RESEARCH PAPER

Differential effects of type and quantity of leaf damage on growth, reproduction and defence of lima bean (*Phaseolus lunatus* L.)

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ABSTRACT

Folivores are major plant antagonists in most terrestrial ecosystems. However, the quantitative effects of leaf area loss on multiple interacting plant traits are still little understood. We sought to contribute to filling this lack of understanding by applying different types of leaf area removal (complete leaflets *versus* leaflet parts) and degrees of leaf damage (0, 33 and 66%) to lima bean (*Phaseolus lunatus*) plants. We quantified various growth and fitness parameters including above- and belowground biomass as well as the production of reproductive structures (fruits, seeds). In addition, we measured plant cyanogenic potential (HCNp; direct chemical defence) and production of extrafloral nectar (EFN; indirect defence). Leaf damage reduced above- and belowground biomass production in general, but neither variation in quantity nor type of damage resulted in different biomass. Similarly, the number of fruits and seeds was significantly reduced in all damaged plants without significant differences between treatment groups. Seed mass, however, was affected by both type and quantity of leaf damage. Leaf area loss had no impact on HCNp, whereas production of EFN decreased with increasing damage. While EFN production was quantitatively affected by leaf area removal, the type of damage had no effect. Our study provides a thorough analysis of the quantitative and qualitative effects of defoliation on multiple productivity-related and defensive plant traits and shows strong differences in plant response depending on trait. Quantifying such plant responses is vital to our understanding of the impact of herbivory on plant fitness and productivity in natural and agricultural ecosystems.

INTRODUCTION

In natural and agricultural ecosystems plants must cope with multiple herbivores simultaneously (Walling 2000). Among different feeding guilds, folivorous herbivores belong to the most common and damaging plant consumers (Zangerl *et al.* 2002). On average, terrestrial herbivores consume approximately 15% of net primary productivity (Cyr & Pace 1993). In particular, herbivorous insects are major plant antagonists and often are serious pests in crop plants (Strauss & Zangerl 2002). While feeding damage imposed by herbivores is generally considered to be detrimental for the plant, plant responses to herbivory have actually been reported to range from negative (Marquis 1984), to neutral (Boege *et al.* 2007), to positive in terms of biomass production and fitness (McNaughton 1979; McArt *et al.* 2013). Beyond the direct negative effect of decreased photosynthetically active leaf area – and thus a reduced supply of photoassimilates – leaf damage might have a broad range of physiological effects that impact the plant. For example, leaf damage can induce the production of chemical or mechanical plant defences (Steppuhn *et al.* 2004; Kariyat *et al.* 2013). Nevertheless, beyond providing enhanced resistance, this induction of defensive traits can result in resource allocation trade-offs with growth and reproduction

(Lucas-Barbosa *et al.* 2011). In particular, nitrogen-based defences, such as alkaloids or cyanogenic compounds, are assumed to be costly for the plant as nitrogen is often a limited nutrient (Ballhorn *et al.* 2011a). The production of carbon-based defences is considered less expensive as plants are generally not carbon-limited and can up-regulate photosynthesis if necessary (Kaschuk *et al.* 2009; Thamer *et al.* 2011). However, the production of carbon-based defensive terpenoids, phenolics or extrafloral nectar (EFN) also frequently occurs with substantial costs, as their biosynthesis requires the expression of multiple enzymes (Koricheva *et al.* 2004; Li *et al.* 2012; Ballhorn *et al.* 2014). In addition to resource allocation constraints, some plant defences also negatively interact with other plant traits at a post-translational level (Ballhorn 2011; Kadow *et al.* 2012). For example, cyanogenesis, the wound-induced release of hydrogen cyanide (HCN) from preformed cyanogenic precursors (HCNp), affects numerous processes in plants as the cyanide ion binds to various metal-containing enzymes including catalases, peroxidases and polyphenol oxidases (Solomonson 1981; Ballhorn *et al.* 2010a, 2013a). Furthermore, plants possess mechanisms to tolerate herbivory through the reallocation of carbon reserves from the roots to the shoots, an increase in branching following the removal of apical dominance or an increase in net photosynthesis rate (Strauss & Agrawal 1999).

