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# Developmental and spatial covariation of nutrients in growing leaves of *Daphne laureola* and their relationships with herbivory

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## Summary

• Here, we studied patterns of covariation of 10 leaf nutrients in expanding and mature leaves of the evergreen shrub *Daphne laureola* (Thymelaeaceae) in southern Spain. Changes in mean values and covariances of nutrients during leaf development may be relevant for plant fitness through herbivory if variation in leaf nutrients influences plant defoliation.

• We analysed the between-population and developmental covariation of leaf nutrients by using common principal components (CPC) analysis. We also studied the relationships between leaf nutrient covariates and natural levels of plant defoliation.

• Plants at our two study sites shared a CPC structure of covariation between concentrations of the leaf nutrients. Trends of nutrient covariation across individual plants were largely determined by between-plant variations in Ca concentration (CPC1), and by an 'overall nutrient status' gradient (CPC2) that was positively associated with major macronutrients (nitrogen, phosphorus and potassium), in both expanding and mature leaves.

• Plant defoliation was positively related to scores on CPC2 for both expanding and mature leaves, indicating greater consumption on plants with balanced, high concentrations of nitrogen, phosphorus, potassium and calcium.

**Key words:** common principal components (CPC), leaf ontogeny, mineral composition of leaves, phenotypic integration, plant–herbivore interactions.



## Introduction

Phenotypic integration can be defined as the pattern of interdependence between organism traits for a given set of morphological, physiological or behavioural characters. Phenotypic integration can originate from genetic constraints (e.g. pleiotropy and linkage), by natural selection favouring certain combinations of functionally related traits over others (Armbruster & Schwaegerle, 1996) and because of developmental interdependence, since different parts and functions of an organism must be coordinated through development to yield a functional whole (Schlichting, 1989; Schlichting & Pigliucci, 1998). Interest in, and empirical data on trait covariation in plants and its evolutionary consequences have increased recently (Waitt & Levin, 1998; Armbruster *et al.*, 1999; Herrera, 2001; Herrera *et al.*, 2002, and references therein). Although most of these studies have dealt with morphological or life history traits whose covariation may directly affect plant fitness (e.g. floral morphology through its influence on pollination success), nonmorphological plant traits that are functionally related may also exhibit distinct patterns of covariation between individual plants. This has been recently documented for leaf nutrient concentrations (Alonso & Herrera, 2001) and to some extent also to nutrients in plant phloem (Linhart *et al.*, 2001), a finding that suggests a number of ecological implications and potential avenues for inquiry (Pugnaire, 2001) since nutrient covariation can also affect plant fitness if related to nutritional deficiencies with consequences for subsequent plant growth and reproduction, or through

herbivory if variation in leaf nutrients influences plant defoliation.

Broad variations in nutrient availability in soils, and in plant physiological responses to this variation, lead to wide ranges of nutrient concentrations in plant tissues of different individuals, species, and ecosystems (Chapin, 1980; Aerts & Chapin, 2000). Furthermore, and superimposed on these differences in mean values, concentrations of different nutrients in leaves tend to covary in characteristic ways across species (Grime *et al.*, 1997; Thompson *et al.*, 1997), suggesting a functional relationship between nutrient concentrations probably arising from interactions between nutrients in uptake processes (Marschner, 1995; Treseder & Vitousek, 2001) or from similarity between plant species in physiological requirements (Garten, 1976). Although studies on patterns of leaf nutrients covariation have mainly focused on interspecific and geographical contexts (Garten *et al.*, 1977; Garten, 1978; Lebreton *et al.*, 1997; Thompson *et al.*, 1997), our recent study on intraspecific variation in leaf nutrients of the deciduous tree, *Prunus mahaleb* (Rosaceae) has shown that the pattern and magnitude of correlations between nutrients across individual plants are not shared by disjoint populations of this species growing at different localities of the same region (Alonso & Herrera, 2001). In addition to drawing attention to the limitations of single-population studies of nutrient covariation, findings of those earlier investigations stress the importance of taking into consideration the hierarchical structure of sampling units (modules, individuals, species, ecosystems) to confirm the existence of meaningful geographical patterns of covariation between nutrients at both the intraspecific and interspecific levels.

One further aspect that needs to be incorporated into studies of intraspecific leaf nutrients covariation are the possible ontogenetic changes in either the extent or pattern of nutrient integration taking place during leaf development. Changes in means and covariances of leaf nutrients during the leaf growing period may be ecologically relevant for at least two reasons. First, possible nutritional deficiencies affecting leaves at this early stage might have consequences for subsequent plant growth and reproduction (Pigliucci, 1997; Pigliucci *et al.*, 1997) and second, it is precisely during the leaf growth period when the impact of insect folivores is usually greatest, even in evergreen species (Alonso & Herrera, 2000, references therein), and thus intraspecific variation in patterns of leaf quality can have immediate effects on defoliation levels. Patterns of nutrient covariation in leaves would be particularly important if host and habitat selection, and/or herbivores' fitness, were related not only to variation in mean concentration of particular nutrients (Bergström & Danell, 1986; Athey & Connor, 1989; McNaughton, 1990), but also responsive to the relative proportions of different nutrients in their food (Clancy, 1992a,b; Cates, 1996; Phelan *et al.*, 1996). Both physiological and ecological aspects are in fact related when we also consider that nutrient imbalance can affect plant resistance and tolerance (Mattson & Scriber, 1987; Herms & Mattson, 1992; Marschner, 1995).

The main goal of this study was to assess, from an integrative perspective, the between-population and developmental patterns of leaf nutrient covariation in expanding and mature leaves of the evergreen shrub, *Daphne laureola* (Thymelaeaceae) in two south-eastern Spanish populations. A secondary objective was to determine whether, in this species, individual variation in folivores' impact was correlated with individual differences in leaf nutrient profiles, since the existence of such a relationship is a prerequisite for interpreting, from an ecological perspective, patterns of nutrient covariation in relation to herbivores. By studying the concentrations of 10 nutrients in expanding and mature leaves of individually marked plants, and assessing average levels of leaf herbivory on the same individuals using data from several years, the following specific questions will be addressed in this paper: (1) Are there predictable bivariate and multivariate patterns of leaf nutrient covariation between *Daphne laureola* individuals at the within-population level (i.e. phenotypic integration with regard to nutrient concentration in leaves)?; if these actually exist, then (2) Are patterns of integration similar in expanding and mature leaves?; (3) Does the developmental component of nutrient covariation remain consistent between populations?; and (4) Is there some predictable relationship between leaf nutrient variation among individual plants and differences in levels of natural defoliation?

