



Original article

Herbivory at marginal populations: Consequences for maternal fitness and vegetative differentiation

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ABSTRACT

Margins of distribution of plant species constitute natural areas where the impact of the antagonistic interactions is expected to be higher and where changes in the dynamics of plant–herbivore coevolution could promote intraspecific differentiation in (co)evolving plant traits. In the present study, we investigated how differences in the average herbivory level affect maternal fitness in core continuous and marginal disjunct populations of *Daphne laureola* in an effort to assess the role of herbivores limiting plant distribution. Furthermore, we investigated intraspecific differentiation in vegetative traits and their potential connection to divergent selection by herbivores in both groups of populations. Our results did not support increased herbivory at the species margin but did support a difference in the effect of herbivory on maternal fitness between core continuous and marginal disjunct populations of *D. laureola*. In addition, herbivores did not exert phenotypic selection consistent with the geographic variation in studied plant traits. Therefore, the geographic variation of vegetative traits of *D. laureola* seems to be consequence of environmental heterogeneity more than result of geographically divergent selection by herbivores.

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1. Introduction

Herbivory can reduce growth, fecundity and survival of plants (Crawley, 1989; Strauss, 1991), thus, it should not be surprising that one of the most prominent sets of adaptations in the history of life is plant defence against natural enemies (Agrawal, 2011). Geographic variation in plant–herbivore interactions has been commonly related to both the magnitude of consumption and the effects of herbivory on plant populations geographically distant (Strauss and Zangerl, 2002; Pennings and Silliman, 2005; Adams and Zhang, 2009). Spatial variation in the intensity of interaction is important because it establishes geographic selection mosaics where intraspecific differentiation processes could occur (Thompson, 1994).

Herbivore abundance can vary spatially and temporally as a result of abiotic (e.g. weather conditions) and/or biotic factors (e.g. abundance of their host plant or natural enemies) (Schoonven et al., 1998). Such variations, while potentially random, can be also the result of highly predictable factors leading changes in plant–

herbivore interactions associated with marginal populations based on several grounds (Maron and Crone, 2006; Gaston, 2009). First, changes in composition and abundance of herbivore fauna have been frequently reported in marginal populations of the host plant (Lewinsohn et al., 2005). Second, local environmental conditions are expected to be furthest from the species' ecological optimum in marginal regions (Hengeveld and Haeck, 1982; Brussard, 1984; Vucetich and Waite, 2003, but see Tsaliki and Diekmann, 2009). Therefore, the negative impact of herbivory on fitness of individuals may be greater in marginal populations and thus could contribute to the maintenance of stable distributional limits (Bruelheide and Scheidel, 1999; Geber and Eckhart, 2005; Maron and Crone, 2006). Finally, marginal populations of plant species frequently are characterised by reduced genetic diversity, which in turn, could decrease the adaptive potential of marginal populations to herbivores (Mopper et al., 1991; Hughes and Stachowicz, 2004; Eckert et al., 2008). Therefore, it could be expected that the margins of plant distributions emerge as natural locations where plant–herbivore interactions and the negative impact for the plant fitness are expected to vary (Maron and Crone, 2006). However, few empirical studies have examined the influence of natural enemies at marginal habitats (Gaston, 2009).

From the herbivore's viewpoint, the quality of a plant depends on a wide variety of phenotypic traits ranging from nutritional and

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secondary chemicals to plant architectural traits. In fact, studies analysing the variation across landscapes in selection by herbivores and in (co)evolving traits have frequently shown that the path of coevolution has proceeded at different rates or in different directions at different sites (Zangerl and Berenbaum, 2003; Muola et al., 2010; Vergeer and Kunin, 2011; Singer and McBride, 2012). In that regard, we propose margins of plant distributions constitute natural areas where one might expect to find *a priori* changes in the selection patterns by herbivores and thus, intraspecific differentiation in (co)evolving phenotypic traits of plant species.

