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Integrating phenological, chemical and biotic defences in ant-plant protection mutualisms: a case study of two myrmecophyte lineages

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Summary. We examined the role of plant phenology in the evolution of anti-herbivore defence in symbiotic ant-plant protection mutualisms. Phenology of the host-plant affects traits of its herbivores, including size, growth rate, development time, and gregariousness. Traits of herbivores in turn determine what traits ants must have to protect their host. Diversity in plant phenological traits could thus help explain the great ecological diversity of coevolved ant-plant mutualisms. We explored the postulated causal chain linking phenology of the plant, herbivore adaptations to phenology, and ant adaptations for protection, by comparing two myrmecophytes presenting strong contrasts in phenology. In *Leonardoxa africana*, a slow-growing understory tree, growth at each twig terminal is intermittent, the rapid flushing of a single leaf-bearing internode being followed by a pause of several months. In contrast, axes of *Barteria nigritana*, a tree of open areas, grow continuously. Analysis of the phenology (kinetics of expansion) and chemistry of leaf development (contents of chlorophylls, lignin, and nitrogen during leaf growth) showed that these two species exhibit strongly contrasting strategies. *Leonardoxa* exhibited a delayed greening strategy, with rapid expansion of leaves during a short period, followed by synthesis of chlorophylls and lignins only after final leaf size has been reached. In contrast, leaves of *Barteria* expanded more slowly, with chlorophylls and lignin gradually synthesised throughout development. Differences in the phenology of leaf development are reflected in differences in the duration of larval development, and thereby in size, of the principal lepidopteran herbivores observed on these two plants. This difference may in turn have led to different requirements for effective defence by ants. The strategy of phenological defence may thus affect the evolution of biotic defence.

Key words. Myrmecophytes – Coevolution – Delayed greening – Leaf growth – Chemical defences – Indirect defence – *Leonardoxa* – Fabaceae – *Barteria* – Passifloraceae

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Introduction

In plants that have evolved symbioses with protective ants, indirect biotic defence provided by these ants is the most conspicuous component of the plant's anti-herbivore defences (Janzen 1966; Heil & McKey 2003). What were the consequences of the evolution of biotic defence for the ancestral defences of ant-plants? Studies motivated by this question have so far concentrated on whether biotic defences have replaced chemical defences (Heil & McKey 2003; Heil *et al.* 2002). The inconclusive nature of results to date suggests the usefulness of a broader conceptual framework. Furthermore, an important component of plant defence strategies, phenology, has been virtually ignored in studies of the defence systems of myrmecophytes. Phenology—temporal patterns in the growth and development of plant vegetative and reproductive organs—is important in defence because of the inherent problems of protecting growing tissues from herbivores. Growth requires high concentrations of the building blocks of cellular constituents and of the enzymes used to construct them. Growing tissues are thus nutrient-rich. Expanding cells and tissues cannot yet be invested with lignin and other rigidifying structural polymers, so that these important defences are excluded in the protection of rapidly growing organs (Hagerman & Butler 1991). Chemical defences are present in young leaves, often in high concentrations (McKey 1979; Kursar & Coley 2003), but are often inadequate to defend against adapted herbivores attracted to these nutrient-rich young tissues devoid of lignified fibres. With mechanical defence ruled out in young leaves and chemical defence often ineffective, two other kinds of defences may plug the gap. (1) Ants opportunistically attracted to food rewards, which are often concentrated on young leaves, provide biotic defence to growing organs of a large number of plant species. (2) Phenology can be adjusted to reduce the probability that growing organs will be attacked by herbivores, or to reduce the cost of attack that does occur (Coley & Kursar 1996).

Phenology is shaped by many different selective pressures, including herbivory (Aide & Londoño 1989; Coley & Barone 1996; Coley & Kursar 1996; Pilson 2000). Phytophagous insects have in turn evolved numerous

adaptations for locating resources whose patchy distribution in space and time largely results from plant phenology (John 2001). One important plant phenological trait is whether growth is intermittent or continuous. Pulses of intermittent growth can result in "satiation" of herbivores dependent on young growth, with many leaves escaping despite the absence of other defences, and in the evolution of insect strategies that maximise their response to "phenological windows of opportunity" (Martel & Krause 2002). Opportunities for exploiting intermittency of growth to achieve escape of young leaves in space and time (Feeny 1976; McKey 1979; Aide 1988) vary with the plant's ecological strategy (Grime 1979). "Competitors" depend on continuous growth to maintain their advantage, whereas "stress-tolerators" concentrate growth in favourable periods. Escape from herbivores, at first perhaps simply a consequence of intermittent growth, could become a new adaptive function of phenology, as selection acts to sharpen the pulsed nature of growth and concentrate growth in periods of low herbivore activity. Such selection on phenology could be especially important in tropical forests, where herbivory on young leaves accounts for a greater proportion of total herbivory than in temperate-zone forests (Coley & Barone 1996).

One phenological trait shaped by such selection is the phenomenon of "delayed greening" (Kursar & Coley 1992a; 1992b; 1992c), in which the expensive machinery of photosynthesis is transported into developing leaves only after they are fully expanded, lignified, and thus relatively invulnerable to herbivores. Delayed greening allows more rapid leaf expansion rates than observed in young leaves that develop gradually, shortening the phenological window of vulnerability. It also reduces the cost when young leaves are attacked (Kursar & Coley 1992a; 1992b; 1992c). The cost of delaying the onset of photosynthesis may explain why delayed greening is restricted to plants in which leaves once mature have a long life expectancy (Kursar & Coley 1992b).

By influencing many traits of herbivores, plant phenological traits can shape the selective pressures acting on coevolving ants and plants. First, plants differing in phenology may be attacked by different subsets of generalist phytophagous insects, intermittent, short-lived pulses of young growth being attacked primarily by small insects with rapid development and high dispersal capacity, or by highly mobile adult stages of insects whose larvae develop on other resources. In contrast, continuous production of young growth enables attack by generalists that are relatively sedentary, or that have longer development times. Secondly, phenology of the host plant influences the evolution of life history traits of its specialist herbivores. Rapid phenological changes in food resources select for rapid development in insects (Kause *et al.* 1999). When the transition of a leaf to maturity is abrupt, selection may favour insects that complete development before this transition. For such insects, the resource packet represented by a leaf is small and short-lived. In contrast, when the transition from young to mature leaves is gradual, successively older instars may be able to eat successively older tissues, even completing final stages of development on fully mature leaves. For such herbivores, the packet of potentially available resources is large and available over a long period. Analogous differences in the size and lifespan of resource packets arise from differences in the rhythm (continuous or intermittent) of growth at each shoot apex and, when growth is intermittent, in the amount of young tissue produced in each pulse and the degree of synchrony in growth between shoots. Thus the phenology of growth should influence traits of herbivores such as development time, size at maturity, gregariousness, and distribution across leaves of different age classes. Relationships between growth phenology of host plants and traits of their herbivores could result from selection (at the intraspecific level) or from purely ecological processes such as competition, or differential colonisation, at the interspecific level. The traits required of ants for effective biotic defence—size of individual workers, number of workers, behaviour, and distribution of patrolling activity over the plant—are thus likely to be highly variable from one system to another. Taken together, these considerations suggest a causal chain linking phenology of myrmecophytic plants, adaptations of their herbivores to phenology, and adaptations of protective ants coevolving with these plants.