## Materials and Methods

The study was carried out February to June 1994–97 at the Sierras de Cazorla, Segura y Las Villas Natural Park (Jaén province), in south-eastern Spain. We studied 56 adult *D. laureola* L. individuals located in two different sites, Roblehondo (1235 m elevation, RH hereafter) and Cuevas Bermejas (1210 m, CB hereafter). The two sites were about 3 km apart within the Guadahornillos river watershed.

### Study species

*Daphne laureola* is a long-lived evergreen shrub that, in the Mediterranean region, occupies preferentially the undergrowth of mixed mountain forests (Nieto Feliner, 1997). Leaves are found only at the distal portion of each branch, forming a single, well-defined rosette ('leaf whorl' hereafter). In the study region, flowering begins in late January, and production of new leaves starts a few weeks later. Expansion of new leaves is rather slow and extends from mid-March to late May. Plants shed their oldest leaves in early summer, when the expansion of new, current-year leaves has finished.

### Leaf sampling and analyses

Mineral composition of *D. laureola* leaves was assessed in 1996 by collecting both expanding (28 April) and fully

expanded (3 June; 'mature leaves' hereafter) young leaves from all marked individuals. Previous year leaves, which could represent the real mature leaves, were not analysed because we had evidence that larvae did not usually consume them (Alonso & Herrera, 2000). On each sampling occasion, four or five leaf whorls were haphazardly selected on each marked plant, and three or four intact (i.e. without signs of herbivory) leaves were collected from each whorl, up to a total of 18 leaves per plant. Leaf whorls used to collect expanding leaves were avoided on the second collection date to reduce possible artefacts. Leaves were removed with scissors, placed into sealed plastic bags and kept in a portable cooler to minimize water loss during transport to the field station. Afterwards, leaves were individually weighed, dried at 45°C until constant mass, and reweighed. Dry leaves from the same plant and collection date were pooled into a single sample and ground in a coffee mill for chemical analyses. Concentrations of five macronutrients – nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca) – and five micronutrients – sodium (Na), copper (Cu), iron (Fe), manganese (Mn), zinc (Zn) – were determined at the laboratories of Instituto de Recursos Naturales y Agrobiología, CSIC, in Seville, Spain. Total N concentration was determined by the Kjeldahl method using a Technicon® BD-40 Digestor Block for mineralization and Technicon AutoAnalyzer® II for determination. For the other elements, samples were incinerated before analysis. Potassium and Na concentrations were determined by flame spectrophotometry, vanadate–molibdate spectrophotometry was used for P determination, and atomic spectrophotometry for the other elements (Pinta, 1973).

## Measurement of natural defoliation

Larvae of noctuid moths (Lepidoptera: Noctuidae) were the only insect herbivores recorded feeding on developing *D. laureola* leaves in two consecutive seasons of intensive scrutiny and occasional observations spread over the 4-yr study period. The most abundant species were *Trigonophora flammea* Esper. and *Noctua janthe* Bkh. (see Alonso & Herrera, 1996 for further details).

The proportion of leaf area removed by the end of the larval growth season (early mid-July) was estimated in the field for every current year's leaf in a series of leaf whorls ($8 < n < 62$) per plant, chosen haphazardly. Individual leaves were classified into one of six defoliation classes according to the per cent leaf area removed: 0, no signs of herbivory; 1, 1–5% area removed; 2, 6–25%; 3, 26–50%; 4, 51–75%; and 5, > 75%. An overall estimate of defoliation for each leaf whorl was obtained as an average using midpoint values of defoliation classes, an estimate that was positively correlated to larval noctuid abundance on plants, as determined by direct censuses (Alonso & Herrera, 1996). A plant-level, mean defoliation figure was obtained by averaging estimates obtained for individual leaf whorls. For the purpose of this study, a long-term, interannual average defoliation per plant was calculated using data from 1994, 1995 and 1997. We did not evaluate defoliation in 1996 because leaf collections from marked plants for chemical analysis could have led to unreliable herbivory estimates. The study years were similar in average defoliation and there was a positive relationship between defoliation experienced by individual plants in consecutive years.

## Data analysis

Unless otherwise stated, analyses were performed using the SAS statistical package (SAS Institute, 1996), and average values are reported as mean ± 1 SD. Before the analyses, data sets were checked for possible outliers by drawing box-whisker plots; values falling out of three times the interquartile range were excluded from the sample. All variables analysed showed no marked deviations from univariate normality. Analyses of variation between expanding and mature leaves in average leaf size and mean concentrations of nutrients at the population level were performed using mixed-model analyses of variance (Proc MIXED), by modelling the data from the same individual plant on different collection dates as repeated measurements. Population, collection Date (April vs June) and Population × Date were included into the model as fixed effects. The covariance structure was defined as compound symmetric (i.e. the Plant within Population random effect was defined as the within-subject term) (Littell *et al.*, 1996).

Patterns of leaf nutrient covariation were analysed in a stepwise fashion, each step being associated with covariation considered from a different perspective. First, we explored static covariation (*sensu* Klingenberg & Zimmermann, 1992) by analysing nutrient correlations across *D. laureola* individuals of the same population and for leaves of the same age (expanding or mature). The overall magnitude of between-individual nutrient correlation was measured as the mean of absolute values of individual nonredundant elements in the correlation matrix (Waitt & Levin, 1998). Second, we performed between-population comparisons of the structure of between-individual covariation of nutrient concentrations by means of common principal components (CPC) analysis (Flury, 1988; Steppan, 1997; Phillips & Arnold, 1999; Alonso & Herrera, 2001). Common principal components analysis is an extension of traditional principal components analysis that allows one to compare the structure of covariation between variables in several independent groups (the two study populations in our case). A common principal components structure would mean that the orientation of the individual principal components is the same in the two study populations, although the variance associated with each one may vary between them. Separate CPC analyses were applied to the two pairs of nutrient covariance matrices corresponding to the two sampling dates. The analyses were carried out with the program CPC, written by Patrick Phillips, University of Oregon, Eugene, OR, USA (available over the World Wide Web at http://www.uoregon.edu/~pphil/software.html).

