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Good neighbors make good defenses: associational refuges reduce defense investment in African savanna plants

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Abstract. Intraspecific variation in plant defense phenotype is common and has wide-ranging ecological consequences. Yet prevailing theories of plant defense allocation, which primarily account for interspecific differences in defense phenotype, often fail to predict intraspecific patterns. Furthermore, although individual variation in defense phenotype is often attributed to ecological interactions, few general mechanisms have been proposed to explain the ubiquity of variable defense phenotype within species. Here, we show experimentally that associational refuges and induced resistance interact to create predictable intraspecific variation in defense phenotype in African savanna plants. Physically defended species from four families (Acanthaceae, Asparagaceae, Cactaceae, and Solanaceae) growing in close association with spinescent *Acacia* trees had 39–78% fewer spines and thorns than did isolated conspecifics. For a subset of these species, we used a series of manipulative experiments to show that this variability is maintained primarily by a reduction in induced responses among individuals that seldom experience mammalian herbivory, whether due to association with *Acacia* trees or to experimental herbivore exclusion. Unassociated plants incurred 4- to 16-fold more browsing damage than did associated individuals and increased spine density by 16–38% within one month following simulated browsing. In contrast, experimental clipping induced no net change in spine density among plants growing beneath *Acacia* canopies or inside long-term herbivore exclosures. Associated and unassociated individuals produced similar numbers of flowers and seeds, but seedling recruitment and survival were vastly greater in refuge habitats, suggesting a net fitness benefit of association. We conclude that plant-plant associations consistently decrease defense investment in this system by reducing both the frequency of herbivory and the intensity of induced responses, and that inducible responses enable plants to capitalize on such associations in heterogeneous environments. Given the prevalence of associational and induced defenses in plant communities worldwide, our results suggest a potentially general mechanism by which biotic interactions might predictably shape intraspecific variation in plant defense phenotype.

Key words: Acacia; *African savannas; associational defense; associational resistance; defense priming; facilitation; herbivory; induced response; inducible defense; physical plant defenses; spines; thorns.*

INTRODUCTION

Herbivory exerts strong selective pressure on plants and has given rise to a diversity of plant defense strategies that reduce tissue loss and mitigate the negative effects of herbivory on plant fitness (Herms and Mattson 1992). A central goal in the study of plant defenses is to explain how variation in defense investment arises and to predict how biotic and abiotic factors affect defense phenotypes (Stamp 2003). Over the past several decades, more than a dozen theories have been proposed to explain interspecific differences in plant defense phenotype, based primarily on evolutionary constraints, resource availability, and physiological trade-offs (e.g., Rhoades 1979, Coley et al. 1985). Several of these theories have broad empirical support and have helped to establish the study of plant defenses as a critical tool for understanding topics in fields such as chemical ecology, co-evolution, and community ecology (Burkepile and Parker 2017).

Intraspecific variation in defense phenotype is also common (Des Roches et al. 2017), affects a variety of ecological processes (Thorpe and Barbosa 1986, Wurst et al. 2008), and often approaches levels commensurate with those observed among species in plant communities or clades (Agrawal and Fishbein 2006, 2008). However, whereas many theories of intraspecific defense variation have been proposed (e.g., McKey 1974, Maschinski and Whitham 1989, Herms and Mattson 1992), they often make conflicting predictions about how phenotype should vary among individuals (see Stamp 2003, Hahn and Maron 2016). In part, these discrepancies result from species- or ecosystem-specific differences in how plants respond to environmental factors; for example, neighbors might increase defense investment in nitrogen-limited systems (Barbosa et al. 2009) but decrease it in systems where herbivores prefer open habitat (Lincoln and Mooney 1984). Identifying which environmental variables have the greatest effect on defense investment across systems is an important step toward developing a predictive understanding of intraspecific variation in defense phenotype (Hahn and Maron 2016).

Interactions between induced resistance and associational effects, two widespread components of plant resistance, represent a potentially general mechanism by which intraspecific variation in plant defense phenotype might arise (Kim 2017). In species capable of induced responses to herbivory,

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consumption triggers increased production of defensive structures and/or chemicals to reduce future tissue loss (Karban and Myers 1989, Karban and Baldwin 1997, Helms et al. 2013). Nearby plants can also decrease (associational resistance) or increase (associational susceptibility) the consumptive damage incurred by their neighbors through a variety of mechanisms, such as reducing the apparency of those neighbors to herbivores (Tahvanainen and Root 1972, Barbosa et al. 2009, Verschut et al. 2017) or repelling herbivores with their own physical and/or chemical deterrents (Hay 1986, Coverdale et al. 2016, Hahn and Orrock 2016). Induced responses and associational effects have been shown to independently affect plant phenotype, vulnerability, and fitness in terrestrial, freshwater, and marine ecosystems (Hay 1986, Hambäck et al. 2014, Louthan et al. 2014, Underwood et al. 2014), but they have typically been studied separately and their interactive effects remain poorly understood (but see Kim 2017). The primary goals of this study are to evaluate whether associational effects and induced resistance interact to produce predictable patterns of defense phenotype and influence components of plant fitness.

The emergence and maintenance of induced defenses require that there be both variable herbivory pressure and reliable cues of future herbivory risk (Karban and Myers 1989, Karban and Baldwin 1997). These features may differ between associational refuges—areas of low browsing pressure created by the physical inhibition of herbivores by neighboring plants (sensu Milchunas and Noy-Meir 2002)—and non-refuge habitats (Maschinski and Whitham 1989, Burghardt 2016). For example, while the intensity and frequency of herbivory is typically low within refuges, herbivory pressure can be significantly more severe and variable in the open habitat immediately outside refuges (e.g., Hay 1986, Coverdale et al. 2016). Inducible resistance may thus be favored in landscapes with discrete refuge patches, because mismatches between defense phenotype and herbivory risk can have deleterious effects on plant fitness (Orrock et al. 2015). Moreover, associational refuges may further affect the speed and/or magnitude of induced responses via defense "priming", the synthesis and storage of precursors necessary for defense production (Douma et al. 2017). Primed plants often respond more strongly and/or rapidly to subsequent herbivore attack, and interactions with neighbors can cause plants to enter a primed state (Frost et al. 2008, Karban et al. 2014, Kim 2017).

In many African savannas, long-lived spinescent trees (e.g., *Acacia* spp.) dominate a patchy overstory layer and can create associational refuges for understory plants by physically impeding herbivores (Coverdale et al. 2016). These refuges are persistent features of the landscape (average lifespan >30 yr; Palmer et al. 2010) and are interspersed within a matrix of grasses, forbs, and subshrubs that contains few plants capable of providing similar antiherbivore protection. Large mammalian herbivores exert strong top-down pressure on African savanna plant communities, which contain species capable of induced responses (Young 1987, Milewski et al. 1991, Palmer et al. 2008). If physically defended trees locally reduce the frequency or intensity of browsing cues below the threshold necessary to trigger induced responses, then associated plants should invest less in defenses than isolated conspecifics. In this way, induced responses may allow plants to match their defense phenotype to local conditions in heterogeneous environments, thereby producing intraspecific variation in defense phenotype.