This strategy of regrowth represents an alternative to defence. In fact, tolerance to herbivory and induced resistance may result in an additional trade-off within plants under herbivory (van der Meijden *et al.* 1988; Núñez-Farfán *et al.* 2007).

Whether acting directly *via* a reduction in photosynthetic tissue and subsequent resource allocation constraints due to a shortage of photosynthates, or indirectly through the cascading effects of damage-induced traits, quantitative information on the effects of leaf area loss *via* herbivory on growth, defence and seed production remains scarce (Gold & Caldwell 1989; Koptur *et al.* 1996; Ballhorn *et al.* 2014). Such quantitative data on the effect of leaf area loss on plant physiology and plant productivity are vital for understanding plant productivity in ecosystems, as well as the establishment of threshold levels for plant protection measures. In this study, we used wild lima bean (Fabaceae, *Phaseolus lunatus* L.) to test the quantitative effects of leaf area removal on multiple traits. Lima bean is an important food crop in South and Central America and Africa, as well as an important commercial crop in the USA (Martínez-Castillo *et al.* 2004). Lima bean is also a model plant used in studies of inducible, indirect plant defences against herbivores and pathogens (Yi *et al.* 2009; Ballhorn *et al.* 2013b). The release of herbivore-induced volatile organic compounds (VOCs) and the secretion of extrafloral nectar (EFN) are well-studied in this species (Arimura *et al.* 2000; Ballhorn *et al.* 2008a; Ballhorn *et al.* 2009, Ballhorn *et al.* 2011a,b; Mumm *et al.* 2008; Radhika *et al.* 2008). In addition to indirect defences, lima bean utilises several direct chemical (*e.g.* cyanogenesis, phenolics, polyphenol oxidase activity) and mechanical (*e.g.* hook-shaped trichomes) defences (Ballhorn *et al.* 2008b, 2013a). Among these traits, lima bean cyanogenesis and EFN represent particularly effective direct and indirect defences against a broad range of generalist and specialist insect herbivores (Ballhorn *et al.* 2007, 2010a,b,c, 2011a).

To better understand the quantitative effects of leaf area loss on plant performance and reproduction, we addressed the following questions: (i) does quantitative and qualitative variation in leaf damage affect production of above- and below-ground biomass; (ii) what are the effects of leaf damage on fruit and seed production; and (iii) are key defences against herbivores differentially affected by variation in type and amount of leaf area removal? The present study provides a clear analysis of quantitative plant responses to leaf damage, including multiple growth, reproductive and defensive-related traits simultaneously.

MATERIAL AND METHODS

Plants

Lima bean plants were grown from seeds obtained from the Institute of Plant Genetics and Crop Plant Research IPK, Gatersleben, Germany. The selected genotype (8067) was used in our previous studies and is characterised by small white seeds and, compared to other cultivated and wild lima bean genotypes, has a moderate concentration of cyanogenic glycosides in leaves (Ballhorn *et al.* 2008a, 2011a,b). Plants were cultivated in the greenhouse with a light regime of 14 h light:10 h dark under a photon flux density of 450–500 $\mu mol \cdot photons \cdot m^{-2} \cdot s^{-1}$ at table height. Light in the chamber was provided by a combination (1:1) of HQI-BT 400 W (Osram) and RNP-T/LR 400 W

(Radium) lamps. Temperature in the greenhouse was 27:16 °C (*i.e.* 27 °C in the light period and 16 °C in the dark period) and we maintained an air humidity of 70–80%. One plant each was grown in pots 8 cm in diameter containing 175 g low nutrient potting soil (Sunshine Mix #1, LC1; SunGro Horticulture, Bellevue, WA, USA). All plants were watered daily and randomly reorganised every 3 days in order to avoid any position or edge effects.

