

Ecological and evolutionary consequences of tri-trophic interactions: Spatial variation and effects of plant density¹

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PREMISE OF STUDY: The factors driving variation in species interactions are often unknown, and few studies have made a link between changes in interactions and the strength of selection.

METHODS: We report on spatial variation in functional responses by a seed predator (SP) and its parasitic wasps associated with the herb *Ruellia nudiflora*. We assessed the influence of plant density on consumer responses and determined whether density effects and spatial variation in functional responses altered natural selection by these consumers on the plant. We established common gardens at two sites in Yucatan, Mexico, and planted *R. nudiflora* at two densities in each garden. We recorded fruit output and SP and parasitoid attack; calculated relative fitness (seed number) under scenarios of three trophic levels (accounting for SP and parasitoid effects), two trophic levels (accounting for SP but not parasitoid effects), and one trophic level (no consumer effects); and compared selection strength on fruit number under these scenarios across sites and densities.

KEY RESULTS: There was spatial variation in SP recruitment, whereby the SP functional response was negatively density-dependent at one site but density-independent at the other; parasitoid responses were density-independent and invariant across sites. Site variation in SP attack led, in turn, to differences in SP selection on fruit output, and parasitoids did not alter SP selection. There were no significant effects of density at either site.

CONCLUSIONS: Our results provide a link between consumer functional responses and consumer selection on plants, which deepens our understanding of geographic variation in the evolutionary outcomes of multitrophic interactions.

KEY WORDS divergent selection; functional response; herbivory; parasitism; plant density; spatial variation

Spatial variation in the strength of species interactions is widespread and responds to heterogeneity in multiple biotic and abiotic conditions (Levin, 1992; Thompson, 2005). Accordingly, a primary goal of ecological research has been to determine the causes of such variation and its effects on species traits, populations, and communities (Gripengberg and Roslin, 2007). In addition, whereas most of the work conducted thus far has addressed geographic variation in pairwise species interactions (Herrera and Pellmyr, 2002), an increasing number of studies have also documented spatial variation in multispecies interactions across two or more trophic levels

(Inouye and Stinchcombe, 2001; Strauss and Irwin, 2004). Such studies have contributed to a more realistic view and better understanding of complex interactions occurring in heterogeneous landscapes.

Spatial variation in species interactions frequently leads to concomitant variation in natural selection originating from such effects and is considered a primary driver of variation within and among species (Thompson, 2005). A number of studies have documented spatial variation in selection from both antagonistic (e.g., Kolb et al., 2007a; Züst et al., 2012) and mutualistic (e.g., Rudgers and Strauss, 2004; Chapurlat et al., 2015) pairwise interactions between plants and arthropods. Similarly, although less investigated, studies have also reported on spatial variation in multispecies interactions whereby selection among pairs of interacting species is modified by another species or by other features of the biotic or abiotic context, thus leading to indirect or diffuse selection (Strauss et al., 2005; terHorst et al., 2015). For instance, studies have shown

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that selection on plant traits by focal herbivores or pollinators varies spatially, depending on the presence of third-party species (e.g., Herrera et al., 2002; Siepielski and Benkman, 2004; Craig et al., 2007; Ågren et al., 2013). However, most of this work has been bi-trophic in nature, and much less is known about the evolutionary consequences of spatially variable species interactions across three or more trophic levels (Rudgers and Strauss, 2004; Estes et al., 2013).

Several features of plant communities or populations, such as patch size (e.g., Bach, 1988; Hambäck et al., 2010), species richness (e.g., Andow, 1991; Loreau et al., 2001), and abundance (Rausher and Feeny, 1980), frequently vary spatially (within or among populations) and lead to divergent outcomes in plant competitive interactions and interactions at higher trophic levels. Of these, variation in producer abundance or biomass is one of the most widespread and frequently studied features of terrestrial plant communities (Underwood and Halpern, 2012). Studies have frequently addressed such effects in terms of density (i.e., individuals per unit area) of a focal plant species while controlling for other features such as patch size and plant species richness (e.g., Shea et al., 2000; Hambäck et al., 2010). These investigations have demonstrated that variation in plant density can have strong effects on plant–plant interactions (e.g., competition) and plant traits, as well as on herbivory by leading to lower or higher damage on focal plants (i.e., resource concentration or dilution effects; Kim and Underwood, 2015). However, the extended consequences of interactions at higher trophic levels (i.e., host–enemy) have received less attention, so our understanding of these dynamics is incomplete (for related work, see Hambäck et al., 2012). Further, the evolutionary consequences of effects of variation in producer density on consumers are largely unknown (for related work, see Sletvold et al., 2013).

The effects of third-party species and plant density on plant–herbivore interactions may operate through changes in species abundances (Wootton, 1994), whereby greater plant biomass (e.g., with increasing plant density) or greater abundance of a third species influences herbivore abundance but the strength or function of plant–herbivore interactions remains unchanged (Mooney and Singer, 2012; Abdala-Roberts and Mooney, 2015). However, third-party species or plant density may also fundamentally alter the strength or function of plant–herbivore interactions through *interaction modifications* (*sensu* Wootton, 1994), which are typically mediated by changes in species traits (i.e., trait-mediated interactions; Schoener and Spiller, 2012) and lead to patterns that cannot be explained by abundance-driven effects alone. For example, plant density may influence herbivore recruitment to plant patches (and, in turn, rates of herbivory) through effects of habitat heterogeneity on herbivore foraging behavior and feeding (Hambäck et al., 2010) or through changes in plant traits influencing herbivores due to stronger plant–plant interactions (e.g., competition) with increasing plant density (Halpern et al., 2014). Similarly, the presence or behavior of predators may influence herbivore foraging patterns and, ultimately, rates of herbivory (Northfield et al., 2012). Nonetheless, most research thus far has failed to test for interaction modifications while controlling for resource abundance. This is a substantial handicap, because measuring changes in the function or strength of species interactions (e.g., across space or under different plant densities), rather than changes in species abundances per se, is ultimately needed to address the evolutionary consequences of species interactions (Benkman, 2013; Vanhoenacker et al., 2013).

