Annals of Botany **112**: 711–720, 2013
doi:10.1093/aob/mct083, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

PART OF A SPECIAL ISSUE ON SEEDLING HERBIVORY

Ontogenetic patterns in the mechanisms of tolerance to herbivory in *Plantago*

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Received: 1 November 2012 Revision requested: 28 November 2012 Accepted: 22 February 2013 Published electronically: 14 April 2013

• *Background and Aims* Herbivory and plant defence differ markedly among seedlings and juvenile and mature plants in most species. While ontogenetic patterns of chemical resistance have been the focus of much research, comparatively little is known about how tolerance to damage changes across ontogeny. Due to dramatic shifts in plant size, resource acquisition, stored reserves and growth, it was predicted that tolerance and related underlying mechanisms would differ among ontogenetic stages.
• *Methods* Ontogenetic patterns in the mechanisms of tolerance were investigated in *Plantago lanceolata* and *P. major* (Plantaginaceae) using the genetic sib-ship approach. Pot-grown plants were subjected to 50 % defoliation at the seedling, juvenile and mature stages and either harvested in the short-term to look at plasticity in growth and photosynthesis in response to damage or allowed to grow through seed maturation to measure phenology, shoot compensation and reproductive fitness.
• *Key Results* Tolerance to defoliation was high in *P. lanceolata*, but low in *P. major*, and did not vary among ontogenetic stages in either species. Mechanisms underlying tolerance did vary across ontogeny. In *P. lanceolata*, tolerance was significantly related to flowering (juveniles) and pre-damage shoot biomass (mature plants). In *P. major*, tolerance was significantly related to pre-damage root biomass (seedlings) and induction of non-photochemical quenching, a photosynthetic parameter (juveniles).
• *Conclusions* Biomass partitioning was very plastic in response to damage and showed associations with tolerance in both species, indicating a strong role in plant defence. In contrast, photosynthesis and phenology showed weaker responses to damage and were related to tolerance only in certain ontogenetic stages. This study highlights the pivotal role of ontogeny in plant defence and herbivory. Additional studies in more species are needed to determine how seedlings tolerate herbivory in general and whether mechanisms vary across ontogeny in consistent patterns.

Key words: Plant defence, herbivory, pulse-amplitude fluorometry, root : shoot ratio, plant allocation, compensation, genetic variation, perennial herbs, maternal genetic families, norm of reaction, phenotypic plasticity, *Plantago lanceolata*, *P. major*, Plantaginaceae.

INTRODUCTION

The intensity of herbivory varies markedly during plant development. These temporal shifts in herbivory paired with developmental changes in plant resource acquisition and allocational priorities (i.e. among growth, storage and reproduction) lead to dramatic ontogenetic patterns of plant defence. Recent reviews have revealed that most plant species examined undergo ontogenetic shifts in defence and have identified general patterns among species (Boege and Marquis, 2005; Barton and Koricheva, 2010). For example, while constitutive secondary chemistry tends to increase from seedling to juvenile to mature plant stages, the inducibility of secondary compounds shows the opposite pattern, decreasing during ontogeny (Barton and Koricheva, 2010). In contrast to ontogenetic patterns in chemical defence, tolerance to herbivory (the maintenance of fitness in damaged plants) shows surprisingly consistent expression among seedlings, juveniles and mature stages (Barton and Koricheva, 2010). This pattern is particularly well supported in herbs; a meta-analysis of data from 36 studies found no evidence that tolerance changes across ontogeny (Barton and Koricheva, 2010), although there are individual cases in which tolerance has been shown to increase (Boege *et al.*, 2007) or decrease (Weltzin *et al.*, 1998; Thomson *et al.*, 2003) across ontogeny. It is particularly striking that early ontogenetic stages seem to tolerate herbivory quite well considering that small size has been predicted to constrain tolerance in seedlings and juvenile plants (Strauss and Agrawal, 1999; Haukioja and Koricheva, 2000; Kelly and Hanley, 2005). Additional research is needed to determine whether seedling tolerance is in fact a general pattern and, more importantly, what mechanisms enable young plants to tolerate herbivory, because it is likely that seedlings and juvenile plants use different mechanisms than mature plants.

Tolerance is measured using the norm of reaction approach; a slope of zero for the relationship between damage and fitness represents complete tolerance (Simms, 2000). As a form of phenotypic plasticity, tolerance should be tested as the effect of damage on fitness within genotypes or, more commonly, within genetic families. However, because experiments that test for tolerance using genotypes or families have such large sample sizes (Boege *et al.*, 2007; Barton, 2008; Stevens *et al.*, 2008; Hochwender *et al.*, 2012), most studies simply test how damage affects a random sample of plants. Unfortunately, omitting the genetic component prevents such

studies from investigating important aspects of the evolution of tolerance, such as tests of genetic variation in tolerance (as significant interaction between genotype and damage on fitness), and identification of mechanisms underlying tolerance via genotypic regression analyses (Mauricio, 2000; Weinig *et al.*, 2003; Stevens *et al.*, 2008; Muola *et al.*, 2010; Hochwender *et al.*, 2012). Thus, studies of tolerance that measure damage effects on fitness within genotypes or genetic families are the most useful for determining which traits and responses are associated with tolerance and whether tolerance can evolve in natural populations.

Despite growing interest in tolerance as a key aspect of plant defence (Strauss and Agrawal, 1999; Nunez-Farfan *et al.*, 2007; Fornoni, 2011), we still have a poor understanding of the mechanisms underlying tolerance (Tiffin, 2000; Fornoni, 2011). Although the following traits are thought to be involved in tolerance to herbivory, the relative importance or generality of any of them remains unknown: increases in photosynthesis (Thomson *et al.*, 2003; Fang *et al.*, 2006); shifts in biomass allocation from roots to shoots (Barton, 2008; Hódar *et al.*, 2008; Stevens *et al.*, 2008); utilization of stored reserves and bud banks (Armstrong and Westoby, 1993; Bossdorf *et al.*, 2004; Gruntman and Novoplansky, 2011; Latzel *et al.*, 2011); and phenological changes such as earlier flowering (Freeman *et al.*, 2003). In addition to such 'induced' mechanisms of tolerance (i.e. traits up-regulated in expression or activity following damage), the constitutive (i.e. 'pre-damage') expression of traits may also act as mechanisms of tolerance if they positively contribute to the maintenance of fitness following damage (Fornoni, 2011). For example, high root : shoot ratios prior to damage can provide plants with additional stored reserves, thereby leading to greater tolerance than plants with low root : shoot ratios (Hochwender *et al.*, 2000; Rivera-Solis *et al.*, 2012). Alternatively, in woody plants (Stevens *et al.*, 2008) and some erect herbs (Tucker and Avila-Sakar, 2010), pre-damage shoot mass is important because of the carbohydrate storage in stems. Although such constitutive mechanisms of tolerance have been largely overlooked in comparison to induced mechanisms, they are likely to be important.