Because phenology has been largely ignored in studies of myrmecophytes, data on such interactions between phenology and biotic defence are lacking. We thus decided to examine the plausibility of these arguments in a comparative study of two myrmecophytes of rainforests of Cameroon. We studied *Leonardoxa africana* Baill. (Aubrév.) subsp. *africana* (Fabaceae: Caesalpinioideae) (McKey 2000)—hereinafter referred to simply as *Leonardoxa*—and *Barteria nigritana* Hook. f. (Passifloraceae). The first is a tree of rainforest understory, while the second, like other members of the genus, is restricted to light-rich environments. Observations prior to the study showed that these two trees differed strongly in many aspects of their vegetative phenology. In fact, they were chosen for this exploratory study because we expected them to present strong contrasts.

Our study falls far short of testing the hypotheses we propose, for two reasons. First, it is restricted to two strongly contrasted lineages. Second, even in this case, data are incomplete, particularly on the crucial second link of the causal chain, traits of herbivores and their relation to plant phenology. The purpose of our study lies elsewhere. By identifying a domain that has been generally neglected in studies of ant-plant protection mutualisms, we hope to stimulate work on other systems that will produce the new kinds of data required for real tests of the new hypotheses we propose.

Materials and methods

Study site and species

The study was conducted near the village of Ebodjé (2°34 N, 9°50 E), in the Campo Forest Reserve (now Campo-Ma'an National Park) in the Southern province of Cameroon. The site is characterised by an equatorial climate with two rainy and two dry seasons per year. Field study took place during the short rainy season, in March-April 1997.

Leonardoxa africana (Baill.) Aubrév. subsp. *africana* (McKey 2000) is a small to medium-sized, slow-growing tree (to 14 m height) of rainforest understory. Trees of this subspecies are associated with the host-specific mutualist ant *Petalomyrmex phylax* Snelling (Formicinae), which live in the tree's swollen, hollow internodes (each internode is a separate domatium with an entrance hole at the apex). Workers of *P. phylax* are very small (2 mm long). Although foliar nectaries, the colony's main source of food, are

active only on mature leaves (McKey 1984; Gaume & McKey 1999), workers of *P. phylax* patrol only young leaves of the plant, which they effectively protect against phytophagous insects (Gaume *et al.* 1997). If not patrolled by ants, young leaves suffer severe attack from sap-sucking hemipterans, microlepidopteran larvae, and adult chrysomelid beetles. Mature leaves are not patrolled, only visited once daily for their nectar (Gaume & McKey 1999), but suffer very little attack from phytophagous insects, indicating the presence of effective chemical and/or mechanical defences (McKey 1984; Gaume *et al.* 1997). Leaves are alternate and paripinnately compound, possessing almost always three leaflet pairs in the population studied.

Patterns of leaf production vary across subspecies of *L. africana*. In the non-myrmecophytic subspecies *L. africana* subsp. *gracilicaulis* McKey (McKey 2000), vegetative growth is highly pulsed. Large flushes of growth (2-4 internodes long) are produced at each twig terminal, and flushing is seasonally synchronized at the individual and population levels. This pattern appears ancestral (Brouat *et al.* 2004). The myrmecophytic subspecies have diverged from it, most markedly the highly specialized myrmecophyte *L. africana* subsp. *africana*. Its pattern of leaf production has been described by McKey (2000). At the scale of a single stem axis, growth is intermittent, with each bout of growth by an apical meristem being followed by a pause of several months. Moreover, each growth bout produces almost always only a single leaf-bearing internode. At the level of the tree crown, growth is staggered and asynchronous across stem axes. While there is seasonal variation in young leaf production, on most individuals some young leaves are present at any time of year.

Barteria nigritana (Passifloraceae) is a small to medium-sized tree (to 15m height) of open habitats, living in thickets on sandy soils of stabilized coastal dunes from Nigeria to Gabon. Like other species of the genus, *B. nigritana* typifies Cook's architectural model, characterised by distinct orthotropic and plagiotropic axes, both displaying continuous growth (Hallé *et al.* 1978). Leaves are simple and alternate. The trunk (a single orthotropic axis) is not occupied by ants, but each horizontal branch (plagiotropic axis) bears at its base a single fusiform swelling (about 10 cm long) extending over several internodes of the branch. Ants bore an entrance hole into the adaxial (upper) surface of the stem at the base of each swelling and occupy these hollow structures. Trees of this species are usually occupied by an undetermined species of *Crematogaster* subgen. *Crematogaster* (Myrmicinae). This species, designated C/F409, has been recorded previously from Nigeria, where it is rarely found foraging on shrubs and cocoa trees (Taylor 2003). While these ants do occupy the tree's domatia, the colony's principal nest site is a large carton nest usually located at several meters height on the main trunk. Workers are about 4-5 mm long. Ants are active on leaves of all age classes, but are especially numerous on young leaves, which bear numerous tiny nectaries on the serrate leaf margins. A row of 3-5 larger nectaries is present on each side of the stem at the node. These nectaries are most pronounced on the orthotropic axis and are active only on young shoots. Observations and experiments show that this *Crematogaster* sp. does provide significant protection against phytophagous insects (Djiéto-Lordon *et al*. 2004).

Kinetics of expansion and proportional growth rate over leaf development

Kinetics of leaf growth was determined by performing repeated measures of leaves over the course of their development. Small young leaves of *Leonardoxa* were not in plentiful supply, and it was impossible to include multiple leaves from each tree. A total of 17 leaves on 11 trees (1-2 leaves per tree) were followed over time for this species. For *Barteria*, we studied a total of 19 leaves on 7 trees (2-3 leaves per tree). For both species, most of the leaves studied were followed from the leaf-bud stage. To compensate for high mortality of young leaves (mainly due to herbivores), and to maintain adequate sample sizes for later stages of leaf development, we added some leaves that had already attained a surface area about half that of a leaf at maturity. Leaf development was followed over a 45-day period. During the first 2 weeks, leaves were measured every 3 days. Near the end of the period of leaf growth, frequency of measurement was reduced to once every 10 days. Measurements were conducted using an electronic calliper (Mitutoyo) of precision ± 0.1 mm. We measured length and greatest width of leaves (or of each leaflet in the case of *Leonardoxa*). Leaf area was estimated using standard curves previously established for leaf area (determined using a scanner [Hewlett-Packard ScanJet 4c associated with the software Dt-Scan ; Delta-T devices, UK]) as a function of the product of length × maximum width. In both species the correlation between logarithms of this index and of measured leaf area was very high (> 150 samples used for each species; $R^2 = 0.997$ for both *Leonardoxa* and *Barteria* [linear regressions; data not shown]).