In the present study, we compared core continuous and marginal disjunct populations of the shrub *Daphne laureola* within its distribution range in the south of Iberian Peninsula. In this region, the primary herbivores of this species are four noctuid caterpillars and no sign of ungulate browsing has ever been observed (Alonso and Herrera, 1996; A.R.C. personal observations). Intraspecific local variation in defoliation levels has been related to plant architecture (Alonso and Herrera, 1996) and leaf nutrient composition (Alonso and Herrera, 2003). Therefore, in the present study we analyse changes in the magnitude and effects of herbivory on plants at a large geographical scale by comparing core continuous and marginal disjunct populations. Specifically we addressed the following questions: (1) do core continuous and marginal disjunct populations of *D. laureola* differ in their level of foliar damage? (2) does foliar damage have stronger negative impact on fitness in individuals of marginal disjunct populations? and (3) is there differential phenotypic selection by herbivores on vegetative traits matching to the geographic variation in these traits?

2. Material and methods

2.1. Study species

D. laureola L. (Thymelaeaceae) is a long-lived evergreen shrub with a disjunct Palaearctic distribution and an association with the main calcareous mountainous ranges of Europe and northern Africa (Meusel et al., 1978). This study was conducted in 2007–2009 in the Baetic Ranges of the southern Iberian Peninsula. Across the Baetic Ranges, *D. laureola* presents a local continuous distribution patch in the Sierra de Cazorla with numerous and largely connected populations (Castilla et al., 2012). The spatial isolation of populations increases towards the eastern and western margins, although the increase is sharper in western edge (Castilla et al., 2012). Therefore, for the purpose of this study, we considered the Sierra de Cazorla populations as the local continuous center (“core region” hereafter) and populations located along western edge as the local disjunct margin (“marginal region” hereafter) of the species’ distribution patch in southern Iberian Peninsula. We studied three accessible populations per region (Table 1; Castilla et al., 2011, for a more detailed description of study populations). At the beginning of the study we randomly marked 25 hermaphrodite and 15 female plants in each study population. Sample size was chosen to reflect the average relative frequency of both sexes in the study region (data not shown), in an effort to avoid potential artifacts due to uncontrolled variation on sample sex ratio.

D. laureola flowers between January and April, shortly before new leaves are produced. Plants consist of a variable number of erect stems that rise from a common trunk at ground level. Leaves are found only at the distal end of each branch, forming a single, well-defined leaf whorl. Leaves of *D. laureola* are consumed by four polyphagous noctuid species (*Trigonophora flammea* Esper., *Noctua janthe* Bkh., *Noctua fimbriata* Schreber, *Pseudenargia ulicis* Staud.), but not by large herbivores (Alonso and Herrera, 1996; A.R.C. personal observations). The period of activity of herbivores finishes when larvae pupate at the end of May.

Table 1

Population features of the six *Daphne laureola* populations studied. Populations were classed into one of the following size categories: <500, 500–1000, 1000–1500, 1500–2000, >2000 reproductive individuals (Castilla et al., 2011).

Population	Coordinates	Region	Altitude (m.a.s.l.)	Size (No. reproductive individuals)	Female frequency (%)
Valdecuevas	37° 54' N 2° 52' W	Core	1380	>2000	25.5
Cañada del Espino	37° 55' N 2° 53' W	Core	1575	500–1000	20
Fuente Bermejo	37° 55' N 2° 50' W	Core	1513	>2000	21
Grazalema	36° 46' N 5° 25' W	Marginal	1229	1500–2000	10
Fuente Molina	36° 41' N 5° 1' W	Marginal	1380	1000–1500	20
Cañada de las Animas	36° 42' N 5° 1' W	Marginal	1333	500–1000	18.5

2.2. Plant phenotypic traits

In 2007, for each marked plant we measured the basal diameter of the supporting stem at ground level using a Mitutoyo digital calliper (basal diameter, hereafter). A previous study showed that basal diameter was negatively selected by herbivores in at least some populations at the core region (Alonso and Herrera, 1996). In addition, we estimated proportion of fruits per flower using five randomly marked inflorescences per individual (“fruit set” hereafter). Both basal diameter and fruit set were estimated at the end of May.

In July of 2009, five intact leaves (i.e. without signs of herbivory) were randomly removed from each marked plant with scissors, placed into sealed plastic bags and kept in a portable cooler to minimize water loss during their transport to the field station. Immediately following, leaves were dried at ambient temperature in sealed containers with silica gel. Dried leaves were individually weighed on an analytical balance Mettler Toledo PL 203.S (± 0.001 g) and their area measured using a leaf area meter LI-3000C (1 mm² resolution; LI-COR, Lincoln, USA). Dry weight and leaf area were used to calculate the specific leaf area mm² g⁻¹ for each sample. Finally, all five dry leaves from the same plant were pooled into a single sample and homogenized to a fine powder using a Retsch MM 301 mill for chemical analyses. We extracted the soluble phenols in 70% methanol from two aliquots of 250 mg of leaf powder per individual. Total phenol content was determined using Folin Ciocalteu assay (Singleton et al., 1999) using gallic acid as standard. We measured at wavelength $\lambda = 765$ nm, and results were expressed as mg gallic acid equivalents per gram of plant material.