Mechanisms of tolerance have rarely been investigated within an ontogenetic context, and there are strong reasons to expect mechanisms to change across ontogeny (Gruntman and Novoplansky, 2011). Seedlings and juvenile plants have limited stored reserves and few lateral buds to be utilized and expressed in response to damage. Moreover, although intrinsic growth rates can be high early in ontogeny, total leaf area and root mass are small in seedlings and juveniles compared with older plants. Thus, in terms of both resource acquisition and utilization of stored reserves, seedlings and juveniles are highly constrained. On the other hand, young plants tend to be quite plastic (Pigliucci, 1998), and so increases in photosynthesis, changes in biomass partitioning via shifts in root versus shoot growth, and phenological changes are feasible mechanisms of tolerance in seedlings and juveniles.

In this study, ontogenetic patterns of tolerance to defoliation were investigated in two widespread perennial herbs, *Plantago lanceolata* and *P. major* (Plantaginaceae). These species are ideal for this study for several reasons. First, as cosmopolitan weeds, both species have successfully established in countless new habitats and communities, and tolerance is increasingly being recognized as a key aspect of introduced plant establishment, allowing plants to thrive despite incurring damage by novel herbivores (Ashton and Lerdau, 2008; Chun *et al.*, 2010). Secondly, dramatic ontogenetic patterns have previously been documented for the chemical resistance of *P. lanceolata* and *P. major*, indicating that ontogeny plays an important role in the defence of these species (Barton, 2007, 2008; Elger *et al.*, 2009; Quintero and Bowers, 2011). Thirdly, although results vary somewhat among studies, likely due to variation among populations sampled and experimental conditions, previous studies have revealed considerable tolerance to damage, particularly in early ontogenetic stages, in both *P. lanceolata* and *P. major* (Hanley and May, 2006; Hanley and Fegan, 2007; Barton, 2008; Hanley, 2012), although none of these studies investigated potential mechanisms underlying tolerance.

To provide new insights into how mechanisms of tolerance shift across ontogeny, an experiment was conducted in which plants were damaged at the seedling, juvenile and mature ontogenetic stages. A subset of damaged and undamaged control plants within each ontogenetic stage was harvested shortly after damage to examine plasticity in photosynthesis, growth, compensation and phenology as potential mechanisms of tolerance. The remaining damaged and undamaged control plants were grown until mature seeds could be collected to quantify tolerance as the slope of the relationship between damage and reproductive fitness within genetic families. Regression analyses were conducted on genetic family means of tolerance and constitutive and induced photosynthetic and growth traits to identify mechanisms of tolerance at the seedling, juvenile and mature plant stages. This study provides the first rigorous assessment of how potential mechanisms of tolerance change over the course of ontogeny using the norm of reaction and genetic sib-ship approach, emphasizing how a physiological and morphological examination of plant responses to damage within a developmental context can give new insights into the evolutionary ecology of plant defence and herbivory.

MATERIALS AND METHODS

Study system

Native to Eurasia (van der Aart and Vulto, 1992), *Plantago lanceolata* L. and *P. major* L. are short-lived perennial herbs with a rosette growth form. Both species produce phenolics and iridoid glycosides, which have been clearly demonstrated to provide defence against herbivores (Bowers and Puttick, 1989; Adler *et al.*, 1995), as well as fungal pathogens (Marak *et al.*, 2002). The two main iridoid glycosides produced by *P. lanceolata* are aucubin and catalpol, which increase substantially during ontogeny, reaching constitutive levels as high as 10–12 % dry weight in mature plants (Bowers and Stamp, 1993; Barton, 2007). *Plantago major* produces only aucubin, and in much lower constitutive levels than *P. lanceolata*, typically producing <1 % dry weight in all ontogenetic stages (Barton and Bowers, 2006; Barton, 2007). Induction of iridoid glycosides in these species is generally weak, but varies across ontogeny, among genotypes, and with respect to herbivore identity (Stamp and Bowers, 2000; Fuchs and Bowers, 2004; Barton, 2008; Quintero and Bowers, 2011). Previous studies have revealed considerable

tolerance to damage in *P. lanceolata* and *P. major*, particularly in early ontogenetic stages (Hanley and May, 2006; Hanley and Fegan, 2007; Barton, 2008; Hanley, 2012).

Both *P. lanceolata* and *P. major* have naturalized populations throughout Hawai'i, although neither is abundant or common except as occasional occupants of roadside communities and lawns. Seeds of both species were collected from roadside populations on the island of O'ahu in Summer 2010. Seeds sampled from maternal plants located at least 2 m apart were stored separately, and sibling seedlings grown from a single maternal plant constitute a 'genetic family'. The total sample size includes 13 genetic families of *P. major* sampled across six different sites, and 14 genetic families of *P. lanceolata* sampled across three different sites.

Herbivore damage is not commonly observed on *P. lanceolata* and *P. major* in Hawai'i, although they are likely to sustain damage from non-native slugs that are abundant in the lowland communities where seeds were collected (Joe and Daehler, 2008). Furthermore, source populations are mown/disturbed several times a year, suggesting that tolerance to damage may have been selected for in these plants.

Germination

Seeds were sown in Promix BX (65–75 % Canadian sphagnum peat moss, perlite, dolomitic and calcitic limestone, macro- and micronutrients, *Glomus intraradices* mycorrhizae inoculum) on 20–21 June 2011 and were germinated outside. Immediately following germination, at the cotyledon stage, seedlings were transplanted into pots filled with Promix BX and a single application of slow-release Osmocote fertilizer. At the time of transplant, seedlings were randomly assigned to either the long-term experiment designed to quantify tolerance or to the short-term experiment designed to measure immediate responses to defoliation and putative mechanisms of tolerance.