Experimental leaf damage

Different methods of leaf area removal were applied to the trifoliate leaves of lima bean to account for different feeding styles of folivorous herbivores, but the overall removed leaf area was similar for both types of damage (Fig. 1). To mimic bulk feeding of mammalian herbivores incorporating whole leaves, leaflets were removed completely at the rachis. Experimental plants of group 1 had one of three leaflets removed per leaf (~33% damage) and group 3 had two leaflets removed per leaf (~66% damage; Fig. 1). To simulate more localised feeding of folivorous insects, leaf tissue was carefully removed from a leaflet lengthwise along the midvein using scissors (Fig. 1). These treatments included group 2 (~33% damage) and group 4 (~66% damage). Care was taken not to disturb the midvein in these treatments. Further, in all treatments extrafloral nectaries located at the base of each leaflet remained undamaged (Fig. 1). One group of plants served as a control with no leaf area removal. Experimental leaf area removal was applied when plants had developed their first fully unfolded secondary leaf about 3 weeks after germination, and damage was continuously applied (once per week) to newly developed but fully expanded leaves throughout the experimental period (13 weeks).

Plant growth parameters

After 13 weeks, when all pods had reached maturity, plant material was harvested. Belowground biomass was cleaned from the potting soil by gently washing roots and rinsing them with water. Aboveground biomass was determined as shoot biomass with exclusion of leaves, as the amount of leaf material was affected by the experimental treatments. Above- and belowground biomass was separated and dried in an oven (IncuMax CV250 Convection Oven; Amerex Instruments, Inc., Lafayette, CA, USA) for 10 days at 70 °C and weighed to the
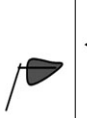
	Group 0	Group 1	Group 2	Group 3	Group 4
% Leaf area removed	0%	33%		66%	
Method of defoliation					

Fig. 1. Method and degree of defoliation of different experimental groups of *Phaseolus lunatus*. Leaf area of plant groups 1 and 2 was reduced by 33%, while leaf area for groups 3 and 4 was reduced by 66%. Removing entire leaflets (groups 1 and 3) is typical for mammalian herbivory, whereas partial removal of leaflets (groups 2 and 4) mimics insect herbivory. Plants of group 0 remained undamaged and served as control (n = 12 plants per group).

nearest 0.001 g (New Classic MF; Mettler Toledo, Zurich, Switzerland).

Reproductive parameters

To quantify effects of leaf area removal on reproductive plant traits, developing pods were monitored continuously (every 3 days) for each plant individual. This continuous observation was required to determine the total fruit production over the growing season, as a significant number of young pods were dropped before reaching maturity. At the end of the experiment all remaining pods were mature and fully ripened. Pods were harvested and pods per plant and seeds per pod and plant were documented, then pods were dried further for 30 days at room temperature and at an ambient humidity of 55%. All seeds were then weighed to the nearest 0.001 g (NewClassic MF; Mettler Toledo).

Leaf cyanogenic potential (HCNp)

Three days before plants were destructively harvested for determination of vegetative and generative traits, leaves were quantitatively analysed for their cyanogenic potential (HCNp; the total amount of cyanide that can be released by a given tissue; Lieberei 1988; Ballhorn et al. 2005). We selected leaves of similar size and age (newly developed, fully expanded leaves on the top of the shoot) to control for ontogenetic variation in HCNp (Ballhorn et al. 2008b). Per leaf, we removed leaf punches of 8 mm in diameter from the centre of the leaf blade randomly right or left of the midvein. Quantification of HCNp was conducted by complete enzymatic degradation of extracted cyanogenic glycosides and subsequent quantitative measurement of released cyanide according to Ballhorn et al. (2005). All steps of preparation were conducted at <4 °C to avoid any premature release of gaseous HCN. For hydrolysis of cyanogenic glycosides in leaf extracts, we used β-glucosidase isolated from young leaves of cyanogenic rubber tree (Euphorbiaceae, Hevea brasiliensis Müll.Arg.) according to Ballhorn et al. (2006), which showed strong affinity to cyanogenic glycosides in lima bean. After 20 min of incubation at 30 °C in gas-tight glass vessels (Thunberg vessels), released cyanide was spectrophotometrically measured at 585 nm using the Spectroquant® cyanide test (Merck, NY, USA). Leaves and plants were harvested between 10:00 and 10:45 h to exclude diurnal effects on chemical composition (Gleadow & Møller 2014).