Comparisons between hypotheses (see below) were performed in a stepwise, hierarchical fashion by means of likelihood ratio tests. The likelihood that a particular model would be not refused was tested against the next lower model in the hierarchy, using the following 'step-up' sequence of hypotheses: unrelatedness → partial components (with increasing number of common components) → common components → proportionality → equality (for a detailed discussion, see Phillips & Arnold, 1999). The null hypothesis in each comparison was always the model indicating the higher level of relatedness of the pair, when the null hypothesis was rejected, then the next lower model in the hierarchy was the one selected. Results obtained from this method were also compared with those based on a 'model building' approach based on Akaike's information criterion (AIC) (Phillips & Arnold, 1999), with a lower value of AIC indicating a better model. The covariance matrices were calculated for all the variables expressed in the same units (mg $g^{-1}$) and transformed logarithmically. This transformation reduced the magnitude of differences between nutrient concentrations and allowed us to apply CPC analysis on the covariance matrix of the 10 study nutrients.

Finally, the third level of analysis involved the exploration of patterns of developmental covariation between nutrients by means of patterned common principal components analysis (dCPC) (Klingenberg *et al.*, 1996). This method, derived from CPC, was originally designed to study covariation between metric characters measured on the same individuals at different growth stages, taking into account that sets of measurements taken on sequential growth stages of the same individual are not statistically independent (Klingenberg *et al.*, 1996). We applied dCPC to study changes in patterns of nutrient covariation between expanding and mature leaves measured on the same plant individuals. The model predicts that the first common principal component (CPC1) at the earlier leaf development stage (expanding leaves) would be more strongly related to the CPC1 of the subsequent developmental stage (mature leaves) than to any other CPC, and the same should also hold true also for CPC2, CPC3, up to CPC*n*. In this study, dCPC was applied to leaf nutrient data from both collection dates and conducted for each population separately by using the DCPC SAS-routine written by C. P. Klingenberg, Department of Biological Sciences, University of Alberta, Edmonton, Canada.

To study the relationship between natural defoliation levels and leaf nutrients, we first calculated the scores of every plant on the dominant CPCs obtained for each collection date at each locality, and on the dominant dCPCs obtained for each population. Scores on a given axis were calculated as the sum of cross-products between the vector of original nutrient concentrations and the corresponding eigenvector. Regression analyses were then used to relate defoliation levels to plant scores on either the CPC- or the dCPC-defined reduced space.

## Results

### Seasonal and between-site variation in mean nutrient concentration

*Daphne laureola* leaves approximately doubled their size from April to June (Fig. 1a). Average concentrations of nutrients also varied between collection dates. Nitrogen and P concentrations exhibited marked declines (Fig. 1c,e), while K, Ca and Mg concentrations increased, from expanding to mature leaves (Fig. 1b,d,f ). For micronutrients, concentrations of Cu and Fe decreased in mature leaves, while Na concentration increased (Table 1). The statistical significance of these variations was tested using repeated measures ANOVA. Differences between dates in average concentrations of all the five macronutrients, Cu and Fe were statistically significant (Table 2), whereas differences between dates in concentrations of Mn, Zn and Na were not. There were also some significant differences between sites in the mean concentrations of some macro- and micro-nutrients (Fig. 1 and Table 1). Calcium, Cu, Fe and Mn concentrations were significantly higher, and Mg concentrations significantly lower, in plants at RH site.

There were some statistically significant Date × Population interaction effects for some nutrients (Table 2), although their interpretation differed between nutrients. For N, the interaction reflected the fact that differences between populations were statistically significant only for mature leaves (Fig. 1c). For Ca and Mg, the significant interaction was due to the magnitude of the difference between populations increasing from expanding to mature leaves (Fig. 1d,f). The seasonal increases in Ca and Mg were most marked in RH and CB sites, respectively (Fig. 1), and the seasonal variation in Mg

**Table 1** Mean, range and coefficient of variation (CV) of micronutrient concentrations (expressed as mg $kg^{-1}$ dry leaf mass) in expanding and mature *Daphne laureola* leaves from the two study sites

| | | Expanding leaves | | | Mature leaves | | |
|---|---|---|---|---|---|---|---|
| Site | Nutrient | Mean | Range | CV | Mean | Range | CV |
| CB | Cu | 10.1 | 6–14 | 19.6 | 8.1 | 4–12 | 21.7 |
| | Fe | 61.9 | 46–102 | 19.6 | 47.0 | 36–63 | 14.3 |
| | Mn | 38.8 | 24–58 | 19.3 | 35.3 | 28–46 | 14.3 |
| | Zn | 63.5 | 37–90 | 21.0 | 62.1 | 41–76 | 17.9 |
| | Na | 132.1 | 0–400 | 85.1 | 155.6 | 0–300 | 48.3 |
| RH | Cu | 11.6 | 7–20 | 26.6 | 9.6 | 7–12 | 12.8 |
| | Fe | 68.3 | 50–98 | 17.2 | 55.1 | 39–80 | 14.6 |
| | Mn | 46.3 | 33–64 | 17.7 | 48.2 | 35–68 | 16.2 |
| | Zn | 62.2 | 44–98 | 18.0 | 63.2 | 39–80 | 13.5 |
| | Na | 153.6 | 0–500 | 89.5 | 217.2 | 0–500 | 52.3 |

CB, Cuevas Bermejas ($n$ = 28 plants); RH, Roblehondo ($n$ = 28 plants). See Table 2 for statistical tests of differences between populations and sampling occasion.

