

Evolution of shade tolerance is associated with attenuation of shade avoidance and reduced phenotypic plasticity in North American milkweeds

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Abstract

Premise: Mismatches between light conditions and light-capture strategy can reduce plant performance and prevent colonization of novel habitats. Although light-capture strategies tend to be highly conserved among closely related species, evolutionary transitions from shaded to unshaded habitats (and vice versa) occur in numerous plant lineages.

Methods: We combined phylogenetic approaches with field and greenhouse experiments to investigate evolutionary constraints on light-capture strategy in North American milkweeds (genus *Asclepias*) and to determine whether colonization of shaded habitats in this heliophilic clade is associated with reduced plasticity and attenuation of the shade avoidance response.

Results: Colonization of shaded habitats has occurred at least 10 times in this genus, including at least once in each major North American clade. Evolutionary transitions between habitats exhibit strong directional bias, with shifts from full-sun to shaded habitats occurring at least three times as often as the opposite transition. In field and greenhouse experiments, sun species responded to shade by increasing internode length, height, and specific leaf area, consistent with the shade avoidance response; paired shade species exhibited reduced plasticity overall, and only one trait (specific leaf area) responded to experimental shade.

Conclusions: Our results suggest that milkweeds colonized shaded environments multiple times using a light-capture strategy distinct from the ancestral (putatively shade avoidant) strategy, including a general attenuation of plasticity in response to variable light conditions. This pattern bolsters the notion that shade avoidance and tolerance represent divergent evolutionary strategies for maximizing performance under qualitatively different types of shade.

KEYWORDS

Apocynaceae, *Asclepias*, evolutionary ecology, milkweed, niche conservatism, phenotypic plasticity, photosynthetically active radiation, RFR ratio, shade avoidance, shade tolerance

The conservation of traits within clades over evolutionary time appears to be a general pattern across the tree of life. Comparative methods have repeatedly supported the notion, for example, that sister taxa tend to be more similar to one another than to randomly selected species from the same clade (“phylogenetic signal” sensu Blomberg and Garland, 2002) and that, in many cases, these similarities are greater than would be predicted by phylogenetic distance alone (“clumping” sensu Fritz and Purvis, 2010; “phylogenetic niche conservatism” sensu Losos, 2008; cf. Harvey and

Pagel, 1991). Traits related to the tolerance of abiotic stress in plants are among those that typically show strong phylogenetic signal (Webb et al., 2002; Ackerly, 2003); for example, strategies for tolerating osmotic stress are often highly conserved within clades and, in extreme cases, can cause lineages to become restricted to narrow habitats corresponding to particular abiotic conditions (e.g., salt tolerance in the marsh genus *Spartina* and drought tolerance in the arid-adapted family *Alzooaceae*). Where they occur, deviations from this pattern—including divergence



among sister taxa and adaptive radiations—represent opportunities to probe the underlying mechanisms of evolution and better understand ecological trade-offs and constraints, including the ability of lineages to colonize new habitats (Weber and Agrawal, 2012).

Like osmotic stress, shade presents a significant physiological challenge for plants (Demmig-Adams and Adams, 1992), and nearly all herbaceous species employ one of two strategies to overcome this challenge: shade avoidance or shade tolerance (Valladares and Niinemets, 2008; Gommers et al., 2013). Shade avoidance is widespread among heliophilic, open habitat species and is typified by a suite of plastic responses—including internode elongation and increased specific leaf area—that are elicited by shade. In particular, shade avoidance responses are typically triggered by a reduction in the ratio of red to far-red (R:FR) wavelengths caused by neighbors intercepting and scattering incoming light, rather than the absolute reduction of photosynthetically active radiation (PAR; Morgan and Smith, 1981; Dudley and Schmitt, 1996; Ruberti et al., 2012). Because plants growing in open habitats tend to perform optimally under conditions of high PAR and because they tend to be similar in stature to their shade-casting neighbors, stem elongation via increased internode length is an effective strategy to “avoid” shaded conditions by vertically outgrowing competitors. In the deep shade typical of forest understoreys, where ambient PAR and R:FR ratio can both be reduced by more than 90% (Jankowska-Blaszczak and Daws, 2007; Agrawal et al., 2012), plants typically exhibit less phenotypic plasticity in response to variation in light availability and spectral quality (Griffith and Sultan, 2005) and instead rely upon a distinct suite of constitutive shade tolerance traits (e.g., reduced chlorophyll *a:b* ratio) that optimize light capture and performance in situ. Despite substantial efforts to elucidate the ecological implications and evolutionary drivers of shade avoidance and tolerance strategies (reviewed by Grime, 1965; Franklin, 2008; Ruberti et al., 2012; Gommers et al., 2013), relatively little is known about the prevalence, evolutionary drivers, and ecological implications of transitions between shade strategies within clades.