2.3. Herbivory incidence

The proportion of leaf area removed was quantified in the leaf whorls for 20% of total stems per plant, except for individuals with ≤ 12 stems, in which the proportion of leaf area removed was quantified in all stems (Herbivory level, hereafter). Herbivory level of individuals was quantified by the end of the larval growth season in two different years (2007 and 2009). We did not find any sign of overcompensation for tissue lost from herbivory (personal observation). In each leaf whorl, leaves were classified into one of 6 herbivory classes according to percentage of 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%. Each herbivory category was represented by its midpoint in the estimation of the herbivory level of leaf whorls (0, 3, 16, 39, 63 and 88 respectively), and weighed by their

frequency to calculate an average proportion of area removed per leaf following the method proposed by Alonso and Herrera (1996). Finally we calculated the mean value of herbivory per individual averaging the herbivory values across leaf whorls.

2.4. Data analysis

Hermaphrodite and female plants frequently differ in the degree of experienced herbivory as well as in the effects of the herbivory on female fitness (Ashman, 2006 and references therein). For that reason, we investigated the differences between sexes in *D. laureola* analysing sexes separately within each region. However, sexes of *D. laureola* did not differ significantly in herbivory level, relationship between herbivory level and maternal component of fitness, vegetative traits and phenotypic selection patterns by herbivores (Castilla, 2012). Therefore, we grouped hermaphrodite and female individuals of each region for getting larger statistical power in the analyses.

All statistical analyses were performed using the SAS statistical package (SAS Institute, 2002). Differences in the level of herbivory between regions were analysed using generalized linear mixed models with a negative binomial function (Procedure GLIMMIX). Effects of region (core vs. marginal) and population were treated as fixed factors. Differences in the level of herbivory in 2007 and 2009 were analysed with separated analyses. In addition, we determined if individuals suffered similar levels of herbivory in different years using a spearman rank correlation.

We studied the relationship between herbivory and fruit set in 2007, using an analysis of covariance (Procedure MIXED). Fruit set was the response variable, showing a normal distribution. Herbivory level used as the continuous explanatory variable and population as the categorical effect.

Differences between regions in basal diameter, phenolic content and specific leaf area of individuals were analysed using generalized linear mixed models (Procedure GLIMMIX) with normal error distribution for phenolic content and negative binomial distribution for both basal diameter and specific leaf area. Population and region effects were treated as fixed.

Phenotypic selection at each region was examined using Lande–Arnold selection gradient models (Lande and Arnold, 1983). We conducted separated phenotypic selection analyses for individuals of core and marginal populations. We quantified phenotypic selection only in 2009 because we only measured foliar traits (specific leaf area and phenolic content) in that year and foliar traits frequently present between-year variation. We used relative values for the response variable herbivory level (estimated by dividing the mean values per individual by the population means). All phenotypic traits were standardized to mean 0 and variance 1, and were used as independent variables. Among population variation in phenotypic selection on foliar morphology and chemistry was evaluated by testing the homogeneity of slopes of the relations of relative level of herbivory to the measures of phenotypic traits among populations with Population \times basal diameter, Population \times phenolic content and Population \times specific leaf area (e.g. Strauss et al., 2005; Rey et al., 2006). We first ran the model with all of the interactions and then excluded the nonsignificant ones (Littell et al., 2006). Preliminary analyses including nonlinear terms, showed that quadratic partial regression coefficients were not significant at any region (data not shown), suggesting that, when present, selection on each particular trait was predominantly directional and linear. Therefore, we focused only on directional selection gradients and did not assess quadratic terms in the phenotypic selection model, as this approach facilitates interpretation of population \times traits interactions. Furthermore, restriction of the analyses to directional selection is also justified in the

present context, because directional selection seems to play a central role in phenotypic diversification at the species level and above (Rieseberg et al., 2002).