Plants were grown at the University of Hawai'i at Manoa campus, in a rooftop growing area of the St John Plant Sciences Building. Plants experienced occasional rainfall, and received supplemental water daily. Although plants were outside, herbivores and pollinators were absent from the rooftop location. Due to space constraints, *P. lanceolata* were grown in full sun, while *P. major* were grown under a shaded canopy. Because light can strongly affect plant defence and growth (Kangasjärvi *et al.*, 2012), the different light conditions used in these experiments confound a species comparison. However, the light conditions correspond with preferred habitats of these species given that *P. major* is common in shaded riparian habitats while *P. lanceolata* is more common in open disturbed habitats (K. E. Barton, unpubl. res.), making these biologically suitable conditions for them to grow. Moreover, this study was not designed around a direct species comparison but rather as a test of the ontogeny of tolerance using two cosmopolitan species that have served as models for plant evolutionary ecology.

Long-term experiment

To calculate tolerance to damage, plants were subjected to 50 % defoliation at the seedling (one true leaf), juvenile (mean five true leaves) and mature (flowering) ontogenetic stages. The defoliation treatments were applied by removing half of all leaves with scissors. All damaged plants, as well as an undamaged control group, were allowed to grow and reproduce until a single harvest time. For *P. lanceolata*, plants were harvested on 11–14 October when all plants were 14 weeks old. Because damage was imposed at different times, the ontogenetic stages had variable lengths of time to recover from and demonstrate tolerance to damage: seedlings had 12 weeks to recover; juveniles had 10 weeks, and mature plants had 7 weeks to recover. For *P. major*, plants were harvested on 1–4 November when they were 17 weeks old. *Plantago major* seedlings had 15 weeks, juveniles had 12 weeks, and mature plants had 7 weeks to recover. All plants were grown in 8·8-L deep pots, and showed no sign of pot-binding effects upon harvest, evidenced by much of the soil remaining uncolonized by roots. Replication per damage group within genetic families ranged from two to four plants (mean 2·8), and the total sample sizes were $n = 159$ for *P. lanceolata* and $n = 146$ for *P. major*.

Plants in the long-term experiment were used to quantify tolerance and to assess two putative mechanisms of tolerance: phenology and compensation. Tolerance was defined as the effect of damage on reproductive output, measured as total seed biomass, combined from seeds extracted from weekly collections of mature capsules and at the final harvest. Phenology was investigated through weekly surveys of plant reproductive status, starting in early August. Flowering state (yes/no) was recorded for all plants, and the date of first flowering was analysed to determine whether shifts in phenology (mean first flowering date) occurred in response to damage. At the final harvest, only above-ground tissue was harvested, oven-dried at 60 °C to constant weight, and weighed to the nearest 0·01 mg. Shoot biomass (leaves) was analysed to determine the degree to which damaged plants replaced lost leaf tissue, defined as compensation.

Short-term experiment

Plants assigned to the short-term experiment were subjected to the same 50 % defoliation treatments at the same three ontogenetic stages as the long-term experiment. In contrast to the long-term experiment, damaged plants and undamaged control plants were harvested as soon as damaged plants had completed maturation of a new leaf (10–20 d later) in order to measure short-term responses as potential mechanisms of damage. Plants damaged at the seedling stage were grown in 540-mL pots; juvenile-stage plants were grown in 4·4-L pots, and mature-stage plants were grown in 8·8-L deep pots to minimize pot-binding effects. In total, the short-term experiment consisted of six treatment groups: (damage + control groups) × 3 ontogenetic classes. Three or four replicate plants (mean 3·2) per genetic family per treatment group gave a total sample size of $n = 261$ for *P. lanceolata* and $n = 249$ for *P. major*.

At the time of harvest, various putative mechanisms of tolerance were measured, including photosynthetic parameters and allocation patterns of growth (biomass partitioning). Photosynthesis was measured on the youngest fully expanded leaf on a subset of plants within each family × treatment

group combination during peak photosynthetic times, 0900–1300 h (Geiger and Servaites, 1994). Net photosynthesis (P_n) and stomatal conductance (C) were measured using the CID-340 hand-held photosynthesis system, which measures gas exchange per leaf area. Due to the large sample size of this experiment, additional photosynthetic data were obtained using pulse amplitude modulated (PAM) chlorophyll fluorometry (Walz Jr). PAM fluorometry measures the fluorescence generated by plants absorbing light for photosynthesis, as well as the heat energy that is dissipated when more light has been absorbed than can be used for electron transport. PAM-generated light saturation curves can be used to quantify three parameters that inform about photosynthesis: (1) photosynthetic light efficiency (also called potential quantum yield; Y_0) which represents the proportion of light that a plant absorbs of the total light available; (2) the maximum photosynthetic electron transport rate under saturating light (ETR_m), which correlates linearly with net photosynthesis under most environmental conditions (Pasquini and Santiago, 2012; Genty *et al.*, 1989; Beer and Axelsson, 2004); and (3) the amount of excess light that is dissipated as heat [non-photochemical quenching (NPQ)], a measure of photoprotection in leaves. PAM fluorometry has the advantage of producing photosynthetic data much faster than gas exchange methods (1–2 min versus 12–15 min, respectively, per sample) and has been broadly used for studies of algae and terrestrial plants (Genty *et al.*, 1989; Baker, 2008). In addition to photosynthesis, chlorophyll content was measured using a Minolta SPAD-502 chlorophyll meter. Due to their small size, it was often impossible to measure photosynthesis on seedlings, and so some photosynthetic data are missing for the seedling stage.

Following photosynthesis measures, shoot and root tissues were harvested, cleaned of debris, oven-dried at 60 °C and weighed. The youngest fully expanded leaf was harvested separately, photographed digitally for area computation with ImageJ (Rasband, 1997–2011), dried and weighed; specific leaf area (SLA) was calculated as area/dry weight ($cm^2\ g^{-1}$) for this leaf. Comparison of damaged versus control plants for shoot biomass reveals the degree to which damaged plants can compensate for the lost leaf tissue; comparisons of damaged versus control plants for root biomass reveals whether allocational shifts from growth below ground or new root growth drive compensation of shoots. Effects of damage on photosynthetic parameters indicate whether damage induces changes in *Plantago* photosynthesis.