The combination of comparative phylogenetic and experimental methods is a powerful tool for elucidating the mechanism and generality of evolutionary patterns (Weber and Agrawal, 2012), and several aspects of plant light-capture strategies make this a promising subject for this approach. First, there is growing consensus that shade avoidance and tolerance represent distinct strategies that have evolved repeatedly in response to qualitatively different shade environments (Grime, 1965; Reich et al., 2003; Franklin, 2008; Gommers et al., 2013). Second, adopting a shade avoidant strategy in deep shade (or, conversely, a shade tolerant strategy in neighbor shade) is maladaptive and has been experimentally demonstrated to reduce performance (Schmitt et al., 1999, 2003; Franklin, 2008; Gommers et al., 2013); the specificity of each response to different types of shade is perhaps best exemplified by the reliable progression from shade avoidant to tolerant strategies over secondary

succession as light availability and R:FR ratio decrease (Horn, 1974). Finally, traits associated with shade avoidance and tolerance are negatively correlated at the species level (Henry and Aarssen, 1997; Valladares and Niinemets, 2008) and tend to be highly conserved within plant lineages (e.g., Patterson and Givnish, 2002; Rueda et al., 2017). Collectively, these characteristics suggest that divergence among sister taxa in shade strategy is likely to be the exception rather than the rule, a prediction consistent with the strong phylogenetic signal typically associated with stress tolerance traits in plants. Where such shifts occur, however, they may allow lineages to rapidly diversify and colonize a broader range of habitats (e.g., Patterson and Givnish, 2002; Edwards and Smith, 2010).

Given that plants producing shade avoidant responses in deeply shaded habitats suffer reduced performance and that shade tolerant plants tend to exhibit lower levels of phenotypic plasticity overall, we predicted that evolutionary transitions from avoidance to tolerance would be associated with strong attenuation of the avoidance response. To test this prediction, we conducted phylogenetic analyses of 100 North American *Asclepias* L. (Apocynaceae) to determine the ancestral light-capture strategy for this clade, the number of transitions between shade strategies, and the strength of phylogenetic conservatism for this trait. We then compared the responses of closely related pairs of milkweeds from different light habitats to light-manipulation treatments mimicking various types of shade in complementary greenhouse and field experiments. We predicted that species found exclusively in full sun habitats (hereafter “sun” species) would exhibit phenotypic plasticity in a variety of light-capture traits in response to experimental shade with reduced R:FR, including in deeply shaded forest plots where shade avoidance potentially represents a maladaptive response driven by macroevolutionary or sensory constraints. Conversely, we predicted that species commonly found in shaded habitats (hereafter “shade” species) would differ from conspecific sun species in their constitutive expression of light-capture traits and would not exhibit plasticity in response to the same shade cues, the latter being consistent with a general loss of plasticity associated with shade tolerance (Valladares and Niinemets, 2008).

MATERIALS AND METHODS

Study species and phylogenetic analyses

The genus *Asclepias* (hereafter “milkweed”) comprises ca. 140 species of perennial, herbaceous plants with centers of diversity in North and South America. Here, we focused on the North American milkweeds (100 are included in the present study), which range from southern Canada to Mexico and the Caribbean. A recent phylogenetic analysis (Fishbein et al., 2018) confirmed the monophyly of four North American clades, three of which (the temperate North American, Sonoran Desert, and Mexican highland clades)

correspond to circumscribed geographic regions within mainland North America. The Incarnatae clade spans the full latitudinal range of the entire North American clade. Across the genus, the majority of species occur in grasslands, open forests, wetlands, and deserts (Agrawal et al., 2009, 2012, 2015; Woodson, 1954).

To determine the ancestral habitat for this clade—and how often the genus transitioned between habitats—we performed complementary phylogenetic analyses using two classification schemes. In the first scheme, we considered species known to occur only in deeply shaded habitat as shade species; all other species were considered sun species. In the second scheme, we employed a less strict definition of shade species by including all species from shaded habitats, including those that also occur in partial shade; all others (i.e., those found exclusively in full-sun habitats) were considered sun species. All designations relied upon a combination of habitat descriptions from a classic milkweed monograph (Woodson, 1954), descriptive reports from the more recent scientific literature (e.g., Agrawal et al., 2009, 2012), citizen science records, and firsthand knowledge of the authors and collaborators with extensive experience identifying this genus in the field.

We then determined the most likely ancestral habitat for the genus separately for each classification scheme using the ace function in the R package ape (Paradis and Schliep, 2015; R Core Team, 2015; v. 3.6.1); we estimated character states using maximum likelihood methods and assumed an equal probability of transition between sun and shade habitats, which were modeled as discrete traits. We also determined the number of transitions between habitats for both classification schemes using the package phytools (Revell, 2012); we again assumed an equal probability of transition between habitats and estimated the average number of transitions across 100 tree simulations. We performed a sign test on each simulation to determine whether there was bias in the direction of transitions between habitats and calculated the average ratio ($\pm 95\%$ CI) of transition directions (sun to shade vs. shade to sun) to determine the magnitude of any bias (following Blanchard and Moreau, 2017). Finally, we estimated D , a metric of phylogenetic signal strength for binary traits, for each classification scheme (number of permutations = 1000; Fritz and Purvis, 2010). Values of $D < 0$ are indicative of phylogenetic niche conservatism (i.e., phenotypic similarity between species pairs greater than expected given phylogenetic distance). Values of $D = 0$ are indicative of phylogenetic signal (i.e., phenotypic similarity between species pairs proportional to phylogenetic distance). Values of $D \approx 1$ are indicative of no phylogenetic signal (i.e., phenotypic similarity between pairs not correlated with phylogenetic distance).