3. Results

3.1. Herbivory and its effect on fitness in core and marginal populations

Herbivory level was highly correlated across two years ($r_s = 0.39$, $P < 0.0001$, $N = 240$; Fig. 1). On average, individuals in the core region suffered more herbivory than those in the marginal region, but the difference was only statistically significant in 2009 ($F_{1,231} = 2.60$, $P = 0.11$ and $F_{1,214} = 4.28$, $P < 0.05$ in 2007 and 2009 respectively; Fig. 2a). Population was the only factor with a significant effect on the herbivory level of individuals in both years ($F_{4,231} = 12.57$, $P < 0.0001$ and $F_{4,214} = 39.40$, $P < 0.0001$ in 2007 and 2009 respectively; Fig. 2b). Among population variation in average herbivory level was greater in the marginal than in the core region. The Grazalema population exhibits the maximum values of herbivory in both years, whereas the Cañada de las Animas and Fuente Molina populations showed the minimum values (Fig. 2b).

In addition, our results showed a difference in the effects of herbivory on maternal fitness between regions (Fig. 3). In core populations, herbivory did not show a significant effect on individual fruit set ($F_{1,112} = 0.42$, $P = 0.5$; Fig. 3). However, in marginal populations fruit set was negatively affected by increased herbivory ($F_{1,114} = 15.92$, $P < 0.0001$; Fig. 3).

3.2. Phenotypic selection by herbivores in core and marginal populations of *D. laureola*

The three investigated phenotypic traits showed significant differences between core and marginal populations (Fig. 4). On average, individuals in marginal populations had larger specific leaf area than those in core populations ($F_{1,209} = 36.67$, $P < 0.0001$; Fig. 4a). Nevertheless, populations also varied widely in the average specific leaf area of their individuals ($F_{4,209} = 5.33$, $P < 0.0001$). Basal diameter was larger in the marginal region than in the core

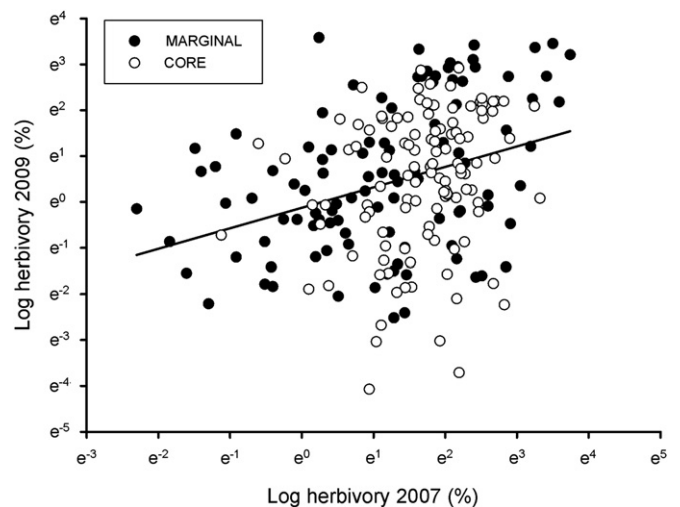


Fig. 1. Relationship between the herbivory levels on individuals of core and marginal populations of *D. laureola* in different years (2007 and 2009). Spearman correlation was used to study the relationship between both years. The herbivory level is expressed as percentage of leaf removed per individual (see material and methods for a more detailed description). White and black dots represent herbivory level scores per individual in core and marginal populations respectively.