We used surveys and experiments in an *Acacia*-dominated savanna in semiarid central Kenya to test how associational refuges influence patterns of intraspecific variation in defense phenotype among several species of physically defended understory plants. We then used manipulative experiments on a subset of these species to investigate whether associational refuges consistently affect defense phenotype by reducing the frequency and/or intensity of browsing damage (and hence induced responses). We hypothesized that plants invest less in spines and thorns when growing within associational refuges (i.e., beneath *Acacia* canopies), and that differences in defense phenotype, including the rate and magnitude of induced responses to browsing, are driven by consistent differences in herbivory damage between associated and unassociated plants. We also sought to evaluate whether associational effects and accompanying phenotypic plasticity would outweigh the potential negative effects of competition with neighbors and therefore result in net fitness benefits for associated plants relative to unassociated conspecifics (see Table 1 for detailed list of hypotheses and predictions).

METHODS

Study site and experimental infrastructure

The Mpala Research Centre and Conservancy (MRC) encompasses 20,000 ha of thorn-scrub savanna in Kenya's Laikipia County, most of which is underlain by infertile red sandy loams (Pringle et al. 2016). The plant community in this ecosystem consists of an overstory dominated by the spinescent trees *Acacia brevispica* (prickles), *A. etbaica* (spines), and *A. mellifera* (prickles), with an understory comprising various species of grasses, forbs, and subshrubs (Goheen et al. 2013, Coverdale et al. 2016). Among the latter, many are physically defended, including *Barleria eranthemoides, B. gracilispina, B. trispinosa*, and *B. spinisepala* (Acanthaceae; spines and spinose sepals), *Asparagus falcatus* (Asparagaceae; thorns), the nonnative *Opuntia ficus-indica* (Cactaceae; spines), and *Lycium europaeum* and *Solanum campylacanthum* (Solanaceae; spines and prickles, respectively). For convenience when referring collectively to multiple physical defense types, we use the generic term "spines" inclusively. MRC also supports more than 20 species of wild large mammalian herbivores, including elephant (*Loxodonta africana*), impala (*Aepyceros melampus*), and dik-dik (*Madoqua cavendishi*), as well as domesticated cattle (*Bos indicus*), sheep (*Ovis aries*), goat (*Capra hircus*), and camel (*Camelus dromedarius*).

The UHURU herbivore-exclusion experiment at MRC (Pringle 2012, Goheen et al. 2013, 2018, Kartzinel et al. 2014) comprises 36 1-ha fenced exclosure (with three size-selective fencing configurations) and unfenced control plots at each of three sites across MRC. These three sites correspond with the largely non-overlapping ranges of three of our focal species: *B. spinisepala* (south), *B. eranthemoides* (central), and *B. trispinosa* (north). All plots were established in 2008, six years prior to this study, and

Table 1. Hypotheses and predictions tested in this study.

Component	Prediction	Approach	Attributes measured	Data
*H*1: Associational refuges reduce herbivory on physically defended plants				
Survey 1	association reduces browsing damage	survey of associated and unassociated plants	browsing damage†	Fig. 1A
Experiment 1	overstory and understory neighbors create associational refuges	neighbor-removal experiment ± herbivore exclosure cages	Δ browsing damage†	Fig. 1B
Experiment 2	spines deter browsers	spine-removal experiment	Δ browsing damage†	Fig. 1C
*H*2: Associational refuges reduce defense investment				
Experiment 3	association and herbivore exclosure reduce spine density	survey of associated and unassociated plants in/out of large-herbivore exclosures	spine density†,‡,§	Fig. 2A,B
Survey 2	association reduces defense investment across species	multi-species survey of associated and unassociated plants	spine density¶,#,‖,††,‡‡	Appendix S1: Fig. S5
Survey 3	association reduces spine length and no. spines per cluster	survey of associated and unassociated plants	spine length†,‡,§; no. spines/cluster†,‡,§	Fig. 2C,D
Survey 4	association reduces biomass allocation to defense	survey of associated and unassociated plants	proportion of dry biomass consisting of defensive tissue†,‡,§	Fig. 2E
*H*3: Associational refuges reduce induced responses				
Experiment 4	browsing induces defense response	simulated browsing on unassociated plants	Δ spine density†	Fig. 3
Experiment 5	long-term herbivore exclusion reduces induced response	simulated browsing on unassociated plants in/out of large-herbivore exclosures	Δ spine density†	Fig. 4A
Experiment 6	association reduces induced response	simulated browsing on associated and unassociated plants	Δ spine density†	Fig. 4B
*H*4: Association and induced responses affect components of plant fitness				
Survey 5	association affects plant size distribution and size: seed-head ratio	transect survey of associated and unassociated plants	canopy area†; no. seed heads†	Fig. 5A,B
Survey 6	association affects key components of plant fitness	survey of associated and unassociated focal plants and immediate vicinity	no. flowers†; seedling density†; adult density†; recruitment†; adult survival†	Fig. 5C-F

Notes: Symbols refer to plant species included in each set of measurements.
†*Barleria trispinosa*, ‡*B. eranthemoides*, §*B. spinisepala*, ¶*Asparagus falcatus*, #*B. gracilispina*, ‖*Lycium europaeum*, ††*Opuntia ficus-indica*, ‡‡*Solanum campylacanthum*.

total-exclosure fences have been maintained continuously to ensure the exclusion of all herbivores >5 kg. Hares and rodents are not excluded by the electrified wires and chain-link fences used in the total-exclosure treatment (Pringle et al. 2011). Here, we utilized a subset of the total-exclosure and unfenced control plots only (see GPS.kmz in Data S1 for coordinates of experimental plots and replicates). For three of our six experiments, we used either a single exclosure plot (Experiments 2 and 5) or one such plot at each of the three sites (Experiment 3), and we treated individual plants as the units of analysis. The lack of replication at this largest scale precluded complete randomization of all treatment combinations in these experiments, as well as the use of split-plot statistical designs. However, given the large size of the experimental exclosures relative to individual understory plants, and the considerable spatial heterogeneity present within each 1-ha plot, we assumed replicate plants to be biologically and statistically independent; that the results of these three experiments were ultimately consistent with those of our other randomized experiments and surveys bolsters our confidence that this assumption did not unduly compromise the robustness of our inferences.

Hypothesis 1: Associational refuges reduce herbivory on physically defended plants

Survey 1.—To determine if association with *Acacia* trees reduced herbivory upon physically defended understory plants, we surveyed browsing damage on associated vs. unassociated *B. eranthemoides*, *B. trispinosa*, and *B. spinisepala* (*n* = 48 plants, eight of each species per neighborhood type; we use "neighborhood" henceforth to refer to association

status). Browsing damage was measured by classifying 10 haphazardly selected stems within each plant as browsed or unbrowsed by mammals. The proportion of browsed stems was recorded for each plant, and the effect of neighborhood was assessed using separate one-factor ANOVA for each species in R (v. 3.3.2; R Core Development Team 2015).

