and climatic treatment and PC1 scores of the additional PCA (without the folivory variable; Fig. S6) as independent variables ($P < 0.001; R^2 = 0.60$), indicated significant effects of the drought treatment ($P < 0.05$) and no significant effect of the PC1 scores ($P = 0.53$). These results imply that the existence of a significant relationship between folivory and the PC1 score coordinates of the PCA is mainly attributable to the fixed effect of drought (Fig. 3).

**Discussion**

**Seasonality and drought**

The foliar stoichiometry and metabolome of *Q. ilex* varied with season (PERMANOVA $P < 0.001$; Figs 1, S4). Seasonal PLS-DA and PCA identified significant stoichiometric and metabolomic shifts between summer and the other seasons, although the differences between seasons were not as marked as those of an earlier study with the Mediterranean shrub *Erica multiflora* (Rivas-Ubach *et al.*, 2012). Trees in spring, the Mediterranean growing season, had high concentrations of metabolites directly related to growth such as amino acids, some RCAAS and some sugars such as pentoses and disaccharides, in accordance with the earlier study (Rivas-Ubach *et al.*, 2012; Fig. 1), even though the total concentration of amino acids did not differ from the levels in winter (Fig. 4a), the coldest season in the Mediterranean basin. In *Q. ilex*, foliar N : P ratios did not present the lowest values in spring as expected from the growth rate hypothesis (Elser *et al.*, 1996; Rivas-Ubach *et al.*, 2012; Figs 1, S4, Table S5), which proposes that organisms with high rates of growth require high concentrations of P (low N : P ratios) to meet the demands of protein synthesis (Elser *et al.*, 2003). Our results are nevertheless in accordance with those of other studies in plants. The high foliar concentrations of amino acids in winter trees might be attributable to accumulation under cold conditions. It has been observed that some amino acids such as proline and glycine are able to buffer the NADP$^+$ : NADPH ratio in plants (Xu *et al.*, 2013) as a consequence of low photosynthetic activity in winter to provide reducing agents for the generation of ATP in mitochondria (Hare & Cress, 1997; Xu *et al.*, 2013). However, Matzek & Vitousek (2009) found no significant relationships between the N : P ratio and increased growth in 14 species of pines, concluding that terrestrial plants must invest in fundamental functions other than growth, such as storage and defense, that often require different investments in N and P (Hermes & Mattson, 1992; Peñuelas & Esterie, 1998).

As observed in the multivariate ordination analyses, the concentrations of the various sugar compounds detected were distributed differentially among the seasons (Figs 1, S4), but droughted trees in winter had the highest concentrations of total sugars (Fig. 4b), which may also be related to their accumulation and allocation from other organs (e.g. wood and lignotubers) in response to a cold environment to leaves or further preparation for the growth season (Grimaud *et al.*, 2013; Xu *et al.*, 2013). *Quercus ilex* is an evergreen tree with slow growth; it presents large lignotubers, swollen woody structures at the base of the stem (James, 1984) that are able to store essential nutrients and metabolites such as carbohydrates to ensure rapid growth after severe stress (Canadell & Zedler, 1995). Our stoichiometric and metabolomic data support the idea that trees, with large woody structures such as trunks, lignotubers, and/or extensive root networks, are able to buffer the metabolomic changes in foliar ontogeny by storing large amounts of nutrients, allowing the allocation of essential resources to photosynthetic organs and thus leaf growth and resistance to severe environmental disturbances (Canadell & Zedler, 1995; Landhauser & Liefers, 2002; Anderegg & Callaway, 2012; Galano *et al.*, 2012).

The largest stoichiometric shift between seasons appeared to be related to the responses to drought conditions (Figs 1, S4, Table S5). Foliar concentrations of K were higher in trees in summer, the driest season. The resulting higher foliar K : P and lower N : K and C : K concentration ratios (Sardans *et al.*, 2013b) were thus in agreement with the findings of other Mediterranean seasonal studies (Sardans *et al.*, 2012a, 2013b). K participates in the water economy of plants (Babita *et al.*, 2010) through osmotic control (Babita *et al.*, 2010; Laus *et al.*, 2011) and improves the functioning of foliar stomata (Khosravifar *et al.*, 2008). These results demonstrate the important role of K in the ecology of terrestrial plants (Cakmak, 2005; Sardans *et al.*, 2012a, 2013b). Moreover, these shifts in K concentrations in summer leaves were accompanied by higher concentrations of sucrose, which can also act as an osmolyte that, together with K, can help to prevent the loss of water through osmotic control (Ingram & Bartels, 1996).