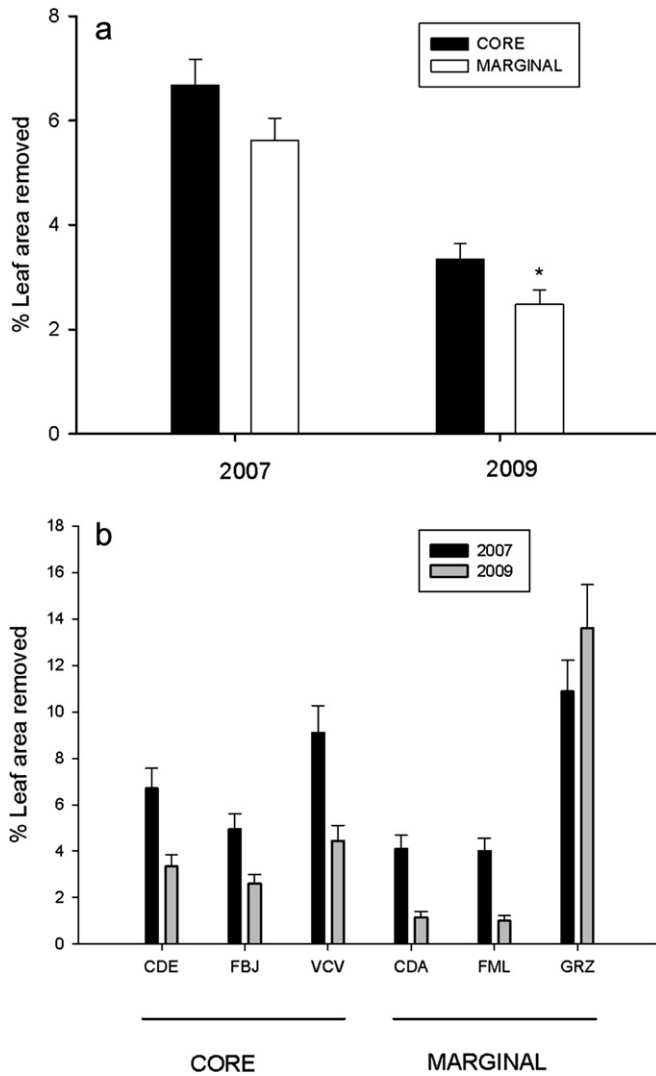


Fig. 2. Differences in the average herbivory level in two different years (2007 and 2009) between regions (a) and populations (b) of *D. laureola*. Statistically significant variation between core and marginal regions is indicated with asterisk ($*P < 0.05$). Herbivory level is expressed as average percentage of leaf removed. Bars represent the least-square means (\pm S.E.) after accounting for region and sex (fixed factors).

region ($F_{1,215} = 23.19$, $P < 0.0001$; Fig. 4b), although there was a high among population variation ($F_{4,215} = 6.69$, $P < 0.0001$). Average phenolic content was lower in marginal populations than in core populations ($F_{1,207} = 297.99$, $P < 0.0001$; Fig. 4c). In addition,

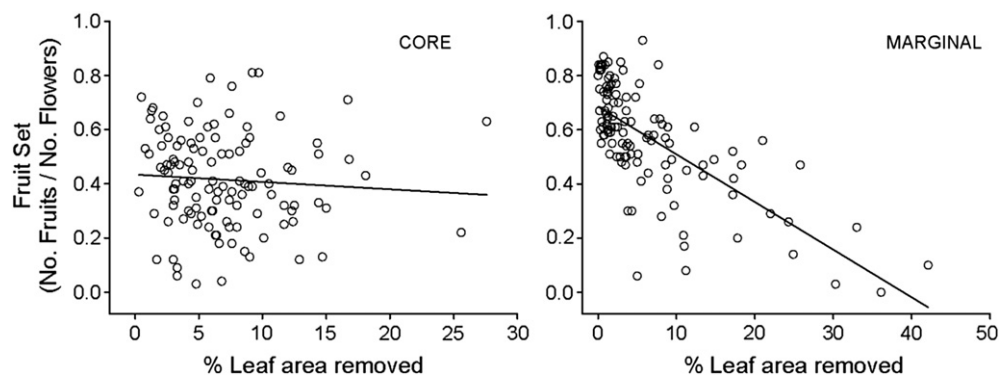


Fig. 3. Fruit set related to herbivory level in individuals of core and marginal populations of *D. laureola*. Fruit set was quantified as the proportion of flowers developing fruits per individual. The herbivory level is expressed as percentage of leaf removed per individual. Each circle corresponds to an individual.

populations differed widely in their average phenolic contents ($F_{4,207} = 10.43$, $P < 0.0001$).

In core populations, plants with lower phenolic content suffered marginally larger herbivory, in contrast to marginal populations where the phenolic content did not have any effect on maternal fitness (Table 2). In marginal populations, herbivores also exerted phenotypic selection on basal diameter but this effect was variable among populations (Table 2). Individuals with larger basal diameter suffered more herbivory in one marginal population (Fuente Molina), but not in either of the other two marginal populations (Fig. 5). In core populations, we did not find any relationship between herbivory level and basal diameter (Table 2). Finally, herbivores did not exert any phenotypic selection on specific leaf area either in core or in marginal populations (Table 2).