To evaluate how macroevolutionary shifts between habitat types affect phenotypic plasticity in milkweed, we then selected two closely related species pairs that differed in their habitat preference in both of our phylogenetic classification schemes: *A. syriaca* (sun) and *A. exaltata* (shade) from the Temperate North American clade, and *A. incarnata* (sun)

and *A. perennis* (shade) from the Incarnatae clade (Figure 1; Appendix S1; Figure S3). In both cases, a single habitat transition was identified between species pairs. Detailed descriptions of all focal species are provided in Woodson (1954) and Agrawal et al. (2009). For *A. syriaca*, *A. incarnata*, and *A. exaltata*, seeds were collected from at least 10 individuals from natural and planted populations at three separate sites within 10 km of Ithaca, New York (42.445°N, 76.502°W) and mixed before planting; for *A. perennis*, seeds were collected from multiple potted plants grown outdoors in Ithaca (originally collected from Leon County, Florida) and similarly mixed before planting. For both experiments, plants were grown as described by Agrawal and Hastings (2019), with the exception that seedlings were initially grown in Lambert LM 111 potting mix (Lambert, Rivière-Ouelle, Québec, Canada) and fertilized immediately after sowing and approximately every 2 weeks thereafter (N:P:K 21:5:20, 150 ppm N [μg/g]). Plants were maintained in 10 cm diameter (~500-mL) pots for the duration of the greenhouse experiment. For the field experiment, plants were initially moved to outdoor mesh cages in 10 cm pots before transplanting into 4-L pots filled with local topsoil. Plants were watered ad libitum for the duration of both experiments.

Greenhouse experiment

To determine whether sun and shade species differ in their response to reduced PAR and RFR, we performed a greenhouse experiment with three shade treatments. Light environments were manipulated using translucent plastic theatrical gels following the methods of McGuire and Agrawal (2005) and Kurashige and Agrawal (2005). The control treatment consisted of open-top, cylindrical tubes made of colorless plastic (0.005 Dura-Lar, Gratix Plastics, Cleveland, OH, USA) that had a negligible (~10% reduction) effect on PAR and RFR compared to ambient light (ambient RFR = 1.04; clear gel RFR = 1.03). Photographs of all treatments are in Appendix S1, Figure S1. The neutral shade treatment consisted of open-top tubes made of the same colorless plastic overlain by a layer of black nylon mesh that reduced PAR by a total of ~60% but did not affect the RFR ratio compared to ambient light or control treatments (neutral shade RFR = 1.03). The neighbor shade treatment consisted of a two-layer tube made up of a colorless control gel and a green plastic gel (#4430 filter, Rosco, Markham, Ontario, Canada) that together reduced PAR by ~60% and shifted the RFR ratio to 0.54; these conditions are comparable to the neighbor shade environment experienced by many milkweeds (Agrawal et al., 2012) in which both PAR and RFR are reduced relative to open habitat. Gels extended >15 cm from the top of each plant to ensure consistent shade manipulation, and tubes were further extended, as necessary, to completely cover plants as they grew. Pairwise comparisons between treatments allows for evaluation of the effects of reduced PAR (neutral shade vs. control) and simultaneously reduced PAR and RFR (neighbor shade vs. control)

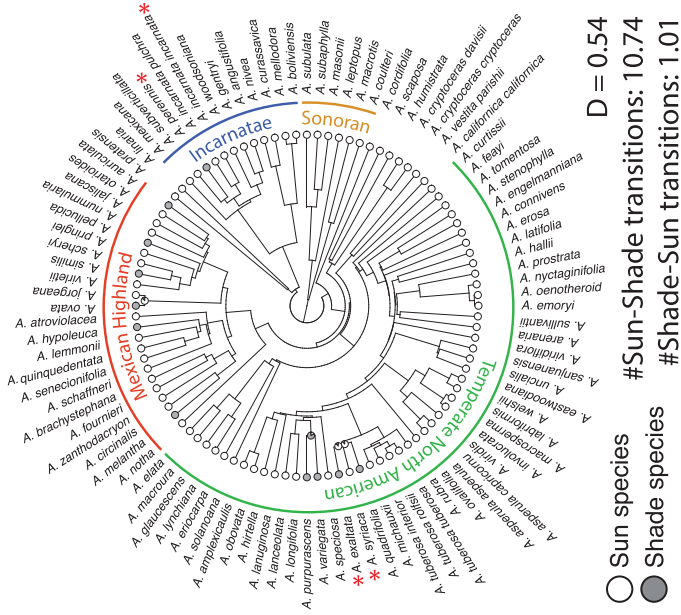


FIGURE 1 Phylogeny showing the distribution of shade (gray) and sun (white) species across 100 North American milkweeds (more restrictive classification shown here; see Appendix S1, Figure S3 for less conservative analysis). Tree modified from Fishbein et al. (2018). Pie charts on internal nodes indicate maximum likelihood estimates of the ancestral habitat; for simplicity, only those nodes with $\geq 5\%$ likelihood of being shade-adapted are shown. Major clades are delineated by colored bars and matching labels. Focal species included in field and greenhouse experiments are denoted with an asterisk (*)

on plant phenotype (Appendix S1, Table S27); we evaluated the effects of each shade treatment on plant performance and light-capture traits using response ratios (neighbor shade: control and neutral shade:control). Previous studies have consistently found that reduced RFR is necessary to elicit shade avoidance responses, and that reduced PAR alone typically does not induce comparable changes (Franklin, 2008; Gommers et al., 2013).