Statistical analyses

Data were analysed using SAS for Windows version 9.2 (Cary, NC, USA). Residuals were examined, and variables were log-transformed to meet assumptions of normality and homoscedasticity. The two species were analysed separately for both the long-term and short-term experiments to avoid heteroscedasticity and to avoid confounding light availability with species. Due to imbalances in replication across groups, type III sums of squares are reported for all analyses. In general, data were analysed with mixed-model ANOVAs in which genetic family and all interactions with genetic family were considered random factors. The significance of each random factor was tested by running the models with and without the random factor of interest, and then calculating the log-likelihood ratio statistics, which can be compared with a chi-square distribution with one degree of freedom (Littell *et al.*, 1996).

Data from the long-term experiment were analysed with mixed-model ANOVAs in which the main factors of damage class (four levels; fixed) and genetic family (random) were tested. Total seed biomass was analysed as the test of tolerance. First flowering date and shoot biomass were analysed to assess whether shifts in phenology and compensation, respectively, were associated with plant responses to defoliation. A significant interaction between damage and genetic family would indicate genetic variation in tolerance, phenology or compensation. Tukey-adjusted least-square mean comparisons were made to determine differences among treatment groups for significant damage effects.

Data from the short-term experiment were analysed with mixed-model ANOVAs in which the main factors of damage (two levels; fixed), ontogenetic stage (three levels; fixed), and genetic family (random) were tested. The interaction between damage and stage was tested to determine whether plant responses vary across ontogeny. All interactions including genetic family were tested and included in the final model only when significant. Response variables analysed include: SLA, P_n, C, Y_0, the maximum photosynthetic electron transport rate under saturating light (ETR_m), NPQ and chlorophyll content (SPAD). All photosynthetic parameters were analysed first jointly in multivariate ANOVAs, testing for effects of damage, ontogenetic stage, genetic family, and their interactions. Because the MANOVAs revealed significant effects (see Results), photosynthetic parameters were subsequently analysed separately with univariate ANOVAs.

To investigate the relationship between potential mechanisms of tolerance and tolerance, genotypic regressions were performed on genetic family means. Tolerance was calculated for each genetic family as the ratio of mean seed biomass for damaged/control plants. Both constitutive and induced mechanisms were investigated. Constitutive traits were inferred from the absolute mean values for undamaged control plants, including root : shoot ratio and ETR_m. Induced traits were calculated as the relative change in damaged plants, calculated as the ratio between the trait mean for damaged/control plants. Induced traits investigated include: shoot compensation and phenology (from the long-term experiment), and root biomass, ETR_m and NPQ (from the short-term experiment). Significant regressions would identify mechanistic traits associated with tolerance. Separate analyses were conducted on data from all three ontogenetic stages for both species to assess the directionality and slope of each relationship at each ontogenetic stage separately.

RESULTS

Long-term experiment

Plantago lanceolata plants tolerated 50 % defoliation at all ontogenetic stages, with no significant differences in seed biomass among treatment groups (Fig. 1A, $F_{3,35\cdot4} = 0\cdot34$, $P = 0\cdot7990$). However, ontogeny did influence shoot

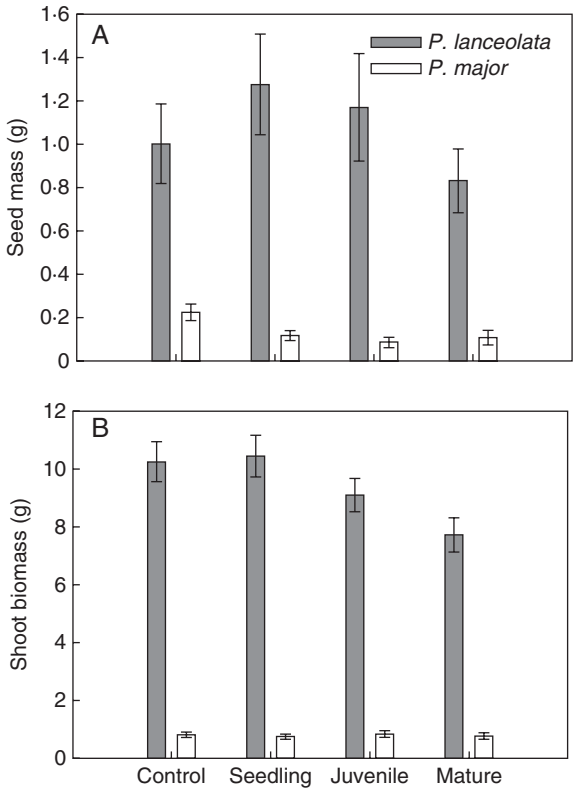

FIG. 1. Patterns of expression for (A) seed and (B) shoot biomass in response to 50 % defoliation in the long-term experiment for both species. Bars are means ± s.e.

compensation in *P. lanceolata*: mature plants were incapable of fully compensating for lost shoot tissue by harvest time, while seedling and juvenile plants were (Fig. 1B, $F_{3,144} = 4{\cdot}56$, $P = 0{\cdot}0044$). Damage had no effect on flowering phenology in *P. lanceolata* ($F_{3,29{\cdot}1} = 1{\cdot}77$, $P = 0{\cdot}1751$). Although genetic families varied significantly for seed ($\chi^2 = 16{\cdot}4$, $P < 0{\cdot}0001$) and shoot ($\chi^2 = 14{\cdot}4$, $P < 0{\cdot}0001$) biomass, there was no variation in the responses of families to damage for seed and shoot biomass (no significant family × damage interactions, $P = 1{\cdot}0$ for both). Genetic families varied in their first flowering date ($\chi^2 = 3{\cdot}3$, $P = 0{\cdot}0346$), and also showed marginally significant variation in how damage influenced flowering date (damage × family interaction: $\chi^2 = 2{\cdot}3$, $P = 0{\cdot}0647$).