4. Discussion

4.1. Herbivory and its effect on fitness in core and marginal populations

Recent theoretical reviews single out herbivory as a factor potentially limiting the distribution range of plant species (Maron and Crone, 2006; Van der Putten, 2012). The role of herbivores in the distribution of invasive species has been widely analysed (Maron and Vilà, 2001), in contrast with the striking scarcity of studies focused on stable limits in native plant species (e.g. Moore, 2009; Vergeer and Kunin, 2011). In this study, we examined the potential role of herbivores in limiting the distribution of *D. laureola* by quantifying the level of herbivory experienced by reproductive individuals and its effect on the maternal component of the fitness in core and marginal populations. Our results did not support the claim that individuals in marginal populations experience higher consumption by herbivores. In fact, individuals from core populations of *D. laureola* suffered larger herbivory level in 2009, whereas core and marginal populations did not differ in the average herbivory in 2007. Our results are in line with other recent studies which document a decrease in herbivory damage in marginal populations (Moore, 2009; Vergeer and Kunin, 2011) and further remark relevance of differences in herbivory among nearby populations.

However, similar levels of herbivory in individuals in core and marginal populations had different effect on their reproductive output. Herbivory negatively affected the fruit set of individuals in marginal populations, but had no effect in core populations. This result suggests a differential tolerance to herbivore damage in core and marginal populations and it seems to support the claim that herbivores play an important role in limiting the reproductive output of marginal populations (Fornoni et al., 2004). Some studies

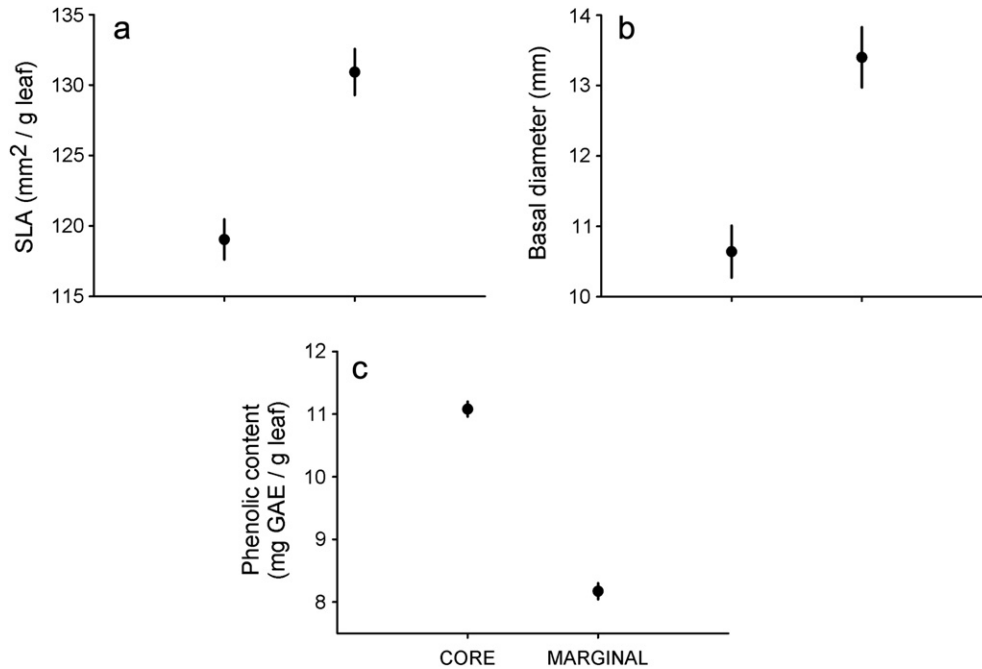


Fig. 4. Regional differences in vegetative traits of *D. laureola*. Three vegetative traits were studied: specific leaf area (SLA), basal diameter, at ground level, of the supporting stem of each plant (BD) and total phenolic content (PC). Dots represent regional least-square means (\pm S.E.) for each vegetative trait.

have shown that even if the magnitude of herbivory is the same among plant populations, variations among populations in its seasonal timing can create variation in its average effects on plant fitness (Knight, 2003). In long-lived plants, late season herbivory should generally be less detrimental than early-season herbivory because current reproductive success relies primarily on resource availability and resource acquisition during early phases of ovary fertilization (García and Ehrlén, 2002). An earlier defoliation by herbivores in marginal disjunct populations than in core populations could explain the different impact of herbivory in both groups of populations. Unfortunately, we did not monitor the progress of herbivory over the period of leaf development, instead we quantified herbivory at the end of the larval growth season. Therefore, we are unable to quantify the interaction between plant phenology and the initiation of herbivory by larvae. Further studies monitoring leaf development and herbivory timing could clarify if the different impact of the herbivory on the maternal fitness is related or not to different timing of herbivore communities in core and marginal disjunct populations of *D. laureola*.