Quantification of extrafloral nectar (EFN)

Extrafloral nectar production (i.e. amount of sugars in EFN) was measured after removal of leaf tissue for HCNp analysis (see above). Young, intact leaves located one position above leaves used for HCNp determination were selected for EFN quantification. Plants were thoroughly sprayed with water to remove all EFN secreted in response to experimental damage of leaves and to set the initial EFN load on plants to 0. When dry, all plants were sprayed with an aqueous solution of 1 mmol·l^{-1} jasmonic acid (JA; Merck) between 08:00 and 09:00 h. Leaves were sprayed with JA solution until completely moistened (20 ml) and allowed to dry (10 min) before being sprayed a second time (20 ml). Twenty-four hours after the priming of experimental plants with JA, plants were washed with water again to remove any nectar produced in the meantime and subsequently damaged with a pestle consisting of 12 blunt steel needles (each with a diameter of 0.8 mm; overall diameter of the pestle: 7 mm) to induce EFN production. This combination of priming EFN through JA followed by physical damage of leaves has been demonstrated to result in the maximum EFN production in previous studies (Heil & Kost 2006). Secreted extrafloral nectar was collected 12 h after mechanical damage was applied, and sugar present in the EFN was quantified as the amount of soluble solids using microcapillaries (PCR micropipettes 1–10 μl; Drummond, Broomall, PA, USA) for determination of volume and a Brix refractometer for determination of sugar concentration (Ballhorn et al. 2013a).

Statistical analyses

All data were checked for normal distribution of residuals and homogeneity of variances. The effects of leaf area removal on plant traits were tested with one-way ANOVAs followed by post-hoc analyses (Tukey's HSD, $P < 0.05$). Interacting effects of type and quantity of leaf damage were tested with two-way ANOVAs. Statistical analyses were conducted with SPSS 21 (SPSS Inc., Chicago, IL, USA).

RESULTS

Leaf area removal had a significant effect on aboveground shoot biomass ($F_{1,55} = 7.204$, $P < 0.05$; Fig. 2A). Control plants had significantly higher aboveground biomass (ranging from 1.3- to 1.6-fold) than all treatment groups. However, differences in shoot biomass among plants with 33% and 66% leaf area removed were not significant. The type of leaf area removal (whole versus half leaflets) did not cause a significant difference in aboveground biomass ($F_{1,55} = 0.001$, $P = 0.978$). Also, there was no interactive effect of the amount of leaf area removed and the type of removal on aboveground biomass ($F_{1,55} = 0.019$, $P = 0.890$). Leaf area removal affected the production of belowground biomass ($F_{1,55} = 11.964$, $P < 0.01$), as the control plants had a significantly higher root biomass (1.1- to 1.4-fold) than the treatment groups (Fig. 2B). Comparing plants of the different treatment groups revealed that plants exposed to high levels of leaf damage (66%) actually had a higher belowground biomass (1.2-fold) than plants with 33% leaf area loss. The type of leaf area removal also had a significant effect on belowground root biomass ($F_{1,55} = 5.475$, $P < 0.05$), while the combined effect of the percentage of leaf area removed and the type of removal was not significant ($F_{1,55} = 0.845$, $P = 0.362$). In plants of treatment group 3 (66%, removal of whole leaflets), root biomass production was not significantly different from the controls (Fig. 2B).

