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HIGHLIGHTED STUDENT RESEARCH

Does investment in leaf defenses drive changes in leaf economic strategy? A focus on whole-plant ontogeny

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Abstract Leaf defenses have long been studied in the context of plant growth rate, resource availability, and optimal investment theory. Likewise, one of the central modern paradigms of plant ecophysiology, the leaf economics spectrum (LES), has been extensively studied in the context of these factors across ecological scales ranging from global species data sets to temporal shifts within individuals. Despite strong physiological links between LES strategy and leaf defenses in structure, function, and resource investment, the relationship between these trait classes has not been well explored. This study investigates the relationship between leaf defenses and LES strategy across whole-plant ontogeny in three diverse *Helianthus* species known to exhibit dramatic ontogenetic shifts in LES strategy, focusing primarily on physical and quantitative chemical defenses. Plants were grown under controlled environmental conditions and sampled for LES and defense traits at four ontogenetic stages. Defenses were found to shift strongly with ontogeny, and to correlate strongly with LES strategy. More advanced ontogenetic stages with more conservative LES strategy leaves had higher tannin activity and toughness in all species, and higher leaf dry matter content in two of three species. Modeling results in two species support the conclusion that changes in defenses drive changes in LES strategy through ontogeny, and in one species that changes in defenses and LES strategy are likely independently driven by ontogeny. Results of this study support the hypothesis that leaf-level allocation to defenses might be an important determinant of leaf economic traits, where high investment in defenses drives a conservative LES strategy.

Keywords Sunflower · *Helianthus* · Tannin · Leaf dry matter content · Toughness

Introduction

Plants mediate the flow of energy into almost all terrestrial ecosystems, and together plants and their herbivores comprise roughly half of the terrestrial species diversity on Earth (Futuyma and Agrawal 2009). Given the magnitude of the herbivory interaction, plant defenses against herbivory are among the most commonly studied plant adaptations, and are a classic model system in evolutionary ecology (Agrawal 2011). Plant defenses have long been studied in the context of plant growth rate and habitat resource availability (e.g., Coley et al. 1985; Endara and Coley 2011), specific limiting and surplus resources (e.g., Tuomi et al. 1988), their optimal allocation to maximize fitness (e.g., Rhoades and Cates 1976; Rhoades 1979), and various ecological and evolutionary unifications of these and other perspectives (e.g., Herms and Mattson 1992; Stamp 2003). The most explanatory unifications of plant defense theory focus on allocation patterns to growth, defense, and reproduction in the maximization of fitness.

Given that so many influential defense hypotheses involve plant C, nutrient, light, and growth rate parameters, it may be possible to advance the field via explicit integration of leaf defense theory with the parallel

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ecophysiological allocation-based hypothesis of the leaf economics spectrum (LES). The LES, one of the principal modern plant ecophysiology paradigms, is rooted in an economic analogy of plant resource use, growth, and reproduction that focuses on C as currency (Mooney 1972; Bloom et al. 1985). The LES describes a continuum of leaf physiology from "resource acquisitive" strategies of low C investment, high nutrient investment, quick-return leaves supporting high growth rates and leaf turnover, to "resource conservative" strategies of high C investment, low nutrient investment, slow-return leaves supporting slower growth rates and leaf turnover (Wright et al. 2004). These opposite ends of the spectrum are predicted to be evolutionary favored in higher and lower resource environments, respectively, and applications of the LES have focused primarily on adaptation to abiotic factors like precipitation, temperature, and soil fertility (e.g., Westoby et al. 2002; Wright et al. 2005; Ordonez et al. 2009; Kikuzawa et al. 2013). As leaf defenses require investments of C and nutrients, and certainly factor into leaf lifespan, they fit easily into the LES framework of investment and return on investment. Furthermore, as the primary productive organ in most plants, leaves are expected to be the most defended tissues, only matched or perhaps exceeded by reproductive tissues (Bazzaz et al. 1987). Even more of a direct connection is the overlap in many of the traits used to define both LES strategy and leaf defense, including structural traits like leaf mass per area (LMA), which is both one of the defining LES traits and a classic trait measured in studies of leaf defense, used as a rough estimate of leaf toughness/palatability (e.g., Hanley et al. 2007; Moles et al. 2013). Likewise, chemical traits like leaf nutrient content, of which N and P content are defining traits of the LES, and traits like C:N ratio have long been used as measures of leaf digestibility/palatability, and relative investment in C-based and N-based defenses are predictions of several defense hypotheses (reviewed in Stamp 2003). Leaf defenses and leaf economic traits must interact, due to their shared interactions with C and nutrient pools, growth rate, environmental resources, and the inclusion of many individual traits as descriptors of both aspects of leaf physiology.

The central trade-off of the LES has been found to exist at multiple ecological and evolutionary scales, including globally (Wright et al. 2004; Heberling and Fridley 2012), across and within specific biomes and communities (Freschet et al. 2010; Pérez-Ramos et al. 2012; Renteria and Jaramillo 2011; Xiang et al. 2013), within groups of closely related species (Dunbar-Co et al. 2009; Santiago and Kim 2009), among populations within species (Brouillette et al. 2014; Grady et al. 2013), and even recently within individuals through time (Mason et al. 2013). The existence of the LES pattern across scales has been suggested to arise from the joint action of selection and genetic constraint (Donovan et al. 2011), and indeed research in model systems has suggested the existence of a common genetic basis for the LES (Vasseur et al. 2012). Very little work has been done on LES trait variation within individuals through whole-plant ontogeny, with most studies that do exist comparing only two stages, typically juveniles and adults (Mediavilla and Escudero 2009; Palow et al. 2012). Though several studies including two or more LES traits have included multiple stages (Niinemets 2004; Ishida et al. 2005; Jullien et al. 2009), these have not had the LES as an explicit consideration (but see discussion below of Mason et al. 2013).

Unlike the LES, no singular global axis of variation has been found for leaf defenses (Moles et al. 2013). Leaf defenses appear to occur in a large number of combinations, though interestingly species appear to either be physically defended, chemically defended, or both, but seemingly never neither (Moles et al. 2013). A few weak global cross-species correlations among individual defenses have been demonstrated, for instance a negative correlation between tannins and ash content; however, research to date suggests that the majority of relationships among individual defense traits will differ strongly with taxonomy and geography, as there exist as many phenotypic combinations of leaf defenses as there are unique suites of herbivores and pathogens (Moles et al. 2013). Also unlike the LES, there have been a moderate number of studies of ontogenetic patterns in defense (reviewed in Boege and Marquis 2005; also in Barton and Koricheva 2010). Once again the problem exists of most studies focusing only on comparisons between two stages, especially in physical defenses (Barton and Koricheva 2010; but see Quintero and Bowers 2012). A synthesis of ontogenetic studies has found that for herbs there is an overall pattern of increasing constitutive secondary chemistry with ontogeny, but there is not enough data to generalize patterns of physical defenses (Barton and Koricheva 2010). This is unfortunate, as physical defenses are probably more likely to be tightly linked to the LES than secondary chemistry, especially qualitative chemical defenses, as physical traits like leaf toughness require large investments in structural C, directly impact LMA, and cannot be broken down and reallocated (Hanley et al. 2007). Of course, because the synthesis of chemical defenses is reliant on both enzyme activity and recently assimilated C, chemical defenses likely depend on the key LES traits of leaf N content and photosynthetic rate. This is especially true for qualitative chemical defenses that typically have high turnover rates and thus maintenance costs (Endara and Coley 2011).

