RESEARCH PAPER

Simulated herbivory does not constrain phenotypic plasticity to shade through ontogeny in a relict tree

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ABSTRACT

Ecological limits to phenotypic plasticity (PP), induced by simultaneous biotic and abiotic factors, can prevent organisms from exhibiting optimal plasticity, and in turn lead to decreased fitness. Herbivory is an important biotic stressor and may limit plant functional responses to challenging environmental conditions such as shading. In this study we investigated whether plant functional responses and PP to shade are constrained by herbivory, and whether such constraints are due to direct effects based on resource limitation by considering ontogeny. We used as a model system the relict tree *Prunus lusitanica* and implemented an indoor experiment to quantify the response of saplings of different ages to shade and herbivory. We measured five functional traits and quantitatively calculated PP. Results showed that herbivory did not constrain functional responses or PP to shade except for shoot:root ratio (SR), which, despite showing a high PP in damaged saplings, decreased under shade instead of increasing. Damaged saplings of older age did not exhibit reduced constraints on functional responses to shade and generally presented a lower PP than damaged saplings of younger age. Our findings suggest that herbivory-mediated constraints on plant plasticity to shade may not be as widespread as previously thought. Nonetheless, the negative effect of herbivory on SR plastic expression to shade could be detrimental for plant fitness. Finally, our results suggest a secondary role of direct effects (resource-based) on *P. lusitanica* plasticity limitation. Further studies should quantify plant resources in order to gain a better understanding of this seldom-explored subject.

INTRODUCTION

Sessile modular organisms, such as plants, require specific mechanisms to cope with highly heterogeneous (both in space and time) environments. Phenotypic plasticity (PP), or the capacity of a given genotype to express different phenotypes under different environmental conditions, is a particularly adequate means for plants to cope with environmental heterogeneity (Bradshaw 1965; Sultan 2000). Plastic responses of plants to contrasting environments have been frequently reported as adaptive (Valladares *et al.* 2007), but this is not always the case and there are still few examples of proven adaptive plasticity that results in a fitness benefit for the plant (Van Kleunen & Fischer 2005). Plasticity levels observed in nature are often lower than expected, and this suggests the existence of costs and limits associated with PP that prevent organisms from exhibiting 'perfect' or 'infinite' plasticity (DeWitt *et al.* 1998; Auld *et al.* 2010); the concept of 'perfect' plasticity refers to the expression of the best trait value in every environment with no cost for having that ability and if no constraints exist (DeWitt *et al.* 1998).

Generally, plastic organisms fail to attain 'perfect' PP because of an inability to consistently produce the optimum (*i.e.* a constraint) or because they pay a high cost for the ability

to be plastic (when actual plasticity is not beneficial or even negative in terms of fitness; DeWitt *et al.* 1998). A potential plastic response in a given trait may be large but the observed plasticity can be lowered by resource limitation or environmental stress (Van Kleunen & Fischer 2005). In particular, the influence of other environmental factors on the plastic responses to a given environmental factor frequently leads to reduced expression of PP, and this has been referred to as the ecological limits to PP (Valladares *et al.* 2007). These ecological limits include both constraints and costs induced by multiple biotic and abiotic factors, which frequently exert their influence simultaneously in complex environments (Valladares *et al.* 2002). Despite growing evidence of the importance of the ecological limits to PP (Quezada & Gianoli 2006; Valladares *et al.* 2007; Gianoli *et al.* 2009), these have been explored in less detail than the internal or intrinsic limits to PP.

Ecological limits to plant PP might be induced, *e.g.* by simultaneous herbivory and shade. Herbivory damage is indeed an important biotic stressor for plants and may interfere with their capacity to respond to shading environments (Callaway *et al.* 2003). Several studies have offered insights on this topic, examining the simultaneous effect of herbivory and shading (Kurashige & Agrawal 2005; Gianoli *et al.* 2007; González-Teuber & Gianoli 2008; Salgado-Luarte & Gianoli 2011). A key

line of evidence (Gianoli *et al.* 2007; Salgado-Luarte & Gianoli 2011) suggests that herbivory can constrain plant functional responses and PP to shading, as they observed that herbivory decreased plant growth rate and final biomass as well as reduced chlorophyll, leaf shape and specific leaf area (SLA) plasticity, among others. Thus, herbivory is expected to interfere with normal plant functional responses to shade. These typically involve increasing SLA and shoot:root ratio (SR) to optimise light capture as part of a net carbon gain maximisation strategy (Givnish 1988) in order to maintain or enhance performance. However, a number of authors have questioned this traditional view (Walters & Reich 1999; Valladares *et al.* 2000; Sánchez-Gómez *et al.* 2006; Valladares & Niinemets 2008), suggesting the existence of a conservative resource-use strategy, through which plants under shade do not maximise growth and instead show high tolerance to low-light stress. In this respect, evidence indicates that plants under shading conditions decrease photosynthetic rate (Amax) as part of a conservative strategy of resource use (Valladares & Niinemets 2008). Nonetheless, the abovementioned hypotheses are not mutually exclusive (Sánchez-Gómez *et al.* 2006). On the basis of the evidence previously mentioned, herbivory is equally expected to constrain plant plastic capacity to face shade (Gianoli *et al.* 2007; Salgado-Luarte & Gianoli 2011). Reduced PP in traits like SLA or SR could compromise the carbon balance and ultimately result in decreased performance of damaged plants in shaded environments (Salgado-Luarte & Gianoli 2011).