The total number of fruits – including fruits that were dropped at some point during the growth period at an unripe stage – produced per plant differed among all groups ($F_{4,55} = 2.591$, $P = 0.046$), and overall was lower in the treatment groups compared to the controls (Fig. 3A). However, the reduction in total production of fruit was only significant for treatment group 3 (66% leaf area removed, whole leaflets), while groups 1, 2 (33% leaf area removed; whole leaflets and half leaflets, respectively) and 4 (66%, half leaflets) did not differ significantly from the controls or group 3 (Fig. 3). The

amount of removed leaf area significantly affected the total number of fruits produced ($F_{4,55} = 2.591$, $P < 0.05$), while neither the type of removal ($F_{1,55} = 0.934$, $P = 0.338$) nor the combined effect of amount of leaf area removal and type of damage caused a significant difference in the total number of fruits ($F_{1,55} = 0.043$, $P = 0.837$). The number of fruits reaching maturity differed among the groups ($F_{4,55} = 15.108$, $P < 0.001$), with the control group having significantly more fruits (1.2- to 1.7-fold) than all treatment groups, while neither type ($F_{1,55} = 0.422$, $P = 0.519$) or amount of damage ($F_{1,55} = 0.750$, $P = 0.390$) significantly affected the number of mature fruits (Fig. 3B). Furthermore, the combined effect of removal type and removal amount was not significantly different among the treatment groups ($F_{1,55} = 0.188$, $P = 0.667$).

In line with the number of fruits, seed number also differed significantly between groups ($F_{4,55} = 9.298$, $P < 0.001$), with plants of the control group having significantly more (1.6- to 1.8-fold) seeds than all treatment groups (Fig. 4A). However, the combined effect of removal amount and removal type did not significantly change the final seed number ($F_{1,55} = 0.227$, $P = 0.635$). Also, the mass of individual seeds differed significantly between groups ($F_{4,480} = 37.315$, $P < 0.001$; Fig. 4B). We observed no significant differences between the controls and group 1, while plants of treatment groups 2 and 3 produced significantly lighter seeds, with plants of group 4 showing a further significant decrease in seed mass (Fig. 4B). Both the amount of removal ($F_{1,480} = 47.025$, $P < 0.001$) and the type of removal ($F_{1,480} = 21.396$, $P < 0.001$) caused significant differences in mass of the seeds. However, the interactive effect of the amount of removal and the type of removal on seed mass was not significant ($F_{1,480} = 0.628$, $P = 0.429$).

Direct and indirect defensive traits were differentially affected by the various treatments. The cyanogenic potential (HCNp) of leaves did not differ significantly among all treatment groups ($F_{4,55} = 0.028$, $P = 0.998$; Fig. 5A), whereas the amount of extrafloral nectar (EFN; measured as amount of produced sugars) showed significant variation in response to treatment ($F_{4,55} = 24.715$, $P < 0.001$; Fig. 5B). The amount of sugar in EFN decreased significantly with increasing leaf area

removed ($F_{1,55} = 43.022$, $P < 0.001$). In plants with 33% removed leaf area, the amount of sugars secreted decreased 1.4-fold and in leaves with 66% leaf area removed, EFN production decreased 3.3-fold (Fig. 5B). The type of leaf damage did not significantly affect EFN production ($F_{1,55} = 2.559$, $P = 0.115$). Furthermore, the interactive effect of amount and type of damage was not significant ($F_{1,55} = 0.026$, $P = 0.873$).

DISCUSSION

Loss of leaf area due to herbivory is commonly assumed to have negative impacts on plant growth, reproduction and fitness (e.g. Mothershead & Marquis 2000). Surprisingly, little quantitative data exist on the effects of leaf area loss on multiple, simultaneously expressed plant traits in a given plant
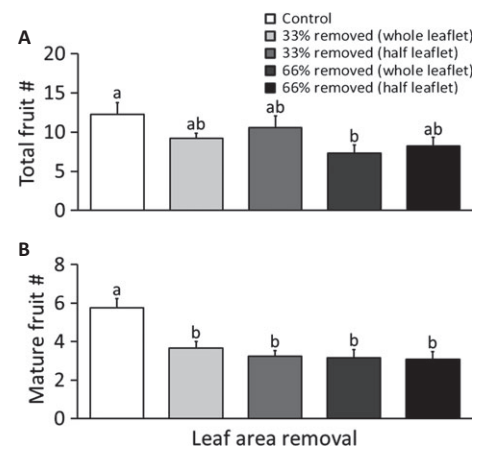

Fig. 3. Effect of leaf area removal on fruit production. The total fruit number (A) represents the number of fruits produced over the experimental period including fruits that were dropped before maturation, whereas mature fruits (B) stayed on the plant and developed seed (mean ± 1 SEM). Letters at the columns indicate significant differences among experimental groups (Tukey's HSD, $P < 0.05$ after one-way ANOVA). n = 12 plants per group.