Before the experiment, all plants were grown in a single growth chamber for approximately 3 weeks. We arranged plants into 14 blocks along two parallel benches in the greenhouse, with each block consisting of 12 plants (4 species $\times$ 3 treatments per block; $N = 168$ plants). Within each block, individuals of each species were matched by initial height and randomly assigned to light manipulation treatments. Initial height, internode length, the number of nodes, and the estimated leaf area of the youngest fully expanded leaf did not differ between treatments for any species (all $P > 0.05$).

Previous tests using the same shade manipulations suggest that leaf surface temperature does not vary between treatments (Kurashige and Agrawal, 2005).

After approximately 4 weeks, we measured seven traits related to performance and light-capture strategy. We measured plant height from the cotyledon to the tip of the stem; for plants (5 *A. incarnata* and 21 *A. perennis*) with more than one stem, we recorded the height of the tallest stem. We also recorded the total number of nodes (all *Asclepias* spp. have paired, opposite leaves), and then calculated the average internode length for each plant by dividing the total stem height (cotyledon to tip) by the number of nodes. We estimated the area of the youngest fully expanded leaf (Cook-Patton and Agrawal, 2014) by measuring leaf length (from base to tip) and the longest orthogonal length and calculating the area of the corresponding oval. We estimated leaf thickness by collecting 6.3-mm-diameter, circular cuttings from the leaf tip (including the midrib) and drying at 60°C

for 2 weeks before weighing each disk; we compared the inverse of the dry mass of each leaf punch as a proxy for specific leaf area (SLA). Finally, we separated and collected all aboveground (i.e., leaf and stem) and belowground (i.e., root) material for each plant, dried at 60°C for 2 weeks, and weighed each separately to estimate above- and belowground biomass.

Field experiment

To determine whether sun and shade species differed in their response to more significant reductions in PAR and R:FR (i.e., those typical of forest understories), we performed a field experiment with two shade treatments that represented the extremes of light conditions that our focal milkweed might naturally experience. Both *A. syriaca* and *A. incarnata* occur at our study site, and *A. exaltata* occurs along forest edges within several kilometers of our site; *A. perennis* is most common in wetter sites in the southeastern United States but can be grown as a perennial in upstate New York. In June 2019, we established three paired, fenced, 4 × 4 m plots along a forest edge–old field transition (42.465°N, 76.444°W); fencing was used to prevent browsing by deer (*Odocoileus virginianus*). Half of the plots ($N = 3$; hereafter “control” plots) were located within old field habitat ~15 m from the forest edge. All aboveground vegetation (primarily tall goldenrod, *Solidago altissima*) was removed from the control plots and from a 50 cm buffer outside the plot perimeters to eliminate neighbor-shade cues (i.e., reduced PAR and R:FR); in this way, the control treatment in the field experiment was comparable to the control treatment in the greenhouse experiment, though ambient PAR was greater in the field. Neighboring vegetation was clipped every 2 weeks to prevent regrowth. The remaining three plots (hereafter “forest” plots) were located in the forest understory ~15 m from the forest edge and were similarly cleared of understory vegetation and fallen tree limbs (Appendix S1, Figure S2). The forest overstory intercepts approximately 98% of ambient PAR at this site (forest PAR = 29 $\mu\text{mol m}^{-2} \text{s}^{-1}$, control PAR = 1504 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and also reduces R:FR ratio by approximately 75% (forest R:FR = 0.26, control R:FR = 1.02). These measurements were taken at a single time and are thus not necessarily representative of the average light regime at this site, but are nonetheless indicative of the relative intensity and spectral quality of light across treatments.

Each plot initially received 25 potted individuals ($N = 75$ plants/treatment; Appendix S1; Figure S2), each of which was matched to a similarly sized conspecific in the paired plot; initial height and internode distance did not differ between treatments (all $P > 0.05$). After approximately 5 and 8 weeks, we estimated the average internode length for each plant by dividing its height (cotyledon to tip) by the total number of nodes. We also calculated the internode length ratio between forest and control plots for each plant pair after 5 weeks. After 12 weeks, we harvested, dried, and

weighed all aboveground biomass for each of the remaining plants to determine how plant performance varied in matched and mismatched light environments. It was not possible to isolate belowground biomass from the denser topsoil used in the field experiment.

Statistical analyses of experimental data

Data from the greenhouse and field experiments were analyzed in R (v. 3.6.1). For the greenhouse experiment, we conducted three complementary analyses. First, we compared the independent and interactive effects of treatment (control, neighbor, and neutral shade), clade (Incarnatae vs. Temperate North American), and type (sun vs. shade species) with a 3-factor mixed effects model using the lmer function in the R package lmerTest (Kuznetsova et al., 2017); block was included as a random effect to account for variation in growing conditions within the greenhouse. We performed separate tests for total number of nodes, leaf area, aboveground biomass, belowground biomass, specific leaf area, height, and average internode length.