As seen in previous studies, a decrease in growth and an increase in tree mortality are among the main consequences of this prolonged moderate drought (Barbeta *et al.*, 2013; Fig. S2). Our seasonal PLS-DA and PCA with metabolomic and stoichiometric data also showed interesting differences between the trees in the drought plots and those in the control plots (Figs 1, S4; PERMANOVA $P < 0.001$). Droughted trees tended to have the same pattern as summer trees on the Component 2 of case plot of PLS-DA (Fig. 1) and PC1 and PC2 of PCA (Fig. S4), in accordance with a previous study (Rivas-Ubach *et al.*, 2012), thus indicating a foliar elemental-metabolomic response to drought independent of plant ontogeny. Summer trees and droughted trees in all seasons tended to have higher foliar concentrations of flavonoids (Figs 1, 4c, S4), the largest group of naturally occurring polyphenols (Strack *et al.*, 1994), but the flavonoid composition differed between summer and the drought treatment. Summer trees had higher concentrations of quinic acid, catechin and luteolin, among other polyphenolics, whereas droughted trees had higher concentrations of catechin, quercitol, homoorientin and quercetin (Figs 1, S4, Table S6). These differences correlated with the differential effects of drought treatment in the different seasons (Fig. 4c). Many biological functions have been ascribed to flavonoids, especially their role as antioxidants (Burda & Oleszek, 2001; Lee *et al.*, 2003). Their antioxidant activity is mainly a result of their role as electron donors (Rice Evans *et al.*, 1997) and their ability to alter the kinetics of peroxidation (Arora *et al.*, 2000). As expected in a water-limited Mediterranean ecosystem, oxidative stress in plants tends to increase under...
conditions of drought (Price et al., 1989; Dat et al., 2000; Munné-Bosch & Pérez-Méndez, 2004; Pérez-Méndez et al., 2004). Quercitol has been associated with the avoidance of osmotic stress by reducing the osmotic potential during drought in Quercus species (Passarinho et al., 2006; Spiess et al., 2012). Our study found no significant differences ($P=0.12$) between choline concentrations in droughted and control trees but did identify a tendency toward higher concentrations in droughted plants (Table S6). Choline is also involved in osmotic protection (McNeil et al., 2001). Drought will probably have a significant impact on the metabolomes of Mediterranean plants, because dry periods will become more frequent and intense in the coming decades, as predicted by projections of climate change (IPCC, 2007).

Droughted trees also presented higher concentrations of total soluble sugars in their leaves (Fig. 4c), supporting the premise that sugars act as osmolytes to prevent the loss of water by regulating water potential and draw water into leaves during periods of drought (Ingram & Bartels, 1996). Control trees in summer did not have significantly lower choline concentrations of sugars relative to droughted trees (Fig. 4b), even though droughted trees had a tendency toward higher concentrations, presenting significantly higher concentrations of sucrose and $\alpha$-glucose. The small differences in foliar sugar concentrations between control and droughted leaves may be mainly attributable to the naturally dry summers of the Mediterranean basin that increased the foliar sugar concentrations in both control and droughted trees (Fig. 4).

**Folivory**

The highest herbivorous activity by insects occurs mainly in spring and early summer in the Mediterranean basin (Powell & Logan, 2005; Bonal et al., 2010), and usually the leaves of trees have accumulated most signs of folivory by the middle of the summer. Interestingly, folivorous activity was higher in the droughted trees in summer (Figs 2, 3). We did not detect any significant difference between control trees and droughted trees in foliar fresh weight, foliar dry weight, foliar water content or foliar size that could explain the higher folivory activity in summer. The small LMA differences between controls and droughted trees were attributable to differences in thickness and not to the foliar density as found in past studies (Ogaya & Pérez-Méndez, 2006), which should not influence consumption through folivory.

GLM analyses for the summer season indicated that the relationship between folivory and PC1 score coordinates of the additional PCA for foliar stoichiometry and metabolomics (Fig. S6) was mainly attributable to the fixed effect of drought, because folivory was not correlated with PC1 within treatments (control, drought; $P>0.05$; Fig. 3). Our results thus suggest that the stoichiometric and metabolomic shifts were mainly caused by the experimental drought, which subsequently indirectly increased the degree of folivory.