Ruellia nudiflora is a short-lived perennial herb distributed throughout southern Mexico. Fruits of this species are attacked by a specialist seed-eating caterpillar (*Tripudia* sp., Noctuidae), which, in turn, is parasitized by wasps that reduce this herbivore's seed consumption (Abdala-Roberts et al., 2010). This seed predator typically exhibits a negative density-dependent functional response (Type II, *sensu* Holling, 1959) whereby the proportion of attacked fruits per plant decreases with increasing fruit output (Abdala-Roberts and Mooney, 2013, 2014), resulting in positive directional selection on reproductive output by this herbivore (Abdala-Roberts et al., 2014); parasitoids, in turn, weaken the selection differential by the seed predator on plant traits (Abdala-Roberts et al., 2014). Previous work has also shown extensive variation in seed predation and parasitism among *R. nudiflora* populations (Abdala-Roberts et al., 2010), and although this plant frequently grows in dense conspecific patches, local densities vary considerably within and among populations (Vargas-Mendoza et al., 2015). Here, we sought to evaluate the effects of *R. nudiflora* patch density on plant–seed predator and seed predator–parasitoid interactions at two sites previously known to differ in seed predation and parasitism (Abdala-Roberts et al., 2010; Moreira et al., 2015). To do so, we analyzed consumer functional responses by measuring the change in seed predator or parasitoid attack with increasing fruit or caterpillar density, respectively. Specifically, we addressed (1) whether there was spatial variation in seed predator and parasitoid functional responses, (2) whether plant density altered such functional responses, and (3) whether density and site effects on plant–seed predator and seed predator–parasitoid interactions led to concomitant changes in natural selection exerted by these consumers on the plant. In addressing these questions, the present study uniquely investigates the ecological and evolutionary consequences of spatial variation in tri-trophic interactions, and of bottom-up effects of resource density on consumers.

MATERIALS AND METHODS

Natural history—*Ruellia nudiflora* Engelm. and Gray Urb. (Acanthaceae) is a short-lived (life span: 2–3 yr) perennial herb distributed from southern Texas (USA) to Honduras (Long, 1977), and in Yucatán (southeast Mexico) it grows across a wide range of abiotic conditions (Ortegón-Campos et al., 2012). This species is self-compatible and produces chasmogamous (CH) flowers that have an open corolla and are visited by insect pollinators; and cleistogamous (CL) flowers that do not open, have reduced corollas, and self-pollinate obligately. Chasmogamous flowers may also self-pollinate autonomously upon corolla dehiscence when anthers come into contact with the stigma. Fruits are dry and dehiscent, and seeds disperse ballistically. Reproduction during the first year of life is strongly skewed toward CL reproduction (Abdala-Roberts and Mooney, 2013), whereas in subsequent years plants invest similarly in each reproductive function (Munguía-Rosas et al., 2012).

Both CH and CL fruits exhibit high levels of attack by larvae of an as-yet-identified species of moth (Lepidoptera: Noctuidae) in the genus *Tripudia*, which are dietary specialists on *Ruellia* (Moreira et al., 2015). Female moths oviposit on recently pollinated flowers and, unless parasitized, a single larva grows inside a developing fruit and usually consumes all the seeds prior to fruit dehiscence ($95 \pm 1.0\%$ of seeds consumed per fruit, on average; Abdala-Roberts and Mooney, 2014). Larval development takes place within a single

fruit, and larvae usually do not move among fruits (L. Abdala-Roberts, unpublished data). Previous work has shown that the proportion of fruits attacked decreases with increasing fruit output (i.e., negative density-dependent attack; Abdala-Roberts and Mooney, 2013, 2014). Seed predator larvae are in turn attacked by several species of parasitic wasps belonging to Braconidae (four species), Ichneumonidae (one), and Pteromalidae (two), as well as one fly species belonging to Tachinidae. Parasitoids stop or reduce consumption by seed predators, and the remaining, uneaten seeds, which are typically found in fruits with parasitized caterpillars, thus represent an indirect positive effect on plant fitness (i.e., a seed “rescue” effect; Abdala-Roberts et al., 2010). Seed predation and parasitism vary extensively across *R. nudiflora* populations, and both remain relatively stable across years in some of the populations studied (Abdala-Roberts et al., 2010; Moreira et al., 2015).

Experimental design and sampling—In mid-July 2015, we collected 10–15 CH fruits from each of 18 plants of two populations situated near the localities of San Pedro Chimay (20°52′12″N, 89°35′6″) and Conkal (21°4′23″N, 89°30′24″), 11 km south and 14 km north of Mérida (Yucatán, México), respectively. The distance between these localities was 25 km. All seeds from a given plant were considered a genetic family composed of a mixture of full- and half-sibs and were germinated immediately after collection. Because seeds rarely disperse >1 m from the parent plant (V. Parra-Tabla, unpublished data), we sampled mother plants that were ≥2 m apart to increase genetic variation among individuals. One month after germination (mid-August 2015), seedlings were transplanted to 1 L plastic bags filled with native soil and remained in a nursery for a 2 mo period under homogeneous environmental conditions to reduce maternal effects. During this period, each plant was fertilized once with 40 mL of a solution (Ferticoral, Impulsora Agroquímica del Sureste, Mexico) containing nitrogen (20%), phosphorus (30%), and potassium (10%) at a concentration of 2 g L⁻¹.