Fig. 2. Effect of leaf area removal on above- and belowground plant biomass. Aboveground biomass (A) was determined as dried shoot weight, belowground biomass (B) represents dry root weight (mean ± 1 SEM). Letters at the columns indicate significant differences among experimental groups (Tukey's HSD, $P < 0.05$) after one-way ANOVA. n = 12 plants per group.
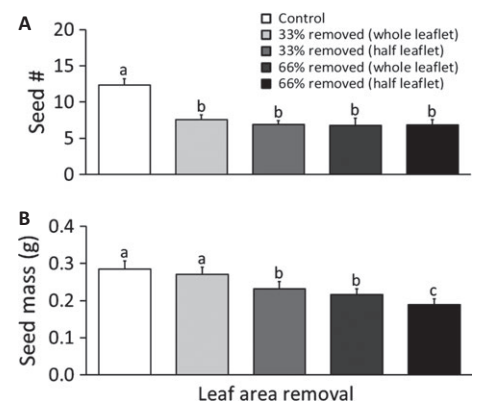
Fig. 4. Effect of leaf area removal on seed production. The total number of seeds (A) and dry weight per seed (B) was measured (mean ± 1 SEM). Letters at the columns indicate significant differences among experimental groups (Tukey's HSD, $P < 0.05$ after one-way ANOVA). n = 12 plants per group.
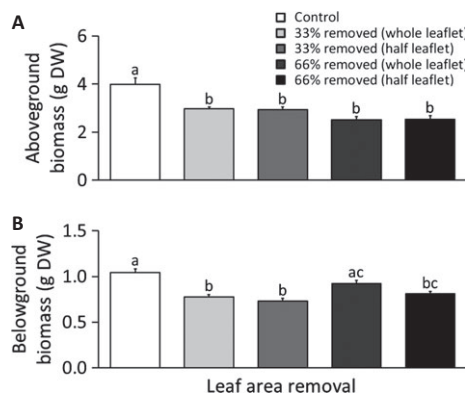
Fig. 5. Effect of leaf area removal on plant defence against herbivores. The cyanogenic potential (A: HCNp; amount of cyanide-containing precursors; a direct chemical defence) and the secretion of extrafloral nectar (an indirect defence) (B) was quantified (mean ± 1 SEM). Letters at the columns indicate significant differences among experimental groups (Tukey's HSD, $P < 0.05$ after one-way ANOVA). n = 12 plants per group.
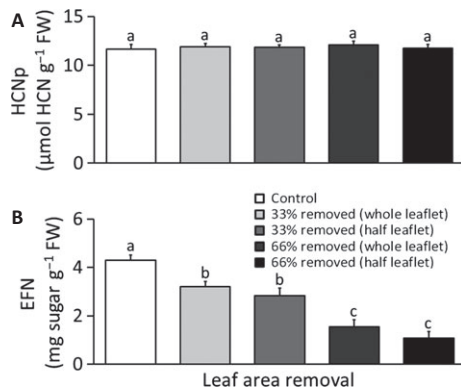
species. In the present study, using wild lima bean (*Phaseolus lunatus*) we show that varying intensities of experimental leaf area removal differentially affected growth and reproduction, as well as defensive plant traits. While some traits did not show a quantitative response to the different treatments, others were affected by the amount and type of damage.

Aboveground biomass production of lima bean was reduced following damage in general but did not show significant variation depending on the extent (33% or 66%) or type (removal of entire leaflets *versus* parts of leaflets) of damage (Fig. 2). In contrast to aboveground biomass, belowground biomass did not decrease at higher levels of defoliation but increased in plants with 66% removed leaf area (treatment 3) compared to plants with 33% leaf area removed (treatments 1 and 2). In accordance with a variation in aboveground biomass, the number of seeds was reduced in damaged plants in general, but was not affected by the different treatments. The mass of individual seeds, however, gradually decreased in response to different levels of leaf area removal and also was affected by the type of damage (Fig. 4).