Defoliation reduced seed biomass at all ontogenetic stages in *P. major*, revealing that this species cannot tolerate damage throughout ontogeny (Fig. 1A, $F_{3,48{\cdot}6} = 4{\cdot}86$, $P = 0{\cdot}0049$). In contrast, all plants had similar shoot biomass at harvest, revealing full shoot compensation for 50 % defoliation at all ontogenetic stages (Fig. 1B, $F_{3,35{\cdot}2} = 1{\cdot}30$, $P = 0{\cdot}2913$). Damage significantly influenced flowering phenology in *P. major* ($F_{3,130} = 13{\cdot}31$, $P < 0{\cdot}0001$). Since defoliation was administered after plants started flowering in the mature treatment group, there was no difference in flowering phenology of control versus mature plants (Tukey least-square mean comparison, $t = 0{\cdot}39$, $P = 0{\cdot}9797$), but plants damaged at the seedling (Tukey least-square mean comparison, $t = 4{\cdot}52$, $P < 0{\cdot}0001$) and juvenile (Tukey least-square mean comparison, $t = 4{\cdot}01$, $P < 0{\cdot}0001$) stages flowered on average 7 and 8 d later, respectively, than control plants. Significant variation among genetic families was detected for shoot biomass ($\chi^2 = 57{\cdot}8$, $P < 0{\cdot}0001$) and first flowering day ($\chi^2 = 53{\cdot}0$, $P < 0{\cdot}0001$), but not for seed biomass ($\chi^2 = 0{\cdot}0$, $P = 1{\cdot}0$). There were no significant genetic family × damage interactions ($P > 0{\cdot}1271$).

Short-term experiment

Plantago lanceolata. Not surprisingly, plants grew, giving rise to a highly significant effect of plant age on shoot and root biomass (Table 1 and Fig. 2). In *P. lanceolata*, damaged plants were incapable of compensating for lost shoot tissue in the time allowed in the short-term experiment, leading to a significant negative effect of damage on shoot biomass (Table 1 and Fig. 2), irrespective of plant age (damage × age interaction was not significant). Defoliation also led to significant reductions in root biomass in the seedling (Tukey least-square mean comparison, $t = 3{\cdot}56$, $P = 0{\cdot}0060$) and juvenile stages (Tukey least-square mean comparison, $t = 5{\cdot}40$, $P < 0{\cdot}0001$), but had no detectable effect on root biomass in the mature stage (Tukey least-square mean comparison, $t = 1{\cdot}56$, $P = 0{\cdot}6289$), leading to a significant interaction between age and damage on root biomass (Table 1 and Fig. 2). SLA significantly increased from the seedling to juvenile stage, and both stages showed a decrease in SLA in response to damage; data were not obtained for mature-phase plants (Table 1 and Supplementary Data Fig. S1). Genetic variation was detected for shoot biomass, and for ontogenetic patterns of root growth (significant age × family interaction; Table 1).

The MANOVA model revealed that *P. lanceolata* photosynthesis parameters jointly responded significantly to age (Wilks' λ $F_{18,130} = 11{\cdot}51$, $P < 0{\cdot}0001$), damage (Wilks' λ $F_{9,65} = 2{\cdot}06$, $P = 0{\cdot}0468$), genetic family (Wilks' λ $F_{117,499} = 1{\cdot}96$, $P < 0{\cdot}0001$), the damage × family interaction (Wilks' λ $F_{117,499} = 1{\cdot}33$, $P = 0{\cdot}0213$), but not to interactions between age × damage (Wilks' λ $F_{18,130} = 1{\cdot}08$, $P = 0{\cdot}3832$), age × family (Wilks' λ $F_{234,575} = 1{\cdot}09$, $P = 0{\cdot}2208$) or age × damage × family (Wilks' λ $F_{234,575} = 1{\cdot}10$, $P = 0{\cdot}1874$). To better understand how plants alter photosynthesis in response to damage, parameters were subsequently analysed individually with univariate ANOVAs.

Photosynthesis changed significantly across ontogenetic stages in *P. lanceolata* (Table 1 and Fig. 3). While the light reactions (potential quantum yield, ETR_m and chlorophyll content) tended to show non-linear trends, peaking in the juvenile stages (Fig. 3A, B, F), seedlings obtained comparably high rates of net photosynthesis by having considerably higher rates of stomatal conductance than either juveniles or mature plants (Fig. 3D). Mature plants tended to perform weakest on all photosynthetic parameters (Fig. 3). NPQ was similar for seedlings and juveniles, and lower in mature plants. NPQ was the only photosynthetic parameter that showed plasticity in response to damage, although this response depended on age (Table 1). Damage increased NPQ at the juvenile and mature stages, but had the opposite effect on seedlings. Only chlorophyll content and stomatal conductance varied significantly among genetic families (Table 1), and

TABLE 1. *Summary of mixed model ANOVA results for the short-term experiment*

Variable	*n*	Age (*F*)	Damage (*F*)	*A* × *D* (*F*)	Family (χ^2)	Significant family interactions
Plantago lanceolata						
Shoot biomass (g)	216	1569·24***	38·24***	0·32	5·9**	
Root biomass (g)	216	632·91***	36·05***	3·33*	0·1	Age × family 9·4**
SLA (g cm^{-2})	179	248·35***	7·00**	0·79	1·5	
Y_0	256	3·70*	1·25	1·40	0·8	
ETR_m	256	7·05**	0·06	1·86	0·6	Damage × family 3·2*
NPQ	256	4·93**	2·74	7·52***	0·1	Damage × family 6·6*
P_n	159	12·60***	0·90	0·29	1·2	
C	159	20·16***	0·16	0·13	7·8**	
SPAD	159	26·40***	1·29	0·21	6·2**	
Plantago major						
Shoot biomass (g)	249	141·09***	43·80***	3·35*	4·2*	Age × family 9·2**
Root biomass (g)	249	82·68***	31·83***	3·30*	2·2	Age × family 12·4***
SLA (g cm^{-2})	150	8·81**	7·73**	0·05	16·8***	
Y	224	1·33	1·17	0·34	0·0	Damage × family 10·9***
ETR_m	224	2·67	0·0	0·74	2·1	
NPQ	224	26·60***	8·50**	2·93*	0·0	
P_n	51	N/A	0·01	N/A	4·9*	
C	51	N/A	0·42	N/A	2·4	
SPAD	107	18·06***	6·51*	0·06	45·6***	

Response variables include photosynthetic light efficiency (Y_0), maximum photosynthetic electron transport rate under saturating light (ETR_m), non-photochemical quenching (NPQ), net photosynthesis (P_n), stomatal conductance (*C*) and chlorophyll content (SPAD). Significance is given as: ***, $P < 0·0001$; **, $P < 0·001$; *, $P < 0·05$. Fixed factors are tested with *F*-test statistics, and random variables are tested using log-likelihood ratio statistics, which can be compared with a chi-square distribution with one degree of freedom.

only NPQ showed significant variation among families in response to damage (family × damage interaction).