The plant defense literature has experienced major upheavals and synthesis over the past two decades, with two major explanatory hypotheses remaining as best supported by evidence (reviewed in Stamp 2003). First, the optimal defense hypothesis has been refined from one

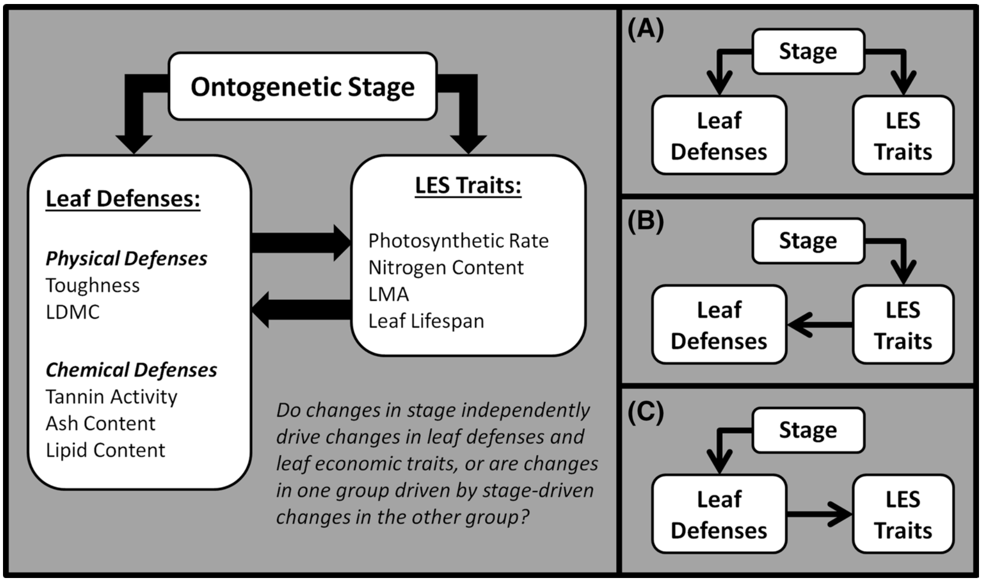

Fig. 1 Conceptual diagram of the interaction between leaf defenses and leaf economics spectrum (*LES*) strategy across ontogeny. Three alternate hypotheses exist: changes in stage independently drive changes in both defenses and LES strategy (*A*), stage directly drives changes in LES strategy and only indirectly drives changes in defense traits through LES strategy (*B*), and stage directly drives changes in defense traits and only indirectly drives changes in LES strategy through defense traits (*C*). *LDMC* Leaf dry matter content, *LMA* leaf mass per area

of the first and most general hypotheses (that plants will only be defended as much as is necessary due to the cost of defense) into a more predictive synthesis of plant apparency and the allocation of defense among tissues, such that the expression of defenses across time and tissues should maximize fitness (reviewed in Stamp 2003). Second, the growth-differentiation balance hypothesis incorporates both the growth rate hypothesis (Coley et al. 1985) and carbon-nutrient balance hypothesis (Tuomi et al. 1988) under a unifying framework of the tissue-level trade-off between growth and all forms of differentiation, including defense (Herms and Mattson 1992; reviewed in Stamp 2003). This hypothesis has been considered the most predictive of existing defense hypotheses (Stamp 2003). Extrapolating existing plant defense theory to whole-plant ontogeny results in several key predictions about plant shifts in defense (as summarized in Boege and Marquis 2005). Based on the optimal defense hypothesis, defenses are costly, and must contribute to plant fitness in excess of their costs, which will vary with plant development. Defenses should thus be favored during stages of increased risk of attack by herbivores and/or stages of highest sensitivity to herbivory. This does not suggest a clear set of predictions for leaf defenses, as risk of attack often *increases* with plant size due to apparency, but the impacts of herbivory tend to *decrease* with plant size, and defenses should track both of these forces through ontogeny and thus not vary much with development (Boege and Marquis 2005). However, it has been argued that defenses likely represent the highest fitness cost in early stages, where their investment comes at the expense of growth during the period of maximum inherent growth rate, so overall defense investment should *increase* with ontogeny (Boege and Marquis 2005). Based on the growth-differentiation balance hypothesis, as a plant develops, increasing shoot:root ratio and slowing growth rate increase the availability of resources (particularly C) that can be allocated to defense, so defenses should *increase* with ontogeny from the juvenile stage through the pre-reproductive stage, after which defenses should be *maintained* for polycarpic plants and *reduced* for monocarpic plants, as resources either need to remain defended to be productive for future reproduction or liquidated for current reproduction, respectively (Boege and Marquis 2005).

The mechanistic relationship between the LES and leaf defenses is less predicted by well-developed defense theory. While physical structural defenses (e.g., toughness) should obviously track the LES based on their shared basis in leaf investment, chemical defenses are less constrained, though quantitative and C-based chemical defenses (e.g., tannins) should more closely track leaf energy investment and thus LES strategy than qualitative and non-C-based chemical defenses (e.g., alkaloids). It is an open question as to whether the investment and return strategy of a set of leaves determines the allocation to defenses in that set of leaves, or whether the allocation to defenses determines the investment and return strategy. As ontogeny progresses, which class of traits determines the other? Three alternate hypotheses are explored in this study, highlighted in Fig. 1. First, ontogenetic stage may independently drive both defenses and LES strategy (hypothesis A). Second, stage may directly drive changes in LES strategy, which in turn drives changes in defenses (hypothesis B). Third, stage may directly drive changes in defense traits, which in turn drive changes in LES strategy (hypothesis C).

This study seeks to investigate the relationship between the LES and leaf defenses, both physical and chemical, across whole plant ontogeny. A recent study (Mason et al.

2013) demonstrated large shifts in LES strategy across four ontogenetic stages in three diverse species of wild sunflower (*Helianthus*) under controlled environmental conditions. This study expands upon those results by exploring ontogenetic patterns in constitutive physical and chemical leaf defenses that accompany the observed strong shifts in LES strategy, and uses exploratory path analysis to test hypothesized functional relationships between these two classes of traits.

Materials and methods

Study system, plant growth, and leaf economic trait measurement

This study builds upon a previous study (Mason et al. 2013) by presenting new data on leaf defenses and novel analyses of the relationships between leaf defenses and the LES. A detailed explanation of study design and LES trait sampling can be found in Mason et al. (2013), which investigated ontogenetic patterns in leaf economic and related leaf traits. Here we provide a concise summary of relevant methods from Mason et al. (2013).