Our stoichiometric results identified no significant relationships between foliar N concentrations and foliar C : N ratios and the degree of folivory, as reported in other studies (Larsson, 1989; Larsson & Björkman, 1993; Rouault et al., 2006), indicating that, in our study, folivory was not influenced only by foliar N concentrations (Choong et al., 1992; Williams et al., 1998). As discussed, droughted trees tended to have higher concentrations of sugars and polyphenols (flavonoids) than did the controls (Fig. 4). Flavonoids such as quercetin have been shown to act as phagostimulants in herbivorous insects (Diaz Napal et al., 2010; Kosonen et al., 2012). Despite the scarcity of supporting evidence, our results also indicated a higher folivorous activity in droughted trees, supporting the premise of the high palatability of some flavonoids that are not directly related to defense but act as antioxidants (Diaz Napal et al., 2010; Kosonen et al., 2012). Also, droughted leaves had higher concentrations of sugars (Fig. 4), especially sucrose and $\alpha$-glucose (Fig. 2), which could thus provide a source of rapid energy for herbivores. The higher concentrations of sugars and some flavonoids found in the foliage of droughted Quercus ilex trees appeared to be related to the increase in herbivorous activity (Figs 3, S7).

Not all the assigned polyphenols, however, provide only antioxidant protection in plants. The role of polyphenolics as a chemical defense against predation by herbivores has been widely reported (Berg, 2003; Lokvam & Kursar, 2005; Treutter, 2006; Kosonen et al., 2012; Rani & Pratyusha, 2013). Moreover, Kosonen et al. (2012) reported that some polyphenolics could be toxic to specialist herbivores but could increase the palatability of plants for generalist herbivores and suggested that climate change may reduce the damage caused by specialist herbivores and increase that caused by generalists. Phenolic acids could thus potentially be used for pest management (Rani & Pratyusha, 2013). It has been reported that even mammals can be affected by the polyphenols of plants. For example, Berg (2003) reported that elevated concentrations of catechin negatively affect the consumption of plants by collared lemmings. In our study, nearly all the assigned polyphenols were associated with drought.

![Fig. 5 Summary of the relationship between foliar metabolic shifts produced by drought and folivory activity.](image)
Most of the assigned flavonoids have antioxidant activity, but droughted trees also had a significantly higher foliar concentration of catechin (Figs 2, S5), thus supporting the idea that plant-induced defenses are not only produced by drought but also by a higher degree of herbivorous attack as many previous studies have suggested (Raubenheimer & Simpson, 2003; Fig. 5).

Our results suggest that seasonal drought influences the stoichiometry and metabolome of plants and that experimental drought indirectly affects folivorous activity by shifting the metabolism of plants, making them more attractive for folivores (Fig. 3). The more severe and frequent droughts predicted for the Mediterranean basin (IPCC, 2007) may thus have an indirect impact on trophic webs by changing the amount of herbivory through foliar stoichiometric and metabolomic shifts (Fig. 3). This impact may lead to more intensive and extensive outbreaks of pests and to possible changes in the distribution of food between specialist and generalist herbivores (Kosonen et al., 2012).

Conclusions and final remarks

Foliar N : P ratios of Q. ilex did not significantly change between seasons and were not lowest in spring, the growing season, as expected from the growth rate hypothesis. Foliar K concentrations, however, did change, demonstrating the importance of K in natural summer droughts. The lack of significant variation in N and P concentrations in Q. ilex may be attributable to the buffering effect of lignotubers in this woody species.

Moderate experimental drought increased the concentrations of sugars and polyphenolic compounds with antioxidant function in the leaves of Q. ilex. Our results showed a relationship between these metabolomic shifts and foliar consumption by herbivores. Herbivores may also induce plant responses, increasing the concentrations of defensive polyphenolic compounds.

Our data indicated that summer and droughted trees could avoid or respond to a reduction in water availability by different means, although both had higher foliar concentrations of flavonoids. Summer trees tended to use more K as an osmolyte than did droughted trees.

The present study is an example of how coupling stoichiometric with metabolomic techniques is useful for identifying the molecular responses of plants to changing environmental conditions such as drought, for understanding the mechanisms and functions that underlie the responses of plants to these new conditions and for interpreting the implications of drought for trophic webs.

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