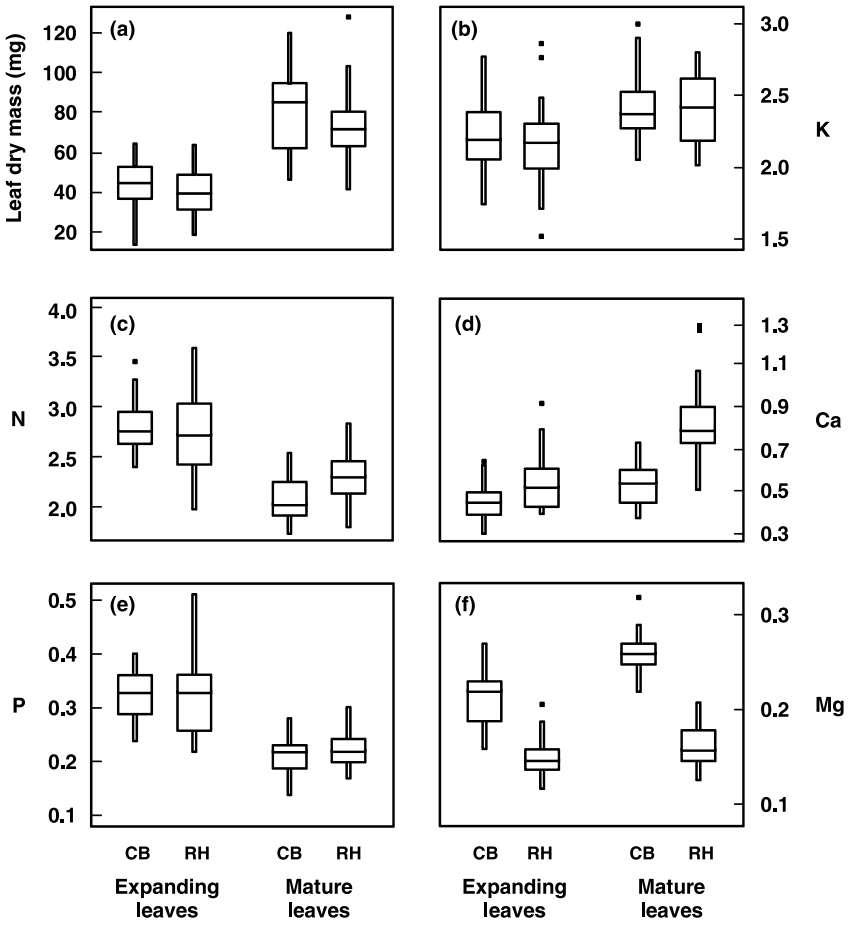


**Fig. 1** Box-and-whisker plots of dry mass and macronutrient concentrations of expanding and mature *Daphne laureola* leaves from Cuevas Bermejas (CB) and Roblehondo (RH) populations, collected in April and June 1996, respectively. The median is represented by the horizontal line, quartiles (25 and 75% percentiles) by wide boxes, and 'whiskers' (observations not greater than 1.5 times the distance between the quartiles) by narrow boxes. More extreme data values are plotted with individual markers. All macronutrient concentrations are expressed as percentage of dry mass. K, potassium; N, nitrogen; Ca, calcium; P, phosphorus; Mg, magnesium.

**Table 2** Summary of results of repeated measures ANOVA testing for the effects of collection date (April vs June), population (Cuevas Bermejas vs Roblehondo ) and their interaction on nutrient concentrations of *Daphne laureola* leaves

| | Collection date (D) | | Population (P) | | D × P | |
|---|---|---|---|---|---|---|
| Nutrient | Wald's $\chi^2$ | *P* | Wald's $\chi^2$ | *P* | Wald's $\chi^2$ | *P* |
| N | 157.8 | < 0.001[1] | 1.2 | 0.28[1] | 7.5 | 0.008 |
| P | 266.9 | < 0.001 | 0.02 | 0.88 | 0.1 | 0.72 |
| K | 34.4 | < 0.001 | 0.5 | 0.50 | 0.4 | 0.53 |
| Ca | 91.1 | < 0.001[1] | 41.0 | < 0.001[1] | 25.5 | < 0.001 |
| Mg | 91.2 | < 0.001[1] | 260.8 | < 0.001[1] | 37.7 | < 0.001 |
| Cu | 25.9 | < 0.001 | 12.1 | < 0.001 | 0.02 | 0.89 |
| Fe | 76.9 | < 0.001 | 11.2 | < 0.001 | 0.3 | 0.57 |
| Mn | 0.4 | 0.55* | 30.7 | < 0.001[1] | 18.1 | < 0.001 |
| Zn | 0.03 | 0.86 | 0.001 | 0.95 | 0.4 | 0.52 |
| Na | 3.8 | 0.05 | 4.4 | 0.04 | 0.9 | 0.35 |

[1]*P*-values for these main effects should be interpreted with caution, given the statistical significance of D × P interactions. See the Results section for details.

concentration in RH site was only marginally significant ($P = 0.04$). Finally, the seasonal change in concentration of Mn had different signs at the two populations, which caused the magnitude of the differences between the populations to increase from expanding to mature leaves (Table 1).

Variation between dates and populations in average per-leaf nutrient contents (i.e. expressed as mg of nutrient per individual leaf ) was also examined. Since all leaves used for chemical analyses had been individually weighed, the average leaf dry mass of each sample could be calculated to obtain an average

per-leaf nutrient content for every individual plant and date. The main difference in the results was in the sign of seasonal changes since, as was expected from the increase in leaf size, the content per leaf of all 10 nutrients analysed increased from expanding to mature leaves. Variation between populations remained. We preferred the analyses of nutrient concentrations because our final aim was more ecologically than physiologically orientated (for discussion, see Koricheva, 1999), largely because concentrations are appropriate estimates of plant quality to herbivores, and also because comparison with interspecific patterns of covariation would be easier in this way.

The N : P ratio increased from expanding ($8.77 \pm 1.17$ and $8.58 \pm 1.14$ for CB and RH, respectively) to mature leaves ($9.76 \pm 1.08$ and $10.46 \pm 1.14$) with differences between dates being statistically significant (Wald's $\chi^2 = 70.82$, $P < 0.0001$ for Date factor in repeated measures ANOVA). Differences between populations in average N : P ratio were statistically significant only in mature leaves ($F = 5.41$, d.f. = 1,53, $P = 0.02$).

## Static nutrient covariation: differences between sites

Correlations between nutrient concentrations are shown in Appendix 1. Concentrations of the different nutrients tended to be more closely interrelated in expanding (overall correlation = $0.29 \pm 0.18$ and $0.28 \pm 0.21$ for CB and RH site, respectively) than in mature leaves ($0.23 \pm 0.16$ and $0.24 \pm 0.18$).