It has been found in other legume species that the percentage of defoliation is negatively correlated to reproductive measures like seed number and seed mass (Nolting & Edwards 1989; Koptur *et al.* 1996; Marshall *et al.* 2005). For example, Koptur *et al.* (1996) reported that increasing the extent of leaf damage significantly decreased the number of fruits, number of seeds and seed mass in *Vicia sativa*. Furthermore, Marshall *et al.* (2005) reported that defoliation in the fabaceous *Sesbania macrocarpa* and *S. vesicaria* resulted in reduced seed mass in these plant species. While in terms of seed mass a similar quantitative response to percentage defoliation was found in lima bean (Fig. 5), other plant growth and reproduction related traits were not quantitatively affected by different degrees of leaf area removal. All treatments – whether 33% or 66% leaf area removed and with either method of defoliation – similarly affected aboveground biomass production and number of seeds. There was only a slight trend with a higher percentage of defoliation more negatively affecting both traits (Fig. 2).

The limited impact of high levels of defoliation compared to lower degrees of leaf area removal observed for some traits may be due to the effect of less self-shading in extensively damaged plants. In a previous study from Gold & Caldwell (1990), experimental removal of leaf area in the tussock grass *Agropyron desertorum* demonstrated a compensatory effect due to the higher light availability to remaining parts. Thus, a reduction of self-shading may have mitigated the negative effects of photosynthetic area loss in lima bean and represents a possible explanation for the lack of quantitative effects observed in our study for aboveground biomass and seed number. Furthermore, in several plant systems, an up-regulation of photosynthesis in response to defoliation has been described (Eyles *et al.* 2011; Pinkard *et al.* 2011). Up-regulation of photosynthesis in response to defoliation is often interpreted as a compensatory mechanism (Quentin *et al.* 2010, 2012), such that increased assimilation of the remaining leaf tissue can at least partially compensate for the loss of photosynthetically active tissue and lost assimilates. This might further explain the non-significant decrease in biomass and seed production when comparing the effects of 33% and 66% of leaf area removed in our system. However, the plants' ability to compensate loss of leaf area through up-regulation likely has limitations. For example, Mauricio *et al.* (1993) found that defoliation of radish (*Raphanus sativus*) led to less flowers and biomass of reproductive tissues depending on the degree of damage. Moderate defoliation damage of 25% to all leaves in *R. sativus* had no fitness effect, while more extensive 50% damage significantly decreased fitness. Thus, there may be a threshold effect where low damage triggers compensation by elevated photosynthetic rates while heavier damage does not (Mauricio *et al.* 1993). However, we could not confirm a general threshold level in our study, as even the higher level of damage (66%) did not result in a quantitative decrease in traits such as aboveground biomass and seed number. In contrast, biomass production of roots even increased in plants exposed to extensive leaf area loss (Fig. 2B). Overall, there is little knowledge on the effects of leaf area loss on root production (Gruner *et al.* 2008), and existing studies show mixed results (Blue *et al.* 2011). In some cases, root production declines with leaf damage due to reallocation processes of nutrients (Brown 1994; Schädler *et al.* 2004). In other cases, however, leaf area removal increased root production (Bardgett *et al.* 1998; Pucheta *et al.* 2004; Nishida *et al.* 2009). The effects of heavy damage on the shoot:root ratio of lima bean we report here may indicate a tolerance strategy of lima bean, as allocation of biomass to the root system supports the regrowth capacity of damaged plants (van der Meijden *et al.* 1988; Strauss & Agrawal 1999). In particular for clonal plant species such as lima bean, which frequently reproduce asexually *via* stolons, the reallocation of resources to belowground plant parts likely represents an important fitness measure (Núñez-Farfán *et al.* 2007).