In late October 2015, plants from seed collected at San Pedro Chimay were transported to a site located 9 km southwest of that source locality (20°47′24″N, 89°37′10″W; hereafter “Aldana”); and plants from seeds collected at Conkal were transported to a site located 6 km southwest of that locality (21°1′27″N, 89°33′15″W; hereafter “Vivero”). In this way, plants from each population were transplanted to nearby sites with biotic and abiotic conditions similar to each corresponding source locality (logistic constraints prevented transplanting plants at the same source locality). Populations sampled within the vicinities of the two experimental sites exhibited marked differences in seed predation and parasitism during previous years (Abdala-Roberts et al., 2010; Moreira et al., 2015), which would lead, presumably, to contrasting selection by consumers on plant traits—and, thus, an opportunity to test for spatially divergent selection. However, we caution that the degree of inter-annual variation in consumer attack rates at these sites is unknown, and this may alter the ecological and evolutionary outcomes of species interactions among sites. In addition, sites vary in temperature seasonality and in soil characteristics, which may influence plant growth and reproduction and, in turn, seed predator and parasitoid recruitment (see Supplemental Data with this article; Appendix S1). At each site, we established a common garden where vegetation (herbs and grasses) was previously cleared and where we planted 24 1 × 1 m plots. Plot size and distance among plots were chosen on the basis of previous work showing that these design

features provide realistic conditions where foraging moths differentiate among plots (Abdala-Roberts and Mooney, 2014; Cuautle and Parra-Tabla, 2014). We established plots with either 9 or 16 plants (low and high density, respectively; $n = 12$ plots in each case; $n = 300$ plants site⁻¹). These density levels are typical for natural populations of *R. nudiflora* (natural range: 4–20 plants m⁻²; Vargas-Mendoza et al., 2015). Low- and high-density plots were randomly interspersed throughout each study site. Distance among adjacent plants was 0.5 and 0.33 m in low- and high-density plots, respectively. Distance among adjacent plots was 1.5 m on average. Although genotype composition was randomly assigned to each plot, we kept genotype number constant across all plots ($n = 9$ of 18 possible genotypes within each plot). Genotypes were similarly represented across plots within each site (range: 11–16 occurrences genotype⁻¹). Plots were watered twice a week during the experiment.

Once a week, from early December 2015 to late January 2016, we collected all mature fruits produced per plant and dissected them under a stereoscope to record seed predator and parasitoid attack, as well as seed number. For each plant, we summed the numbers of fruits sampled, fruits attacked by the seed predator (i.e., number of seed predators), and attacked fruits with a parasitoid (i.e., number of parasitized herbivores) and then calculated mean values per plot for statistical analyses. Calculations were based on fruit and insect abundance data pooled across all sampling periods; we also pooled specimens across species in the case of parasitoid abundance (>90% cases with 1 parasitoid caterpillar⁻¹). More than 95% of fruits collected were CL, and results did not change if we excluded CH fruits from the analyses; therefore, we did not differentiate between fruit types. As a result, and because CL fruits obligatorily self-pollinate, site or density effects on fruit initiation and output in this study were not influenced by pollinators. In late January 2016, we estimated plant size in terms of area (i.e., cover) by multiplying the plant’s largest diameter (from tip to tip of the largest pair of opposite leaves) by the plant’s smaller diameter, perpendicular to the former.

Testing for plant density effects and site variation in interactions

To test for site variation and density effects on plant growth and reproduction, we performed general linear models that included the effects of site (fixed), density (fixed), and their interaction on plant size and fruit number using mean values per plot (average across plants within each plot). An influence of density on growth or reproduction points to potential effects of interactions among neighboring plants at high density (e.g., competition, facilitation), whereas site effects are suggestive of site-specific differences in productivity associated with biotic (soil microorganisms) or abiotic (water availability, soil type) conditions (Ortegón-Campos et al., 2012).

To test for plant density effects on and spatial variation in interactions, we ran models for seed predator and parasitoid abundance using plot-level mean values, which included the main effects of density and site, as well as additional terms to describe consumer functional responses and the dependency of such responses on such factors (see below). After careful inspection of consumer functional responses at each site, we found that recruitment of seed predators to fruits at Aldana was a nonlinear relationship estimated by a quadratic regression model in which the linear ($t = 24.04$, $P < 0.0001$) and quadratic ($t = -2.22$, $P = 0.037$) terms of fruit number were significant predictors of seed predator abundance and in which

a quadratic model had a better fit than a linear model (AIC linear = 48.8, AIC quadratic = 45.7; a model with lower AIC values [$\Delta\text{AIC} > 2$] is considered a better-fitting model; Burnham and Anderson, 2002). By contrast, seed predator recruitment at Vivero was best described by a linear relationship because the quadratic term for fruit number was not significant ($t = 1.65$, $P = 0.12$) in the quadratic model, and both models exhibited a similar fit (AIC linear = 10.6, AIC quadratic = 9.7). Following the same criteria, parasitoid recruitment to seed predators was best described by a linear model at both sites, so the quadratic term was dropped ($\Delta\text{AIC} < 1$ in both cases). Based on these results, for the seed predator model we included the linear and quadratic terms of fruit number to test for linear and nonlinear responses (respectively) to resource abundance, given that nonlinearity was detected at one site, as well as two-way interactions of the linear and quadratic terms of fruit number with site and density. These interactions tested for a change in the function of plant–seed predator interactions across sites or levels of plant density after accounting for fruit number (i.e., an interaction modification). Including the quadratic term of fruit number tests whether the relationship between fruit number and seed predator number is nonlinear, having accounted for the linear relationship based on the linear term for fruit number. Similarly, the parasitoid abundance model was similar to that for seed predators, except that instead of fruit number we included the linear term of seed predator number, which is the resource to which these consumers respond, as well as the two-way interactions of seed predator number with density and site. We did not include the quadratic term of seed predator number, because parasitoid recruitment was linear at both sites (see above). We had previously removed the site \times density interaction from both models because it was nonsignificant ($F \leq 1.21$, $P \geq 0.27$). Likewise, the three-way interactions between fruit number or fruit number², site, and density (seed predator model) and between seed predator number, site, and density (parasitoid model) were nonsignificant ($F \leq 2.09$, $P \geq 0.16$), indicating that density effects remained consistent across sites, and were thus also previously removed to simplify the models.

All models were performed with PROC MIXED in SAS version 9.2 (SAS Institute, Cary, North Carolina, USA), under the assumption of normally distributed residuals, which was met in all cases. We report least-square means (\pm SE) as descriptive statistics.