In brief, three very different species of *Helianthus* were selected for study in order to maximize ecological variation: the erect annual *Helianthus annuus*, the erect perennial *Helianthus mollis*, and the basal rosette perennial *Helianthus radula*. In addition to differing in growth form and life history, these species are also separated geographically, and are also placed in distantly related clades based on the most recent phylogeny of the genus (Timme et al. 2007). For each species, intraspecific variation was incorporated by selecting multiple populations for study from across the range of each species ($n = 3$ for *H. annuus* and *H. radula*, $n = 2$ for *H. mollis*), with seed obtained either directly from wild populations or obtained from the USDA Germplasm Resources Information Network [see Mason et al. (2013) for more information on seed sources].

Plants were grown from seed in growth chambers at the UGA Plant Biology greenhouses under controlled temperature and humidity with supplemental lighting, in 4-L pots with daily watering and slow-release complete fertilizer to provide conditions conducive to rapid growth and development. Plants were spread across three growth chambers in a randomized complete block design, with two replicates per population per growth chamber, for a grand total across species of 47 plants due to one irreplaceable mortality.

The goal of Mason et al. (2013) was to assess trait differences in leaves produced across plant ontogeny, while standardizing for leaf age. Each plant was sampled on four dates during plant growth and development, with dates chosen separately for each species in order to sample new leaves produced during four distinct ontogenetic stages for each species. Thus, differences in phenology resulted in different dates for different species, but all members of a species were always assessed together on the same dates. For *H. mollis* and *H. radula*, the four stages sampled were juvenile (J), pre-reproductive (PR), flowering (F), and fruiting (FR). For *H. annuus*, which does not produce new leaves while fruiting and has a more uniform phenology, the pre-reproductive stage was split into two stages, not budding (NB) and budding (B). On each sampling date, measurements were made on most recently fully expanded leaves. Photosynthetic rate was measured with a LI-COR 6400 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE), with chamber conditions of 400 p.p.m. CO_2 and 2000 μmol m^{-2} s^{-1} light intensity. This leaf was excised immediately after measurements, scanned with a digital scanner, and dried at 60 °C with a forced-air drying oven for 96 h before being weighed for dry mass. Leaf area was obtained from digital leaf scans using ImageJ (Schneider et al. 2012), and used to calculate LMA as the ratio of leaf dry mass per leaf area. LMA was used to convert photosynthetic rate to a mass basis (A_{mass}). The leaf was then ground into a fine powder for CHN analysis, which was performed using Micro-Dumas combustion (NA1500; Carlo Erba Strumentazione, Milan) at the University of Georgia Analytical Chemistry lab in order to obtain leaf N concentration (N_{mass}). At the time of photosynthetic measurements, a newly expanding leaf was tagged with string to track leaf lifespan (LL), which was defined as the number of days until complete leaf senescence, defined as 100 % loss of greenness. For a more detailed description of plant growth, sampling design, and leaf economic trait measurements, see Mason et al. (2013).

There has been some debate recently over the use of mass-basis versus area-basis leaf economic traits, as mass normalization requires the use of LMA to convert A_{area} to A_{mass} and thus photosynthesis tends to correlate better with LMA on a mass versus an area basis (Osnas et al. 2013; Lloyd et al. 2013). It has been argued from a primarily statistical standpoint that some traits like photosynthesis are more "naturally" expressed on an area basis, and that mass normalization results in statistical artifacts (Osnas et al. 2013; Lloyd et al. 2013). However, this has been criticized as lacking a biological basis, and that both normalizations are informative, and in particular that mass normalization is necessary if leaf economics are to be reflective of leaf investment, return on investment, and thus overall plant growth (Westoby et al. 2013; Poorter et al. 2014). The nuances of this debate are beyond the scope of this study. The primary goal here is to consider relative investment in leaf defenses relative to position on the LES, so standard mass normalization is employed.

Measurement of defense traits

Five leaf defenses were selected for study in relation to the LES: tannin activity, ash content, lipid content, leaf dry matter content (LDMC), and leaf toughness. The first three of these are typically considered quantitative or qualitative chemical defenses, while the last two are typically considered physical defenses (Moles et al. 2013; Wright and Cannon 2001). Tannins are C-based plant secondary metabolites, specifically polyphenols, noted primarily for their protein precipitation capacity but also more recently for their oxidative activity (Salminen and Karonen 2011). Tannins reduce herbivory in a number of ways, including reducing leaf protein digestibility, causing digestive system damage, and ultimately by interfering with metabolism and growth (Roslin and Salminen 2008; Shimada 2006; Spalinger et al. 2010; reviewed in Moles et al. 2011). Leaf ash content is primarily composed of silicate phytoliths and calcium oxalates, which increase the abrasiveness of leaves and reduce growth rates and disrupt digestion of herbivores (Korth et al. 2006; Massey et al. 2006; Hanley et al. 2007; reviewed in Moles et al. 2011). Leaf lipid content is a proxy for oils, resins, and cuticular waxes, all of which have been shown to deter or harm herbivores through both chemical and physical effects (Coley et al. 1985; Marko et al. 2008; Lincoln 1985; reviewed in Moles et al. 2011). LDMC and leaf toughness are common measures of leaf palatability and digestibility, with higher dry matter content and toughness correlating with reduced herbivory and reduced herbivore growth rates (Scriber 1977; Elger and Willby 2003; Hanley et al. 2007; Kitajima and Poorter 2010; Kitajima et al. 2012). Together the defense traits of focus in this study represent some of the leading axes of variation in leaf defenses globally (Moles et al. 2013).

Several commonly used metrics of leaf palatability in studies of plant defense were not considered defenses for the purposes of this study, either because they are defining LES traits (LMA, N content) or mathematically linked to LES traits (C:N ratio). In many defense studies, LMA has long been used as an easy to measure proxy for physical defense traits like leaf toughness and LDMC. Despite this, the most detailed studies of C-based physical defenses have shown leaf-level LMA to be a composite trait defining leaf investment, composed of a large number of smaller-scale physical traits on mass and volume bases, some of which are defensive and some of which are not (see Kitajima et al. 2012). Furthermore, leaf nutritional quality is not a defense trait in the sense that decreasing nutrient investment does not represent a clear allocation of resources away from growth or reproduction.

Defense traits were all assessed on the same leaf as LES traits, so that defense sampling is paired to leaf economic traits. Tannin activity, ash content, and lipid content were all assessed on dried and ground leaf tissue, the same tissue used for CHN analysis. Tannin activity was assessed using the radial diffusion method (Hagerman 1987), which compares the protein-precipitation capacity of sample leaf tissue extracts against a tannic acid standard ($C_{76}H_{52}O_{46}$, CAS no. 1401-55-4). Leaf ash content was assessed by combusting a sample of ground leaf tissue in a muffle furnace at 600 °C for 12 h, and then calculating the proportion of mass remaining relative to the original sample. Leaf lipid content was assessed by extracting the lipid-soluble fraction of a sample of ground leaf tissue with petroleum ether (b.p. 40–60 °C), discarding the extract, drying the remaining sample at 60 °C, and calculating the proportion of mass lost from the original sample. The protocols for leaf ash content and leaf lipid content were adapted from those of Moles et al. (2011). Leaf toughness was assessed using a penetrometer on fresh leaves, giving the amount of force needed to puncture the leaf lamina with a millimeter-wide flat-tipped needle. Three measurements were taken on different parts of the leaf and averaged. LDMC was calculated as the proportion of leaf dry mass per leaf fresh mass.