Second, we calculated response ratios of the same seven traits between pairs of treatments (neighbor shade:control and neutral shade:control) within blocks ($N = 14$) to quantify and compare plastic responses to shade consistent with a shade avoidance strategy (Commers et al., 2013); the neighbor shade:control ratio represents plant responses to reduced PAR and R:FR, while the neutral shade:control ratio represents plant responses to reduced PAR alone. Response ratios were compared with separate linear mixed models for each response variable, with species as a fixed effect and block as a random effect. We assessed the effect of species with chi-square difference tests and report Holm-Bonferroni corrected P -values to account for multiple comparisons involving the control treatment. We then calculated estimated marginal means and 95% confidence intervals (CI) for each species and trait combination using emmeans (Lenth, 2021) and evaluated pairwise differences between species using Tukey's honestly significant difference (HSD) tests. This analysis allowed us to test two related predictions: (1) that macroevolutionary transitions between habitats result in distinct light-capture strategies between closely related species (indicated by a significant main effect of species and pairwise differences between clade-mates in post hoc tests) and (2) that sun species exhibit plastic responses consistent with the shade avoidance response (and that shade species lack such responses). Because the neighbor shade and control treatments differ in both PAR and R:FR, we consider a response ratio (and 95% CI) greater than 1 for this comparison to be indicative of a shade avoidance response (Commers et al., 2013). We included the second response ratio (comparing neutral shade and control treatments) to evaluate whether the same responses can be elicited by reduced PAR alone. We again consider a response ratio (and 95% CI) greater than 1 to be indicative of shade avoidance.

Finally, to assess species-level differences in the constitutive expression of these same seven traits, we compared mean values of each trait from the control treatment with separate mixed models, with species as a main effect and block as a random effect. Full model outputs, confidence intervals, and post hoc comparisons can be found in Appendix S1, Tables S1–S22.

For the field experiment, we used separate 3-factor ANOVA to assess the independent and interactive effects of treatment (control vs. forest), type (sun vs. shade species), and clade (Incarnatae vs. Temperate North American) on average internode length (5 and 8 weeks after experiment initiation) and aboveground biomass (12 weeks after experiment initiation). Pairwise post hoc comparisons were made using Tukey's HSD. As in the greenhouse experiment, we also calculated the internode length ratio between forest and control plots after 5 weeks and estimated the mean and 95% CI for each species using emmeans; we considered a forest:control response ratio (and 95% CI) greater than one to be indicative of a shade avoidance response. Full model outputs, confidence intervals, and post hoc comparisons can be found in Appendix S1, Tables S23–S26.

RESULTS

Phylogenetic comparisons

Our more restrictive classification scheme included 11 shade species (and 89 sun species; Figure 1), while the less conservative classification included an additional fourteen shade species (Appendix S1, Figure S3). In both cases, ancestral state reconstructions strongly supported full sun as the ancestral habitat for North American milkweeds (>99% likelihood of shade avoidance at the root). Phylogenetic signal for habitat was intermediate ($D = 0.54$) for the more restrictive scheme and differed significantly from both $D = 0$ and $D = 1$; phylogenetic signal was comparable for the less conservative scheme ($D = 0.50$), but was not significantly different from $D = 1$, a result consistent with a lack of phylogenetic signal. Collectively, these results indicate that habitat (shade vs. sun) does not show strong phylogenetic signal ($D = 0$) or phylogenetic niche conservatism ($D < 0$) in North American milkweeds. We consider these results to be consistent with moderate phylogenetic signal, though significantly less than would be predicted by the null expectation of Brownian motion evolution (sensu Fritz and Purvis, 2010).

For the restrictive classification scheme, 90% of simulations showed significant bias ($P < 0.05$) in favor of transitions from sun to shaded habitats, with approximately eleven (95% CI: 9.1–14.0) times more transitions from sun to shade than shade to sun. Analysis of the less conservative scheme indicated that the clade may have experienced up to 24 total transitions between habitats, with a more modest, but still significant, bias in favor of transitions from sun to shade (mean = 3.3; 95% CI: 3.1–3.6, $P < 0.05$) of

simulations. There were more transitions to shaded habitat in all simulations of both classification schemes.