Testing for evolutionary effects of site variation and plant density—Previous work with *R. nudiflora* has shown that changes in the function of density-dependent recruitment to fruits by the seed predator (i.e., functional response) leads to concomitant changes in the strength or mode of selection by this herbivore on fruit number (Abdala-Roberts and Mooney, 2014). Thus, given that fruit number is a component of fitness and is expected to be under positive directional selection, changes in the function of seed predator recruitment between sites or between low and high planting densities should lead to differences in the strength of selection by the seed predator on fruit output. In addition, based on previous work, we also expected that parasitoids would, in turn, influence such patterns of selection by the seed predator (Abdala-Roberts et al., 2014). Substantial variation among plant families was found for fruit number at both sites (Aldana: 2.3-fold, $F_{17, 257} = 4.42$, $P < 0.0001$; Vivero: 3.5-fold, $F_{17, 259} = 11.83$, $P < 0.0001$; data from present study), which indicates ample potential for natural selection to act on this trait. Trade-offs between chasmogamous and cleistogamous reproduction have been reported previously for this species and

may contribute to maintain variation in reproductive output (Abdala-Roberts et al., 2014). Likewise, temporal variation in the strength of seed predator selection is also expected to contribute to variation in fruit output, as well as variation in parasitoid attack, given that parasitoids have been shown to dampen seed predator selection on this trait (Abdala-Roberts et al., 2014).

Plant fitness under different trophic scenarios—To test for effects of site variation and density on consumer selection, it is necessary to first separate the selective effects of seed predators and parasitoids on fruit number and then to determine which of these consumers are influenced by these factors and how. Experimentally excluding seed predators and parasitoids to test for their individual and combined effects on plant fitness is not feasible; seed predators cannot be excluded without affecting pollinators (in the case of CH flowers), and parasitoids cannot be excluded without affecting seed predators and pollinators. Nonetheless, it is feasible to measure the impacts of these consumers without experimental manipulation by estimating plant fitness under different “trophic scenarios” (Abdala-Roberts and Mooney, 2014; Abdala-Roberts et al., 2014). The nature of this system allows for measuring the impacts of seed predators and parasitoids without experimental manipulation, for two reasons. First, unparasitized seed predators consume virtually all seeds in a fruit, whereas parasitized seed predators do not. The seeds remaining in a fruit following parasitoid attack (i.e., seed rescue) thus represent a good proxy for parasitoid indirect effects on plant fitness. Second, undamaged fruit size is correlated with seed number (Abdala-Roberts et al., 2014), and there is no difference in size between fruits attacked and those not attacked by seed predators (Abdala-Roberts and Mooney, 2013). Based on this, seed number in fruits not attacked can be used to estimate potential seed number (i.e., fitness) in the absence of seed consumption and, therefore, the number of seeds lost to seed predators.

First, we estimated potential seed number in the absence of seed predator effects (*mono-trophic* scenario) by multiplying the number of fruits produced per plant (sum of weekly counts) by the corresponding plant family mean for seed number in fruits that were not attacked (fruit size is correlated with seed number and there is no detectable difference in the size of fruits attacked and not attacked by seed predators; Abdala-Roberts and Mooney, 2013). Second, fitness in the presence of seed predators alone (*bi-trophic* scenario) was estimated by assuming that all seeds rescued by parasitoids were instead lost to herbivory (i.e., potential seed number—seeds consumed assuming no seed rescue). Third, the observed number of seeds per plant represented fitness in the presence of both seed predators and parasitoids (*tri-trophic* scenario). For each site, we calculated family means for seed number (i.e., fitness) under each trophic scenario, separately for each level of density, and then calculated relative fitness by dividing these values by the mean fitness across families specific to each density and trophic scenario. Based on this approach, we were able to estimate selection on fruit number in the absence of consumer effects (mono-trophic), as well as seed predator selection (comparing mono- vs. bi-trophic) and parasitoid effects on seed predator selection (comparing bi- vs. tri-trophic) on this trait. Seed number is a good predictor of *R. nudiflora* fitness because it is highly correlated with percent germination, which is typically $>90\%$ in this species (Ortegón-Campos et al., 2012). We used plant family means in all cases because genotypic selection analysis avoids environmentally induced correlations between measured and unmeasured traits, which may occur with

estimates of plant fitness in phenotypic selection analyses (Stinchcombe et al., 2002). In calculating plant fitness estimates, an important assumption is that resources are not diverted away from damaged fruits, because this might result in an overestimation of seed predator impacts on plant fitness given that plants divert resources from damaged fruits toward undamaged fruits or subsequent fruit production. Accordingly, data from the present study as well as from previous work (Abdala-Roberts and Mooney, 2014; Abdala-Roberts et al., 2014) indicated that resource reallocation was not present or was weak at most.

Selection analyses—We estimated directional selection on fruit number by means of simple linear regression in which relative fitness was predicted by fruit number (Lande and Arnold, 1983) and ran models under each trophic scenario separately for each site and density, for a total of 12 regressions. We also reran these regressions including the quadratic term of fruit number to test for stabilizing and disruptive selection, but there was no evidence of such selection and therefore we focused exclusively on directional selection. We calculated standardized selection gradients ($\beta\sigma$) by multiplying original slope values by the population standard deviation for fruit number specific to each site and provide standard errors in each case (Stinchcombe, 2005).

After running the univariate selection models, we ran analysis of covariance (ANCOVA) models to test for changes in selection by consumers across sites and levels of plant density. First, we performed an ANCOVA across all sites, densities, and trophic scenarios using the full data set, modeling relative fitness as dependent on fruit number, trophic scenario, site, density, all two-way interactions, and the three-way interactions between fruit number, trophic scenario, and site and between fruit number, trophic scenario, and density. The site \times density \times trophic scenario interaction and the four-way interaction were not included, to avoid making the models overly complex and to facilitate model convergence. We also reran the ANCOVA model including the quadratic term of fruit number and its interactions with site, density, and trophic scenario, but there was no evidence of nonlinear selection and we therefore focused only on the former model testing for directional selection. The two-way interactions of fruit number with trophic scenario, site, and density tested for changes in strength of directional selection between trophic scenarios (i.e., selective effects of seed predators and/or parasitoids), sites, or levels of density, respectively. However, of highest relevance were the three-way interactions (1) fruit number \times trophic scenario \times site and (2) fruit number \times trophic scenario \times density, which tested whether selection by seed predators and/or parasitoids changed between sites or levels of plant density. Briefly, the results of this ANCOVA showed a significant fruit number \times trophic scenario \times site interaction, but no fruit number \times trophic \times density interaction (Appendix S3). Therefore, we focused on and further investigated the specific changes in consumer selection across sites (but not densities). First, we tested for differences in selection gradients among trophic scenarios separately within each site by running component ANCOVAs (based on subsets of the full data set), which compared pairs of trophic scenarios at a time. For each site, we modeled relative fitness as dependent on fruit number, trophic scenario, and the fruit number \times trophic scenario interaction to test for selective effects of seed predators and parasitoids. Second, we tested for changes in selection across sites separately under each trophic scenario by comparing pairs of trophic scenarios at a time and modeled relative fitness as

dependent on fruit number, site, and the fruit number \times site interaction. In both cases, we included density as a fixed effect to account for this factor in the model.