Until now no study has dealt with assessment of the relative importance of direct *versus* indirect effects on plasticity constraints. Herbivory could decrease the ability of a plant to face shade through direct and indirect effects, although these are not mutually exclusive (Gianoli *et al.* 2009). The direct pathway acts through reduction of photosynthetic area and thus carbon assimilation, which reduces the amount of resources available to compensate for stress and compromises the capacity to express metabolic responses to shading (deep shade physiology; Valladares *et al.* 2007). Direct effects have been shown to influence plant performance in traits such as growth, reproduction and survival (Baraza *et al.* 2004; Atala & Gianoli 2009), but to our knowledge no studies have reported direct effects on PP expression. The indirect pathway acts through compensatory responses to herbivory, such as induction of costly chemical defences or changes in resource allocation, which involve trait expression that is opposite to that elicited by shade conditions. For instance, leaf wounding may elicit both an increase in proteinase inhibitors, which may deter herbivores (Broadway *et al.* 1986), and a decrease in levels of indole-acetic acid (Thornburg & Li 1991), which may limit plastic responses to shade. Thus, in order to disentangle the relative importance of direct *versus* indirect effects, it could be crucial to consider the developmental trajectory when quantifying PP (Watson *et al.* 1995). There are many plant traits related to resource acquisition and allocation (*e.g.* SR, storage capacity) that change during ontogeny (Boege *et al.* 2007). Thus, the sensitivity of reaction norms can be different according to plant age, and constraints in herbivory-mediated plant responses to shade could be buffered through ontogeny due to an expected increase in reserve storage (Bryant *et al.* 1991; Farnsworth 2004; Boege & Marquis 2005; Boege *et al.* 2007), which would indicate that direct effects (resource-based) are predominant.

In this study, we tested, through a greenhouse experiment, whether herbivory constrains plant functional responses and PP to shade. To achieve this goal, we first analysed the variation in key physiological and morphological trait responses to shade between damaged and intact plants. Second, we examined the effect of herbivory on whole PP, by analysing the differences in reaction norms and quantitative plasticity measures between damaged and undamaged saplings. We additionally investigated the nature of the constraints by testing for existence of an ontogenetic effect, which would buffer changes due to an increase in reserve storage. We used as a model system, the relict tree *Prunus lusitanica,* an intermediate light-demanding species that is frequently restricted to shaded environments under current climate conditions (Pulido *et al.* 2008). Specifically, we tested the following predictions: (i) simulated herbivory will constrain sapling functional responses to shade; (ii) the herbivory-mediated constraint on functional responses to shade will be less pronounced in older saplings; and (iii) simulated herbivory will decrease plant plasticity to shade. We postulate that constraints on PP to shade are mainly due to direct effects (due to lack of resources) if damaged older saplings present a lower restriction of phenotypic responses than damaged younger saplings.

MATERIAL AND METHODS

Study species

The Portuguese laurel cherry (*P. lusitanica*) is a perennial lauroid tree of subtropical origin. At present it occurs in Macaronesian mountain cloud forests and also in the Iberian Peninsula, where the species grows in riparian forests under drought-prone Mediterranean climate (Pulido *et al.* 2008; Calleja *et al.* 2009). Saplings of this species (Fig. 1) cannot tolerate continuous drought, which forces them to establish only in river belts (Pulido *et al.* 2008). In addition, sapling performance and survival in favourable moisture environments is higher under intermediate values of light found in small forest gaps (10–20% full sun irradiance), survival being intermediate at higher irradiances (60% full sun) and extremely low at irradiances below 2% full sun (Pulido *et al.* 2008). Therefore, deep shade environments can be considered stressful for saplings of *P. lusitanica*, and such environments have been shown to negatively affect stomatal conductance, photosynthetic rate and photochemical efficiency (Pulido *et al.* 2008). Additionally, *P. lusitanica* saplings suffer considerable herbivory damage in their natural habitat (A. Pardo & F. Pulido, unpublished; Fig. 1).