Phenotypic responses to light manipulation

Across the seven light-capture traits measured, several broad categories of responses to shade emerged in the greenhouse experiment (Figure 2). Shade treatment had no effect on the total number of nodes at the level of species (i.e., all 95% CI overlapped 1 for neighbor shade:control and neutral shade:control comparisons; all $\chi^2_{(3,168)} < 4.07$, $P > 0.50$) or type (treatment × type effect: $F_{2,143} = 0.10$, $P = 0.90$; Appendix S1, Tables S1–S3). Plasticity in individual leaf area followed a similar pattern, with only *A. syriaca* responding to neighbor shade by slightly decreasing leaf area (upper 95% CI = 0.986); changes in leaf area in response to shade also did not differ between species (all $\chi^2_{(3,168)} < 2.31$, $P > 0.51$) or type ($F_{2,143} = 0.09$, $P = 0.92$; Appendix S1, Tables S4–6). Shade significantly reduced both aboveground (Appendix S1, Tables S7–S9) and belowground biomass (Appendix S1, Tables S11, S12) for all species (all $\chi^2_{(3,166)} > 8.44$, $P < 0.038$), but this response did not vary between sun and shade species (all $F_{2,141.1} < 1.68$, $P > 0.19$) and is consistent with light limitation under both shade treatments. Neighbor shade elicited a strong increase in specific leaf area (Appendix S1, Tables S13–S15) relative to ambient light for all species (all means and 95% CI > 0), but this response did not vary by species ($\chi^2_{(3,168)} = 4.74$, $P = 0.19$) or type ($F_{2,143} = 0.30$, $P = 0.74$) and was not elicited by neutral shade (reduced PAR) alone. Finally, height and internode length showed significant variation in plasticity in response to neighbor shade at the level of species (all $\chi^2_{(3,168)} > 15.45$, $P < 0.002$) and type (all $F_{2,143} > 4.75$, $P < 0.011$). Both sun species increased height and internode length in response to neighbor shade, whereas shade species did not; the magnitude of these responses was significantly greater for *A. syriaca* than its clade-mate *A. exaltata* (see gray bars in Figure 2). Overall, both sun species exhibited a number of plastic responses to neighbor shade (reduced PAR and R:FR; see white dots in Figure 2) consistent with the shade avoidance response: increased specific leaf area, internode length, and height (*A. syriaca* only). Moreover, the lack of plasticity in these same responses to neutral shade (reduced PAR only; see black dots in Figure 2) indicates that reduced R:FR is necessary to elicit shade avoidance responses in milkweed. In contrast, responses to reduced R:FR and/or PAR in shade species were typically muted relative to paired sun clade-mates and, for the only trait for which shade species exhibited a response consistent with shade avoidance (specific leaf area), the response in each paired sun species was greater in magnitude. Species differed in the constitutive expression of all seven performance and light-capture traits (Appendix S1, Figure S4, Table S22), with the two sun species having greater aboveground biomass, taller stems, more nodes, and reduced internode lengths.

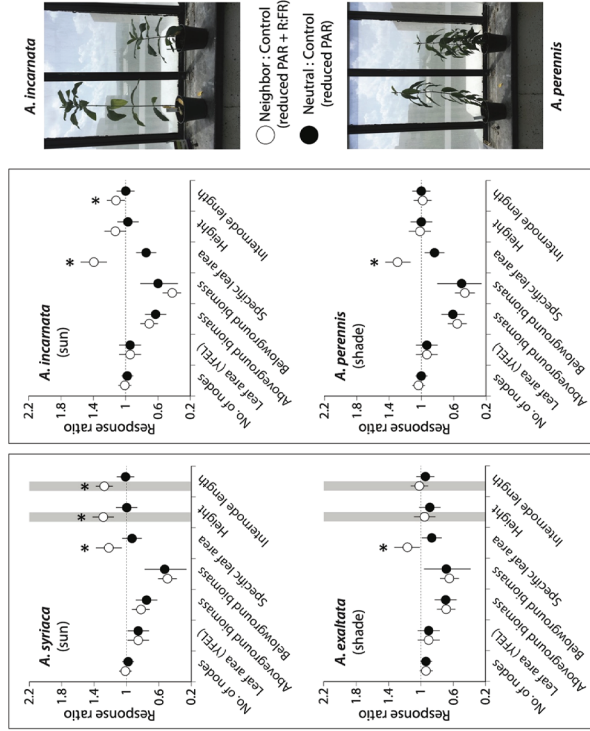


FIGURE 2 Response of seven light-capture and performance traits to experimental shade manipulation across four milkweed species in a greenhouse experiment. Two response ratios are presented to assess how each species responds to reduced PAR and RFR: white dots represent plant responses to simultaneously reduced PAR and RFR (i.e., the response ratio between the neighbor shade and control treatments); black dots represent plant responses to reduced PAR alone (i.e., the response ratio between the neutral shade and control treatments). Response ratios were calculated separately for each species by dividing the trait values of plants in each shade treatment (neighbor or neutral shade) by the trait value of the plant in the control treatment in the same block ($N = 14$ blocks). Species pairs from the Temperate North American (left) and Incarnatae (middle) clades are grouped for comparison. Within each pair, sun species are shown on the top. Leaf area estimates are from the youngest fully expanded leaf (YFEL) on each plant. Data are means and 95% CI; values >1 are denoted by an asterisk (*) and are consistent with the shade avoidance response. Significant pairwise differences between species within a clade are denoted by gray boxes (Tukey's HSD). Inset photos show typical phenotypes for *A. incarnata* (a sun species) and *A. perennis* (a shade species) under full sun (control treatment; right of photo) and reduced PAR and RFR (neighbor shade treatment; left of photo)