Experiment 1.—To parse the relative contributions of spinescent *Acacia* trees and understory vegetation to the creation of associational refuges beneath tree canopies, we performed a neighbor-removal experiment with four randomized treatments: unmanipulated control, overstory removal, overstory + understory removal, and a procedural control to isolate any effects of neighbor removal per se from its effect on herbivory, wherein overstory and understory vegetation was removed around focal plants that were enclosed inside individual wire-mesh cages to prevent herbivore access ($n = 40$ plants, 10 per treatment; Appendix S1: Fig. S1). We used *B. trispinosa* as a focal understory species and measured browsing damage immediately after the experimental manipulation and again after one month. Changes in browsing damage were analyzed using ANOVA, and Tukey's HSD post-hoc tests were used to assess the statistical significance ($P \leq 0.05$) of differences between treatments.

Experiment 2.—Physically defended understory subshrubs such as *B. trispinosa* have been documented in large-herbivore diets at MRC (Pringle et al. 2014, Kartzinel et al. 2015). We conducted a spine-removal experiment to test the efficacy of *B. trispinosa* spines against large mammalian herbivores. We identified 24 unassociated plants and initially randomly assigned each plant to one of four spine-removal treatments: unmanipulated controls, 50% spine removal, 100% spine removal, and 100% spine removal within individual wire-mesh herbivore-exclusion cages (a procedural control to isolate any effects of spine removal per se from its effect on herbivory; Appendix S1: Fig. S2). However, the eight procedural-control cages were vandalized within three days of experiment initiation, so we replaced these replicates with an equivalent number of individuals (each with 100% of spines removed, but without individual cages) inside the nearest UHURU total-exclosure plot in northern MRC (~200 m away from the other replicates); although the exclosures themselves were therefore not replicated in this experiment (i.e., individual replicate plants were retained as the units of analysis), we nonetheless expected this treatment to fulfill the intended procedural-control function. Spines were removed at their base with scissors, with care taken to minimize damage to other tissues. Changes in browsing damage over one month were compared across treatments using one-factor ANOVA, with Tukey's HSD post-hoc tests used for pairwise comparisons between treatments.

Hypothesis 2: Associational refuges reduce defense investment

Experiment 3.—To determine the effect of associational refuges on defense phenotype, we surveyed associated and unassociated individuals of *B. eranthemoides*, *B. trispinosa*, and *B. spinisepala*, both inside and outside a single 1-ha total-exclosure plot in each of the central, north, and south regions of MRC, respectively ($n = 40$ plants per species, 10 each per neighborhood in each exclosure treatment; Appendix S1: Fig. S3). We included contrasts of associated and unassociated plants within exclosures in an effort to distinguish effects of large-mammal herbivory from any effects that might arise from abiotic factors (e.g., light, soil moisture, temperature); we again treated individual plants as the units of replication and analysis in this experiment. For all individuals, we estimated spine density by averaging the number of spines or spinose sepals on the terminal 10 cm of one to three haphazardly selected branches per plant. The independent and interactive effects of association and herbivore exclusion on spine density were analyzed with separate two-factor ANOVA for each species.

Survey 2.—We evaluated the extent and consistency of association-related intraspecific variation in defense phenotype across species by surveying the density of physical defenses on associated and unassociated individuals from five additional species representing four plant families ($n = 40$ plants per species, 20 in each neighborhood type). For species with defenses originating along the stem (*A. falcatus*, *B. gracilispina*, and *L. europaeum*), we characterized defense phenotype by averaging the number of spines on the terminal 10 cm of four stems. For *S. campylacanthum*, which produces prickles along the top and bottom of the leaf mid rib, we averaged the total number of prickles on four leaves per plant. For the cactus *O. ficus-indica*, we counted the total number of spines on a single terminal paddle and estimated spine density by dividing the number of spines by paddle area. The effect of association on defense phenotype was compared using separate ANOVA for each species.

Survey 3.—To determine whether association affects spine morphology, we quantified the length of *Barleria* spines and the structure of spine clusters (for *B. eranthemoides* and *B. trispinosa*, which produce four-rayed axillary spines) or spinose sepals (for *B. spinisepala*; Balkwill and Balkwill 1997). We quantified spine lengths of associated and unassociated plants by measuring five spines per plant ($n = 50$ plants per species, 25 in each neighborhood type). We quantified defense structure for a randomly selected subset of these individuals ($n = 10$ plants per neighborhood per species) by counting the number of spines within five spine clusters or sepals per plant. Spine lengths and counts were averaged for each individual and analyzed as a function of association using separate ANOVA for each species.

Survey 4.—To test the hypothesis that association reduces the proportion of biomass allocated to defense, we surveyed the relative weights of defensive and non-defensive tissues in these same three *Barleria* species ($n = 50$ plants per species, 25 in each neighborhood type). We clipped the terminal 10 cm of a single branch, separated the spines from the remaining leaf and stem tissue, and dried all samples to constant weight at 60°C. We then weighed the dried tissues separately for each replicate plant and estimated relative defense investment by dividing the mass of defensive tissue by the total mass of each clipping. The proportion of biomass allocated to defense was analyzed as a function of association using separate ANOVA for each species.

Hypothesis 3: Associational refuges reduce induced responses

Experiment 4.—To evaluate the inducibility of defenses in *B. trispinosa*, and thus the potential for intraspecific variation in defense phenotype to result from interactions between induced responses and associational effects, we randomly assigned 24 unassociated plants to three simulated browsing treatments: unmanipulated controls, 25% leaf clipping, and 50% leaf clipping ($n = 8$ plants per treatment). *Barleria trispinosa* leaves grow in clusters of four, and browsing was simulated using scissors, with approximately one-half of individual leaf biomass removed (by cutting transversely across the leaf) from either one (25% treatment) or two (50% treatment) of the leaves in each cluster (Appendix S1: Fig. S4A–D). Changes in spine density over one month were analyzed as a function of experimental treatment using ANOVA, and Tukey's HSD post-hoc tests were used for pairwise comparisons between treatments.

Experiment 5.—To determine if a long-term (six-year) history of differences in browsing pressure affects induced responses to herbivory, we simulated browsing damage on unassociated *B. trispinosa* plants both inside and outside one of the 1-ha total-exclosure plots in the northern site of the UHURU experiment (Appendix S1: Fig. S4E). To control for any short-term differences in herbivory between exclosure treatments that might occur during the course of the experiment, we enclosed half of the replicates in each plot within individual wire-mesh cages, with a mesh size (~15 mm) small enough to exclude rodents and lagomorphs that can pass through the UHURU fence (see Appendix S1 for locations of all replicates). All plants had 50% of their leaves clipped transversely (as described above) to simulate natural browsing damage; changes in spine density over one month were compared as a function of large-scale exclosure treatment, individual-level caging treatment, and their interaction using two-factor ANOVA.