For each species, an estimated curve (Boltzmann's sigmoid) of the kinetics of growth in surface area was determined using the software GraphPad Prism (v. 4.00 for Windows, GraphPad Software, San Diego California USA). To enable comparisons independent of leaf size (mature leaves varied in size among individuals and between the two species), values for each growing leaf were expressed as the proportion of the final area at maturity of that leaf. The curves are defined by four parameters: (i) the y-intercept of the origin (the proportional area at which the first measures of the leaf were made); (ii) the asymptote (here, final leaf area at maturity); (iii) the point on the x-axis when 50 % of the leaf's final area is reached (V_{50}); (iv) the slope value (growth rate) at V_{50}. To test for differences between species in kinetics of leaf growth, it was necessary to determine first whether variances of slopes of curves during the expansion phase were equal in the two species. Because their variances were found to be unequal ($F^8_{16} = 18.007$, $P < 0.001$), we used a Welch's approximate *t*-test (Sokal & Rohlf 1980) to compare slopes of the estimated curves in the two species at different stages of growth. We also calculated the proportional growth rate (PGR) during each day of development, by the expresion $\lambda = \frac{P(t+1)}{[P(t+1)-P(t)]}$, with P representing the percentage of final leaf area, at time t (in days).

Chlorophyll concentrations over leaf development

To compare the phenology of chlorophyll synthesis between *Leonardoxa* and *Barteria*, we determined the contents of chlorophylls a and b in leaves at different stages of development, from very young to mature. We arbitrarily defined eight developmental stages for *Leonardoxa*, and 9 for *Barteria*, based on size and aspect. Because in each species leaves showed some variation in size at maturity, size alone was not sufficient to define these stages. The criteria we used are presented in Table 1. For each species, a total of from 6 to 10 leaves (except for stage 4 for *Leonardoxa*, from which only three leaves were sampled), collected opportunistically from several individuals, were haphazardly sampled for each stage. Each leaf sampled was immediately placed in a Nalgene® bottle with a known volume of 95% ethanol, in order to completely dissolve chlorophylls and prevent their degradation by polyphenols. The bottles were covered with aluminium foil to prevent alteration of these pigments by light, and kept in darkness until their analysis.

Analyses of chlorophyll contents were conducted at the Centre Pasteur in Yaoundé, Cameroon, within 15 days after sample collection. Preliminary tests conducted in the laboratory at Montpellier showed that this period was more than sufficient to completely dissolve chlorophylls, and led to no significant degradation of chlorophylls if bottles were kept in darkness (L. Amsellem, unpublished data). Chlorophylls were measured by absorption at wave-lengths of 649 nm and 665 nm using a spectrophotometer (Beckman DU-40). Contents of chlorophylls a and b were estimated from the optical densities (OD) measured at these two wave-lengths by using the following equations (Lichtenthaler & Wellburn 1983):

$$[\text{chlorophyll a}]\ (\text{in } \mu g \text{ / ml of solution}) = (13.95 \times OD_{665}) - (6.88 \times OD_{649}).$$
$$[\text{chlorophyll b}]\ (\text{in } \mu g \text{ / ml of solution}) = (24.96 \times OD_{665}) - (7.32 \times OD_{649}).$$

For simplicity, we report the results as the summed contents of chlorophylls a and b. This was justified by the fact that the ratio

Table 1. Definition and description of the different stages of leaf development for *Leonardoxa* and *Barteria*.

Species	Developmental stage	Age (d)	Mean age(d)	Length (mm)	Description
				(Leaflet)	
Leonardoxa	1	1–3	1	< 3	
	2	3–5	4	3–10	Leaflets limp and pendant
	3	5–7	6	10 20	" " " "
	4	7–9	8	20–50	" " " "
	5	9–13	11	> 50	Leaflets still pendant, very pale green and tender, ≥ 90 % of final length
	6	13–18	16		As for stage 5, but have reached final size
	7	18–25	21		Leaflets begin to open and assume final position, become green, and toughen
	8	> 25	25		Leaflets fully opened, bright green, tough
				(Leaf)	
Barteria	1	1 3	2	< 25	
	2	3–6	5	25 50	
	3	6–11	8	50 70	
	4	11–14	13	70 95	
	5	14–16	15	95 130	
	6	16–19	17	130 170	
	7	19–25	22	≥170	≥90 % of final length
	8	25–60	33		Final size, appear young (surfaces bright green, shiny)
	9	> 60	43		Appear older (surface of leaf less shiny)

between the two chlorophylls ($R = \frac{\text{chlorophyll a}}{\text{chlorophyll b}}$) was constant across developmental stages within each species ($R = 2.594 \pm 0.223$) for *Barteria*; $R = 2.063 \pm 0.350$ for *Leonardoxa*).

We determined dry mass of leaves and their area, using a scanner (Hewlett-Packard ScanJet 4c). This allowed estimation of the mean concentration of chlorophylls per unit mass (in $mg \cdot g^{-1}$ leaf) at each stage of leaf development.

For each species, we first tested the normality of distribution for residuals of fitted models concerning concentrations of chlorophylls over leaf development with a Shapiro-Wilk test. We then performed an ANOVA (SAS Institute 1996, version 6.12; PROC GLM) to compare concentrations of chlorophylls over leaf development between different stages. Because of the design of our sampling strategy (leaves were taken from several individuals both within each stage and among stages), a repeated-measures ANOVA could not be performed. To compare the time course of chlorophyll accumulation in the two species, Tukey-Kramer tests between all pairwise comparisons among mean values of chlorophyll concentration of different stages (adjustment for multiple comparisons by least squares method) allowed assignment of stages to statistically homogeneous groups for each species.

Lignin concentration over leaf development

Young leaves were not large enough to be analysed individually, so for each developmental stage a number of young leaves (up to 50 leaflets sampled on more than 10 individuals, for Stage 1 of *Leonardoxa*) had to be pooled. To ensure that results were comparable, we thus also used pooled samples for stages of mature leaves (7 and 6 leaves per stage, for *Barteria* and *Leonardoxa* respectively). Samples of dried leaves were ground into fine powder (1 mm² mesh) using a Cyclotec® mill.

We used two different approaches to estimate lignin contents over leaf development. First, using powdered leaf samples prepared as described above, we determined neutral detergent fibre and acid detergent fibre using the chemical methods of Van Soest (1963). Neutral detergent fibre includes the main cell wall components, essentially hemicelluloses and pectins. Acid detergent fibre includes lignin and the fraction of cellulose encrusted by lignin. Again, young leaves were too small to be analyzed individually, and pooled samples of leaves were used for each developmental stage. For *Leonardoxa*, the limited quantity of material available required pooling of leaves from stages 1-3 into a single sample. Secondly, using aliquots of the same set of leaf samples, we estimated lignin content spectroscopically. Using Near Infrared Reflectance Spectroscopy (NIRS), we screened samples in a spectrum of reflectance of wavelengths comprised between 400 and 2500 nm, sampled every 2 nm (Joffre *et al.* 1992; Gillon *et al.* 1993), and then selected the wavelength for which the correlation coefficient between reflectance and lignin contents obtained according to the chemical method was highest. Comparison of results using spectroscopic and chemical methods to quantify lignin enabled us to assess whether the method used affected the patterns observed.