Plant material and greenhouse environment

Saplings of *P. lusitanica* of different ages (1, 2 and 3 years old) were used in this study. All saplings were grown from seeds collected from a population located close to Castañar de Ibor (Cáceres province, central Spain; 39°37′34.27″ N, 5°25′1.28″ W). Three-year-old saplings were grown from fruits collected in early December 2004, 2-year-old saplings from fruits collected in December 2005 and 1-year-old saplings from fruits collected in December 2006. Seeds were sown in plastic containers in a greenhouse at the University of Extremadura Campus in Plasencia (Cáceres province; 40°1′39″ N, 6°5′27″ W). In April 2007, 120 saplings of each age

Fig. 1. Saplings of *P. lusitanica* growing in the forest understorey in western Iberia. Left: Undamaged saplings. Right: Sapling showing foliar damage by insects.

were transplanted to 500-cm³ pots. A soil substrate (pH 6.5) consisting of a 3:1 volume mixture of peat and washed river sand was used in all pots. The glasshouse was covered with shade cloth to reduce irradiance to that observed in the field site from which plant material was collected. Thus, values of photosynthetically active radiation (PAR) measured with a LiCor ACS sensor (LCi; ADC BioScientific, Hoddesdon, UK) were 1711 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at full sun and 424 $\mu mol \cdot m^{-2} \cdot s^{-1}$ inside the glasshouse (80% reduction). One month after transplantation, sapling pots were placed on two rectangular metal containers (300 cm × 200 cm × 10 cm). These containers were permanently filled with a 5-cm deep water table to ensure that all saplings were kept at field capacity and did not experience water shortage.

Experimental design and treatments

An indoor glasshouse experiment was conducted to evaluate the response of saplings to the combined stress induced by very low irradiance and simulated herbivory throughout ontogeny. We used a fully factorial design with two levels of light, three levels of herbivory and three levels of age. Eight replicates (saplings) were used for each cell of the design (8 × 2 × 3 × 3; N = 144).

One month after acclimation to the new moisture environment, saplings were split in two separate blocks, each consisting of 72 saplings, with 24 saplings of each age class. One block was thereafter exposed to an optimum light environment (control treatment), while the other was exposed to a shade-stress light environment (stress treatment). Light treatments were established using different number of layers of neutral shade cloth supported on metal frames. Values of PAR at midday were 424 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 38 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the control and stress treatments, respectively. Thus, irradiance values represented 20% and 2% of full sunlight in the control and stress treatment, respectively.

One month after setting the light environments, the 24 saplings in each light × age combination were assigned to one of three treatments of simulated herbivory, differing in the proportion of entire leaves removed. In the 'severe damage' treatment two-thirds of the leaves were removed by cutting their petioles with a pair of scissors, while in the 'partial damage' treatment one-third of leaves was removed. Finally, all leaves were left undamaged in the 'intact' treatment serving as control. This procedure was used instead of cutting portions of leaves because it allowed later measurements on the remaining entire leaves and because it better mimicked mammalian herbivory observed in natural conditions (Pulido & Pardo personal observation).

Plant morphological and physiological measurements

We used two physiological and three morphological integrative measures of plant response to experimental treatments: maximum photosynthetic rate (Amax, $\mu mol\ CO_2\ m^{-2} \cdot s^{-1}$), water use efficiency (WUE, $\mu mol\ CO_2\ mol\ H_2O^{-1}$), relative growth rate (RGR), specific leaf area (SLA, $mm^2 \cdot mg^{-1}$) and shoot:root ratio (SR, g). Ecophysiological measurements to evaluate sapling response to the cumulative effects of all experimental treatments were evaluated at the end of the experiment, 4 months after initial transplantation. We measured Amax and WUE using a portable differential IRGA (LCi; ADC BioScientific). For RGR, we measured size (stem height and number of leaves) of saplings 1 month after light treatments (before defoliation) and 2 months after defoliation. RGR was computed as ($\ln G_2 - \ln G_1)/(t_2 - t_1)$, G_1 and G_2 being the height or number of leaves at time 1 (t_1) and time 2 (t_2). SLA was measured by dividing leaf area by leaf dry weight. Leaf area was calculated with the image analysis software Image J (Schneider *et al.* 2012), then leaves were dried and weighed on a precision balance (ST 71 model; Gram precision, Barcelona, Spain) to obtain the SLA index. Similarly, at the end of the experiment, saplings were removed from pots and air-dried to obtain measures of stem, leaves and root biomass to the nearest 0.001 g with a precision balance (Gram precision). SR was computed by dividing total biomass of aerial parts (stem + leaves) by root biomass.

Estimation of phenotypic plasticity

Phenotypic plasticity for the studied traits (Amax, WUE, SLA and SR) was estimated quantitatively using the relative distance plasticity index (RDPI; Valladares *et al.* 2006). RDPI has the advantage of not assuming any particular distribution of the data and thus significantly increasing the power of the statistical analysis (Valladares *et al.* 2006), having been recently used successfully (Scoffoni *et al.* 2015). First, the relative distances (RD) were determined for all possible pair-wise combinations of saplings assigned to different treatment levels. RD were obtained by dividing the absolute phenotypic distances between individuals of same genotype and different environments (absolute difference among trait phenotypic values) by the sum the two phenotypic values. The RD is calculated as: RD = Abs $(X_{control} - X_{shade})/X_{control} + X_{shade}$; where $X_{control}$ is the trait value for each individual under control light treatment and X_{shade} is the trait value for each individual under shade treatment. Then the RDPI was obtained by averaging the RD (adding all RD values and dividing by total number of distances), which ranges from 0 (no plasticity) to 1 (maximal