As plants were grown in growth chambers and thus protected from exposure to herbivores and disease, all defense traits assessed and ontogenetic patterns observed in this study should be considered constitutive. One potential caveat to this statement is the longitudinal nature of this study, where plants were sampled repeatedly through time. It is possible that the removal of individual leaves for sampling in each stage may represent a source of defense induction and thus error in estimates of constitutive defenses across ontogeny. However, sampling in this study represented a proportionally small amount of leaf removal by total leaf area that was performed mechanically, with leaves excised cleanly flush to the stem. Studies investigating defense induction by mechanical damage have largely employed either partial mechanical damage to still-attached leaves (whereby damaged leaves represent a source of ongoing damage signaling) or severe to complete plant defoliation, neither of which are analogous to the sampling here, and even in these studies induction has not been consistently demonstrated (Hanley et al. 2007; Lieurance and Cipollini 2013). Given the known intraspecific trait variation in wild *Helianthus* species (Donovan et al. 2014), a longitudinal design was deliberately chosen in order to eliminate a key source of error that would occur under a cross-sectional design: ontogeny-by-genotype confounding, where sampling different groups of plants at different stages results in comparisons of plants with different genotypes among stages.

Statistical analysis

Statistical analyses were performed separately for each species. As a goal of this study was to investigate relationships

Table 1 Eigenvectors of traits comprising the first principal component axis (*PCA1*) for each species, as well as the proportion of variation explained by PCA1. Note the similarity in trait loadings across species

LES trait	PCA1 (*Helianthus annuus*)	PCA1 (*Helianthus mollis*)	PCA1 (*Helianthus radula*)
A_{mass} (nmol CO_2 g^{-1} s^{-1})	0.61322	0.57387	0.61742
LMA (g m^{-2})	−0.51024	−0.54422	−0.40724
N_{mass} (% dry mass)	0.55100	0.57462	0.57192
LL (days)	−0.24499	−0.21050	−0.35477
Proportion of variation explained	50.7 %	71.9 %	53.6 %

LES Leaf economics spectrum, A_{mass} photosynthetic rate on a mass basis, *LMA* leaf mass per area, N_{mass} leaf N concentration, *LL* leaf lifespan

between leaf defenses and the LES, a principal components analysis (PCA) was performed using A_{mass}, N_{mass}, LMA, and LL in JMP Pro version 11.0 (SAS, Cary, NC). This analysis included all individuals of a species across all sampled stages, effectively describing the LES within each species. This generated a value for each sampled leaf on the first principal component axis that is representative of relative position on the LES within each species (hereafter "LES PCA"). The LES PCAs performed for each of the three species were remarkably similar in their trait loadings (Table 1), and explained between 51 and 72 % of the variation in the four leaf economic traits. These LES PCA values were then used in subsequent analyses.

In order to investigate how leaf defenses change with ontogeny, a repeated-measures ANOVA was employed for each trait via PROC MIXED in SAS version 9.3 (SAS). Species were analyzed separately with block and ontogenetic stage as main effects. Populations were pooled for analysis at the species level to minimize effects of missing data, as some individuals did not have enough leaf tissue to obtain data for all defense traits at all stages (Appendix S1), and because there were no consistent population differences across stages within any species. Tukey post hoc tests were used to test for differences among stages. Furthermore, in order to examine how defense and the LES covary through ontogeny, correlations between all five defense traits and the LES PCA were calculated for each species. Unlike leaf economics traits, leaf defenses have not been found to form a unified global axis of variation (Moles et al. 2013), and indeed defenses here did not form strong suites or syndromes, so leaf defenses were not collapsed by PCA and were considered independently in relation to leaf economic strategy.

Exploratory path analysis

Given the strong correlations observed between several defense traits and the LES, the next logical step was to investigate whether these strong correlations were representative of an underlying directional causation in either direction. While in a non-manipulative experiment it is arguably impossible to demonstrate causation, it is possible to test whether the data better support a model where both traits are independently driven by ontogenetic stage, a model where stage-driven changes in the LES drive changes in defenses, or a model where stage-driven changes in defenses drive changes in the LES (Fig. 1). In order to test which of these three distinct classes of models is better supported by the data, exploratory path analysis was implemented using the specification search tool in AMOS version 5 (AMOS, Spring House, PA). This form of structural equation modeling generates and compares models with all possible combinations of directional arrows among variables (Grace 2006). For each species, models were constructed that contained five variables: LES PCA, the three defense traits that most strongly correlated with the LES PCA in that species, and ontogenetic stage. The three most correlated defense traits were used, rather than all defense traits, due to the computational difficulty of performing exploratory path analysis with more than five variables, and because defenses weakly correlated with the LES are unlikely to be mechanistically related to the LES. Ontogenetic stage was included as an ordinal variable with four levels. Optional path arrows were drawn from every variable to every other variable in both directions, with the exception of the stage variable, which was constrained to always be an exogenous variable (i.e., only have path arrows originating from it, not pointing to it). This constraint was implemented because leaf trait sampling was selected by stage, and stages progress in a fixed order. Specification search resulted in 65,536 models per species. All acyclic models with positive degrees of freedom were retained, and absolute model fit was assessed with a χ^2 goodness-of-fit test (Grace 2006). Models that were not rejected by the χ^2-test were sorted by standardized Bayesian information criteria, with all models within two units of the best model retained as equivalent best models. Standardized path coefficients for all equivalent best models were estimated with maximum likelihood. All equivalent best models were categorized by which of the three hypotheses in Fig. 1 they supported, based on whether stage directly drove changes in the LES PCA as well as directly drove changes in at least one defense trait (hypothesis A), stage directly drove changes in LES PCA and only indirectly drove changes in defense traits through the LES PCA (hypothesis B), or stage directly drove changes in defense traits and only indirectly drove changes in the LES PCA through defense traits (hypothesis C). In this way, the hypothesis of "independent action" (hypothesis A) is the most inclusive hypothesis, as it allows for myriad combinations of direct and indirect effects of stage on both defenses and LES PCA, so long as a direct effect of stage exists on both the LES PCA and at least one defense trait. In contrast, the two directional hypotheses (hypotheses B and C) require very specific situations where no direct effects of stage exist on one class of trait, and the other class mediates the effect of stage. This categorization process thus provides a rather conservative way to evaluate the modeling support for the two directional hypotheses (B and C). Additionally, consensus path diagrams were constructed for each species in order to visualize differences in general patterns among the three study species, based on a majority-trends analysis of the presence and directionality of paths across equivalent best models.

Results

Ontogenetic patterns in leaf defenses

As expected from the individual trait results of Mason et al. (2013), the LES PCA showed significant changes across ontogenetic stages (repeated-measures ANOVA, $p < 0.0001$ for all three species), driven by significant differences between juvenile and later stages for all three species (Tukey, $p < 0.05$). It should be noted that this variation within individual plants through time spans up to two-thirds of globally reported cross-species variation in LES traits (Mason et al. 2013).