Nitrogen concentrations over leaf development

Aliquots of the powdered samples used for estimating lignin contents over leaf development were also used to determine nitrogen contents, using a chromatograph (Perkin Elmer elemental analyser [EP 2400 CHN]). For each species, each stage was thus represented by a single measure, giving the average nitrogen content in a pooled sample of leaves from several trees (8-15, depending on the stage of development).

Larval development of the principal lepidopteran herbivores of Leonardoxa *and* Barteria

To examine the relationship between phenology of leaf development and traits of herbivores that shape some of the requirements for effective biotic defence, we compared development time, gregariousness, and size of the principal lepidopteran herbivore associated with each plant. An unidentified microlepidopteran caterpillar (Pyraloidea) was the most frequent insect found attacking young leaves of *Leonardoxa*. Caterpillars of the butterfly *Acraea zetes* (L.) (Nymphalidae: Acraeinae) were by far the most abundant herbivore

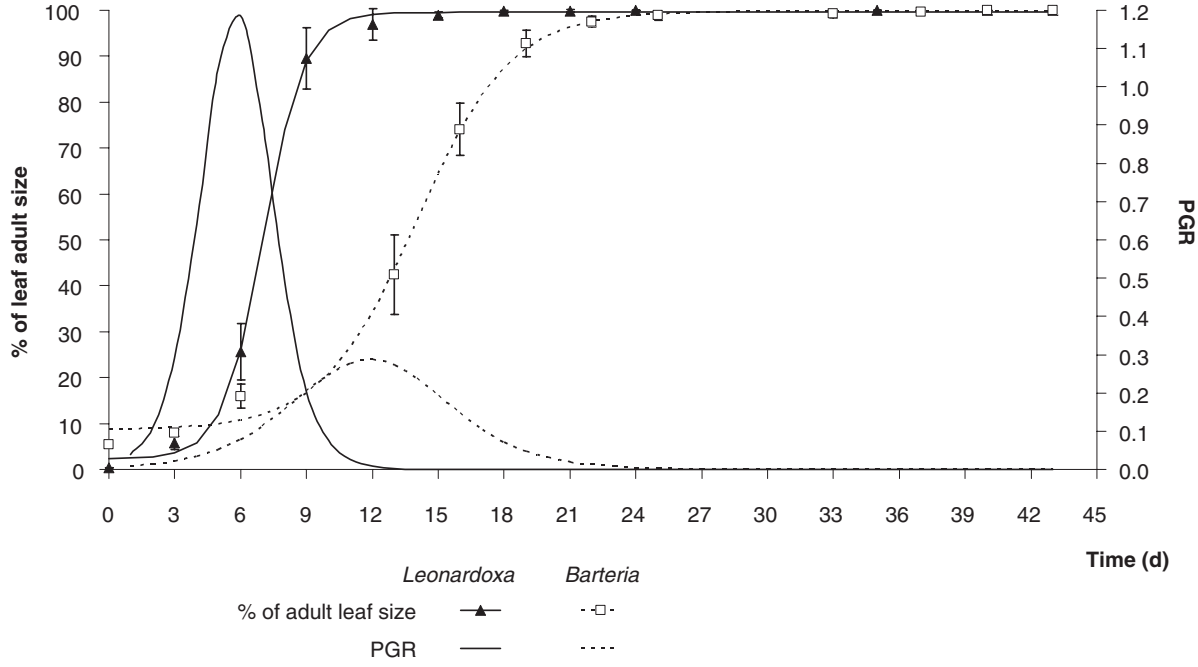

Fig. 1 Kinetics of expansion and proportional growth rate (PGR) over leaf development of *Leonardoxa* and *Barteria*

encountered on *Barteria*. We searched for eggs, counting the number present on each young leaf. Eggs were collected and allowed to hatch, larvae (n = 9) were placed on young leaves of the host, protected from predators in gauze bags, and their development followed. Time to pupation was recorded. We collected cephalic capsules at each moult, allowing determination of the duration of each larval instar and estimation of size and growth rate.

Caterpillars of both species always began their development on young leaves. To determine whether they could complete development on mature leaves, we took caterpillars (n = 8) of different instars from young leaves and placed them on mature leaves, again enclosing them in gauze bags. We compared development time and survivorship with those of caterpillars at equivalent stages that were transplanted from one young leaf to another.

Results

Kinetics of expansion and proportional growth rate over leaf development

The pattern of cumulative increase in leaf area over time was very different in the two species (Fig. 1). Slopes during the growth phase were very different (approximate *t*-test, $t = 5.712$, $P < 0.001$), confirming that *Leonardoxa* leaves grew significantly faster than did those of *Barteria*. Proportional growth rates, calculated from the curves for each species, are also given in Fig. 1. Leaves of *Leonardoxa* completed their expansion after 15 days of growth, whereas those of *Barteria* required 25 days to complete expansion. The actual difference was even greater, because the smallest leaves of *Barteria* we measured had already reached 8.77 % of their area at maturity, whereas the smallest leaves of *Leonardoxa* were only 2.38 % of their final size. Equations for Boltzmann's sigmoid and estimates for the four parameters are given for the two species in Table 2.

Proportional growth rates were also very different between the two species (see Fig. 1). They show that leaves of *Leonardoxa* grew much more rapidly during a much shorter time than did those of *Barteria*, expansion of which was spread out over a much longer period of time. The highest proportional growth rate observed for *Leonardoxa* was > 1 (indicating that leaves more than doubled in area from one day to the next during this period of greatest expansion). In contrast, proportional growth rate reached a maximum value of only 0.28 for *Barteria*.

Chlorophyll concentration over leaf development

Pattern of accumulation of chlorophyll during leaf development was very different in the two species (Fig. 2 and Table 3). In young leaves of *Leonardoxa*, concentration of chlorophylls remained low (< 2.3 $mg.g^{-1}$) throughout the entire period of leaf expansion (through 15 days old; stages 1-6), began to increase (doubling in 6 days from stages 6 to 7), and was still increasing in the oldest cohort of leaves examined in this species (24 days old; stage 8). In contrast, in young leaves of *Barteria*, concentration of chlorophylls was already high (2.3 $mg.g^{-1}$) in the youngest leaves examined (stage 1) and continued to increase slowly during expansion. Shapiro-Wilk tests showed that distributions of residuals of fitted models concerning concentrations of chlorophylls over leaf development were normal, both for *Leonardoxa* and *Barteria* (W = 0.99, $P = 0.64$; and W = 0.97, $P = 0.07$, respectively). ANOVAs showed that in both species, there was a highly significant effect of developmental stage on concentration of chlorophylls ($F^{7}_{59} = 101.12$, $P < 10^{-4}$ for *Leonardoxa* ; $F^{8}_{63} = 30.02$; $P < 10^{-4}$ for *Barteria*). Homogeneous groups deduced from Tukey-Kramer tests confirmed, however, that the patterns of

Table 2. Expression of Boltzmann's sigmoid curve, and associated estimated parameters (means ± standard errors) for developing leaves of *Leonardoxa* and *Barteria* over time. Estimated leaf area, over time, as proportion of final leaf size $= \frac{\text{Bottom}+(\text{Top}-\text{Bottom})}{1+e^{\left[\frac{V_{50}-t}{\text{Slope}}\right]}}$, where t is time (in days)

	Estimated mean ± SE	
Parameters	*Leonardoxa*	*Barteria*
Bottom (y-intercept of the origin ; estimated size [%] at beginning of measures)	2.329 ± 1.060	8.638 ± 1.474
Top (estimated size [%] at end of measures)	99.56 ± 0.5038	99.84 ± 1.002
V_{50} (time when half of final size is reached) (days)	7.048 ± 0.078	14.03 ± 0.205
Slope[1] (steepness of the curve)	0.925 ± 0.052	2.149 ± 0.207
R^2	0.9991	0.9975

[1]The parameter estimated in the equation is actually inversely related to slope. A smaller value thus denotes a steeper slope.