For the sake of simplicity, we only report results for the interaction terms (not for main effects) from the ANCOVA models testing for pairwise differences in consumer selection within each site and for site differences in selection under each trophic scenario. In addition, ANCOVA models for the full data set and component ANCOVAs comparing trophic scenario gradients within each site included the effect of plant family (treated as random) to control for using multiple estimates of fitness for each family (one per trophic scenario). In all cases, data were normally distributed. ANCOVA and regression models were performed with PROC MIXED and PROC REG (SAS 9.2), respectively.

RESULTS

Site and density effects on plant growth and reproduction—A total of 4271 fruits were collected during the study (Vivero, $n = 3143$; Aldana, $n = 1128$). There was no effect of density on either plant size or fruit output, but there were site differences in fruit output (Appendix S2): mean fruit number per plot was $2.8\times$ greater at Vivero (10.48 ± 0.67 fruits) than at Aldana (3.75 ± 0.44). Plant size did not vary between sites (Appendix S2), and there were no interactive effects of site and density on plant size or fruit output (Appendix S2).

Site and plant density effects on consumer interactions—**Seed predators**—We recorded a total of 3771 seed predator larvae (88% of fruits sampled were attacked). We found a marginally significant fruit number \times site interaction and a significant fruit number² \times site interaction (Table 1), indicating that the linear and nonlinear functions (respectively) of seed predator recruitment differed between sites (albeit weakly in the former case). Consistent with previous work in this system, we found a saturating recruitment function of the seed predator to fruits at Aldana (seed predators = $-0.027*\text{fruit number}^2 + 1.10*\text{fruit number} - 0.21$; Fig. 1A). By contrast, at Vivero the functional response was linear (seed predators = $0.87*\text{fruit number} + 0.08$; Fig. 1B). When expressed in fractional

TABLE 1. Results from general linear models testing for effects of site and *Ruellia nudiflora* density on plant–seed predator and seed predator–parasitoid interactions (i.e., interaction modifications). Significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.10$) results are in bold and italics, respectively. SP = seed predator.

Response variable	Source	df	F	P
SP number	Site (S)	1, 39	3.02	<i>0.090</i>
	Density (D)	1, 39	0.05	0.822
	Fruit number	1, 39	20.85	<0.0001
	Fruit number ²	1, 39	0.18	0.675
	Fruit number*D	1, 39	0.02	0.900
	Fruit number*S	1, 39	3.64	<i>0.063</i>
	Fruit number ² *D	1, 39	0.11	0.745
	Fruit number ² *S	1, 39	4.13	0.048
	Parasitoid number	Site (S)	1, 42	0.52
Density (D)		1, 42	0.14	0.707
SP number		1, 42	296.21	<0.0001
SP number*D		1, 42	0.01	0.926
SP number*S		1, 42	0.85	0.361