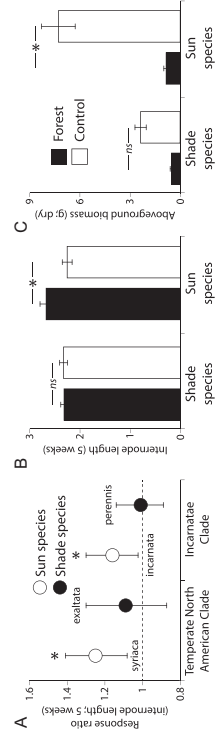


FIGURE 3 Comparison of internode elongation and aboveground biomass between sun and shade species in a field experiment. (A) Species-level comparison of the internode length ratio between forest and control plots after 5 weeks. Data are means and 95% CI; values >1 are denoted by an asterisk (*) and are consistent with the shade avoidance response. (B) Comparison of internode length plasticity in response to deep shade (reduced PAR and RFR) between sun and shade species ($N = 2$ species/type) after 5 weeks. (C) Comparison of aboveground biomass plasticity in response to deep shade after twelve weeks. Data in (A) and (B) are means $\pm$ SEM; statistically significant pairwise differences in (B) and (C) are denoted with an asterisk (*)

In the field experiment, sun and shade species exhibited consistent differences in internode length and aboveground biomass: internode length increased in forest plots relative to control plots by 5 weeks for both sun species (*A. syriaca* 95% CI: 1.08–1.41; *A. incarnata* 95% CI: 1.02–1.30), but did not vary in paired shade species (*A. exaltata* 95% CI: 0.87–1.30; *A. perennis* 95% CI: 0.89–1.14; treatment $\times$ type interaction: $F_{1,108} = 9.59$, $P = 0.003$; Figure 3A, B). This effect persisted 8 weeks after the initiation of the experiment (Appendix S1, Table S25). Similarly, forest shade significantly reduced aboveground biomass in sun species but had no effect on biomass in shade species (treatment $\times$ type interaction: $F_{1,79} = 11.45$, $P = 0.001$, Figure 3C). All species exhibited signs of physiological stress associated with habitat mismatches: some individuals of both sun species exposed to forest shade elongated to the point of stem collapse, while shade species exposed to full sun exhibited increased anthocyanin production, leaf yellowing, and early leaf drop.

DISCUSSION

There is growing evidence that shade avoidance and shade tolerance represent distinct evolutionary strategies for coping with qualitatively different types of shade (e.g., Schmitt et al., 1999; Reich et al., 2003; Franklin, 2008) and that there is little overlap in the suite of constitutive and plastic traits associated with each strategy across species (but see Gommers et al., 2013). The results of our greenhouse and field experiments support this notion. Paired sun and shade species differed in numerous constitutive light-capture traits, with sun species exhibiting several traits (e.g., shorter internode length and greater height) that are likely adaptive in competitive, light-rich environments (and, conversely, maladaptive in light-poor environments; see Appendix S1, Figure S4). Sun species also exhibited several plastic responses to reduced PAR and RFR—including increased internode length, height, and specific leaf area—that are consistent with a shade avoidance strategy. In all cases, reduced RFR was necessary to elicit plastic responses: none of our focal species exhibited responses consistent with shade avoidance in response to reduced PAR alone (compare white and black dots in Figure 2). Specific leaf area was the only trait to vary across light conditions in our shade species, albeit to a lesser extent than for paired sun species, which is also consistent with previous reports of leaf area plasticity in shade-tolerant plants (Gommers et al., 2013). While the costs of adopting a shade-avoidance response in deeply shaded habitat are well-documented (Schmitt et al., 1999, 2003), the cost of reduced plasticity in competitive light environments is less well understood. The results of our field experiment, in which we removed all understory neighbors, likely underestimate these costs because shade species did not have to compete with neighbors for light. Collectively, our results show that macroevolutionary transitions from full sun to partial or complete shade in *Asclepias* are accompanied by changes in both constitutive and plastic light-capture traits, and that the

nature of these changes is broadly consistent with a shift from shade avoidance to shade tolerance. In this regard, our results agree with previous reports of strong negative correlations between shade avoidance and shade tolerance traits across species (Henry and Aarssen, 2001; Valladares and Ninemets, 2008), but represent a surprisingly large number of transitions between habitats relative to other clades (e.g., Patterson and Givnish, 2002).

The loss of plasticity in shade species may be at least partially explained by the costs of maintaining phenotypic plasticity per se in environments with reduced spatial or temporal variability in abiotic conditions (Agrawal et al., 2002; Walls et al., 2005), as well as the fact that many plastic responses are maladaptive in deeply shaded habitats. The fact that both shade species increased specific leaf area (but not internode length or height) in deeply shaded habitats in the field experiment, however, indicates that plasticity in some light-capture traits may be adaptive in deeply shaded habitats even as other shade avoidance responses are not (Schmitt et al., 2003): plasticity in leaf thickness, in particular, may allow shade tolerant species to produce larger leaves at comparable (or lower) resource costs (Gommers et al., 2013).