Experiment 6.—To test the prediction that long-term reductions in herbivory caused by association with well-defended neighbors would diminish induced responses to browsing (analogous to the hypothesized effects of long-term experimental herbivore exclusion in *Experiment 5*), we simulated browsing damage on associated and unassociated *B. trispinosa* in a factorial experiment. Associated and unassociated plants were evenly and randomly split between unclipped control and 50% browsing treatments ($n = 40$ plants, 10 in each treatment × neighborhood combination; Appendix S1: Fig. S4F), and the independent and interactive effects of simulated browsing and association on changes in spine density over one month were compared using two-factor ANOVA.

Hypothesis 4: Association and induced responses affect components of plant fitness

Survey 5.—We estimated the size distribution of all associated and unassociated *B. trispinosa* along eighteen 50 × 4 m transects in northern MRC by modeling canopy area as an ellipse (Pringle et al. 2014). In addition, to determine how reproductive output varies with canopy area, we counted the total number of seed heads (each containing 10–15 seeds) per plant on 150 plants along the transects ($n = 75$ plants per neighborhood); we then assessed the effects of association on seed production using ANCOVA, with canopy area as the covariate.

Survey 6.—We tagged 480 *B. trispinosa* ($n = 240$ associated and unassociated) and counted the total number of flowers on each plant at the time of tagging and again after one year. We quantified densities of seedlings and adults by counting the number of *B. trispinosa* within a 1-m radius of each of the 480 focal plants; individuals <10 cm in height were classified as seedlings and plucked immediately after the initial survey, whereas individuals >10 cm tall were classified as adults and were not plucked. After one year, we estimated neighborhood-specific recruitment rates by counting the number of new seedlings within 1 m of each associated and unassociated focal plant (*B. trispinosa* seeds are dispersed via explosive dehiscence, such that seedlings are most likely to recruit in the same neighborhood as their parent plant). We estimated survival rates of mature plants by resurveying the number of adult *B. trispinosa* in the same area over the same interval (several replicate focal plants were lost prior to resurvey and were excluded from subsequent analyses). We compared all components of plant fitness between neighborhood types using separate one-factor ANOVA.

RESULTS

Hypothesis 1: Associational refuges reduce herbivory on physically defended plants

Survey 1.—Association significantly reduced the percentage of stems browsed on all three *Barleria* species: associated individuals incurred 74.6–93.8% less browsing than unassociated conspecifics (all $F_{1,14} \geq 20.90$, $P < 0.0001$; Fig. 1A).

Experiment 1.—Removing overhanging *Acacia* branches from associated *B. trispinosa* increased the percentage of stems with browsing damage from 2% to 27% over one month ($F_{3,36} = 19.11$, $P < 0.0001$; Fig. 1B); the additional removal of understory neighbors did not significantly increase browsing damage relative to plants with overstory removal only (Tukey's HSD; $P = 0.21$). Browsing damage did not appreciably increase over one month for either the unmanipulated controls or the procedural controls with total neighbor removal inside individual cages (leftmost and rightmost bars, respectively, in Fig. 1B), and these treatments were statistically indistinguishable (Tukey's HSD, $P = 0.89$).

Experiment 2.—Removing 50% or 100% of spines from unassociated plants increased the percentage of stems with browsing damage from 2.5% to 30.0% and from 0% to 31.3%, respectively, and both spine-removal treatments exhibited significantly higher browsing damage after one month than did either control treatment ($F_{3,28} = 10.19$, $P = 0.0001$; Tukey's HSD, $P < 0.001$ for both pairwise contrasts with unmanipulated controls); unmanipulated controls and procedural controls with 100% spine removal inside a total-exclosure plot exhibited negligible changes in
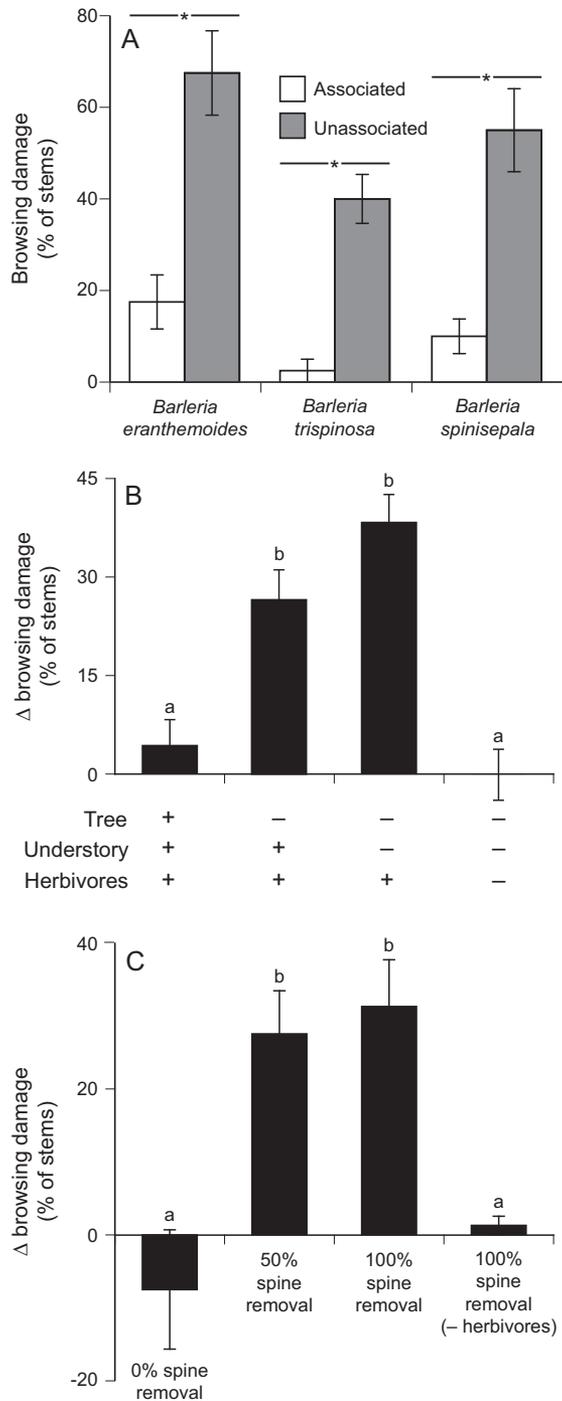
FIG. 1. Effects of association and spines on browsing damage. (A) Standing levels of herbivory damage on associated vs. unassociated *Barleria eranthemoides*, *B. trispinosa*, and *B. spinisepala* in unfenced areas accessible to large herbivores. Asterisks denote statistically significant differences ($P \leq 0.05$) between neighborhoods. (B) Change in browsing damage after one month on associated *B. trispinosa* in the following treatments (left to right, indicated by + and −): unmanipulated control, overstory removal only, overstory + understory removal, and procedural control comprising overstory + understory removal with individual herbivore-exclusion cages. (C) Change in browsing damage after one month for unassociated *B. trispinosa* with 0%, 50%, and 100% of spines removed; the −Herbivore replicates were located within a long-term 1-ha herbivore-exclusion plot. Data are means ± 1 SE. In panels B and C, different lowercase letters indicate statistically significant differences between treatments.

browsing damage (Fig. 1C) and were statistically indistinguishable from each other (Tukey's HSD, $P = 0.73$).