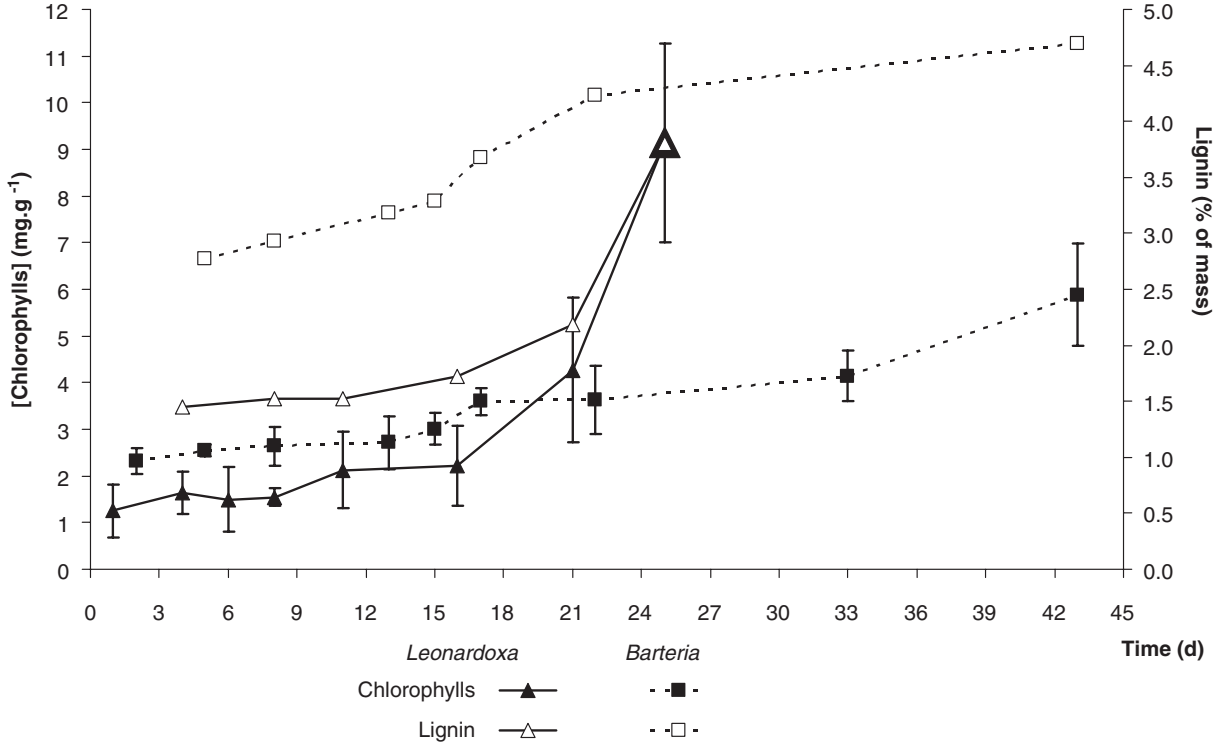

Fig. 2 Concentrations of chlorophylls (mg . g^{-1}) and lignin (% of dry leaf mass) over leaf development in *Leonardoxa* and *Barteria*

chlorophyll accumulation were different in the two species (Table 3). In *Leonardoxa*, developmental stages formed two non-overlapping homogeneous groups, stages 1 to 6 (before completion of leaf expansion) with low values, and stages 7 and 8 (after leaf expansion) with high values. In *Barteria*, in contrast, chlorophyll concentration increased gradually during leaf development, so that homogeneous groups overlapped. Only stage 9 (fully mature leaves over 40 days old) was clearly separated from all the others.

Lignin concentration over leaf development

In each species, the pattern of increasing concentration of lignin in young leaves roughly paralleled that observed for chlorophyll, as shown in Fig. 2. In *Barteria*, lignin concentration was already relatively high in the youngest leaves examined (almost 3 % of leaf dry mass), and increased relatively steadily during leaf expansion, reaching 4.7 % at maturity (the sample for stage 8 of *Barteria* was lost due to an error in experimental protocol). In contrast, in *Leonardoxa* lignin concentration was low and relatively invariable (about 1.5 %) through stage 6, and then rapidly increased, reaching 3.8 % at stage 8 and apparently still increasing at this final sample date.

For all developmental stages of both species, lignin concentration as estimated by chemical analysis (Van Soest 1963) was strongly negatively correlated (R^2 = 0.69) with reflectance of samples at wavelength 2116 nm

Table 3. Mean Leaf Area (MLA), Mean Leaf Mass (MLM), Mean Concentration of Chlorophylls (MCC), and Homogeneous Groups (HG) for chlorophyll concentration deduced from pairwise Tukey-Kramer tests on least square means for the different developmental stages of leaves of the two myrmecophyte species. Values of estimated parameters given are mean ± standard deviation.

Species	Leaf stage	Sample size	Mean age (d)	MLA (cm²)	MLM (g)	MCC (mg/g)	HG
Leonardoxa	1	10	1	1.9 ± 0.3	0.011 ± 0.002	1.3 ± 0.6	a
	2	10	4	4.1 ± 0.2	0.021 ± 0.001	1.6 ± 0.5	a
	3	9	6	7.7 ± 1.2	0.038 ± 0.006	1.5 ± 0.7	a
	4	3	8	15.9 ± 6	0.074 ± 0.025	1.5 ± 0.2	a
	5	8	11	60.2 ± 29.2	0.244 ± 0.107	2.1 ± 0.8	a
	6	10	16	212 ± 39.6	0.766 ± 0.13	2.2 ± 0.9	a
	7	9	21	241.9 ± 70.6	0.862 ± 0.23	4.6 ± 1.2	b
	8	8	25	252.2 ± 62.8	0.896 ± 0.2	10.0 ± 1.2	c
Barteria	1	6	2	0.8 ± 0.3	0.015 ± 0.006	2.3 ± 0.3	a
	2	6	5	4.7 ± 2.1	0.069 ± 0.027	2.5 ± 0.1	a
	3	6	8	7.1 ± 2.2	0.097 ± 0.026	2.6 ± 0.4	a, b
	4	10	13	16.5 ± 3.7	0.202 ± 0.039	2.7 ± 0.6	a
	5	10	15	27.2 ± 7	0.309 ± 0.069	3 ± 0.3	a, b
	6	10	17	51.7 ± 11.5	0.538 ± 0.102	3.6 ± 0.3	b, c
	7	7	22	91.9 ± 11.7	0.883 ± 0.097	3.6 ± 0.7	b, c
	8	8	33	142.8 ± 13.2	1.289 ± 0.103	4.1 ± 0.5	c
	9	9	43	166.1 ± 21.8	1.466 ± 0.164	5.7 ± 1.0	d