Patterns of multiple covariation can be better described by principal components. Our two study populations shared a CPC structure of covariation between nutrient concentrations of both expanding and mature leaves (Table 3). In both cases, the AIC value of CPC structure was the lowest one and, thus, CPC was the preferred model. In addition, going upwards from the simplest comparison (i.e. unrelatedness), we have to reject, in both sampling dates, proportionality against CPC (Table 3). However, for expanding leaves there were some inconsistencies, for example the five CPC structure, CPC5, was also statistically rejected against CPC4. Yet the AIC value of CPC4 was higher than the corresponding figure for the complete CPC structure (87.8 vs 77.7), which suggested that plants from both sites effectively shared all principal components and not only the first four (these first four components explained most of the observed variance), and that the CPC model was the one better fitting the data. In both expanding and mature leaves, differences between sites in the variation along the CPC axes (i.e. differences in the eigenvalues; Appendix 2) tend to rule out the possibility of proportionality or equality between site covariation matrices.

The patterns of covariation between nutrients of expanding leaves (Appendix 2) indicated that the two populations shared two CPCs that each explained more variance than a single variable. The first, CPC1, corresponded to a gradient mainly defined by variation in Ca concentration, and the second, CPC2, associated positively with most of the macronutrients. The CPC3 reflected a gradient correlated positively with P concentration and negatively with K concentration, and this CPC was particularly relevant in the CB site, where it accounted for 14.4% of variance. Finally, CPC4 mainly reflected variation between plants in Na concentration. Together, the four CPC with the highest eigenvalues

**Table 3** Summary of results of common principal components (CPC) analysis testing for the degree of relatedness of nutrient covariance matrices of the two *Daphne laureola* populations studied, for both expanding and mature leaves

| Model | | Expanding leaves Decomposition of log-likelihood ratio | | | | Mature leaves Decomposition of log-likelihood ratio | | | |
|---|---|---|---|---|---|---|---|---|---|
| Higher (H0) | Lower (H1) | $\chi^2$ | d.f. | *P* | AIC | $\chi^2$ | d.f. | *P* | AIC |
| Equal | Proportional | 3.6 | 1 | 0.06 | 89.1 | 10.0 | 1 | 0.001 | 97.0 |
| Proportional | CPC | **27.7** | **9** | **0.001** | 87.5 | **35.8** | **9** | **< 0.001** | 89.0 |
| **CPC** | CPC(8) | 0.1 | 1 | 0.75 | **77.7** | 0.9 | 1 | 0.34 | **71.1** |
| CPC(8) | CPC(7) | 1.3 | 2 | 0.52 | 79.6 | 0.3 | 2 | 0.86 | 72.3 |
| CPC(7) | CPC(6) | 1.0 | 3 | 0.80 | 82.3 | 6.8 | 3 | 0.08 | 76.0 |
| CPC(6) | CPC(5) | 3.2 | 4 | 0.53 | 87.3 | 2.7 | 4 | 0.60 | 75.2 |
| CPC(5) | CPC(4) | 14.3 | 5 | 0.01 | 92.1 | 2.0 | 5 | 0.85 | 80.5 |
| CPC(4) | CPC(3) | 7.9 | 6 | 0.24 | 87.8 | 10.5 | 6 | 0.10 | 88.5 |
| CPC(3) | CPC(2) | 3.4 | 7 | 0.85 | 91.9 | 5.7 | 7 | 0.58 | 90.0 |
| CPC(2) | CPC(1) | 17.4 | 8 | 0.03 | 102.5 | 9.8 | 8 | 0.28 | 98.3 |
| CPC(1) | Unrelated | 9.1 | 9 | 0.43 | 101.1 | 12.5 | 9 | 0.19 | 104.4 |
| Unrelated | | | | | 110.0 | | | | 110.0 |

Results relevant to the 'step-up' (decomposition of the log-likelihood ratio) and 'best fitting' (based on the Akaike information criterion (AIC) a measure of lack of fit) testing approaches used. The 'Model' column indicates the two hypotheses under comparison by means of the likelihood ratio test. CPC denotes the full common principal components model, and CPC(*n*) are partial components hypotheses having *n* common components. The decomposition of the log-likelihood ratio shows the $\chi^2$ values corresponding to the comparison between consecutive models in the hierarchy. Data shown in bold type indicate the H0 rejected and the model with lowest AIC at each collection date.

explained 85% and 91.5% of the variance between plants in CB and RH, respectively.

For mature leaves, the gradients defined by CPC1 and CPC2 were similar to the ones described above for expanding leaves, CPC3 was mostly associated with K concentration, and CPC4 showed a negative relationship between concentration of Na and Mg (Appendix 2). Together, the four CPC with the highest eigenvalues explained 84.8% and 93.4% of the variance between plants in CB and RH sites, respectively.

## Developmental nutrient covariation: differences between leaf growth stages

Although some results from the previous section suggest a common structure of nutrient covariation between dates, developmental patterns of individual variation were not entirely consistent between populations. At RH, the dCPC model had lower AIC than the full model that considered the data from the two developmental stages as independent (AIC = 262 and 310, respectively). A $\chi^2$ test also indicated that the dCPC model fitted the data better than the full model ($\chi^2 = 202$, d.f. = 135, $P < 0.0001$). This means that individual plant scores in a particular CPC for expanding and mature leaves were correlated, thus denoting stable relationships between the concentrations of nutrients through leaf development of individual plants. Not surprisingly, therefore, the biological meaning of the eigenvectors associated with the dCPC was similar to that found in expanding leaves (Appendix 2): dCPC1 was positively related to Ca concentration, dCPC2 to abundance of major macronutrients (mainly N, P and K), dCPC3 indicated a negative correlation between concentrations of K and P, and dCPC4 was positively correlated with Na.

Nutrient covariation of plant individuals during leaf growth followed distinct patterns in the plants at the CB site. Patterned common principal components was also found to explain satisfactorily the seasonal covariation of leaf nutrients at the CB site. The AIC value of the dCPC model was smaller than the corresponding value for the full model (272 vs 310, respectively), and rejection of the latter was also supported by a statistically significant chi-squared test ($\chi^2 = 232.8$, d.f. = 135, $P < 0.0001$). The biological meaning of eigenvectors associated with dCPC were, however, not exactly the same found for static covariation. While dCPC1 also in this case reflected variation in Ca concentrations, the dCPC2 differed in that it reflected a negative correlation between concentration of Mg and N that was not apparent in the previous analyses. The dCPC3 was positively correlated with P, K and Na concentrations, but not correlated with N, and dCPC4 showed a negative relationship between K and P concentrations.