plasticity). Phenotypic plasticity in response to light variation was studied as a function of sapling age (1, 2 and 3 year old) and sapling damage (intact, partial and severe defoliation). RDPI was calculated for each trait × age × damage combination and used for graphical interpretation. The RD values for all possible pair-wise combinations for each trait studied were then statistically analysed as a function of herbivory damage and age. RDPI can be used as a complementary PP measure to reaction norms in this study, as RDPI has the advantage of allowing powerful statistical comparisons among herbivory treatments and sapling ages and of being particularly suitable for complex and non-linear responses to environment variation (Valladares *et al.* 2006).

Statistical analyses

The effect of light environment, herbivory treatment and age on plant ecophysiological and morphological traits (Amax, SR, WUE, RGR and SLA) was analysed through generalised linear models (GLM). GLM were also employed to analyse the effects of age, herbivory and the interaction among these two factors on the phenotypic plasticity (measured as relative phenotypic distances, RD) for each trait (SLA, WUE, SR and Amax). For both sets of analyses we fitted separate models for each trait studied. Normality and homocedasticity assumptions were tested prior to analyses using the Shapiro-Wilk and Levene's test, respectively. Variables were log-transformed or squared-root transformed to meet normality. We used the R environment, version 3.1.1 (R Core Team 2014), for all statistical analyses, with the functions 'lm', 'aov' and 'TukeyHSD' from the stats package.

RESULTS

Plant functional responses

Results from our greenhouse experiment showed that simulated herbivory had little effect on functional responses to light availability. No significant light x damage interaction was found for any of the studied traits (Table 1), except for a marginally significant effect for SR ($P = 0.055$). Regarding ecophysiological measures, Amax was significantly affected by light and age but not by herbivory damage (Table 1). Amax decreased significantly under shade but similarly for the different herbivory treatments and age classes (Fig. 2). Moreover, Amax was higher in older than younger saplings, as theoretically expected (Fig. 2). WUE was negatively affected by light treatment, but did not significantly differ under different levels of herbivory, or different sapling ages (Table 1). No second- or third-order interactions were observed for either ecophysiological variable.

With respect to morphological variables, SR was significantly affected by herbivory treatment and age (Table 1). We also found an age × light significant interaction: the effect of shade on SR changed significantly with ontogeny; only 1-year-old saplings increased SR under stressful shade conditions (Fig. 2). There was also a marginally significant light x damage interaction for SR ($P = 0.055$, not shown in Table 1), with severely damaged saplings having lower SR values than undamaged saplings under stressful light conditions (Tukey test: $P < 0.0001$; Fig. 2). A significant third-order interaction was also detected: the effect of light treatment on SR depended on the level of herbivory damage, and this relationship changed through ontogeny. When damage was severe, 1-year-old saplings allocated more biomass to shoots under stressful light conditions, but older saplings did not (Tukey test: $P < 0.000$; Fig. 2). In addition, RGR (height) was significantly affected by light and age, but there was no effect of herbivory: no second- or third-order interactions (Table 1, Fig. 2). In contrast, RGR (leaves) was significantly affected by light, age and herbivory, and there were significant age × herbivory and light × age × herbivory interactions (Table 1). Finally, a significant effect of light and age was detected for SLA (Table 1). All saplings increased SLA under low light conditions (Fig. 2), and there was a significant interaction among light × age and age × herbivory (Table 1).

Phenotypic plasticity

The effect of the herbivory treatment on PP was analysed both statistically and graphically for RDPI (Table 2, Fig. 3), as well as through examination of the variation in reaction norms (Fig. 2). Certain deviations among PP results shown in Figs 2 and 3 (in Amax and WUE particularly) are due to RDPI being an integrated quantitative measure influenced by the existence of small phenotypic distances between some pairs of saplings, as well as because RDPI is an absolute plasticity value that does not take into account the sign of reaction norms. The effect of herbivory on plasticity of ecophysiological traits (Amax and WUE) was highly significant (Table 2). Severely damaged saplings showed significantly higher Amax plasticity than partially damaged or undamaged saplings (Tukey test: $P < 0.047$; Fig. 3). Likewise, severely damaged saplings had higher WUE plasticity than partially damaged ones (Tukey test: $P = 0.030$). Among morphological traits, we detected a significant effect of herbivory on SR and SLA plasticity (Table 2). Severe damaged saplings had a

Table 1. Effect of light treatment, age and damage on maximum photosynthetic rate (Amax), RGR height and leaves, WUE, shoot:root ratio (SR) and SLA. F-values are shown with statistical significance. Data were log- or square root-transformed to meet normality assumptions.