Hypothesis 2: Associational refuges reduce defense investment

Experiment 3.—Both association with *Acacia* (all $F_{1,36} \geq 56.44$, $P < 0.0001$) and long-term herbivore exclusion (all $F_{1,36} \geq 9.46$, $P \leq 0.003$) independently reduced spine density for *B. eranthemoides*, *B. trispinosa*, and *B. spinisepala* (Fig. 2A,B). The interaction between herbivore exclusion and association with *Acacia* was statistically significant for two of the three species (*B. eranthemoides*, $F_{1,36} = 13.87$, $P = 0.0007$; *B. spinisepala*, $F_{1,36} = 11.34$, $P = 0.002$; *B. trispinosa* $F_{1,36} = 2.06$, $P = 0.16$); on average, unassociated plants within exclosure plots had 26.2% fewer spines than unassociated conspecifics outside exclosures, while values for associated plants remained approximately constant across exclosure treatments (compare bars across Fig. 2A,B). This suggests that, at least for the former two species, long-term relaxation of herbivory pressure diminished the effect of association on defense (see *Surveys 2*, *Survey 3*, and *Survey 4* for similar tests of the effects of long-term browsing relaxation on defense phenotype and morphology).

Survey 2.—The effect of association on defense phenotype was similar in magnitude across all five species surveyed: spine density was 39.1–78.4% lower in associated individuals than in unassociated conspecifics for each species (all $F_{1,38} \geq 16.03$, $P \leq 0.0003$; Appendix S1: Fig. S5).

Survey 3.—For all three *Barleria* species, spines were significantly shorter on associated plants than unassociated plants (all $F_{1,48} \geq 29.50$, $P < 0.0001$, Fig. 2C). Associated *B. eranthemoides* and *B. trispinosa* also had significantly fewer spines per cluster than did unassociated conspecifics ($F_{1,18} \geq 8.18$, $P \leq 0.01$); the number of spines per sepal was likewise lower in associated vs. unassociated *B. spinisepala*, albeit with marginal statistical significance ($F_{1,18} = 3.77$, $P = 0.068$; Fig. 2D).

Survey 4.—Across all *Barleria* species, relative defensive tissue biomass was 34.7–68.2% lower in associated individuals (all $F_{1,48} \geq 21.48$, $P < 0.0001$; Fig. 2E,F).

Hypothesis 3: Associational refuges reduce induced responses

Experiment 4.—Initial spine density did not vary between simulated browsing treatments (average initial spine density: 29.6 ± 0.8 spines/branch; data not shown). The magnitude of the induced response over one month was proportional to the intensity of simulated browsing damage: there was a negligible net change in spine density in the unclipped controls, whereas the 25% clipping treatment induced a mean increase in spine density of 16.0% (from 30.5 to 35.3 spines/branch), and the 50% clipping treatment induced a mean increase in spine density of 38.0% (from 28.3 to 39.1 spines/branch; $F_{2,21} = 19.09$, $P < 0.0001$; Fig. 3).

Experiment 5.—Simulated browsing caused a 10-fold greater increase in spine density among *B. trispinosa* plants
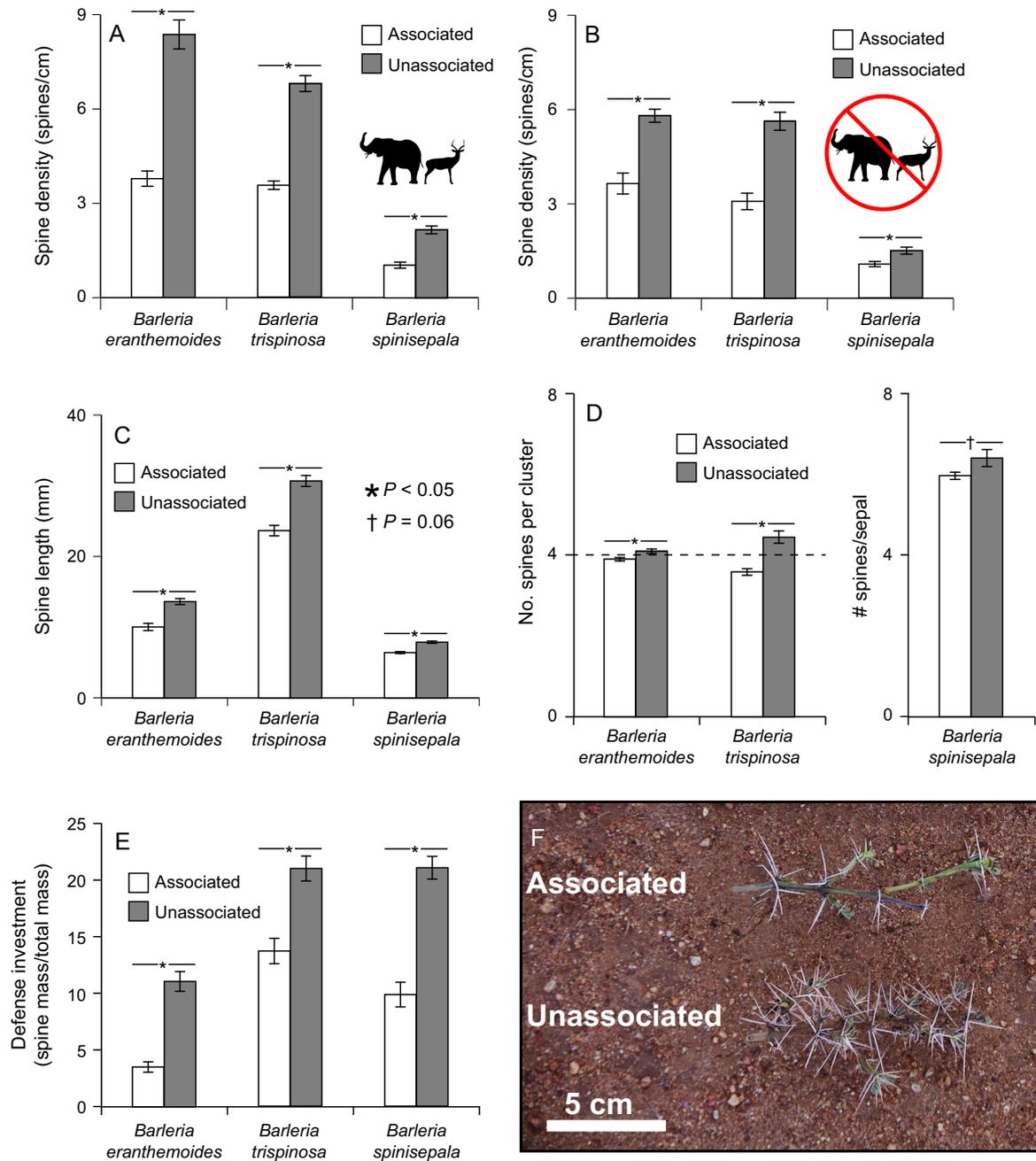
FIG. 2. Effects of association and large-herbivore exclusion on defense phenotype in three species of *Barleria*. (A) Density of spines or spinose sepals on 10-cm long branches of associated and unassociated plants in unfenced control plots. (B) Density of spines or spinose sepals on 10-cm long branches of associated and unassociated plants within fenced exclosure plots that excluded all herbivores >5 kg. (C) Average spine length on associated and unassociated plants. (D) Morphology of individual *Barleria* defense structures. Dashed line denotes four spines per cluster, a morphology that is considered typical of *B. eranthemoides* and *B. trispinosa*; *B. spinisepala* produces spinose sepals. (E) Ratio of defensive vs. non-defensive tissue dry biomass in terminal 10-cm stem clippings. (F) Photograph of representative clippings from associated and unassociated *B. trispinosa*, with leaves removed to expose spines. Data are means ± 1 SE. Asterisks denote statistically significant differences ($P \leq 0.05$) between neighborhoods.