## Nutrient concentrations and defoliation

Mean plant defoliation levels did not differ significantly between populations in any of the study years (analyses not shown) or in average values for the study period (4.47 ± 2.5% and 4.51 ± 2.4% for CB and RH, respectively; $F = 0.005$, d.f. = 1,54, $P = 0.9$). This allowed us to combine the herbivory data from both populations into a single sample, thus enhancing statistical power to detect some possible relationship between defoliation level and the static patterns of nutrient covariation (CPCs).

Plant defoliation level was positively related to CPC scores obtained for both expanding and mature leaves. Significance of the relationships was clear for the CPC2 at both collection dates (Table 4a), indicating that plants with a relatively high concentration of major macronutrients (N, P and K; Appendix 2) experienced significantly higher defoliation levels. The relationship with CPC1 was less clear, as it was only barely significant in expanding leaves and nonsignificant in mature leaves, hence tending to rule out an effect of Ca concentration on plant defoliation.

The analysis of the relationship between defoliation levels and developmental patterns of nutrient covariation revealed a significant relationship only in RH (Table 4b). At this site, individual variation in dCPC1 and dCPC2 scores explained

**Table 4** Summary of results of linear regression analyses relating 3-yr average defoliation levels for individual *Daphne laureola* plants and their scores on the main independent gradients of between-individual variation in nutrient composition, as revealed by (A) analysis of static patterns of nutrient covariation in expanding and mature leaves, the two study populations combined; and (B) analysis of developmental patterns of nutrient covariation at each population

| | Effect | *b* (SE) | *F* | d.f. | *P* | Model $R^2$ |
|---|---|---|---|---|---|---|
| (A) Static covariation | | | | | | |
| Leaf stage | | | | | | |
| Expanding | CPC1 | 3.39 (1.72) | 3.88 | 1,48 | 0.05 | 0.14 |
| | CPC2 | 4.54 (2.06) | 4.86 | 1,48 | 0.03 | |
| Mature | CPC1 | −0.99 (1.59) | 0.39 | 1,49 | 0.54 | 0.13 |
| | CPC2 | 6.33 (2.36) | 7.21 | 1,49 | 0.01 | |
| (B) Developmental covariation | | | | | | |
| Population | | | | | | |
| CB | dCPC1 | −0.89 (1.74) | 0.26 | 1,49 | 0.61 | 0.01 |
| | dCPC2 | 3.18 (4.12) | 0.60 | 1,49 | 0.44 | |
| RH | dCPC1 | 4.5 (1.23) | 13.37 | 1,48 | < 0.001 | 0.23 |
| | dCPC2 | 3.52 (1.53) | 5.32 | 1,48 | 0.02 | |

23% of within-population variance in defoliation level. The sign of this relationship was the same as described above, with plants with a foliage richer in major macronutrients being more heavily defoliated, but on this occasion differences between plants in Ca concentration were also highly significant. By contrast, there were no significant relationships between defoliation and the developmental patterns of covariation in CB population (Table 4b). It should be remembered here that the patterns obtained from dCPC analysis in CB site were different from those observed at RH site and the static covariation patterns common to both sites. Thus, absence of a significant relationship between defoliation and dCPC at CB does not invalidate the relationships previously found, although it suggests that herbivores did not actually discriminate between plants on the basis of differences in Ca concentration itself but, most likely, in relation to the other covariation gradients that were absent in CB plants.

## Discussion

### Nutrient composition

Final-sized, mature leaves of *Daphne laureola* in our study sites had, on average, higher concentrations of N (2.1% and 2.3% for CB and RH, respectively) and P (0.22% at both sites) than usually found for evergreen species (Aerts & Chapin, 2000), and they were also higher than previously reported for this species in southern France (Lebreton *et al.*, 1997). These observations may suggest that, in our study area, this species was growing under particularly good conditions from the viewpoint of mineral status and nutrition, but they may also be a consequence of the fact that we used only first-year leaves in our analyses. Although our mature leaves collected in June had already reached their final size, the concentration of nutrients in older leaves could give different figures because of further development of cell walls and variations in cell content composition (Garnier *et al.*, 2001).

Only mean concentrations of P, K and Zn, in both expanding and mature leaves, failed to show statistically significant differences between populations. All the other nutrients had significantly different concentrations at the two sites, these differences being more marked in mature than in expanding leaves, particularly for N. These findings are consistent with those from other studies that have similarly shown significant variation in mean nutrient concentration between populations of a single species (e.g. Phelan *et al.*, 1996; Alonso & Herrera, 2001; Linhart *et al.*, 2001), whose magnitude may in some cases exceed that of interspecific variation (Thompson *et al.*, 1997). The difference in concentration of leaf nutrients was not always positive for the same population and the main contrast between both populations was in fact due to an inverse relationship between concentrations of Ca and Mg (Fig. 1), with plants at CB site being richer in Mg and poorer in Ca. A similar inverse relationship between Ca and Mg concentrations was found in a previous study of geographic variation in leaf nutrients of *Prunus mahaleb* in the same region (Alonso & Herrera, 2001), and it most likely reflects differences between localities in the relative proportions of dolomite (calcium magnesium carbonate) and calcite (calcium carbonate) as major components of the limestone bedrock prevailing in the area (González Parra *et al.*, 1985a,b).

By comparing the seasonal changes in average concentration (on a per-mass basis) and content (on a per-leaf basis) of leaf nutrients, we can deduce differences in the metabolism of leaf nutrients that probably reflect physiological mechanisms and source–sink relationships (Weetman, 1989; Koricheva, 1999). Both N and P decreased in concentration (Fig. 1) but increased in absolute content as leaves developed and grew in size, which suggests a dilution of both macronutrients that could not be acquired by leaves at the high rate of biomass accumulation. The same pattern has been previously described in four taiga tree species (Chapin & Kedrowski, 1983), and confirms for *D. laureola* that leaf nutritional value to herbivores on a per-mass basis is highest at the beginning of leaf flush, as found for many other species (Raupp & Denno, 1983; Lowman, 1992). Moreover, the significant change in the N : P ratio from expanding to mature leaves reveals that the dilution effect was relatively higher for P, hence suggesting that N was more efficiently acquired and/or more efficiently retranslocated from previous year leaves (Chapin & Kedrowski, 1983). A second group of nutrients including K, Ca, Mg, Cu and Fe, which have multiple functions in enzyme activation, protein synthesis, photosynthesis and osmoregulation (Marschner, 1995), increased in both concentration and content during the leaf development period studied here, which suggests that these nutrients could probably be limiting leaf growth rate. Finally, Mn, Zn and Na did not change their concentration but increased in content, indicating that acquisition of these nutrients kept pace with leaf growth.