Trait	Light (df = 1)	Age (df = 2)	Damage (df = 2)	A × L (df = 2)	A × D (df = 4)	L × D (df = 2)	L × A × D (df = 4)
Amax	113.338***	3.168*	0.765 ns	1.900 ns	0.140 ns	0.688 ns	0.697 ns
SR	1.111 ns	25.062***	22.402***	8.533***	0.661 ns	3.025 ns	4.051**
WUE	7.267**	0.442 ns	0.000 ns	0.629 ns	0.187 ns	0.030 ns	0.422 ns
RGR height	6.152*	12.467***	1.686 ns	0.314 ns	0.469 ns	0.320 ns	1.017 ns
RGR leaves	34.610***	7.017**	24.146***	1.734 ns	2.593*	0.837 ns	4.208**
SLA	158.583***	17.742***	1.461 ns	5.930*	2.664*	1.908 ns	0.679 ns

$*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns $P > 0.05$.
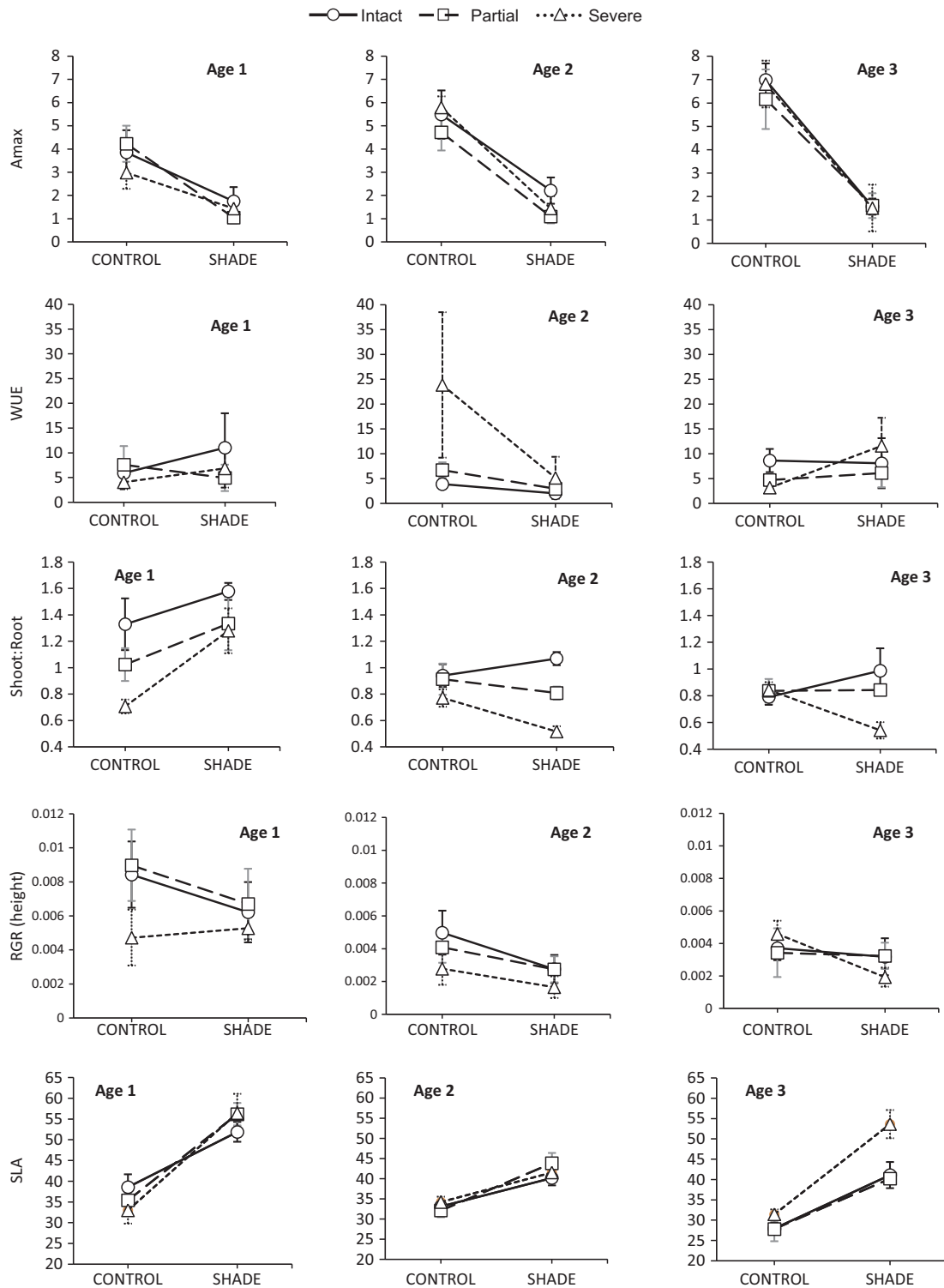
Fig. 2. Contrasting reaction norms to shade in undamaged (intact), partially damaged (partial) and severely damaged (severe) saplings of *P. lusitanica* across ontogeny (age 1, 2 and 3 years). Traits shown (mean ± SE): Amax (μmol CO_2 $m^{-2} \cdot s^{-1}$), WUE (μmol CO_2 mol H_2O^{-1}), SR (g), RGR (mm) and SLA (mm^2 mg^{-1}).