outside vs. inside the long-term, large-scale herbivore exclosure ($F_{1,28} = 64.82$, $P < 0.0001$, Fig. 4A); in contrast, there was no significant main effect of short-term individual-scale caging on induced responses ($F_{1,28} = 1.39$, $P = 0.26$). However, the induced response of plants inside the large-scale exclosure was further reduced by individual cages (exclosure × cage interaction: $F_{1,28} = 5.49$, $P = 0.026$), potentially due to hare and/or rodent browsing on uncaged plants

within exclosure plots during the trial (see *Experiment 6* for a similar test of the effects of long-term browsing release on induced responses).

Experiment 6.—Leaf clipping increased spine density by 38.0% (from 28.4 to 39.2 spines/branch) for unassociated *B. trispinosa*. The same treatment had a negligible effect (from 25.7 to 26.2 spines/branch) on individuals growing in

association with *Acacia* trees (clipping × neighborhood interaction: $F_{1,36} = 32.11$, $P < 0.0001$, Fig. 4B), again indicating that a lifetime history of growing in a low-herbivory environment mutes the induced response of associated plants to a browsing event. Unclipped individuals similarly exhibited negligible changes in spine density over the same time period, regardless of neighborhood type.

Hypothesis 4: Association and induced responses affect components of plant fitness

Survey 5.—The total number and mean canopy area of associated and unassociated *B. trispinosa* (respectively, $n = 662$ and 574; $5,548 \pm 305$ and $5,498 \pm 287$ cm^2) were similar across all transects (Fig. 5A); since tree cover is <50% in northern MRC (see GPS.kmz in Data S1 for locations of transects), the majority of understory habitat falls outside associational refuges. The total number of seed heads per plant was strongly correlated with canopy area ($R^2 = 0.86$) for both associated and unassociated plants; larger plants produced more seeds ($F_{1,146} = 856.1$, $P < 0.0001$), and unassociated plants produced slightly more seed heads on average than associated plants of equivalent size (e.g., 115 vs. 94 seed heads for a 1-m^2 plant; $F_{1,146} = 36.4$, $P < 0.0001$; Fig. 5B). The slope of the relationship between reproductive output and canopy size, however, did not differ between associated and unassociated plants (Fig. 5B; neighborhood × canopy-size interaction: $F_{1,146} = 0.27$, $P = 0.60$).

Survey 6.— There was no significant effect of association on the average number of flowers per plant in either survey year (2016: 0.33 ± 0.06 flowers/plant, $F_{1,478} = 0.007$, $P = 0.93$; 2017: 0.23 ± 0.06 flowers/plant $F_{1,374} = 0.049$, $P = 0.82$). In contrast, the initial densities (individuals/m^2) of *B. trispinosa* adults ($F_{1,478} = 82.72$, $P < 0.0001$; Fig. 5C) and seedlings ($F_{1,478} = 111.50$, $P < 0.0001$; Fig. 5D) were approximately twofold and fivefold greater, respectively, around associated plants than unassociated plants. Similarly, adult survival ($F_{1,374} = 6.36$, $P = 0.012$; Fig. 5E) and new seedling recruitment ($F_{1,374} = 22.82$, $P < 0.0001$; Fig. 5F) were significantly greater in associated neighborhoods: average adult density increased by 0.14 ± 0.12 seedlings/m^2 around associated plants, but decreased by 0.28 ± 0.11 seedlings/m^2 around unassociated plants during the same interval (Fig. 5E), while the density of new seedling recruits was nearly thirty-fold greater beneath tree canopies than in the unassociated neighborhoods between trees (Fig. 5F).

DISCUSSION

Our results show that two common mechanisms of plant defense, associational refuges and induced resistance, interact to consistently reduce individual defense investment and modulate defense phenotype in several species of African

FIG. 3. Induced resistance in *B. trispinosa* following two levels of simulated browsing. Change in spine density was measured one month after experimental manipulation; initial spine density prior to manipulation averaged 3.0 ± 0.08 spines/cm and did not differ significantly among treatments. Data are means $\pm$ 1 SE.