For defense traits, much more variation in developmental trajectories was observed among species. In *H. annuus*, tannin activity, leaf toughness, and LDMC all increased significantly from juvenile to later stages, while lipid content significantly decreased after the not-budding stage, and ash content significantly decreased after the juvenile stage before increasing again during flowering (Fig. 2; Table 2). In *H. mollis*, leaf toughness and LDMC both significantly increased after the juvenile stage, while tannin activity increased after the juvenile stage to peak in the pre-reproductive stage and fall back to juvenile levels by fruiting (Fig. 2; Table 2). Ash content in *H. mollis* significantly decreased after the juvenile stage, while lipid content decreased between the juvenile and reproductive stage, before increasing back to juvenile levels by flowering (Fig. 2; Table 2). In *H. radula*, leaf toughness, tannin activity, and lipid content all significantly increased between the juvenile and later stages, while ash content and LDMC did not show significant differences by stage (Fig. 2; Table 2).

All of the five defense traits of interest showed significant correlations with LES PCA across ontogeny in at least one species. In *H. annuus*, all five defense traits were significantly correlated with LES PCA, with toughness, LDMC, and tannin activity correlated most strongly (Fig. 3). In *H. mollis*, all defenses except for lipid content were significantly correlated with LES PCA, with toughness, LDMC, and ash content correlated most strongly (Fig. 3). In *H. radula,* only toughness, LDMC, and tannin activity were significantly correlated with LES PCA (Fig. 3).

Exploratory path analysis

The exploratory path analysis resulted in 15 equivalent best models for *H. annuus*, five for *H. mollis*, and 28 for *H. radula*. All of the equivalent best models for *H. annuus* and *H. mollis* supported hypothesis C that stage-driven changes in defense traits drove changes in LES PCA (Table 3; Fig. 4; Figs. S1, S2). In *H. annuus*, all of the equivalent best models contained LDMC as a trait linking stage to the LES, while 8/15 contained leaf toughness and 5/15 contained tannin activity as mediating traits (Table 3; Fig. 4; Fig. S1). In *H. mollis*, all of the equivalent best models also contained LDMC as a trait linking stage to the LES, with one model also containing ash content as a mediating trait (Table 3; Fig. 4; Fig. S2). In *H. radula*, one-quarter of equivalent best models supported hypothesis C that stage-driven changes in defense traits drove changes in LES PCA, while three-quarters supported hypothesis A of independent effects of stage on defenses and LES (Table 3; Fig. S3). In models that supported hypothesis C, all contained leaf toughness as a mediating trait, 4/7 contained tannin activity, and only 2/7 contained LDMC (Table 3; Fig. S3). Consensus path diagrams for each species highlight these general patterns (Fig. 4).

Discussion

Ontogenetic patterns in leaf defense

Across all three species, defenses generally increased with ontogeny from the juvenile stage forward, in keeping with both the optimal defense and growth-differentiation balance hypotheses (Boege and Marquis 2005). Under the latter hypothesis, defenses are expected to be reduced during reproduction in monocarpic species and maintained during reproduction in polycarpic species (Boege and Marquis 2005). Despite the difference in life history between the

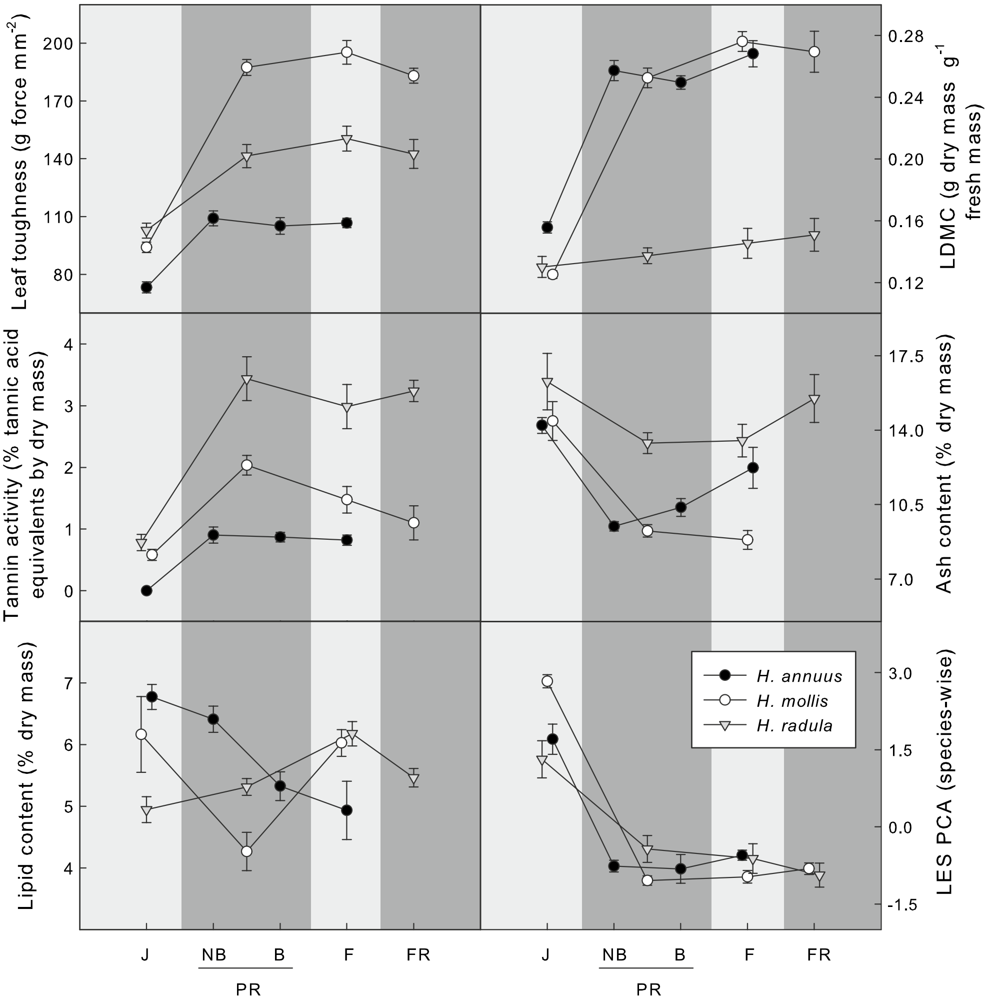

Fig. 2 Changes in leaf defense traits and LES strategy across ontogenetic stages. *Closed circles* represent *Helianthus annuus*, *open circles* represent *Helianthus mollis*, and *triangles* represent *Helianthus radula*. Stages shown are juvenile (*J*), not budding (*NB*), budding (*B*), pre-reproductive (*PR*), flowering (*F*), and fruiting (*FR*), as applicable. *Error bars* represent SEs. Repeated-measures ANOVAs for each trait can be found in Table 2. Note that LES principal components analysis (PCA) is the first principal components axis of photosynthetic rate, N content, leaf mass per area, and leaf lifespan calculated separately for each species (species wise), trait loadings for which can be found in Table 1. Higher values of the LES PCA indicate a more resource-acquisitive strategy. For other abbreviations, see Fig. 1

erect annual *H. annuus* and the erect deciduous perennial *H. mollis*, these species differed during reproduction only in the behavior of tannin activity and ash content. Between *H. mollis* and the evergreen basal rosette perennial *H. radula* there were large differences in the behavior of LDMC, tannin activity, lipid content and ash content during reproduction. This may indicate that differences in growth form are more important than life history in explaining ontogenetic patterns of defense in *Helianthus*.