significantly higher PP in SR than partially damaged or undamaged samplings (Tukey test: $P < 0.001$; Fig. 3), as also observed from the steeper reaction norms (Fig. 2), especially in 1-year-old saplings. Additionally we observed a change in direction of the reaction norms for SR in damaged saplings in ages 2 and 3 with respect to 1-year-old saplings (Fig. 2). Finally, we observed higher SLA plasticity in the partial and severe herbivory treatments with respect to the undamaged treatment (Tukey test: $P < 0.001$; Fig. 3), as also observed from the steeper reaction norms in Fig. 2.

Table 2. Effect of age and damage on maximum photosynthetic rate (Amax), WUE, shoot:root ratio (SR) and SLA phenotypic plasticity (calculated as relative distances, RD). F-values are shown with statistical significance. Data were log- or square root-transformed to meet normality assumptions.

Trait plasticity	Age (df = 2)	Damage (df = 2)	A × D (df = 4)
Amax	10.863***	7.100***	4.056**
WUE	2.917 ns	4.202*	3.051*
SR	17.033***	19.105***	5.985***
SLA	60.211***	19.841***	5.281***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns $P > 0.05$.

Analysis of the effect of sapling age on PP revealed a significant effect on Amax, SR and SLA plasticity, but not on WUE (Table 2). Two-year-old saplings showed higher Amax plasticity than 1-year-old or 3-year-old saplings (Tukey test: $P < 0.001$; Fig. 3). Moreover, we observed lower SR plasticity in 2- and 3-year-old saplings with respect to 1-year-old saplings (Tukey test: $P < 0.001$; Fig. 3), and higher SLA plasticity in 1- and 3-year-old with respect to 2-year-old saplings (Tukey test: $P < 0.001$; Fig. 3). Additionally, the age × damage interaction was significant for all traits (Table 2). The effect of herbivory on Amax plasticity changed significantly with ontogeny, with PP decreasing sharply only in partially damaged 3-year-old saplings. Partially damaged 2-year-old sapling had higher Amax PP than 3-year-old saplings (Tukey test: $P < 0.001$; Fig. 3). Moreover, the effect of herbivory treatment on SR plasticity changed in 3-year-old saplings, with partially damaged saplings having lower plasticity values than undamaged saplings (Fig. 3). Additionally, partially damaged 2- and 3-year-old saplings had lower SR plasticity than 1-year-old saplings (Tukey test: $P < 0.01$; Figs 2 and 3). Finally, severe and partially damaged 2-year-old saplings had a lower PP in SLA than 1-year-old saplings (Tukey test: $P < 0.01$; Figs 2 and 3).

DISCUSSION

Effect of herbivory on functional responses to shade

Several ecophysiological studies have shown that herbivory damage can constrain functional responses to low light, causing plants to deviate from the optimal phenotype that allows better performance in the shade (Blundell & Peart 2001; Rogers & Siemann 2002; Norghauer *et al.* 2008). This could ultimately explain the often-observed larger fitness losses due to herbivory in shade environments (Salgado-Luarte & Gianoli 2011). However, our results do not show consistent support for this pattern. No significant evidence was found for simulated herbivory constraining *P. lusitanica* functional responses to shade (only marginally for SR), contrary to initial expectations of our first prediction. In fact, herbivory damage did not significantly affect Amax, WUE, RGR or SLA functional responses to shade. Physiological traits like Amax and related WUE (assimilation/transpiration) have been shown to decrease under shade, probably as part of a conservative strategy of resource use (Valladares & Niinemets 2008). In contrast, morphological traits such as SLA, SR and RGR are expected to increase under shade in order to contribute to the maintenance of a positive carbon budget and maximisation of growth to maintain plant fitness (Givnish 1988; Valladares & Pearcy 1998). However, both control and damaged saplings showed similar functional responses to shade conditions, except for SR. Severely damaged saplings showed a decrease in SR under shade instead of an increase like undamaged saplings. It has been suggested that reducing SR after herbivory or mechanical damage could be a strategy to reduce the risk of mortality at the expense of growth (Blundell & Peart 2001).