Plantago major. Both plant ontogenetic stage and defoliation significantly influenced shoot and root biomass in *P. major* (Table 1 and Fig. 2). However, the magnitude of the reduction in shoot and root biomass by defoliation was greater in seedlings and juveniles than it was in mature plants (Fig. 2), giving rise to significant age × damage interactions (Table 1). SLA was not calculated in mature plants due to logistical constraints, but from the seedling to juvenile stage, there was a significant increase in SLA (Table 1 and Supplementary Data Fig. S1). In both early ontogenetic classes, SLA was significantly reduced in damaged plants compared with controls (Table 1). Genetic variation was observed for both shoot biomass and SLA, and genetic families differed significantly in their ontogenetic patterns of shoot and root growth (Table 1).

The MANOVA model revealed that *P. major* photosynthesis parameters jointly responded significantly to age (Wilks' λ $F_{6,50} = 12·09$, $P < 0·0001$), damage (Wilks' λ $F_{6,50} = 3·31$, $P = 0·0081$), genetic family (Wilks' λ $F_{72,278} = 2·63$, $P < 0·0001$), age × family (Wilks' λ $F_{72,278} = 1·45$, $P = 0·0181$) and age × damage × family (Wilks' λ $F_{72,278} = 1·35$, $P = 0·0463$), but not to interactions between damage × family (Wilks' λ $F_{72,278} = 1·12$, $P = 0·2616$) or age × damage (Wilks' λ $F_{6,50} = 1·32$, $P = 0·2670$). To understand better how plants alter photosynthesis in response to damage, parameters were subsequently analysed individually with univariate ANOVAs.

Seedling leaves were too small for CID photosynthetic measurements, and mature plants were not measured with either CID or SPAD due to logistical constraints, so for net photosynthesis and stomatal conductance, data are only available for the juvenile stage. All fluorescence parameters are available for all three ontogenetic stages, and chlorophyll content data are available for seedlings and juveniles (Fig. 3). Both NPQ and chlorophyll content increased significantly over ontogeny, while ETR_m and potential quantum yield showed no ontogenetic pattern (Table 1 and Fig. 3). Only NPQ and chlorophyll content were affected by damage (Table 1), and in opposite patterns. Chlorophyll content was reduced in damaged plants compared with controls (Table 1), irrespective of plant ontogenetic stage (damage × age interaction not significant). In contrast, damage induced higher levels of NPQ in the mature stage (Tukey least-square mean comparison $t = 3·66$, $P = 0·0042$), but had no detectable effect on NPQ at either the juvenile (Tukey least-square mean comparison $t = 1·42$, $P = 0·7137$) or seedling stage (Tukey least-square mean comparison $t = 0·15$, $P = 1·0000$). Significant variation among genetic families was observed for chlorophyll content, and as an interaction with damage for potential quantum yield (Table 1).

Mechanisms of tolerance

Most regression analyses were not statistically significant, likely due to the general absence of significant genetic variation for tolerance, or the mechanistic traits studied (Table 1). However, some statistically significant associations were observed, identifying key mechanistic traits driving tolerance to defoliation. In *P. major* seedlings, tolerance is significantly related to the constitutive root : shoot ratio (slope = 4·04, $R^2 = 0·6232$, $P = 0·0013$), and in *P. major* juvenile plants there was a significant positive relationship with the induction of NPQ (slope = 0·586, $R^2 = 0·3599$, $P = 0·0302$). In