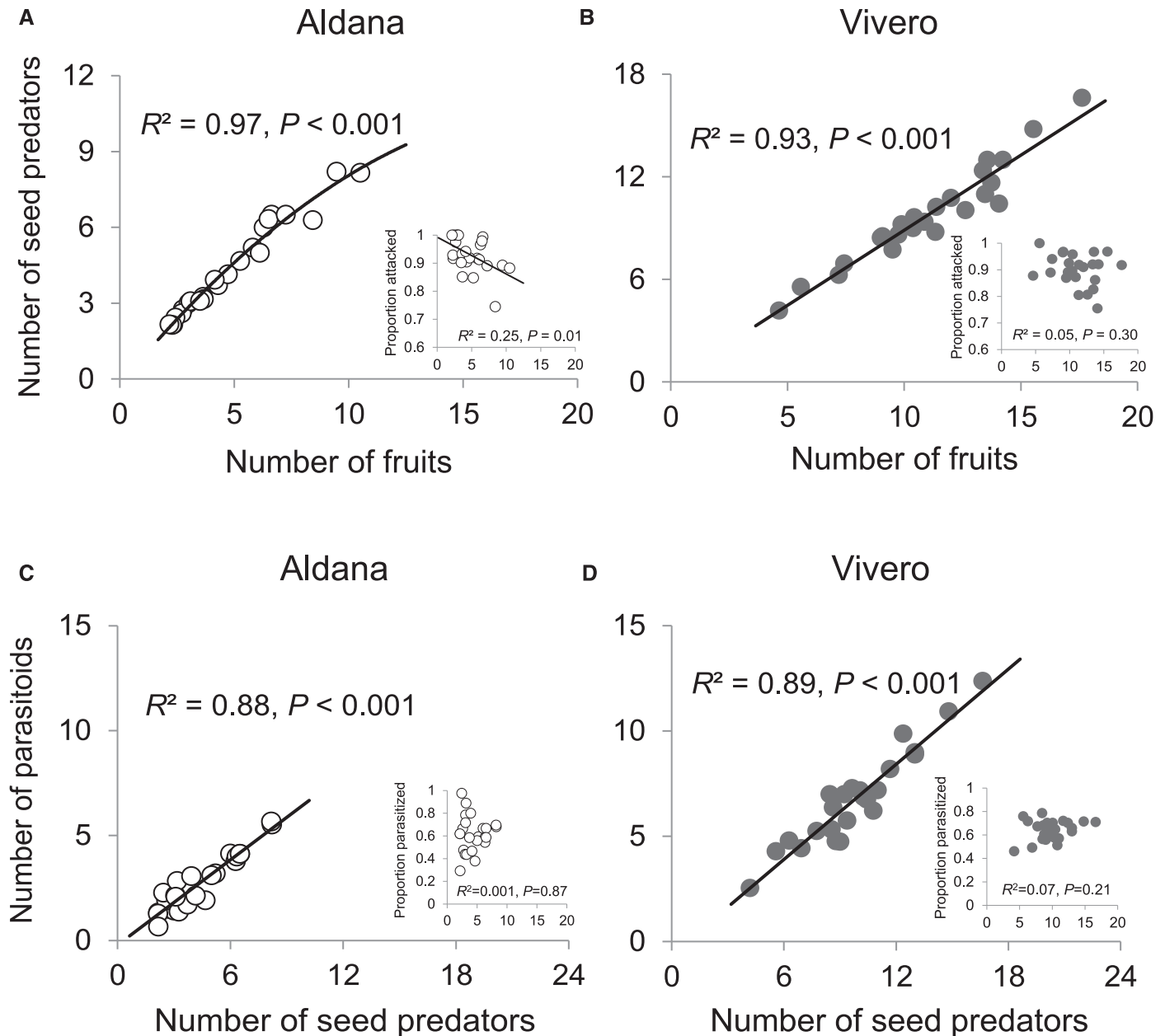


FIGURE 1 Site-specific relationships (A, B) between the number of fruits produced by *Ruellia nudiflora* and seed predator abundance and (C, D) between seed predator abundance and parasitoid abundance at our two study sites, Aldana and Vivero. Predicted relationships are based on quadratic (A) or linear (B–D) regression models derived from inspection of model fit and the significance of the quadratic term in each case. Note the difference in the scale of the y-axis for panels A and B. Inset graphs in A and B depict fractional responses between the number of fruits and the proportion of attacked fruits. Inset graphs in C and D depict fractional responses between the number of seed predators and the proportion of parasitized seed predators. Dots represent plot-level means ($n = 24$ site⁻¹). The fractional response shown in inset A remained significant after removing the lowest value for proportion of attacked fruits.

terms, the proportion of attacked fruits at Aldana decreased linearly with increasing fruit number, which is the hallmark of negative density-dependence (proportion of attacked fruits = $-0.013 \times \text{fruit number} + 0.99$; Fig. 1A, inset), whereas at Vivero the relationship was not significant, indicating no density-dependence (proportion of attacked fruits = $-0.004 \times \text{fruit number} + 0.94$; Fig. 1B, inset). Contrary to expectations, plant density did not influence the function of seed predator recruitment to fruits (high density:

seed predators = $0.840 \times \text{fruit number} + 0.270$ [Appendix S4, panel A]; low density: seed predators = $0.889 \times \text{fruit number} + 0.108$ [Appendix S4, panel B]) as indicated by nonsignificant fruit number or fruit number² \times density interactions (Table 1).

Parasitoids—We recorded a total of 2548 parasitoid specimens (68% of seed predator larvae were parasitized). There was no evidence of site variation in the function of seed predator–parasitoid

interactions; the linear function of parasitoid recruitment to seed predators was similar at both Aldana (parasitoid number = $0.675 \times \text{seed predator number} - 0.217$; Fig. 1C) and Vivero (parasitoid number = $0.753 \times \text{seed predator number} - 0.62$; Fig. 1D), as shown by a nonsignificant site \times seed predator number interaction in each case (Table 1). When expressed in fractional terms, there was no relationship between seed predator number and the proportion of parasitized seed predators at either site (Fig. 1C and D, insets; parameter estimates not shown), indicating a consistently density-independent response by parasitoids. In addition, we found no effect of plant density on the function of parasitoid recruitment to seed predators (high density: parasitoid number = $0.739 \times \text{seed predator number} - 0.39$ [Appendix S4, panel C]; low density: parasitoid number = $0.735 \times \text{seed predator number} - 0.55$ [Appendix S4, panel D]), as indicated by a nonsignificant density \times seed predator number interaction (Table 1).

Evolutionary effects of site variation and plant density—We found positive directional selection on fruit number at both sites and levels of plant density under all three trophic scenarios, though selection gradients were generally stronger at Aldana than at Vivero (Table 2). At Aldana, the bi-trophic selection gradient tended to be stronger than the mono-trophic gradient, whereas the tri-trophic and the bi-trophic gradients were similar (Table 2). By contrast, selection at Vivero was similar under all trophic scenarios, except for the nonsignificant bi-trophic selection gradient at low density (Table 2). On the other hand, selection gradients were similar between levels of plant density at both sites (Table 2). These findings agree with the ANCOVA model based on the full data set, indicating site differences in consumer selection but no effect of density on such dynamics (Appendix S3; also see Methods). We further assessed spatial variation in consumer selection by means of component ANCOVAs comparing selection gradients among trophic scenarios within each site, as well as across sites under each trophic scenario.

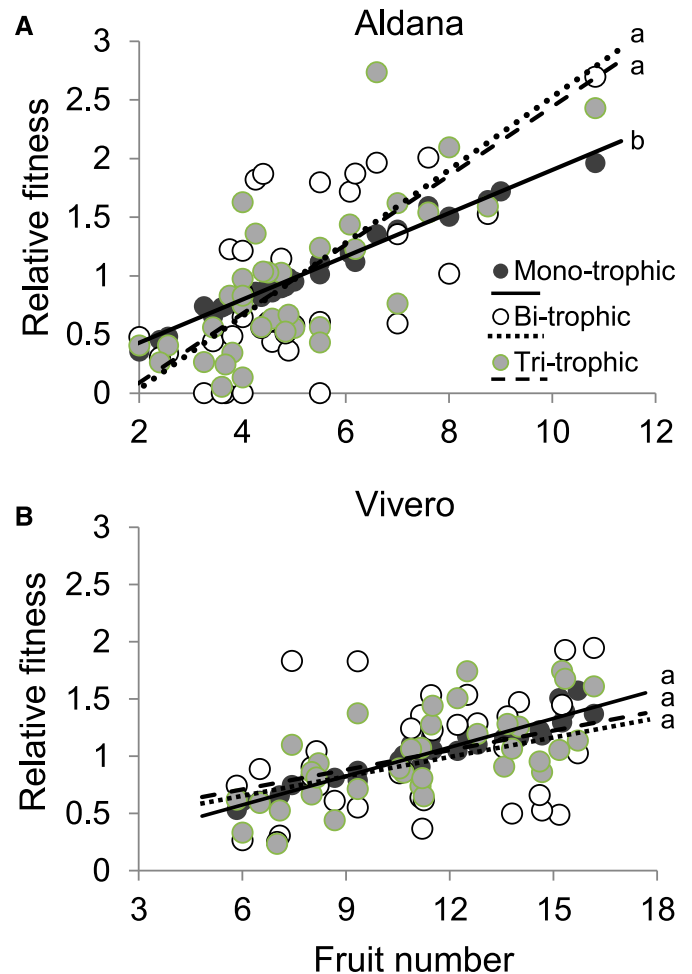
Aldana—In comparing the mono- and bi-trophic scenarios, we found that accounting for seed predator effects resulted in a significant