Table 2

ANCOVA analyses comparing patterns of selection by herbivores on vegetative and foliar traits in core and marginal populations of *D. laureola*. Dependent variable was the level of herbivory. BD, PC and SLA represent basal diameter, phenolic content and specific leaf area respectively. Independent variables were standardized to mean 0 and variance 1. Significant relationships ($P < 0.05$ or smaller) are in boldface. Marginally significant relationships ($0.05 \leq P < 0.09$) are in italics. Only linear selection gradients were considered (see Material and Methods). Results show the standardized selection coefficient (b) and its standard error mean (SE).

Effect	Core				Marginal		
	d.f.	$F_{(108)}$	P	b (SE)	$F_{(101)}$	P	b (SE)
BD	1	2.64	0.11	-0.18 (0.11)	0.71	0.40	-0.19 (0.12)
PC	1	3.90	0.05	-0.21 (0.11)	2.71	0.10	-0.13 (0.08)
SLA	1	0.11	0.74	-0.04 (0.11)	1.26	0.27	-0.09 (0.08)
Pop	2	0.03	0.97		0.11	0.89	
BD \times Pop	2				7.31	<0.01	

4.2. Phenotypic selection by herbivores in core and marginal populations of *D. laureola*

Herbivores frequently exert phenotypic selection on the vegetative traits of plant species, although the intensity of such phenotypic selection varies geographically (Gómez, 2005; Pennings et al., 2009; Muola et al., 2010; Vergeer and Kunin, 2011). This geographic variation in phenotypic selection could lead to intraspecific differentiation among plant populations in phenotypic traits important for plant–herbivore interactions (e.g. Herrera et al., 2006 for selection by pollinators). Our results showed that all of the vegetative traits we investigated did differ between core and marginal populations of *D. laureola*, but phenolic content of leaves was the only trait that resulted in congruent phenotypic selection patterns based on maternal fitness of individuals. Herbivores exerted negative selective pressure on the phenolic content of leaves of individuals in core populations, whereas herbivores did not exert any selection on this trait in marginal populations. This result seems to match with the geographic variation in the chemical composition of *D. laureola* populations, with an increased concentration of phenolic compounds in populations where herbivores exert negative phenotypic selection on this trait. However, we must be cautious for two reasons. First, phenotypic selection on phenolic content of leaves in core populations was only marginally significant. Second, the geographic variation in leaf phenolic content of leaves and specific leaf area suggest that there exists geographic variation in the light environment of the understory (see below). On the other hand, neither specific leaf area nor basal diameter geographic variations matched the phenotypic selection patterns by herbivores in core and marginal populations.

Individuals of core populations had lower average basal diameters and leaves with lower specific leaf area but higher phenolic content than those individuals of marginal populations. Differences in specific leaf area and phenolic content could be related to contrasting light regimes in core and marginal populations of *D. laureola*. Plants growing in shaded environments frequently had