Hence, our results disagree with predictions of the often-invoked compensatory continuum hypothesis (Hawkes & Sullivan 2001), which states that plant tolerance to herbivory
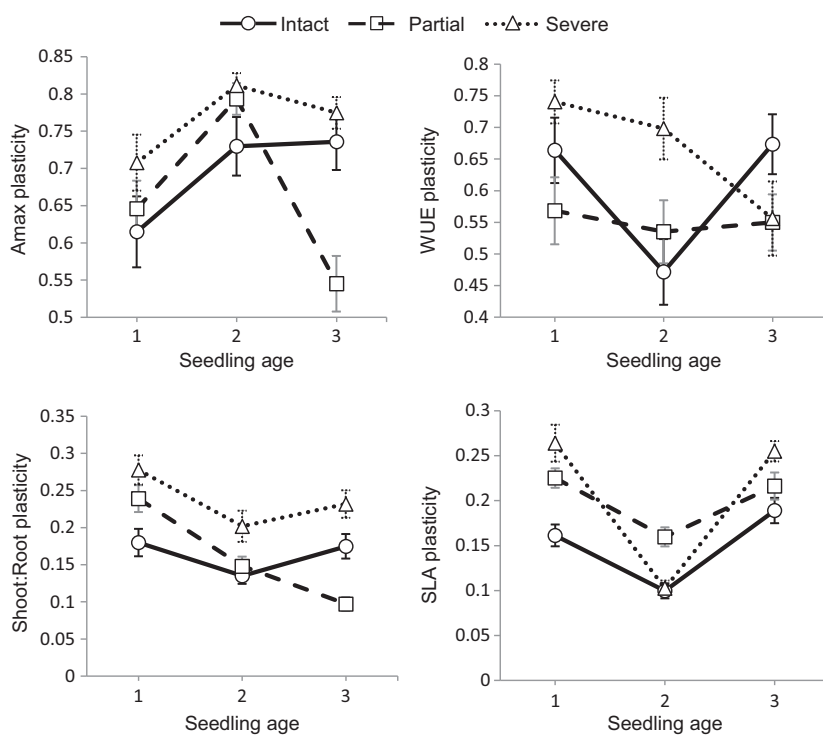

Fig. 3. Amax, WUE, SR and SLA phenotypic plasticity (relative distance plasticity index, RDPI) across sapling age, represented for each of the three herbivory treatments: undamaged (intact), partially damaged (partial) and severely damaged (severe). RDPI values are shown ± SE.

should be larger in high resource, low competition or otherwise benign environments. Instead, our results are consistent with the more recently formulated hypothesis of the limiting resource model (LMR; Wise & Abrahamson 2005, 2007). Thus, if leaf herbivory (simulated herbivory in our case) does not interfere significantly with the plant's ability to capture light for photosynthesis (likely due to the increase in SLA in damaged saplings that could compensate), an equal tolerance to herbivory at different levels of light would be expected according to the LMR.

Herbivory-mediated functional responses to shade through ontogeny

Ontogenetic changes in physiological and morphological traits were observed in our study; the effect of sapling age on all measured traits being highly significant. We observed that Amax increased in older saplings, whereas SR and SLA decreased. Nonetheless, damaged older saplings did not show a lower constraint on functional responses to shade than damaged younger saplings, as initially expected from a potential increase in reserve storage, but rather the opposite. When damage was severe, 1-year-old saplings increased SR and RGR (leaves) under shade, but older saplings did not. This result disagrees with the common view that the impacts of herbivore damage decrease as plants develop, as larger plants are generally thought to have increased access to resources (Warner & Cushman 2002; Boege & Marquis 2005). For instance, the commonly observed increase in SR under shade is the result of reallocation of resources and biomass from belowground tissue to aboveground structures in order to maximise light capture to maintain fitness (Stowe et al. 2000). In our case, undamaged saplings of P. lusitanica increased SR under shade across all ages, although they reached higher values in 1-year-old saplings (Fig. 2). However, when damage was severe, 1-year-old saplings allocated more biomass to shoots over roots under shade, but older saplings did not (Fig. 2). Other species, such as Prosopis glandulosa, have also been shown to have better tolerance to damage in young than older seedlings, likely as a result of ontogenetic variation in allocation to above- and belowground tissues (Weltzin et al. 1998).

Effect of herbivory on PP to shade

Our findings showed that simulated herbivory did not largely constrain P. lusitanica plastic expression to shade, suggesting no major ecological limits to PP are induced by these two simultaneous stressors in this relict tree species. These results broadly disagree with previous studies, which have shown that plant responses to light competition and herbivory interact negatively as a result of both ecological and physiological trade-offs (Cipollini 2004; Ballaré 2009; Salgado-Luarte & Gianoli 2011). In contrast, our findings are in agreement with the results of Kurashige & Agrawal (2005), who showed that plant responses to herbivory did not affect subsequent responses to light competition in Chenopodium album. In our study, damaged plants showed lower plasticity to reduced light availability than undamaged plants exclusively in a few cases: Amax and WUE in 1-year-old saplings as well as Amax, WUE and SR in 3-year-old saplings (see Figs 2 and 3). In contrast, quantitative plasticity values (RDPI) and reaction norms in all

other cases were higher and steeper, respectively, in damaged plants. Nevertheless, it should be noted that in the case of SR, reaction norms in damaged plants showed an opposite trend with respect to undamaged saplings. Possible explanations for the lack of a strong reduction in plasticity in damaged plants could be related to the weak effect of herbivory on light capture capacity, as previously discussed, or due to decreased cost of defensive traits against herbivory under stressful shaded conditions. Empirical evidence has shown a decrease in costs of resistance and tolerance to herbivory in competitive environments (Defence Stress Benefit Hypothesis; Siemens et al. 2003), indicating that additional functions of defensive traits may benefit plants under competition, reducing the total cost of such traits (Boege 2010).