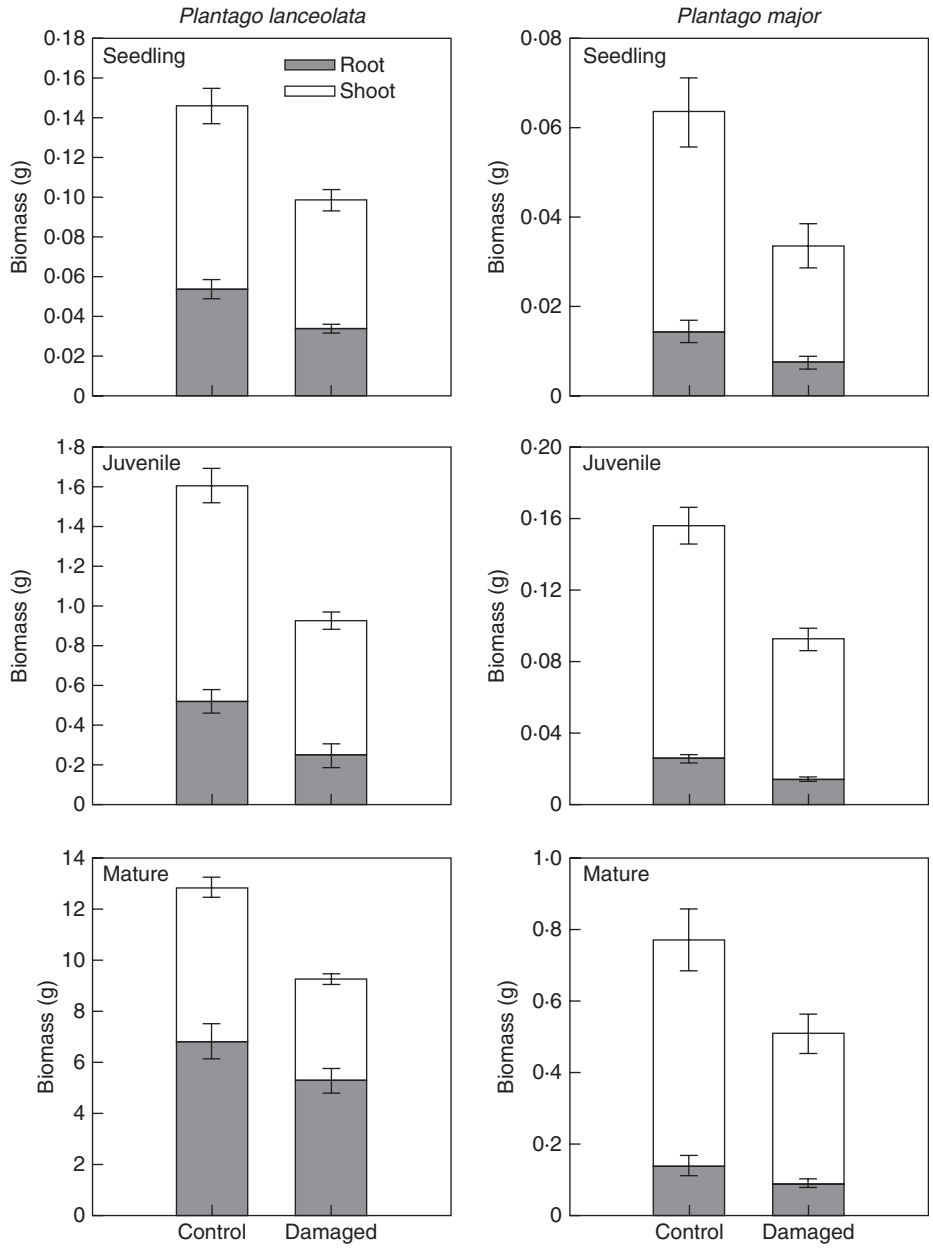

FIG. 2. Biomass allocation patterns in the short-term experiment for both species at seeding, juvenile and mature stages. Bars are means ± s.e.

P. lanceolata, juvenile plants that were induced to flower earlier had significantly higher tolerance compared with plants that responded to damage with a phenological delay (slope = −1·20, $R^2 = 0{\cdot}3748$, $P = 0{\cdot}0261$). In *P. lanceolata* mature plants, a significant association was again detected between tolerance and constitutive root : shoot ratio, but, in contrast to the pattern observed for *P. major* seedlings, mature *P. lanceolata* plants demonstrated a negative association between tolerance and root : shoot ratio (slope = −0·826, $R^2 = 0{\cdot}3091$, $P = 0{\cdot}0485$), indicating that tolerance was enhanced by a higher allocation to shoot growth. No other traits were significantly related to tolerance at any ontogenetic stage in either species (Supplementary Data Table S1).

DISCUSSION

This study revealed that ontogeny played a strong role in plant responses to damage in both *P. lanceolata* and *P. major*, although the species differed markedly both in the degree of tolerance and in the mechanisms underlying tolerance. In *P. lanceolata*, biomass partitioning and phenology were key mechanisms of tolerance, and in *P. major*, a different kind of biomass partitioning and photosynthesis were key mechanisms. These results add to the growing body of literature demonstrating how ontogeny plays a fundamental role in plant herbivory and defence (Boege and Marquis, 2005; Barton and Koricheva, 2010; Boege *et al.*, 2011; Koricheva and Barton, 2012).

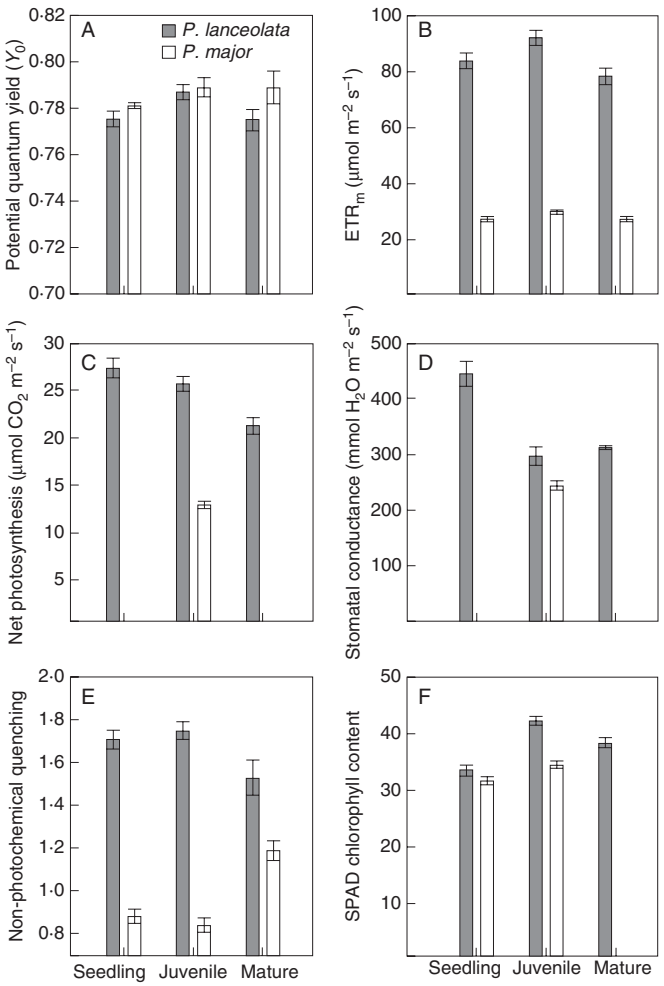

FIG. 3. Ontogenetic patterns in constitutive rates of photosynthetic parameters in seedlings, juvenile and mature stages of both species. Bars are means ± s.e.

In *P. lanceolata*, plants in all ontogenetic stages demonstrated full tolerance of 50 % defoliation, expressed as similar seed production among treatment groups damaged at the seedling, juvenile and mature stages with undamaged control plants. However, plants showed limited plasticity in many of the functional traits assayed, particularly the photosynthetic parameters measured. Only NPQ varied in response to defoliation, leading to an increase in NPQ in juveniles and mature plants, and a decrease in seedlings. NPQ has been previously implicated in tolerance to herbivory (Thomson *et al.*, 2003), where a decrease following defoliation was thought to reflect greater light-use efficiency in order to maximize regrowth. Similarly, in *P. lanceolata* seedlings, a decrease in NPQ may enhance tolerance, but the increase observed later in ontogeny is likely to be linked to a general stress response of these plants induced by damage.

Defoliated *P. lanceolata* plants altered biomass partitioning following damage, leading to a reduction in root biomass in seedlings and juveniles and a significant reduction in SLA across all ontogenetic stages. A reduction in root growth following defoliation is thought to allow plants to maximize regrowth and replacement of lost shoot tissue and is a commonly reported response to herbivory (Mabry and Wayne, 1997; Barton, 2008; Stevens *et al.*, 2008; Hochwender *et al.*, 2012). In contrast, mature *P. lanceolata* plants did not reduce root growth and were incapable of fully compensating for the removed tissue, indicating that biomass allocation is less plastic in older ontogenetic stages (Gedroc *et al.*, 1996), and that this constrains compensation in older plants. The significant reduction in SLA observed among all ontogenetic stages is not likely to contribute to tolerance given that smaller leaf area would most likely reduce photosynthesis and growth.