TABLE 2. Selection on *Ruellia nudiflora* fruit number at each study site and planting density, under different “trophic” scenarios: mono-trophic (M) selection on fruit number assumes no seed predator or parasitoid effects on seed output; bi-trophic (B) selection accounts for seed predation but not seed rescue by parasitoids; and tri-trophic (T) selection accounts for both seed predator and parasitoid effects on seed number. Both raw (β) and standardized (β_{σ}) selection gradients (\pm SE) are reported, as well as model R^2 values.

Site	Density	Selection	β	β_{σ}	R^2
Aldana	High	M	$0.183 \pm 0.004^{**}$	0.834 ± 0.108	0.98
		B	$0.243 \pm 0.044^{**}$	1.108 ± 0.200	0.65
		T	$0.255 \pm 0.024^{**}$	1.162 ± 0.109	0.87
	Low	M	$0.191 \pm 0.005^{**}$	0.870 ± 0.022	0.98
		B	$0.453 \pm 0.117^{**}$	2.065 ± 0.533	0.49
		T	$0.370 \pm 0.093^{**}$	1.687 ± 0.424	0.51
Vivero	High	M	$0.084 \pm 0.006^{**}$	0.638 ± 0.045	0.93
		B	$0.121 \pm 0.023^{**}$	0.919 ± 0.174	0.63
		T	$0.116 \pm 0.019^{**}$	0.881 ± 0.144	0.69
	Low	M	$0.083 \pm 0.006^{**}$	0.630 ± 0.045	0.92
		B	-0.008 ± 0.041	0.061 ± 0.311	0.003
		T	$0.056 \pm 0.024^*$	0.425 ± 0.182	0.25

* $P < 0.05$, ** $P < 0.001$.

$1.8 \times$ increase in the magnitude of directional selection, indicating that seed predators strengthened selection on fruit number (significant fruit number \times trophic scenario interaction, mono- vs. bi-trophic comparison; $F_{1,50} = 5.01$, $P = 0.029$; Fig. 2A). However, we



Trophic scenario	Site comparison
Mono-trophic	Aldana > Vivero
Bi-trophic	Aldana > Vivero
Tri-trophic	Aldana > Vivero

FIGURE 2 Site-specific (A = Aldana, B = Vivero) patterns of directional selection on *Ruellia nudiflora* fruit output under three trophic scenarios: in the absence of seed predator and parasitoid effects (*mono-trophic*), accounting for seed predator effects but not parasitoid seed rescue (*bi-trophic*), and accounting for both seed predator and parasitoid effects (*tri-trophic*, i.e., observed fitness). Note the difference in scale of the x-axis between panels. Different letters next to each regression line indicate significant differences ($P < 0.05$) between trophic selection gradients within each site. The legend below the panels specifies differences in the strength of directional selection between sites for each trophic scenario (in all cases selection was stronger at Aldana). Dots are genotype means ($n = 18$ site $^{-1}$). Analyses were conducted using genotype means for low and high density at each site, and we included density in each model to account for this effect.

found that the bi- and tri-trophic selection gradients did not differ ($F_{1,50} = 0.20$, $P = 0.658$), indicating that parasitoids did not alter selection by seed predators (Fig. 2A). The mono- and tri-trophic selection gradients differed significantly ($F_{1,50} = 6.52$, $P = 0.013$; Fig. 2A), also showing that parasitoids did not dampen seed predator selection as this would have made the mono- and tri-trophic selection gradients similar.

Vivero—We found that selection under the bi-trophic scenario did not differ from selection under the mono-trophic scenario ($F_{1,50} = 0.95$, $P = 0.334$), indicating that the seed predator did not strengthen selection on fruit number (Fig. 2B). In addition, the bi- and tri-trophic gradients did not differ, which means that parasitoids did not alter seed predator selection ($F_{1,50} = 0.65$, $P = 0.423$; Fig. 2B), and the mono- and tri-trophic gradients did not differ either ($F_{1,50} = 0.06$, $P = 0.815$; Fig. 2B).

Between-site comparison—We found that positive directional selection on fruit number was significantly stronger at Aldana than at Vivero under all three trophic scenarios ($F_{1,42} \geq 19.06$, $P \leq 0.001$; Fig. 2A, B).

DISCUSSION

We found spatial variation in plant–seed predator interactions, whereby the function of seed predator recruitment to *R. nudiflora* fruits was negatively density-dependent (saturating) at Aldana but density-independent at Vivero. By contrast, the function of parasitoid recruitment to caterpillars was density-independent and this response remained unchanged across sites. Such differences in the seed predator’s functional response led, in turn, to site differences in seed predator selection on fruit output, such that this herbivore selected for increased fruit output at Aldana but did not exert selection on this trait at Vivero. These findings thus provide a direct link between changes in herbivore functional responses and herbivore selection on plant traits, which contributes to explain spatial variation in the evolutionary outcomes of this interaction. By contrast, parasitoids did not alter seed predator selection and this finding was consistent between sites, showing that the third trophic level did not contribute to spatial variation in plant–seed predator evolutionary dynamics. On the other hand, we found no evidence of plant density effects on the function of these interactions, and this result was consistent across sites, suggesting that other unmeasured biotic or abiotic factors are responsible for within- and between-site variation in herbivore and parasitoid functional responses and their effects on plant fitness. Together, these results provide unique evidence of the evolutionary consequences of spatial variation in tri-trophic interactions and offer a powerful framework for describing and predicting such dynamics.