Nonetheless, high plasticity in the measured traits might not always imply benefit for plant fitness. Physiological plasticity (i.e. plasticity in traits related to gas exchange, photochemical efficiency and water relations, such as Amax and WUE) may allow plants to adjust to variations in light intensity (Sultan 2000), and has been linked to enhanced capacity to colonise gaps and grow under high irradiances, whereas morphological plasticity (in traits like SLA or SR) has been linked to enhanced capacity to survive and grow in the understorey (Sánchez-Gómez et al. 2006). However, being highly plastic in some functional traits to light availability may be associated with costs and risks that compromise long-term survival (Valladares & Niinemets 2008). Recent studies have shown that shade tolerance may be associated instead with a conservative resource-use strategy expressed through reduced plasticity (Walters & Reich 1999; Valladares et al. 2000). In support of this line of evidence, the findings of a comparative study of four Iberian tree species showed that the most plastic species in response to light had the highest seedling mortality in deep shade (Sánchez-Gómez et al. 2006). However, certain morphological traits such as SLA in shade-tolerant species can adjust to light variation more strongly than such traits in shade-intolerant species (Sánchez-Gómez et al. 2006; Valladares & Niinemets 2008). Generally, species originating and still surviving in dense subtropical forests, such as P. lusitanica, can be expected to exhibit shade tolerance. However, it has been shown that deep shade has important negative effects for this species, with significantly lower survival rates under deep shade compared with moderate or high irradiances (Pulido et al. 2008). The fact that even damaged plants of P. lusitanica were able to show a wide PP in Amax might contribute to the ability of this species to occupy diverse light environments in nature (from high to moderately low irradiances). Yet, its lack of tolerance to deep shade (Pulido et al. 2008) could be related to excessive plasticity in Amax, ultimately not being beneficial. The large plasticity in traits like Amax and SR in P. lusitanica, the latter in addition showing opposite negative reaction norms in damaged saplings (SR decreasing under shade instead of increasing in damaged older saplings), could lead to decreased fitness, particularly in damaged plants, under shade. Thus, large detrimental plasticity in physiological and morphological traits to shade could contribute to explain the higher mortality of P. lusitanica saplings under deep shade observed in the field (Pulido et al. 2008), where plants suffer considerable herbivory (A. Pardo & F. Pulido unpublished).

Effect of herbivory on PP through ontogeny

Older plants are theoretically assumed to be able to acquire more resources to store and use for herbivory resistance through an inherent increase in plant size (larger resource-acquiring organs such as roots and foliage area), change in functional priorities, and increase in the carbon nutrient balance, storage capacity and SR across ontogeny (Bryant *et al.* 1991; Farnsworth 2004; Boege & Marquis 2005). Our results show that the herbivory-mediated effect on PP to shade varied throughout ontogeny, but no decrease in plasticity restriction with plant age was observed. Instead, damaged older saplings generally showed lower plastic response in Amax, SR and SLA than damaged younger saplings. Thus, our findings suggest that the phenotypic response to shade mediated through herbivory in *P. lusitanica* is not primarily limited by direct effects, namely a decrease in resource availability due to the reduction in photosynthetic area and carbon assimilation. Nevertheless, the fact that no major constraints on plasticity due to herbivory were found, suggests that, at present, it is not possible to make conclusive inferences on the relative importance of direct *versus* indirect effects on plasticity limitation in *P. lusitanica*. Quantification of resources would be desirable in future studies in order to gain a better understanding of this seldom-explored subject.

The central finding of this study is that simulated herbivory does not largely constrain ecophysiological performance or plastic phenotypic responses to shading through ontogeny in the relict tree *P. lusitanica,* contrary to initial expectations. These findings suggest that herbivory-mediated constraints on plant PP may not be as widespread as previously thought. However, large plasticity in physiological and morphological traits such as Amax and SR (biomass allocation to shoot over roots in response to shade changing in damaged plants) could have negative consequences for plant fitness and be somewhat responsible for the higher mortality of *P. lusitanica* saplings under deep shade observed in the field (Pulido *et al.* 2008), where saplings suffer considerable herbivory damage (A. Pardo & F. Pulido unpublished). Further studies are needed to elucidate the potential simultaneous influence of herbivory damage together with other abiotic stress factors, such as drought, on plant PP limitation (but see Quezada & Gianoli 2006; Gianoli *et al.* 2009), as well as additional studies that quantitatively measure plant resources in order to gain a better understanding of the relative importance of direct effects on plant plastic limitation.

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