### Nutrient covariation

The magnitude of the covariation between *D. laureola* leaf nutrients across individual plants, measured as the mean of absolute values of correlation coefficients in each population and date, decreased from expanding to mature leaves, although in both developmental stages it was always within the range of phenotypic correlations reported for other phenotypic plant traits (Waitt & Levin, 1998).

We found a common structure of leaf nutrients covariation in the two study sites. We are confident of this result despite our sample sizes being lower than desirable because, in general, CPC analysis tends to underestimate the degree of structure that matrices share (Houle *et al.*, 2002) (i.e. it is possible that the relationship between covariance matrices of both sites might be even higher). Nevertheless, this finding alone is insufficient to conclude that the observed structure is a

species-specific, constant feature reflecting consistent phenotypic integration of leaf nutrient concentrations. Our previous study of leaf nutrients covariation in *P. mahaleb*, based on data from five different populations (Alonso & Herrera, 2001), revealed that different pairs of populations of that species showed widely different degrees of agreement between their nutrient covariance matrices (Pigliucci, 1992), and it was only when all five populations were considered simultaneously that we failed to detect a nutrient covariance structure common to all of them. These small-to-moderate differences between populations in patterns of nutrient covariations can be due to either environmental or genetic causes (Linhart *et al.*, 2001), not easily distinguishable in natural conditions, and whose determination deserves further studies.

Expanding and mature leaves were similar in that the main trends of nutrient covariation across individual plants were largely determined by between-plant variations in Ca concentration (CPC1), and by an 'overall nutrient status' gradient (CPC2) reflecting differences between plants in the concentrations of each of the three major macronutrients (Appendix 2). This second gradient would be equivalent to the 'size factor' commonly found in allometry studies (Somers, 1986), where it usually explains the largest proportion of total variation (even more than 99%). This was clearly not the case for the leaf nutrients studied here, where we found that 'nutrient status' component was the second axis of variation and explained a maximum of 35% of total intrapopulation variance in nutrient concentrations. Departure from multiple isometry has also been found for other plant traits. For example, studies of variation in leaf shape during plant growth showed strong deviation from multivariate isometry in heteroblastic plant species (Jones, 1993), and also different traits characterizing blossom morphology exhibited different magnitudes of ontogenetic variation (Armbruster, 1991).

The developmental component of covariation is particularly relevant to plant fitness because natural selection does not necessarily act on mature traits (Roach, 1986). In particular, the variation of nutrients during leaf development may affect plant growth and reproduction (Pigliucci, 1997; Pigliucci *et al.*, 1997) or herbivores' impact (Cates, 1996; Phelan *et al.*, 1996) and, thus, be subjected to natural selection. We found different patterns of developmental covariation in leaf nutrients in our two study populations, despite the fact that they shared a CPC structure of covariation at the two leaf growth stages studied. Consistency between developmental and static patterns of covariation in the RH site indicated that the variation among plant individuals in this population reflected the same covariations between nutrients maintained at individual level during leaf growth, which suggests a low environmental influence on the covariations between nutrients observed. Although its causes are not evident, the decoupling between static and developmental covariation in the CB site at least suggests that differences between populations in the eigenvalues of CPC had some ecological basis.

### Nutrient concentrations and defoliation levels

Literature on plant–herbivore interactions is full of examples in which herbivores' feeding patterns at scales from within-individual plants (Bowers & Stamp, 1992) to lineages (Farrell & Mitter, 1998; Berenbaum, 2001) are related to differences in plant secondary compounds (for a review, see Rosenthal & Berenbaum, 1991). The relationships between insect herbivory and plant primary metabolites have been less studied despite primary metabolites (i.e. carbohydrates, mineral nutrients, proteins and vitamins) being fundamental for insect herbivores' feeding behaviour and physiology (Scriber & Slansky, 1981; Mattson & Scriber, 1987; Slansky, 1992; Berenbaum, 1995). Mineral nutrients other than N are probably the least-studied elements of insect nutrition, although some evidence exists suggesting that insects may also respond to their variation and covariation (Clancy, 1992a,b; Cates, 1996; Phelan *et al.*, 1996; Linhart *et al.*, 2001). We believe this is the first study in which intraspecific variation and covariation of leaf nutrients during leaf development have been related to natural patterns of defoliation.

Our analyses of the relationship between natural defoliation and leaf composition were conducted on the assumption that leaf nutrient concentrations of individual plants in different years did not differ (Garnier *et al.*, 2001) or were positively correlated. A positive relationship between years in a perennial plant would reasonably be expected if either genotype, location (i.e. soil and shading) or both are the main determinants of leaf nutrient composition. In that case, herbivory and leaf composition could be measured in different years, which would reduce the risks of modifying defoliation records as a result of plant manipulation (Cahill *et al.*, 2001), but would, at the same time, decrease the probability of detecting any relationships if other unstudied factors were stronger determinants of defoliation (Cronin *et al.*, 2001). Despite this, we found a static structure of nutrient covariation common to the two study populations that could be partly related to the average natural defoliation levels experienced over a 3-yr period. The significant relationship between nutrient covariation patterns and plant defoliation did suggest that if nutrient concentrations of *D. laureola* were associated in a predictable way, herbivores could select plants based on these clues. In particular, results suggested that herbivores discriminated between nutrient-balanced individuals, consuming more leaf surface from plants with high concentration of macronutrients (N, P, K and Ca).