Overall, tannin activity and toughness stand out as the traits that most consistently increased with ontogeny. This is consistent with predictions of both the growth-differentiation balance hypothesis and the optimal defense hypothesis (reviewed in Stamp 2003), as these defenses are primarily C based and likely require substantial energetic investments. LDMC also falls into this category, and is in line with predictions for *H. annuus* and *H. mollis*, though interestingly not for *H. radula*. This is likely due to the rather fleshy nature of the leaves of *H. radula*, which border on succulent and must derive their ontogenetically increasing toughness from a mechanism other than LDMC.

Conversely, ash and lipid content did not consistently increase with ontogeny. In fact, in *H. annuus* and *H. mollis* ash content was higher earlier in ontogeny, while *H. radula* did not significantly differ among stages. Ash content (a proxy for silicates, calcium oxalates, and other mineral deposits) has been suggested to be defensive, but also has been suggested to be a cheap replacement for structural C in acquisitive-strategy LES leaves (Cooke and Leishman 2011). Regardless of function, ash content

Table 2 Summary of repeated-measures ANOVAs for defense traits. *F*-values and *p*-values correspond to the "stage" term in each ANOVA, while Tukey differences describe which stage means differ significantly from one another via Tukey post hoc test ($p < 0.05$), where the main effect of stage was significant. In *H. annuus*, the pre-reproductive stage is split into not budding (*NB*) and budding (*B*), as the fruiting stage does not produce new leaves

	F-value	*P*-value	Tukey differences
H. annuus			
Tannin activity	35.28	<0.0001	J < NB, B, F
Ash content	20.00	<0.0001	J, F > NB, B
Lipid content	14.26	<0.0001	J, NB > B, F
Leaf toughness	27.50	<0.0001	J < NB, B, F
LDMC	89.12	<0.0001	J < NB, B, F
H. mollis			
Tannin activity	11.55	<0.0001	J < PR > F, FR (J = FR)
Ash content	18.90	0.0199	J > PR, F
Lipid content	9.89	0.0043	J, F > PR
Leaf toughness	113.49	<0.0001	J < PR, F, FR
LDMC	73.86	<0.0001	J < PR, F, FR
H. radula			
Tannin activity	21.96	<0.0001	J < PR, F, FR
Ash content	2.62	0.1456	–
Lipid content	5.41	0.0138	J < PR, F, FR
Leaf toughness	15.52	<0.0001	J < PR, F, FR
LDMC	1.18	0.3353	–

LDMC Leaf dry matter content, *J* juvenile, *PR* pre-reproductive, *F* flowering, *FR* fruiting

does not reflect a substantial C investment other than the cost of uptake, transport, and mineral deposition in leaf tissue, and thus allocation to ash content should not be as constrained by C limitation as C-based defenses. This explanation does not apply to leaf lipid content, however, as cuticular waxes and essential oils are certainly C based and should be subject to the predictions of the growth-differentiation balance hypothesis. The increase with ontogeny seen in *H. radula* fits this prediction, but the decrease seen in *H. annuus* and the decrease and subsequent increase seen in *H. mollis* do not. This may be in part due to the fact that leaf lipid content reflects both structural defenses (in the form of cuticular waxes), as well as quantitative and qualitative chemical defenses in various oils and resins. It is important to note that cuticular waxes, which likely make up a large proportion of total leaf lipid content in these species, likely serve other functions besides defense, such as reducing transpirational water loss. This multi-functionality can be found in many defensive traits, a prime example being trichomes that can prevent water loss, frost, radiation damage, and other functions in addition to providing herbivore defense (Levin 1973; Ehleringer 1984; Liakoura et al. 1997; Agrawal et al. 2004). It is quite likely that these non-defensive functions may lack the same selective regimes expected to shape ontogenetic patterns of defense under the growth-differentiation balance and optimal defense hypotheses, and so traits with multiple functions may be less likely to shift as predicted.

Other specific defenses not measured in this study, especially N-based and other qualitative defenses, may show ontogenetic patterns opposite to those predicted by the growth-differentiation balance and optimal defense hypotheses, and might play an important role in reducing herbivory in young plants where C-based, quantitative, and physical defenses are incompatible with fast growth (Barton and Koricheva 2010). The shifts observed in ash content in *H. annuus* and *H. mollis* might be an example of this pattern. In any case, the observed shifts in physical defense fit well with existing expectations of the LES spectrum, as allocation to physical defenses can play an important role in the extension of leaf lifespan in conservative-strategy leaves (Kitajima et al. 2012). Interpreting existing defense theory in light of the LES, it likewise makes sense that there would be energetic constraints on expensive defenses in acquisitive-strategy leaves, especially when defenses are largely immobile (as with physical defense).

Does allocation to defense mediate leaf economic strategy?

This study supports the idea that allocation to certain defenses, particularly physical and quantitative chemical defenses, might be important determinants of leaf economic strategy in some species. This makes conceptual sense, as genetic or environmental mechanisms allocating immobile physical and quantitative C-based chemical defenses to a leaf inherently increase the investment cost of the leaf, for which extended leaf lifespan is needed to repay these costs. Additionally, leaf toughness, waxes, fibers, or other sclerophylly-inducing traits will simultaneously dilute leaf N content and contribute to self-shading at the whole-leaf level, resulting in reduced instantaneous photosynthetic rate. Together these mechanisms force the leaf economic strategy of the leaf to the conservative end of the spectrum.

While investment in expensive defenses may force a leaf to the conservative end of the LES, conceptually the converse of this statement is not necessarily true, as leaves on the conservative end of the spectrum need not necessarily be more conservative by virtue of investment in leaf defenses. Investment in any number of other traits may be associated with conservative LES strategy, such as thick supportive petioles, succulence, or non-structural C storage. This of course assumes that the defining LES trait of LMA is not itself considered a strict defense; if LMA were considered a defense trait in and of itself there could not