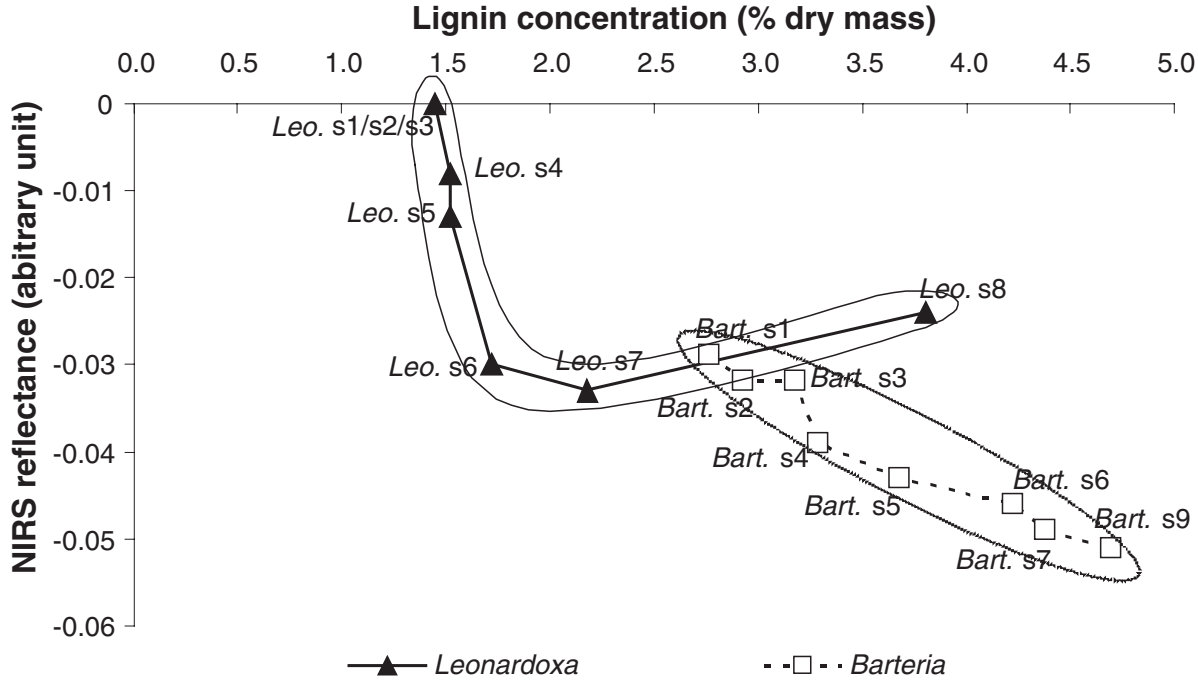

Fig. 3 Comparison of results between chemical (Van Soest, 1963) and spectroscopic (NIRS reflectance at wavelength 2116 nm) methods of estimating lignin concentration over leaf development of *Leonardoxa* and *Barteria*. Leaf developmental stages are indicated for each species

determined using NIRS (expressed as the second derivative of the spectrum at 2116 nm (see Brinkmann *et al.* [2002] for methods and interpretation of results), as shown in Fig. 3. We thus focused on this wavelength to estimate relative lignin content over leaf development. However, the correlation was much stronger, and the relationship much more regular, among different leaf developmental stages of *Barteria*, reflecting the gradual change over leaf development in this species. In contrast, for leaves of *Leonardoxa* at different developmental stages, two points (stages 7 and 8) strongly departed from the relationship evident from the other points (through stage 6). The form of the relationship for *Leonardoxa* thus appeared to be strongly affected by changes occurring after full leaf expansion (stages 7 and 8).

Nitrogen concentration over leaf development

Nitrogen concentrations of young leaves of the two species over the course of development are presented in Fig. 4. During early stages of development, nitrogen concentrations

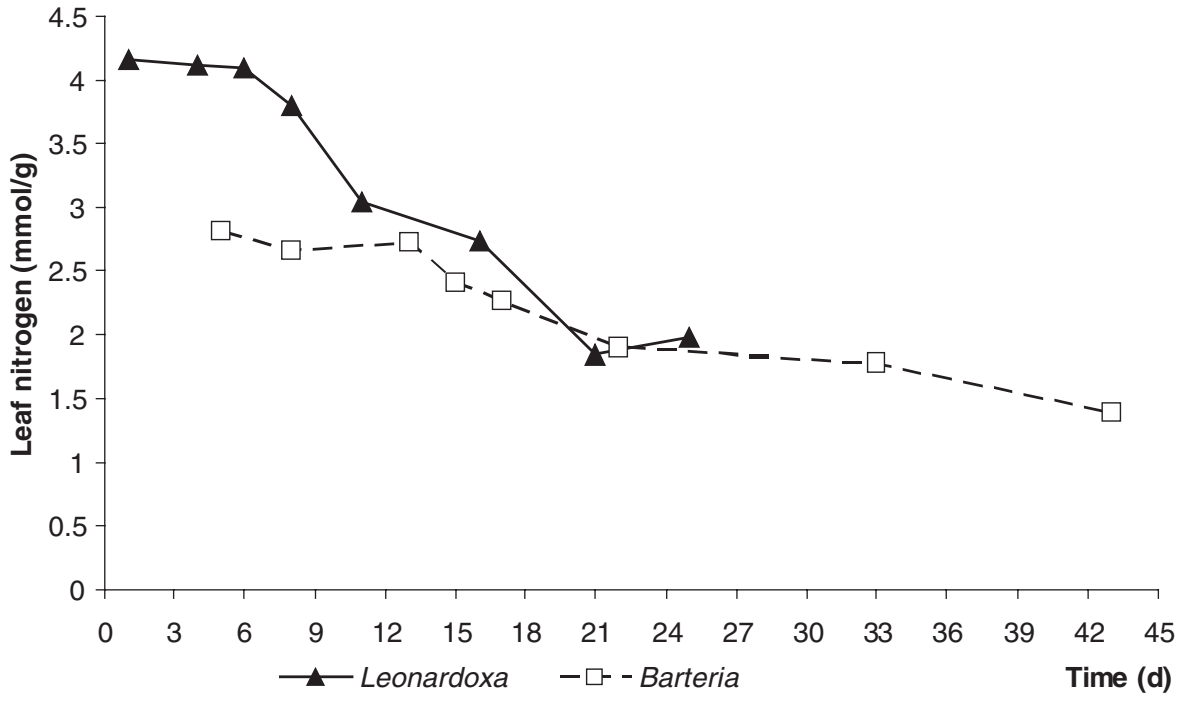

Fig. 4 Mean contents of nitrogen (mmol·g^{-1}) over leaf development of *Leonardoxa* and *Barteria*

of young leaves of *Leonardoxa* (4.16 mmol·g^{-1}, stage 1) were about 1.5 times higher than those of *Barteria* (2.81 mmol·g^{-1}, stage 2). In both species, nitrogen concentration decreased as leaves expanded, but more markedly so in *Leonardoxa*, so that nitrogen concentration in leaves of the two species was comparable by the time they neared maturity (mean of 1.91 mmol·g^{-1} for stages 7 and 8 of *Leonardoxa*, and 1.55 mmol·g^{-1} for stages 8 and 9 of *Barteria*). Because in each species only a single bulk sample of young leaves could be analyzed for each developmental stage, statistical comparison of differences between species and among developmental stages is impossible. However, we believe the values we obtained merit confidence, as they represent a mean value for leaves of 8-15 different individuals at each stage.