The dual discrimination hypothesis proposed that 'secondary chemicals are involved principally in host–plant recognition and nutrients principally in feeding-site selection' (Berenbaum, 1995). In this study, we found that defoliation levels at both study sites were similar despite plants at both populations differing in their nutrient composition, suggesting that herbivores did not use nutrients to discriminate between *D. laureola* plant populations. At the more local scale,

however, herbivores consumed more on plants characterized by balanced and high concentrations of macronutrients, and this trend was stronger in the population where these nutrients maintained their covariation patterns along leaf growth. Therefore, leaf nutrients may be relevant for insect herbivores at the scale of plants within populations and should receive greater study in order to understand the microevolutionary processes of plant–insect interactions.

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## Appendix 1

Pearson product-moment correlation coefficients between nutrient concentrations in expanding and mature leaves of *Daphne laureola* at our two study sites. In each correlation matrix, figures in bold type denote statistically significant correlations at a table-wide $\alpha = 0.05$, and values in italic type are marginally significant ones (at $\alpha = 0.15$), tested using sequential Bonferroni tests (Rice, 1989)

| | Expanding leaves | | | | | | | | | Mature leaves | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Site | N | P | K | Ca | Mg | Cu | Fe | Mn | Zn | N | P | K | Ca | Mg | Cu | Fe | Mn | Zn |
| CB | | | | | | | | | | | | | | | | | | |
| P | 0.27 | | | | | | | | | **0.63** | | | | | | | | |
| K | 0.36 | 0.25 | | | | | | | | −0.02 | 0.07 | | | | | | | |
| Ca | −0.52 | −0.26 | −0.32 | | | | | | | −0.37 | −0.31 | −0.31 | | | | | | |
| Mg | −0.16 | 0.17 | −0.07 | 0.40 | | | | | | −0.12 | −0.02 | −0.01 | 0.27 | | | | | |
| Cu | −0.03 | 0.29 | 0.51 | 0.00 | 0.07 | | | | | 0.06 | 0.10 | −0.27 | 0.10 | −0.34 | | | | |
| Fe | 0.32 | 0.48 | 0.42 | −0.27 | 0.23 | *0.54* | | | | 0.09 | 0.48 | 0.14 | −0.08 | −0.18 | 0.14 | | | |
| Mn | −0.09 | 0.06 | 0.16 | 0.43 | **0.58** | 0.09 | 0.20 | | | −0.42 | −0.44 | 0.07 | 0.40 | 0.33 | −0.14 | −0.29 | | |
| Zn | 0.03 | 0.43 | 0.46 | 0.27 | 0.43 | **0.72** | **0.61** | 0.52 | | 0.15 | 0.26 | 0.41 | −0.17 | −0.20 | 0.42 | 0.37 | 0.16 | |
| Na | −0.19 | 0.15 | 0.01 | 0.36 | 0.18 | 0.40 | 0.06 | 0.33 | 0.17 | *−0.58* | −0.38 | 0.02 | 0.29 | −0.01 | 0.31 | −0.01 | 0.26 | 0.10 |
| RH | | | | | | | | | | | | | | | | | | |
| P | **0.76** | | | | | | | | | **0.64** | | | | | | | | |
| K | 0.49 | 0.36 | | | | | | | | 0.51 | 0.35 | | | | | | | |
| Ca | −0.18 | −0.22 | 0.10 | | | | | | | 0.01 | −0.16 | 0.33 | | | | | | |
| Mg | 0.22 | 0.49 | 0.50 | 0.16 | | | | | | 0.11 | 0.20 | 0.26 | 0.37 | | | | | |
| Cu | 0.44 | **0.66** | 0.17 | 0.01 | 0.38 | | | | | −0.41 | −0.20 | −0.32 | 0.05 | 0.10 | | | | |
| Fe | 0.43 | **0.61** | 0.26 | −0.16 | 0.50 | 0.45 | | | | **0.67** | **0.66** | 0.47 | 0.05 | 0.26 | −0.18 | | | |
| Mn | −0.04 | −0.23 | 0.24 | 0.42 | 0.01 | −0.07 | −0.01 | | | 0.12 | −0.01 | 0.05 | 0.09 | 0.06 | −0.06 | −0.01 | | |
| Zn | 0.31 | *0.59* | 0.17 | 0.18 | 0.33 | *0.56* | **0.67** | 0.01 | | 0.33 | 0.15 | 0.50 | 0.35 | 0.15 | −0.32 | 0.16 | 0.35 | |
| Na | 0.14 | 0.08 | 0.31 | 0.11 | 0.14 | 0.30 | 0.06 | 0.05 | 0.10 | −0.26 | −0.28 | 0.23 | 0.34 | −0.38 | 0.04 | −0.18 | 0.02 | 0.16 |

## Appendix 2

Eigenvectors corresponding to the four common principal components (CPCs) with the largest associated eigenvalues resulting from the CPC analysis of nutrient concentrations of expanding and mature. The main table entries are the correlation coefficients between individual nutrients and the corresponding CPC. To facilitate reading, coefficients with absolute values > 0.4 are shown in bold type, and those with values < 0.1 are shown as dashes

| | Expanding leaves | | | | Mature leaves | | | |
|---|---|---|---|---|---|---|---|---|
| Nutrient | CPC1 | CPC2 | CPC3 | CPC4 | CPC1 | CPC2 | CPC3 | CPC4 |
| Ca | **0.872** | 0.350 | – | −0.292 | **0.817** | **0.484** | −0.256 | – |
| N | −0.331 | **0.419** | −0.272 | −0.234 | −0.387 | **0.517** | −0.360 | 0.263 |
| P | −0.333 | **0.509** | **0.526** | −0.379 | −0.346 | **0.435** | −0.262 | – |
| K | – | **0.530** | **−0.625** | 0.342 | – | **0.478** | **0.821** | 0.145 |
| Na | 0.118 | 0.300 | **0.454** | **0.771** | 0.217 | −0.114 | 0.154 | **0.729** |
| Mg | – | 0.264 | 0.221 | – | – | 0.256 | 0.193 | **−0.611** |
| Cu | – | – | – | – | – | – | – | – |
| Fe | – | – | – | – | – | – | – | – |
| Mn | – | – | – | – | – | – | – | – |
| Zn | – | – | – | – | – | – | – | – |
| Proportion of variance (%): | | | | | | | | |
| CB | 43.7 | 17.3 | 14.4 | 9.6 | 50.3 | 14.9 | 17.7 | 5.5 |
| RH | 36.7 | 35.3 | 7.5 | 12.0 | 41.9 | 33.9 | 6.2 | 11.4 |