FIG. 4. Effects of long- and short-term disparities in herbivory pressure on induced responses in *B. trispinosa*. (A) Change in spine density on the terminal 10 cm of *B. trispinosa* branches following simulated browsing of unassociated plants within and outside a long-term fenced exclosure that excluded all herbivores >5 kg. Individual cages were placed around one-half of the replicates in each long-term herbivory regime to control for ongoing herbivory during the one-month trial and test the relative effects of short- vs. long-term relaxation of herbivory on induced responses. (B) Change in spine density on the terminal 10 cm of *B. trispinosa* after one month following simulated browsing on plants outside herbivore exclosures with long-term disparities in herbivory pressure created by association (or lack thereof) with *Acacia* trees. Initial spine density did not differ significantly among treatments in panel B; initial spine density was lower within the total exclosure plot in panel A, but did not differ between caging treatments within exclosure types. Data are means $\pm$ 1 SE. Different lowercase letters indicate statistically significant differences between treatments.
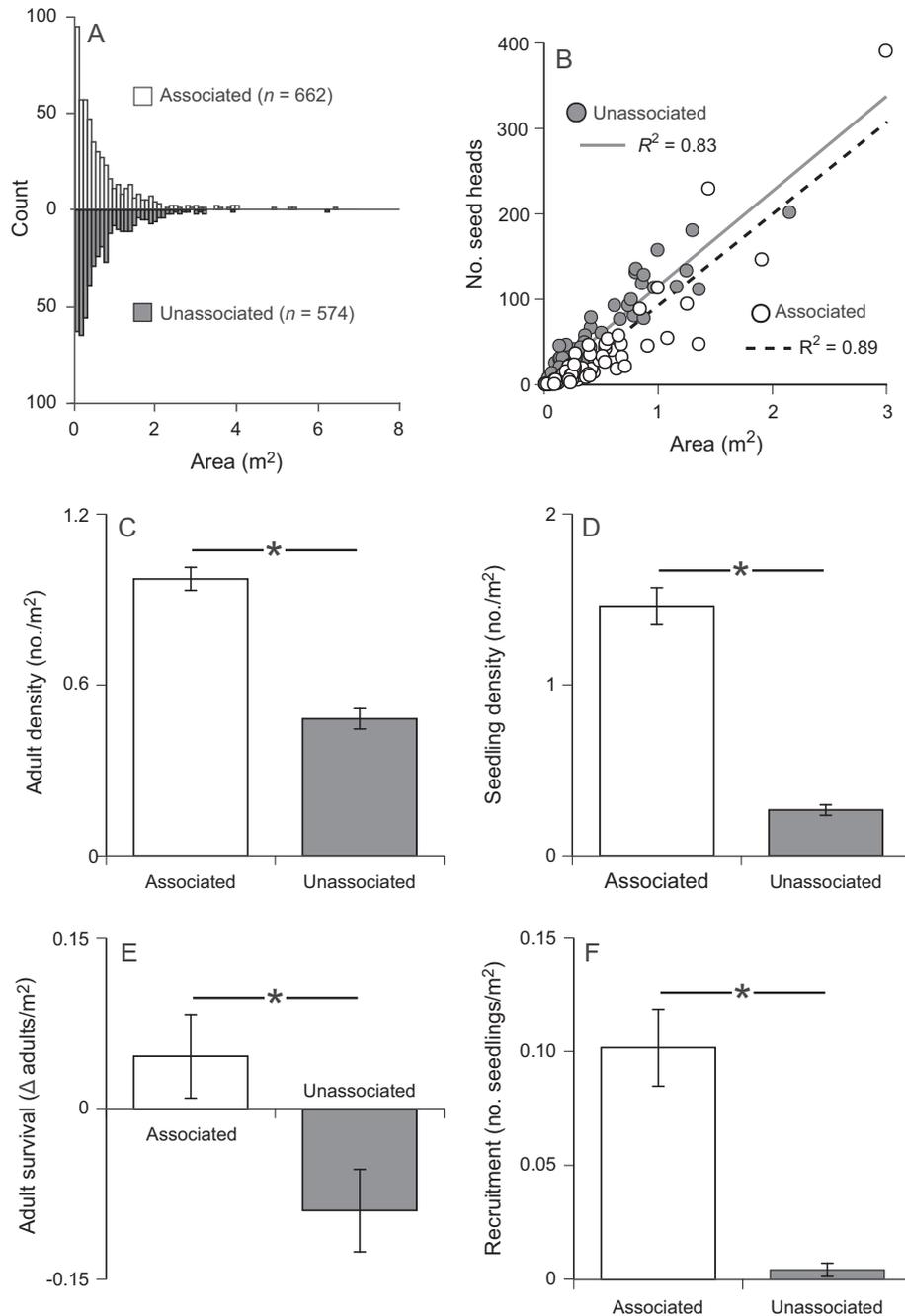
FIG. 5.   Effects of associational refuges on components of plant fitness. (A) Distribution of *Barleria trispinosa* canopy areas for associated and unassociated plants. (B) Correlation between canopy area and reproductive output (number of seed heads) for associated and unassociated plants. For comparison, ordinary least-squares regression lines are plotted separately for each neighborhood type. (C) Initial densities of adult *B. trispinosa* in associated and unassociated neighborhoods in 2016. (D) Initial densities of *B. trispinosa* seedlings in associated and unassociated neighborhoods in 2016. (E) Change in adult densities (a proxy for survival) after one year. (F) Numbers of newly recruited seedlings after one year. Panels C–F show results of surveys conducted within 1-m radius areas around tagged focal plants in each neighborhood type. Data are means ± 1 SE. Asterisks denote statistically significant differences ($P \leq 0.05$) between neighborhoods.

savanna plants. By decreasing the frequency and intensity of browsing cues necessary to trigger defense priming and induced responses, associational refuges contribute to predictable patterns of intraspecific phenotypic variation, wherein plants within refuges are significantly less defended than unassociated conspecifics. This mechanism also appears to allow plants to calibrate their resource allocation to local browsing conditions and locally enhance their fitness within a heterogeneous risk environment. In other words, induced responses (a cost-saving component of resistance over which plants have some control) can be differentially deployed depending on the associational environment (which plants cannot control) to buffer the negative impacts of herbivory (for unassociated plants) or competition (for associated plants). At least for this focal assemblage, our results indicate that associational refuges confer net fitness

benefits that outweigh any negative effects of competition, a kind of silver-spoon effect (sensu Grafen 1988) for understory plants that reflects the distribution of long-lived overstory neighbors across the landscape.

Below, we synthesize our main findings to address how associational refuges affect defense phenotype and plant fitness, and we highlight several promising topics for future research on the interactive effects of associational refuges and induced resistance.

How do associational refuges affect defense phenotype?

Our results suggest that association indirectly reduces spine density by decreasing browsing damage on plants capable of induced responses to herbivory. These results agree with a previous study at MRC (Coverdale et al. 2016), which found that overstory neighbors reduce herbivory, presumably through physical inhibition of browsing mammals; decreased apparency conferred by understory neighbors did not significantly reduce browsing damage (Louthan et al. 2014). The efficacy of associational refuges against large mammalian herbivores is underscored by the finding that associated plants incurred similar amounts of browsing damage to plants enclosed within wire-mesh cages during our neighbor-removal experiment (Fig. 1B). Surveys of associated and unassociated individuals, which quantified browsing damage accumulated over longer time-spans, likewise revealed that associated individuals experienced significantly less browsing damage than unassociated conspecifics (Fig. 1A).

In a series of simulated-browsing experiments with the common understory shrub *B. trispinosa*, we found that browsing reliably induced the production of new defensive spines, but that the magnitude and speed of induction varied as a function of contemporary and long-term differences in browsing intensity. Simulated browsing induced a proportional response in unassociated plants, with more heavily damaged individuals producing greater densities of defensive spines (Fig. 3). However, a consistent absence of browsing, whether as a result of long-term herbivore exclusion (Fig. 4A) or association with *Acacia* trees (Fig. 4B) significantly dampened the induced response to identical browsing damage. Notably, the short-term protection provided by individual cages during our one-month clipping experiment did not affect the speed or magnitude of induced resistance, suggesting that plants integrate information accumulated over longer timespans into their response to browsing.