Larval development of the principal lepidopteran herbivores of Leonardoxa *and* Barteria

Eggs of the unidentified microlepidopteran on *Leonardoxa* were laid singly, and only on young leaves. Unfortunately, too few caterpillars of different developmental stages were available to allow rearing of this species. However, our observations suggest that larval development requires on the order of 10-14 days under optimal conditions. Size of larvae at maturity was small. For the single last-instar larva measured, the cephalic capsule was 1.26 mm in width and 1.12 mm long.

Acraea zetes on *Barteria* contrasted strongly with respect to all these traits. Eggs were laid in large clusters, up to 80 or more (in this study) on a single young leaf. These eggs were much larger than those of the microlepidopteran on *Leonardoxa*. Seventy caterpillars were reared from egg to pupation, going through five instars and requiring on average 25 days. Whereas early-instar larvae (instars 1-3) were encountered only on young leaves, late-instar larvae fed on progressively older and tougher leaves, and in all cohorts studied, caterpillars completed their development eating fully mature leaves. First-instar larvae of *A. zetes* were larger than those of the microlepidopteran on *Leonardoxa*, and their size at maturity was much larger, with cephalic capsules of last-instar larvae averaging 3.16 ± 0.2 mm in width and 2.37 ± 0.14 mm in length (n = 3).

Caterpillar-transfer experiments confirmed that even final-instar larvae of the microlepidopteran on *Leonardoxa* were unable to use mature leaves of this plant as food. All of the 8 caterpillars transferred to mature leaves died within 4 days, having ceased to eat. In contrast, the 10 control caterpillars transplanted from one young leaf to another continued to eat and grow. Although *Petalomyrmex* ants attracted to young leaves succeeded in entering some bags (they did not enter bags on mature leaves) and killed caterpillars, when the experiment was terminated at 8 days, four of the 10 caterpillars on young leaves were still alive, and had grown considerably. In contrast, fourth- and fifth-instar larvae of *A. zetes* confined in bags with mature leaves of *Barteria* continued to eat and grow and suffered no mortality, apart from four caterpillars, all sampled from the same cohort, that were killed by parasitoids (tachinid flies), presumably acquired before they were enclosed in the bags. Their growth and survivorship did not differ significantly from those of caterpillars confined on young leaves (data not shown).

Discussion

Our results show that patterns of young-leaf development are very different in these two myrmecophytes, and that

these differences are reflected in the contrasting life histories of insect herbivores frequent on each plant. Patterns of leaf development could thus have helped to shape the gregariousness, duration of development, and size of insect herbivores, which in turn can affect the requirements for effective biotic defence by the plant's symbiotic ants. A single causal thread may thus connect the phenology of plant growth to the biology of their insect herbivores, and thereby to the evolution of traits of plant-ants such as worker size and number and the distribution of their patrolling activities on the plant (Meunier *et al.* 1999). Phenology thus not only contributes to defence, it may also shape the kind of biotic defence that evolves in protection mutualisms.

Contrasting strategies of leaf development in myrmecophytes

Our results demonstrate two strongly contrasting patterns of leaf development in these two myrmecophytes. *Leonardoxa africana* exhibits delayed greening. As in other plants with this type of leaf development (Kursar & Coley 1992a), very young leaves have high levels of nitrogen and little fibre, rate of leaf expansion is high, and concentrations of fibre and chlorophyll both increase abruptly following leaf expansion. Like other plants in which delayed greening has been demonstrated (Kursar & Coley 1992b), *Leonardoxa* is a forest-understorey tree with leaves that are long-lived (several years) once mature (McKey 1984). In contrast, young leaves of *Barteria* develop gradually. As in other plants with gradual leaf development (Kursar & Coley 1992c), nitrogen concentration is lower in very young leaves than in plants with delayed greening, leaf expansion is slower, and concentrations of fibre and chlorophyll increase gradually throughout leaf development. *Barteria nigritana* is a light-demanding tree of the canopy of low-stature littoral forest. While lifespan of its leaves is unknown, comparison between the two species of the rhythm at which young leaves are produced on twig terminals and old leaves are lost on older parts of twigs suggests that it is probably considerably shorter than in *Leonardoxa*. These results illustrate the variation to be found in the phenology of young-leaf growth among myrmecophytes, and suggest that this variation follows the same ecological patterns as those identified in other plants.

Has the evolution of biotic defence led to evolutionary adjustment of the phenology of growth?

The evolutionary effect of enhanced biotic defence on phenological defences appears to have been different in these two plants, which also differ in their ancestral phenologies. *Barteria nigritana* is closely related to *B. fistulosa* Mast. (Breteler 1999). Although quantitative data are lacking, the latter species, a highly specialized myrmecophyte (Bequaert 1922; Janzen 1972; Breteler 1999), appears very similar to *B. nigritana* and *B. solida* Breteler (a non-myrmecophyte) in its phenology of vegetative growth (D. McKey, pers. observ.). In all three species, shoot apices virtually always bear some young leaves, suggesting continuous growth. Based on qualitative observation of toughness and colour (D. McKey, pers. observ.), young leaves of *B. solida* and *B. fistulosa* appear to show gradual development, like those of *B. nigritana*. Thus, it appears that in *Barteria*, phenological traits of the non-myrmecophytic ancestor have evolved little in myrmecophytes, presumably because they posed no conflicts with the new system of enhanced biotic defence.

In contrast, in *Leonardoxa* at least two traits of ancestral phenology were transformed during the evolution of myrmecophytism. The number of young leaves and internodes produced by an axis in each pulse of growth was strongly reduced, and the ancestral synchrony of growth across axes in the non-myrmecophyte was greatly relaxed with myrmecophytic specialisation (McKey 2000). As a result, the total area of young leaves that must be patrolled by ants at any one time is reduced. This suggests that components of phenological defence that were incompatible with effective biotic defence have been replaced by the latter. A phenology that satiates herbivores could also exhaust protective ants! However, another ancestral component of phenological defence has been retained by the myrmecophytes. While quantitative data are again lacking, observations of leaf colour, toughness, and expansion rate suggest that young leaves of the non-myrmecophyte *L. africana* subsp. *gracilicaulis*, like those of the myrmecophyte, exhibit delayed greening. As noted above, delayed greening is frequently combined with intermittent and synchronous production of large quantities of young leaves (Coley & Kursar 1996). Why has this component of phenological defence, in contrast to others, been retained in *Leonardoxa*? This question is addressed in the following section.