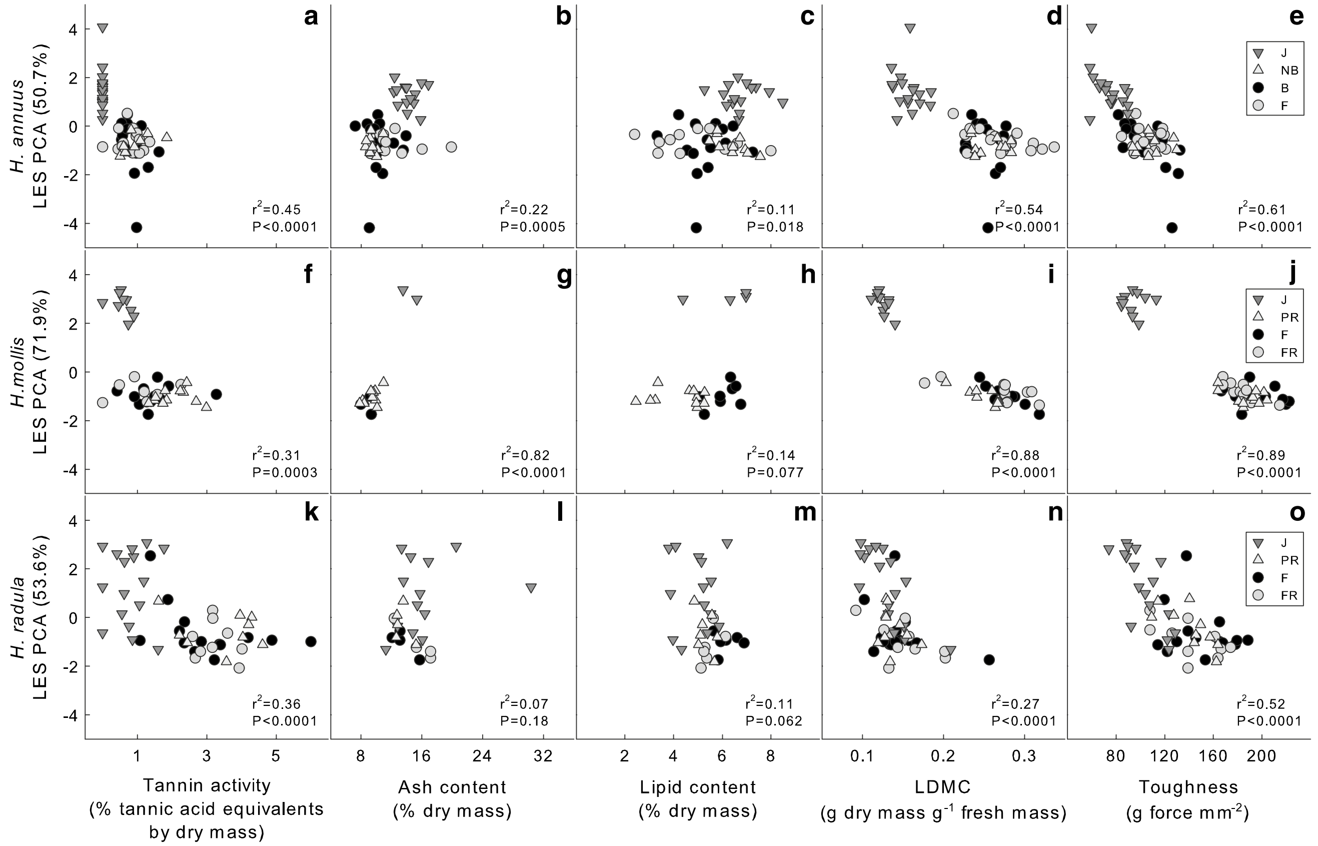

Fig. 3 Correlations between LES strategy and five defense traits across ontogenetic stages in *H. annuus* (**a–e**), *H. mollis* (**f–j**), and *H. radula* (**k–o**). Stages shown are J, NB, B, PR, F, and FR, as applicable. Percentages next to LES PCA indicate percentage of variation in the four LES traits explained. *Higher values* of the LES PCA indicate more resource-acquisitive strategy. For abbreviations, see Figs. 1 and 2

be a meaningful distinction between the LES and physical defenses. Evidence suggests that LMA is not a direct contributor to leaf toughness or reduced herbivory, and that it is better considered a trait that can be altered by investment in specific defenses like cellulose content and fracture toughness (Kitajima et al. 2012). Under this paradigm, shifts to conservative LES strategy may typically be correlated with increased investment in defenses, but not always.

Results of the exploratory path analysis indicate that for *H. annuus* and *H. mollis*, ontogenetic shifts to a more conservative LES strategy are mediated by changes in LDMC, as well as possibly toughness or tannins in *H. annuus*, and possibly ash content in *H. mollis*. The shift in LDMC in both species represents a replacement of water in juvenile leaves with structural matter in later stage leaves, resulting in decreased palatability and digestibility as well as a likely decrease in cytoplasm-to-cell wall ratio, which has been previously suggested to underlie the LES (Shipley et al. 2006). While this stage-driven increase in LDMC drives the increase in toughness in all equivalent best models for *H. annuus* (Fig. 4; Fig. S1), it does not directly drive leaf toughness in any of the equivalent best models for *H. mollis* (Fig. 4; Fig. S2). Instead, in *H. mollis* the stage-driven change in LDMC drives the change in LES strategy, which in turn drives the change in toughness (Fig. 4; Fig. S2). While LDMC appears to drive changes in the LES in both *H. annuus* and *H. mollis*, this is not the case for *H. radula*. In this species LDMC does not significantly change through ontogeny, though LES strategy does, and no equivalent best models suggest stage-driven changes in LDMC drive any other traits (Fig. 4; Fig. S3). In fact, for *H. radula* only five models suggest that LDMC contributes to LES strategy, versus 22 that suggest LES strategy alone drives LDMC (Fig. 4; Fig. S3). These findings do not support LDMC as a universal LES-driving trait (Shipley et al. 2006). The difference between *H. radula* and the other two species in LDMC may have to do with the fleshy nature of *H. radula* leaves, which are stiff and inflexible due to turgor and apparently do not derive their toughness from LDMC.

As two-thirds of the equivalent best models support independent actions of stage on LES strategy and defense characteristics (hypothesis A) in *H. radula*, the dynamics of