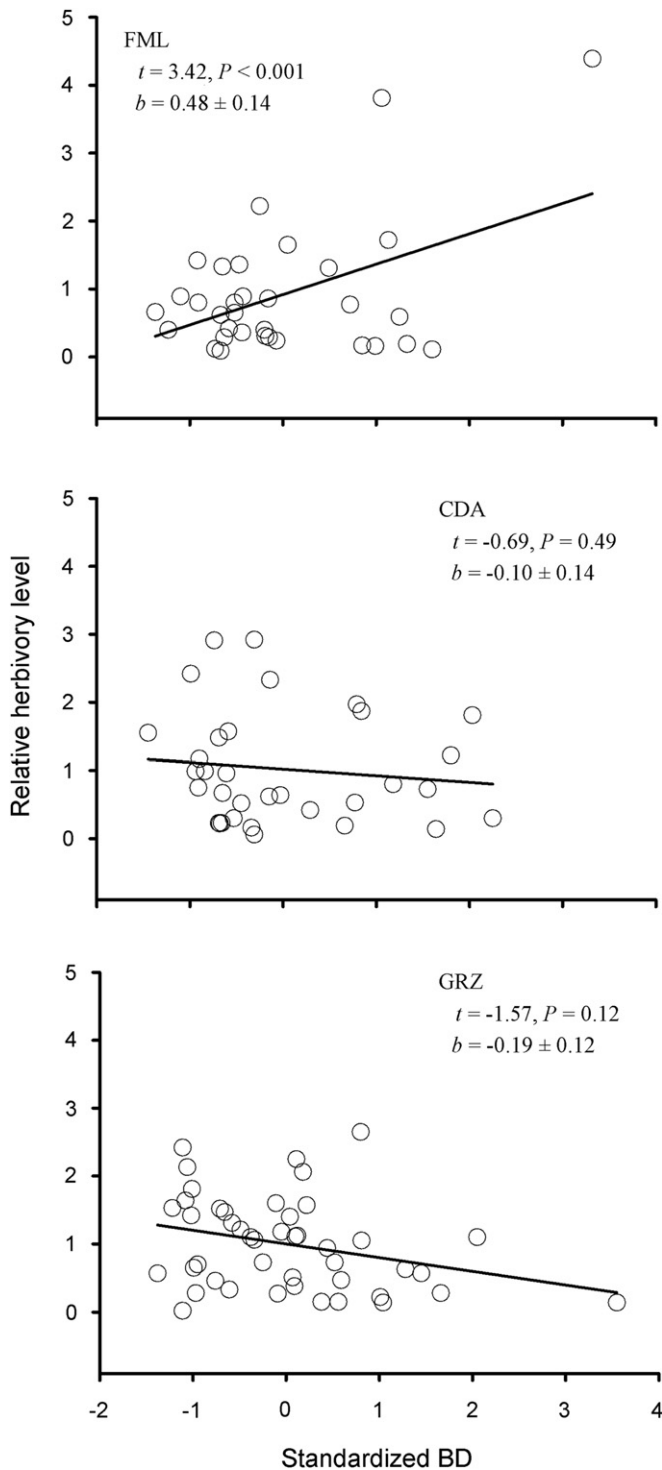


Fig. 5. Standardized linear phenotypic selection gradients for the basal diameter (BD) in individuals of marginal populations of *D. laureola* (Fuente Molina, Cañada de las Animas, Grazalema). Dependent variable was the relative fruit set, estimated by dividing the mean values per individual by the population means.

leaves with larger specific leaf area to efficiently capture light, a potentially limiting resource (Chen et al., 1996; Meziane and Shipley, 1999; Barber and Marquis, 2011). Light exposure can also alter leaf quality, especially in plants with carbon-based defences, resulting in plants with lower phenolic content in leaves in shaded environments (Nichols-Orions, 1991; Dudt and Shure, 1994; Barber

and Marquis, 2011). In the core region, *D. laureola* grows in the understory of mixed forests characterized by patchy light availability (Alonso and Herrera, 2008). In marginal areas, individuals of *D. laureola* are established in the shrub stratum of closed fir forests, *Abies pinsapo* Boiss, which are more characterized by a homogeneous and closed canopy (Arista, 1995). Larger specific leaf area and lower phenolic content in the leaves of individuals of marginal populations could be a consequence of living in shadier environments.

The results of the present study suggest that herbivores did not have a relevant role promoting intraspecific differentiation in the vegetative traits of adult plants among *D. laureola* populations. However, we have only studied the effects of foliar herbivory on adult plants. The relevance of herbivory could differ among different life stages (e.g. Bruelheide and Scheidel, 1999). For instance, both caterpillars and slugs seem to be important mortality agents for *D. laureola* seedlings (C.A. unpubl. data), but we did not analyse their effect in the present study.

4.3. Concluding remarks

Results of the present study showed important differences in the herbivory level among nearby populations more than clear regional divergence. In addition, our results ruled out that regional differentiation in vegetative traits is the result of divergent phenotypic selection by herbivores in *D. laureola*. Rather, contrasting light regimes because of differences in the species composition of the tree canopy in core and marginal populations of *D. laureola* could be related to regional differences in foliar traits.

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