In our study, seed mass – an important reproductive trait – was affected by the quantity and type of damage. Removing parts of leaflets had stronger negative effects on seed mass than removal of entire leaflets (Fig. 4). The stronger effects of partly damaged leaves may be due to a decrease in photosynthetic activity in leaf areas adjacent to the site of damage caused by a leakage of nutrients, metabolites and water through damaged tissue. Similar effects have been demonstrated in other legumes such as soybean (Ostlie & Pedigo 1984). Such changes in seed

traits can have a far-reaching impact on plant fitness. Seed weight and size have been identified as plant traits strongly influencing the dispersal, establishment, survival and growth of seedlings (Harper *et al.* 1970; Harper 1977; Westoby 1998; Weiher *et al.* 1999; Moles & Westoby 2004), particularly at early seedling stages (Leishman *et al.* 2000; Coomes & Grubb 2003). Fenner & Thompson (2005) showed that large seeds had an increased probability of establishment under detrimental conditions. Generally, seedlings developing from large seeds cope better than those of smaller-seeded species under competition (Parrish & Bazzaz 1985; Rees 1995), drought, nutrient limitation (Lee & Fenner 1989; Jurado & Westoby 1992; Leishman & Westoby 1994) and depth of seedling emergence (Gulmon 1992; Peterson & Facelli 1992; Vázquez-Yanes & Orozco-Segovia 1992).

The secretion of extrafloral nectar (EFN; an indirect defence) showed a strong quantitative response to leaf area reduction, while the type of damage did not affect EFN production. However, similar to seed mass, we observed a higher impact of removing parts of leaflets compared to entire leaflets, but the differences between the two types of damage were not significant for each degree of defoliation (66% *versus* 33%; Fig. 5B). Like floral nectar, EFN can be an expensive defence, although the general costs are yet unknown (Ballhorn *et al.* 2013a). Floral nectar can constitute an estimated 20% of the energy in total crop yield in the legume *Medicago sativa* (Southwick 1984), and EFN is also secreted in amounts of several milligrams of sugars per gram leaf mass over 24 h (Ballhorn *et al.* 2014). While plants can reabsorb EFN (Nepi & Stpiczyńska 2008), there is no known mechanism by which non-consumed EFN can be re-absorbed. This further increases the costs of EFN, potentially making EFN secretion a significant sink for carbohydrates. Under conditions of continuous removal of leaf area leading to massive reductions of photosynthetic material, as in our experiment, the costly production of EFN might be impacted from resource allocation constraints. Alternatively,

EFN secretion might be actively down-regulated, as it is diurnally in lima bean through exposure to red or far-red light (Radhika *et al.* 2010) in response to extreme defoliation. This would allow for reallocation of carbon to seed production and would fit the predictions of the Optimal Defence Hypothesis, which states that organisms allocate defences in a way that maximises fitness (McKey 1974, 1979; Rhoades 1979; Radhika *et al.* 2008).

In contrast to indirect defence *via* EFN secretion, direct defence through cyanogenesis was not affected by the extent or type of experimental defoliation. Among all treatments, we observed no significant changes in the cyanogenic potential (HCNp) compared to the controls (Fig. 5A). This is in line with previous studies, in which cyanide precursor concentrations have been found to remain constant in plants that have endured different degrees of herbivory (Ballhorn *et al.* 2006). Furthermore, as the synthesis of cyanogenic precursors is highly demanding for nitrogen, which in contrast to photosynthetic carbohydrates was similarly available to all experimental plants, limited effects of leaf area removal on HCNp observed in our study can be explained.

When interpreting the results of our study from an ecological perspective, it should be noted that this experiment exaggerated the effects of herbivory. In nature, folivory frequently does not lead to more than 10–25% reduction in leaf tissue (Coley & Barone 1996; Koptur *et al.* 1996). However, it is predicted that with increasing global temperatures, insect populations could shift or increase in certain areas, resulting in an increase in herbivory on some plant populations (Bale *et al.* 2002; Ballhorn *et al.* 2010c).

Our data indicate fundamentally different effects of leaf damage on various key measures of plant performance and fitness. This finding shows that multiple plant traits need to be evaluated to determine the impact of herbivory on plant fitness and productivity in natural and agricultural ecosystems.

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