Although it is possible that consistently low browsing pressure causes associated *B. trispinosa* (and, analogously, those within long-term herbivore exclosures) to lose the ability to induce defenses altogether, longer-term studies involving repeated and/or more intense simulated-browsing events would be required to demonstrate this; we instead conservatively interpret our results to indicate that such plants have a greater damage threshold for defense induction and/or a longer temporal lag in spine production following browsing damage. This result is consistent with previous studies of induced-response priming in other systems (Frost et al. 2008). Given the intense mammalian herbivory pressure in many African savannas, including potentially lethal levels of consumption by species such as elephants (Pringle et al.

2015), unassociated plants may be particularly vulnerable to browsing-induced mortality if induced resistance is not sufficiently rapid following initial attack (Orrock et al. 2015); to our knowledge, the doubling of spine density that we observed in *B. trispinosa* within one month of simulated browsing is among the most rapid and dramatic examples of physical defense induction ever documented (Barton 2016). Priming is thus a likely mechanism by which unassociated (and, analogously, unfenced) plants produced such rapid responses, and it provides a plausible explanation for the observed intraspecific variation in the magnitude and speed of induced resistance to browsing in *B. trispinosa* (Frost et al. 2008, Douma et al. 2017). Likewise, the muted response of associated plants to simulated browsing is consistent with the hypothesis that maintaining the ability to induce defenses, including remaining in a primed state, can be energetically costly and lead to trade-offs with other vital functions (DeWitt et al. 1998).

We predicted that the combination of reduced browsing cues and a diminished induced response in associated plants would lead to consistent intraspecific patterns of defense phenotype in savanna plant communities, with associated plants producing fewer spines and thorns than isolated conspecifics. Our surveys revealed that, across eight common understory species, the density of physical defenses was, on average, ~150% greater in isolated individuals than in associated conspecifics. This pattern held for both native and nonnative (*O. ficus-indica*) species, and across diverse plant families employing a variety of physical defense structures (thorns, prickles, spines, and spinose sepals). For a subset of these species, we also found that association with well-defended *Acacia* trees caused reductions in spine length and in the number of spines per cluster. Collectively, reductions in spine length and density, as well as changes in spine-cluster morphology, contributed to a two- to threefold decrease in absolute defensive tissue biomass in associated plants.

Thus, our results suggest that repeated browsing led to a proliferation of physical defenses on unassociated plants via rapid induced responses, potentially facilitated by defense priming. For associated plants, in contrast, well-defended neighbors appear to reduce the frequency and/or intensity of browsing cues below some critical threshold necessary for defense induction. We suggest that this ecological mechanism produces the superficially counterintuitive pattern of higher browsing damage on unassociated plants (Fig. 1A), despite their conspicuously greater spine density (Fig. 2A,F).

How does association affect plant fitness?

Associational refuges are commonly assumed to enhance fitness (Agrawal 1998, Louthan et al. 2014), but may be deleterious if resource competition between neighbors outweighs the benefits of reduced herbivory (Hay 1986, Barbosa et al. 2009). Though it is difficult to accurately measure true lifetime fitness for long-lived perennial plants such as *B. trispinosa* in natural populations (Campbell 2000), comparisons of key vital rates across habitats can provide useful insights into the relative impacts of herbivory on plant fitness (Pringle et al. 2014).

The available evidence suggests that the net effect of association on plant fitness is almost certainly positive. Adult

density and survival in our study system were significantly greater beneath tree canopies, as were seedling density and the recruitment of new individuals (Fig. 5C–F). Because *B. trispinosa* disperses seeds through explosive dehiscence, which allows seeds to disperse ~1 m from parent plants, the majority of associated seedlings are likely to be the progeny of associated adults (and vice-versa); nonetheless, some proportion of seeds may disperse out of the adult neighborhood, such that seeds of unassociated plants could land within associational refuges. The only detrimental effect we detected was a marginal (~20%) reduction in seed production that was far smaller in magnitude than the multiple-fold positive effects on survival and recruitment; flower production, and hence the male component of fitness, did not differ between habitats.

We contend that association-driven plasticity in plant defenses, in addition to matching defense phenotype with local risk conditions, may also allow associated plants to compensate for increased competitive costs by allocating a greater proportion of resources to reproduction and avoiding unnecessary investment in intrinsic defenses. Thus, one benefit of inducible resistance in associated plants may be a greater ability to overcome the constraints of increased competition and/or abiotic stress through reduced defense investment.

Future Directions and Conclusions

Our results suggest several promising avenues for future research on the fitness effects, ecological mechanisms, and molecular underpinnings of intraspecific variation in defense phenotype. A multi-year demographic study aimed at estimating robust λ values for associated and unassociated plants, parsing the relative strength and mechanism(s) of positive and negative interactions between neighbors, and accounting for the effects of induced defenses on reproductive output, would be useful (e.g., Palmer et al. 2010, Louthan et al. 2017). Likewise, future investigations might account for the effects of abiotic factors on associational refuges; for example, fire and rainfall can both independently and interactively influence savanna tree density and morphology, and hence the distribution and efficacy of refuge habitats (Staver et al. 2012, Charles-Dominique et al. 2016). Future work should also focus on disentangling the independent and interactive effects of abiotic factors such as light quantity and wavelengths, nutrient availability, and soil moisture on defense phenotype, and how environmental conditions affect defense priming and constrain the speed and magnitude of induced resistance (Reed et al. 1993, Tegelberg et al. 2004, Burghardt 2016). Similarly, the genetic and/or epigenetic mechanism(s) that underpin intraspecific variation in defense phenotype, including differences in the rate and magnitude of induced responses, remain largely unknown. Understanding the relative contributions of genetic and epigenetic mechanisms to observed differences in plant phenotype remains a promising avenue for future research (Barton 2016).

Accurately predicting patterns of intraspecific defense variation across biotic and abiotic gradients requires the integration of ecological interactions, physiological mechanisms, and microevolutionary processes into existing theories of plant defense (Hahn and Maron 2016). Here, we show that,

by reducing the frequency and intensity of browsing damage, associational refuges cause predictable variation in defense phenotype for species that respond to browsing by inducing defenses. Individual plasticity in defense phenotype, including induced resistance to browsing, allows plants to produce fitness-enhancing phenotypes in heterogeneous environments (Barton and Koricheva 2010), and associational refuges may contribute to the creation of predictably heterogeneous risk landscapes in ecosystems worldwide. In African savannas, the distribution and abundance of associational refuges is determined by a combination of biotic and abiotic factors (Sankaran et al. 2005, Staver et al. 2011, Charles-Dominique et al. 2016), while the prevalence of induced resistance is driven largely by intense, yet spatiotemporally variable, top-down pressure by large mammalian herbivores (Augustine 2010). The co-occurrence of associational effects and induced resistance is not unique to savannas: associational refuges and induced defenses are found in plant communities from settings as diverse as coral reefs, pine forests, and agro-ecosystems. Future work should explicitly evaluate the generality of such patterns across ecosystems and continue to explore the molecular basis and ecological consequences of intraspecific variation in plant defense.

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Supporting Information