Table 3 All equivalent best models for each species

Species	Model	*df*	χ^2	*p*-value	BIC$_0$	Hypothesis supported	Mediating traits
H. annuus	29,227	4	4.5	0.34	0	(C) Defense- > LES	LDMC
	12,891	4	4.61	0.33	0.1	(C) Defense- > LES	LDMC, tough
	12,907	4	4.61	0.33	0.1	(C) Defense- > LES	LDMC, tough
	28,703	4	5.1	0.28	0.59	(C) Defense- > LES	LDMC, tough
	28,719	4	5.1	0.28	0.59	(C) Defense- > LES	LDMC
	45,103	4	5.1	0.28	0.59	(C) Defense- > LES	LDMC, tannins
	29,274	3	1.1	0.78	0.72	(C) Defense- > LES	LDMC, tough
	29,290	3	1.1	0.78	0.72	(C) Defense- > LES	LDMC
	41,563	4	5.67	0.23	1.16	(C) Defense- > LES	LDMC, tough, tannins
	45,658	3	1.76	0.62	1.38	(C) Defense- > LES	LDMC, tough, tannins
	45,614	3	2.06	0.56	1.68	(C) Defense- > LES	LDMC, tannins
	45,618	3	2.06	0.56	1.68	(C) Defense- > LES	LDMC, tannins
	12,383	4	6.2	0.18	1.7	(C) Defense- > LES	LDMC, tough
	12,399	4	6.2	0.18	1.7	(C) Defense- > LES	LDMC
	12,447	4	6.2	0.18	1.7	(C) Defense- > LES	LDMC, tough
H. mollis	26,893	6	5.09	0.53	0	(C) Defense- > LES	LDMC
	10,518	5	2.27	0.81	0.96	(C) Defense- > LES	LDMC
	10,509	6	6.34	0.39	1.25	(C) Defense- > LES	LDMC
	10,574	5	3.12	0.68	1.82	(C) Defense- > LES	LDMC, Ash
	26,894	5	3.12	0.68	1.82	(C) Defense- > LES	LDMC
H. radula	20,139	4	6.13	0.19	0	(A) Independent	–
	36,523	4	6.13	0.19	0	(A) Independent	–
	3,931	4	6.16	0.19	0.02	(A) Independent	–
	3,947	4	6.16	0.19	0.02	(A) Independent	–
	3,995	4	6.16	0.19	0.02	(A) Independent	–
	35,675	4	6.2	0.18	0.06	(C) Defense- > LES	Tannins, tough
	35,739	4	6.2	0.18	0.06	(C) Defense- > LES	Tannins, tough
	20,251	4	6.31	0.18	0.18	(A) Independent	–
	20,267	4	6.31	0.18	0.18	(A) Independent	–
	36,651	4	6.31	0.18	0.18	(A) Independent	–
	37,722	3	2.77	0.43	0.57	(C) Defense- > LES	LDMC, tannins, tough
	37,786	3	2.77	0.43	0.57	(C) Defense- > LES	LDMC, tannins, tough
	2,908	5	10.81	0.06	0.75	(C) Defense- > LES	Tough
	2,972	5	10.81	0.06	0.75	(C) Defense- > LES	Tough
	20,140	3	3.19	0.36	0.99	(A) Independent	–
	36,524	3	3.19	0.36	0.99	(A) Independent	–
	5,978	3	3.75	0.29	1.55	(C) Defense- > LES	Tough
	6,042	3	3.75	0.29	1.55	(A) Independent	–
	22,298	3	3.91	0.27	1.71	(A) Independent	–
	3,948	3	3.94	0.27	1.74	(A) Independent	–
	20,268	3	4.1	0.25	1.89	(A) Independent	–
	36,652	3	4.1	0.25	1.89	(A) Independent	–
	20,314	3	4.18	0.24	1.98	(A) Independent	–
	20,330	3	4.18	0.24	1.98	(A) Independent	–
	36,698	3	4.18	0.24	1.98	(A) Independent	–
	36,762	3	4.18	0.24	1.98	(A) Independent	–
	36,778	3	4.18	0.24	1.98	(A) Independent	–
	38,825	2	0.27	0.88	1.99	(A) Independent	–

Equivalent best models were defined as models that passed a χ^2 goodness-of-fit test ($p > 0.05$), and were within two standardized Bayesian information criteria (*BIC*$_0$) units of the best-fitting model. Each model is indicated as supporting one of the three conceptual hypotheses (*A*–*C*) described in Fig. 1. For models consistent with defenses driving leaf economics spectrum (*LES*) strategy, the specific defense traits linking stage and LES traits are listed as mediating traits

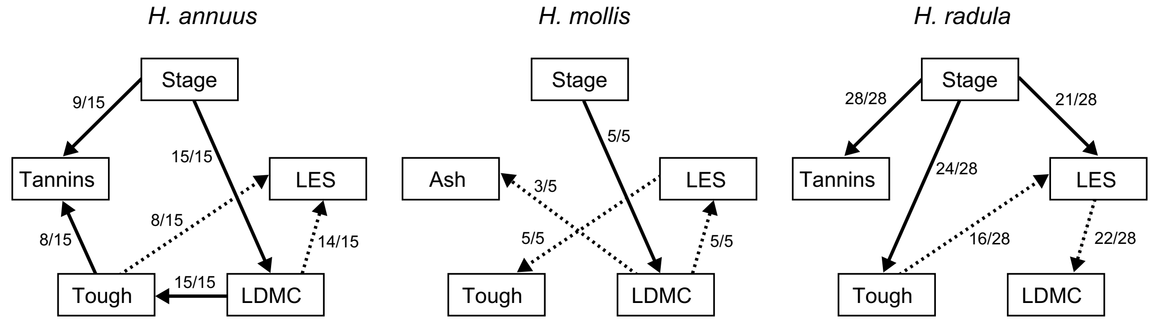

Fig. 4 Consensus path diagrams of equivalent best models for each of the three study species (15 for *H. annuus*, five for *H. mollis*, 28 for *H. radula*), based on a majority-trends analysis. Paths shown were present in a majority of equivalent best models, with the proportion of models in which a path is present listed *next to the path*. Positive effects are represented by *solid arrows*, and negative effects by *dotted arrows*

this species provide support for the idea that defenses and LES are mostly functionally independent axes, rather than tightly linked mechanistically. However, it is apparent from the unequivocal modeling results in *H. annuus* and *H. mollis* (supporting hypothesis C), that these functionally independent axes do interact strongly in many if not most circumstances, with investment in leaf defenses driving LES strategy. Furthermore, not a single equivalent best model in any species supports the hypothesis that LES strategy drives investment in leaf defenses (hypothesis B), which suggests strongly that LES strategy does not determine leaf defense investment.

Combining inferences from across all three species, defenses and LES strategy appear to constitute two functionally independent axes of trait variation, where investment in defenses may or may not drive LES strategy across ontogeny in a given species. It seems that when LES strategy and defenses do interact, the interaction is primarily in one direction, with LES strategy adjusting to the level of investment in leaf defenses, resulting in an alignment of defenses with LES strategy in combinations that maximize fitness.

Ontogeny as a scale of inquiry

While the relationships seen here between LES strategy and leaf defenses are compelling and may at first appear applicable to plant variation across populations and species, it is important to remember the scale of inquiry of this study. Phenotypes observed within plants through time occur within the same genetic background, and thus trait covariation is due to differential gene expression through time. This means that correlations observed between defenses and LES strategy with ontogeny are attributable to the pleiotropic, epistatic, or other interactions of specific gene variants through time, and thus that trait relationships observed at this scale may not necessarily hold among individuals, populations, or species. However, large defense and LES variation with ontogeny within a single genetic background has strong implications for the study of the genetic control of these important traits. Through the use of gene expression analysis (e.g., RNAseq), and the concomitant examination of ontogenetic trajectories in phenotypic traits, it may be possible to identify the genetic basis of complex traits in non-model systems. In this way, ontogeny may yet prove to be a useful scale at which to uncover the genetic basis of the LES (Mason et al. 2013), as well as specific leaf defenses that shift strongly with ontogeny.

While the relationships observed here among LES traits and leaf defenses with ontogeny may or may not hold across scales, the LES itself is an example of trait covariation that does hold across scales, from ontogenetic to cross-species (e.g., Wright et al. 2004; Heberling and Fridley 2012; Freschet et al. 2010; Xiang et al. 2013; Dunbar-Co et al. 2009; Brouillette et al. 2014; Mason et al. 2013). The relationship between LES strategy and leaf defenses in this study conforms to the expectations of the growth-differentiation balance and optimal defense hypotheses, so if the expectations of these two major defense hypotheses hold across scales, then total leaf investment in physical and quantitative C-based chemical defenses should covary with LES strategy across scales as well. Further explicit study of the relationship between LES traits and total leaf defense investment is needed to better address the interaction between leaf defenses and LES strategy across scales.

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Conflict of interest The authors declare no conflict of interest.

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