There is evidence that juvenile *P. lanceolata* plants rely on flowering time as a mechanism of tolerance. Flowering time was a genetically variable trait in *P. lanceolata*, and, although defoliation had no overall significant effect on flowering time, in many families defoliation delayed flowering. The genetic regression analyses revealed that families that were delayed the least by defoliation (i.e. damaged plants flowering near the same time as control plants) tolerated defoliation the best. Other studies have found that defoliation significantly delays flowering and, in general, this has a negative effect on tolerance (Freeman *et al.*, 2003; Hanley and May, 2006; Hanley and Fegan, 2007), particularly when a delay occurs during a change in seasons that prevents defoliated plants from attracting pollinators or setting seed (Brody and Irwin, 2012). Because this experiment was conducted in Hawai'i where *P. lanceolata* flowers year-round (K. E. Barton, pers. obs.) and because *P. lanceolata* is primarily wind-pollinated, a delay in flowering probably does not affect fitness in the long-term.

In contrast to general predictions (Strauss and Agrawal, 1999; Kelly and Hanley, 2005), but in agreement with previous research on *P. lanceolata* (Barton, 2008; Hanley, 2012), *P. lanceolata* seedlings fully tolerated 50 % defoliation. Although regression analyses failed to elucidate the mechanism underlying this result, it is clear that seedlings have the highest constitutive rates of photosynthesis and show plasticity in biomass partitioning via a reduction in root growth following defoliation, both of which could contribute to damage tolerance. Moreover, rather than individual traits driving tolerance in seedlings, it may be a suite of traits associated with the fast growth rates inherent to seedlings, particularly of weedy species such as *P. lanceolata* (Storkey, 2004).

Unlike *P. lanceolata*, tolerance was low in *P. major*, with seed output reduced by approximately half in defoliated plants, irrespective of the timing of defoliation. Surprisingly, though, defoliated plants fully compensated in shoot growth, achieving similar biomass to undamaged control plants at the final harvest. It thus appears that in *P. major* vegetative compensation has a higher priority than reproduction in the short term. Because this is a perennial plant, the long-term consequences of defoliation on reproductive output would reveal the effects on life-time fitness.

Although tolerance did not vary among ontogenetic stages in *P. major*, the mechanisms underlying tolerance did. Like *P. lanceolata*, biomass partitioning was important, although in very different ways. In *P. major* seedlings, a larger investment in roots before damage was associated with tolerance. This result is consistent with previous research that constitutive root growth enhances tolerance in herbs (Welter and Steggall, 1993; Mabry and Wayne, 1997; Hochwender *et al.*, 2000),

likely due to stored reserves in roots and greater nutrient uptake, both of which are important to support the increase in growth following defoliation (Moreira *et al.*, 2012). In the case of *P. major* seedlings, stored reserves are likely to be minimal, and so nutrient uptake may be the driving factor here. Furthermore, like *P. lanceolata*, biomass partitioning was plastic in response to defoliation, particularly in seedlings and juveniles, decreasing root growth and SLA in response to defoliation.

Photosynthesis was plastic in response to defoliation in *P. major*, although it is difficult to interpret these changes in the context of tolerance. Defoliation led to a decrease in leaf chlorophyll content (SPAD), possibly in response to the stress of defoliation and probably not contributing in a positive way to regrowth. Moreover, defoliation led to an increase in NPQ. Although it seems plausible that the increase in NPQ observed here results from a generalized stress response also responsible for the decrease in leaf chlorophyll content, the genotypic regression analyses suggest that NPQ might actually play an important role in *P. major* tolerance. In juvenile *P. major*, a greater induction of NPQ was found to be positively related to damage tolerance. Thus, it may be that very responsive plants are better at avoiding light stress via heat dissipation, and that this is associated with overall better performance following defoliation. This novel result is intriguing and suggests that a greater integration of primary and secondary chemical changes following defoliation will provide new insights into induced plant responses to defoliation and tolerance (Babst *et al.*, 2009).

There were no clear mechanisms of tolerance for *P. major* mature plants. It may be that selection by herbivores is only important early in ontogeny, leading to significant relationships between mechanistic traits and tolerance early and then a lack of relationships later (Muola *et al.*, 2010). Indeed, field studies in the native range of *P. major* report high levels of herbivore-mediated seedling herbivory and mortality by slugs (Hanley *et al.*, 1995*a*, *b*; Hanley *et al.*, 1996), and in Hawai'i, non-native slugs are also important seedling herbivores (Joe and Daehler, 2008). Field surveys measuring levels of herbivory and the effects of herbivory across ontogeny would shed further light on ontogenetic shifts in selection pressure in these introduced populations.

In summary, this study provides compelling evidence that mechanisms of tolerance change across ontogeny, and that these ontogenetic patterns even differ between closely related species with similar life histories. Photosynthetic light reactions, biomass partitioning and phenology were related to tolerance to defoliation, but at different ontogenetic stages. Ontogenetic patterns in mechanisms of tolerance most likely occur due to shifts in herbivore selection pressure from the seedling to juvenile and mature stages and also as a consequence of developmental trajectories in plant physiology, growth and plasticity. Similar experiments conducted on other species are needed to confirm that mechanisms of tolerance differ across ontogenetic stages (Tucker and Avila-Sakar, 2010; Gruntman and Novoplansky, 2011). In addition to enriching our knowledge about the nature of ontogenetic patterns in plant herbivory and defence, such research will highlight the value of integrating plant development with ecology to gain new insights into plant evolutionary ecology.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: patterns of induction in specific leaf area by 50 % defoliation in the seedling and juvenile ontogenetic stages for *P. lanceolata* and *P. major*. Table S1: summary of results from genotypic regression analyses to investigate the relationship between potential mechanistic traits and tolerance to damage.

ACKNOWLEDGEMENTS

The author thanks V. Gibson, M. Moffit, R. Hoan and R. Ormond for greenhouse and laboratory assistance; and Michael Hanley and two anonymous reviewers for advice and constructive feedback on the manuscript. This work was supported by the College of Natural Sciences, University of Hawai'i at Mānoa.

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