Leaf development and larval development of insect herbivores

The principal herbivores encountered on these two plants showed marked contrasts in life history, and these appeared to be related to patterns of young leaf development. Although our study was brief, the principal herbivores are among those shown to be most important in longer-term studies of both *Leonardoxa* (McKey 1984, Gaume *et al*. 1997) and *Barteria* (Djiéto-Lordon *et al*. 2004). While first-instar larvae of *Acraea zetes* fed only on tender young leaves of *Barteria*, as larvae grew they ate successively tougher leaves. Such a pattern is frequent in chewing insect herbivores (Hochuli 2001). Owing to the destruction of young leaves, near the end of their development larvae usually fed only on mature leaves. Duration of larval development of *A. zetes* is longer than the time to expansion of a *Barteria* leaf. We suggest that this pattern of development is permitted by the gradual transition to maturity of leaves of this species. Because of the absence of any abrupt transition, resources potentially available to larvae thus include not only the young leaves, essential for the early stages, but also the plant's mature leaves. This permits not only larger size and longer duration of development of individual larvae, but also the deposition of larger clutches of eggs. Female *A. zetes* lay clutches of up to 150 eggs on an individual young leaf. In contrast, caterpillars of the unidentified microlepidopteran attacking *Leonardoxa* were much smaller in size, fed strictly on tender young leaves, and completed their development in a much shorter period, less than the time required for maturation of the leaf. We suggest that such a pattern of development is required by the short period of expansion of leaves of this species and the abrupt nature

of the chemical and physical changes that quickly follow expansion. The resource packet available is represented solely by the young leaf, and larval development must match leaf development. The high nitrogen content and low lignin content of expanding leaves may facilitate this rapid development. Furthermore, only very rarely was more than one larva found on a young leaf, suggesting that eggs are usually deposited singly. The small size of resource packets should favour females that avoid sibling competition by avoiding deposition of multiple eggs on a leaf.

Other phytophagous insects observed on young leaves of *Leonardoxa* were also very small. These included psyllids, hemipterans that are characterised by rapid development and highly effective dispersal by means of flight and passive transport (e.g., Peck [1994]). These traits appear to be important in their strategy of moving around from one packet of young growth to another. Another frequent herbivore on young leaves of *Leonardoxa* was the adult stage of the chrysomelid beetle *Monolepta* sp. (Galerucinae). Larvae of *Monolepta* spp. usually develop on grass roots, and the adults appear to be generalist feeders on young leaves (Gaume *et al.* 1997). These highly mobile insects also can rapidly move among localised and ephemeral packets of young growth, such as the young leaves of *Leonardoxa*.

Leaf development, herbivore traits, and requirements for successful biotic defence

Patterns of growth and development of young leaves thus influence, directly or indirectly, many traits of herbivores associated with a given plant. These include growth rate, duration of larval development, size at maturity, gregariousness, and distribution of feeding activity over leaves of different age classes. Across and within insect and other animal species, duration of development is correlated with size at maturity (Roff 2000), although some intraspecific studies have shown that selection for rapid growth can weaken trade-offs between development time and final size (Kause *et al.* 1999). Differences in these traits of herbivores mean that the traits of ants required for effective biotic defence will also vary from one system to another. These traits include the size of individual workers, recruitment and other behaviours, and the distribution of patrolling by ants among leaves of different age classes. Greater investment in patrolling and defence of the plant will be favoured by selection to the extent that it confers a fitness payoff to ants. High investment will be favoured if health of the plant (and fitness of its ant colony) depend on a large worker force patrolling leaves of all age classes. Lower investment in patrolling and defence will be favoured if phenology and chemistry combine to restrict herbivores to young leaves, which can be protected by a smaller force of ants. This investment can also be subdivided among many small workers, if abrupt leaf development imposes rapid development and small maximum size of phytophagous insects.

In *Leonardoxa*, delayed greening presents no obvious incompatibility with biotic defence. Furthermore, its maintenance has probably been key to a distinguishing feature of biotic defence in *Leonardoxa*, namely its complete restriction to young leaves. Workers of *Petalomyrmex phylax*, the host-specific mutualist of *L. africana* subsp. *africana*, are much smaller (total length ca. 2 mm) than those of the *Crematogaster* sp. (total length ca 4-5 mm) associated with *B. nigritana*. We postulate that across different myrmecophyte systems, the type of leaf development, gradual or with delayed greening, will be associated with development time and thereby also with the range of larval size of their respective herbivores. In consequence, leaf development will also be associated with the size and other traits of workers of each plant's mutualistic ants.

What evidence exists to test these hypotheses? First, comparative studies strongly suggest that worker size often does evolve during evolutionary specialisation of plant-associated ants. For example, workers of *P. phylax* are much smaller than those of their less specialised closest relative, *Aphomomyrmex afer* Emery, and their reduction in size is postulated to have been driven by coevolutionary pressures such as those examined here (Meunier *et al.* 1999). In contrast, in some other systems specialisation is associated with evolutionary increase in worker size. For example, *Tetraponera aethiops* F. Smith (Pseudomyrmecinae), the host-specific mutualistic ant associated with *Barteria fistulosa*, has the largest workers known for the genus (P. Ward, pers. comm.). Leaf development in *B. fistulosa* Mast., a much more specialised myrmecophyte than its congener *B. nigritana* (Janzen 1972; Breteler 1999; Djiéto-Lordon *et al.* 2004), is very similar to that in the latter species (D. McKey, pers. observ.), and the two plants share many of the same large phytophagous insects, including *A. zetes*. While selective pressure by mammalian herbivores, which eat both young and mature leaves of this plant (McKey 1974), has been emphasized in explaining the significance of large size in *T. aethiops* (Janzen 1972), the presence of large insect herbivores, favoured by the plant's growth phenology, should not be neglected.

Conclusion

The aim of this study was to document contrasting patterns of leaf development in two myrmecophytes and to examine their possible consequences. To our knowledge, this is the first study to examine interactions between biotic and phenological defence of myrmecophytes. The large differences in leaf developmental patterns that we demonstrate appear to have important consequences for development of phytophagous insects. By influencing size, gregariousness, distribution of feeding activity across leaf age classes, and other traits of insects, leaf development patterns could thus indirectly influence the evolution of many traits of ants important in biotic defence. Scattered information on other systems is consistent with the hypotheses developed from our two-species comparison. However, testing the generality of these hypotheses will require comparative studies that document the pertinent traits of insect herbivores attacking myrmecophytes in more detail than most studies so far. Ecological analyses of the composition of herbivore faunas could be combined with phylogenetic analyses that simultaneously track the evolution of phenology and of chemical and other direct defences in myrmecophytes, and of traits affecting biotic defence by their

associated ants. Such studies integrating all the various defences of myrmecophytes into a single conceptual framework could not only teach us much about the comparative biology of protection mutualisms, but could also yield insights into the role of phenology in plant defence generally.

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