Traits involved in habitat specialization and stress tolerance are typically under strong stabilizing selection (Ackerly, 2003) which, in combination with other ecological and evolutionary processes (Prinz et al., 2001; Losos, 2008; Wiens et al., 2010; Crisp and Cook, 2012), may result in strong phylogenetic signal or niche conservatism. Community-based investigations of shade and other stress tolerance traits at continental scales, for example, often report patterns of evolutionary conservatism (e.g., Prinz et al., 2001; Hawkins et al., 2014; Rueda et al., 2017). Clade-based comparisons of shade tolerance are comparable: in a study of the core Liliaceae, for example, Patterson and Givnish (2002) report phylogenetic niche conservatism for five traits associated with shade tolerance, and similar results have been noted in Cyperaceae (Waterway et al., 2009). Contrary to these reports, we found that light-capture strategy exhibited weak phylogenetic signal in milkweeds. This disparity may result, in part, from the broad phylogenetic coverage ($>70\%$ of extant species) and high resolution (species- vs. generic-level comparison) of our study relative to previous analyses of the evolution of shade tolerance. Phylogenetic resolution can significantly influence the interpretation of phylogenetic signal for stress tolerance traits (Seger et al., 2013), and neither coarser clade-based nor community-based comparisons would have detected the shifts in stress tolerance strategies between closely related species that we observed in *Asclepias*. Although there are ample biological reasons to believe that stress tolerance traits should tend to be conserved within clades, our results highlight the need to be cautious in interpreting the results of community- and clade-based comparisons, particularly when these involve coarser phylogenetic coverage or resolution.

Another possible explanation for the disparity between our results and previous studies is that, over

macroevolutionary timescales, there appears to be a strong directional bias in the transition between shade strategies in the milkweeds: transitions from sun to shaded habitats were at least three times (and as much as eleven times) as common as the opposite transition. For clades whose ancestral strategy is shade tolerance (such as the Liliales), similar transition biases would reduce the likelihood of transitions to full sun habitats, potentially leading to strong phylogenetic signal at the level of genera or families (Ackerly, 2003; Wiens et al., 2010). It is also possible that the lower overall prevalence of shade species in *Asclepias* may have contributed to this pattern, though we do not consider this likely given that roughly one-quarter of the species in our less conservative analysis were designated as occurring in shaded habitats; estimates of *D* (and related, nonbinary metrics of phylogenetic signal) are robust to low trait prevalence, particularly when more than 50 total species are considered (Fritz and Purvis, 2010). More likely, in our opinion, is that the general loss of plasticity associated with shade tolerance (Figure 2) is an evolutionarily challenging transition to reverse (i.e., Dollo's Law; but see Collin and Miglietta, 2008) and that the likelihood of reversions is further reduced by antagonisms between separate components of the critical phytochrome signaling pathway associated with shade avoidance and tolerance (Gommers et al., 2013; Molina-Contreras et al., 2019). The identification of genus-level clades with differing ancestral habitat preferences and propensities to transition may help to unravel general constraints and drivers in the evolution of light-capture strategies.

Within the milkweeds, the only clade that does not appear to have colonized shaded habitats in our conservative classification scheme is the relatively small (6 species) Sonoran Desert clade. One possibility for the lack of shade species in this clade is that drought tolerance traits may constrain the colonization of shaded habitats: the Sonoran Desert milkweeds have among the narrowest leaves (1–6 mm) of any species in the genus (Woodson, 1954), and many shade tolerant species rely upon increased specific leaf area (relative to shade avoidant congeners) to maximize light capture in light-limited environments (Gommers et al., 2013). It is also possible that the relative scarcity of deeply shaded habitat in this region precludes the evolution of shade tolerance, but we suggest that this is unlikely to be the primary driver of this pattern for two reasons: (1) drought and shade tolerance show some of the strongest negative phylogenetic correlations of any stress tolerance traits across diverse plant lineages (Demmig-Adams and Adams, 1992; Rueda et al., 2017), and (2) shade tolerance appears to have evolved numerous times in the Mexican Highland clade, which encompasses an arid region in central Mexico and Central America with comparable light conditions. Understanding how co-occurring biotic and abiotic stressors constrain macroevolutionary transitions among shade strategies is a promising direction for future research.

Coupling experimental and phylogenetic techniques can help elucidate the ecological mechanisms underlying

evolutionary patterns (Weber and Agrawal, 2012). Although our experimental results support the notion that shade avoidance and tolerance are interspecific alternatives (Franklin, 2008; Valladares and Ninemets, 2008; Gommers et al., 2013), this may be primarily driven by adaptive specialization more so than trade-offs between strategies per se (Agrawal, 2020). Our results suggest that North American milkweeds have colonized deeply shaded habitats multiple times and that these events are associated with a general attenuation of phenotypic plasticity and a specific loss of several core shade avoidance responses. For *Asclepias*, these transitions appear to have occurred relatively unencumbered by evolutionary constraints, but additional studies of stress tolerance traits in other clades with comprehensive phylogenetic coverage and species-level resolution are necessary to determine the generality of this pattern.

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AUTHOR CONTRIBUTIONS

T.C.C. and A.A.A. conceived the research and designed the research plan. T.C.C. performed the research and analyzed the data. T.C.C. wrote the manuscript with input from A.A.A. Both authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.t1g1jw27> (Coverdale and Agrawal, 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix 1. Supplementary figures and tables detailing the experimental design of the greenhouse (Figure S1) and field (Figure S2) experiments, the results of the less conservative phylogenetic comparison (Figure S3), the constitutive expression of light capture and performance traits (Figure S4), and statistical analyses of the greenhouse (Tables S1–S22, S27) and field (Tables S23–26) experiments.

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