Spatial variation in tri-trophic interactions and effects of plant density—We found that the function of seed predator recruitment to fruits at Aldana resembled a Type II functional response (*sensu* Holling, 1959), whereby the proportion of attacked fruits decreased with increasing fruit output. This result is in agreement with previous work showing that this herbivore forages in a negative density-dependent manner (Abdala-Roberts and Mooney, 2013, 2014). However, at Vivero the seed predator exhibited a density-independent response in which the proportion of attacked

fruits was unrelated to fruit output. These findings indicate fundamental differences across sites in the function of plant–seed predator interactions, which are expected to lead to spatially divergent ecological (and evolutionary) outcomes. In addition, we found that parasitoids recruited to seed predators in a density-independent fashion, which also supports previous findings (Abdala-Roberts and Mooney, 2013, 2014); and, contrary to seed predators, such response was consistent across sites. These results suggest that parasitoids are less sensitive to biotic or abiotic factors driving site variation in seed predator responses. More broadly, these findings emphasize the value of using functional responses to describe changes in the strength of species interactions (Abdala-Roberts and Mooney, 2015). However, determining which site-specific biotic or abiotic factors influence consumer functional responses is key to achieve a predictive understanding of these ecological dynamics (Kolb et al., 2007b; Sletvold et al., 2013).

Although previous work has shown that plant density is an important driver of plant–herbivore interactions (e.g., Hambäck et al., 2010; Halpern et al., 2014; Kim and Underwood, 2015), there was no detectable influence of *R. nudiflora* density on either seed predator or parasitoid functional responses. The absence of density effects may be explained in two nonexclusive ways. First, density did not influence plant growth or reproductive output, suggesting weak effects on plant–plant physical interactions, which therefore likely precluded density effects on consumers mediated by changes in plant traits (Kim and Underwood, 2015). Second, density presumably did not influence habitat (patch-level) heterogeneity, another important source of variation in species interactions leading to resource concentration or dilution effects (Kim and Underwood, 2015). By contrast, previous work has shown that increasing patch-level genotypic diversity in *R. nudiflora* influences the function of seed predator recruitment to fruits (Abdala-Roberts and Mooney, 2014), presumably due to an increase in patch-level (physical or plant-based) heterogeneity influencing seed predator behavior (Cuautle and Parra-Tabla, 2014). Although our findings suggest that *R. nudiflora* density has weak effects on tri-trophic interactions in this system, it is important to note that we included only two levels of plant density, whereas recent work has emphasized testing for multiple levels of plant density to fully address these effects on consumers (Kim and Underwood, 2015). Therefore, further work is needed to robustly evaluate the nature and magnitude of plant density effects in this system.

It is also worth noting that seed predation was extremely high at both sites (percentage of fruits attacked: Aldana, 92%; Vivero, 89%), and for the first time in 10 yr studying this system, we found evidence of density-independent seed predator recruitment to fruits (at Vivero). In addition, we also found that seed predator density-dependence at Aldana was weaker (i.e., less satiation and stronger herbivore recruitment with increasing fruit number) compared with previous work (Abdala-Roberts and Mooney, 2013, 2014). Based on this, it is possible that effects of habitat features such as (but not restricted to) plant density acting at small (patch-level) spatial scales become less important when the system is “saturated” with herbivores. In turn, the fact that there were site differences in the seed predator’s functional response (even under consistently high seed predation), suggests that there are other factors, operating at larger spatial scales, that drive site variation in these interactions. For instance, previous work in this system has shown that soil fertility influences the seed predator’s functional response (Abdala-Roberts et al., 2014) and could play an important role in

driving site variation in plant–seed predator interactions (for examples with biotic factors, see Kolb et al., 2007a; Sletvold et al., 2013).

Spatial variation in tri-trophic evolutionary dynamics—During the past two decades, evidence has mounted for spatial variation in the evolutionary outcomes of species interactions (reviewed by Thompson, 2005; Kolb et al., 2007a). However, many of these studies have failed to address the mechanisms driving changes in species interactions and their evolutionary effects. In this sense, our results provide a direct link between changes in the function of consumer recruitment to resources and concomitant variation in the strength of selection exerted by such consumers. Changes in consumer functional responses through interaction modifications represent a major driver of evolutionary change from species interactions (Mooney and Singer, 2012; Abdala-Roberts and Mooney, 2015), and our findings emphasize the value of using functional responses for understanding and predicting the evolutionary consequences of spatial variation in species interactions (for theoretical work, see Abrams, 2000).

We found that site differences in the function of plant–seed predator interactions resulted in parallel differences in the selective effects of the seed predator on *R. nudiflora* fruit output. By contrast, plant density did not alter the seed predator’s functional response and therefore had no influence on consumer selection on the plant. In comparing study sites, our results indicated that seed predators at Aldana recruited in a density-dependent manner whereby the proportion of attacked fruits decreased with increasing fruit output and, therefore, that seed predators strengthened positive selection on fruit number (see also Abdala-Roberts and Mooney, 2013, 2014). Although the degree of density-dependence at this site was moderate in relation to previous work (i.e., weaker saturation in consumer recruitment with increasing fruit output), these findings suggest that even small variations in the seed predator’s functional response can result in substantial changes in selection by this herbivore. Previous studies have similarly found positive directional selection by seed predators on reproductive output (e.g., Leimu et al., 2002), whereas others have shown opposite trends in which positive density-dependent attack by herbivores selects for smaller or intermittent fruit output (e.g., Miller et al., 2008; see studies in Elzinga et al., 2007). By contrast, at Vivero seed predators did not exhibit density-dependence (i.e., the proportion of attacked fruits was independent of fruit number), and the herbivore therefore did not strengthen selection on fruit number (i.e., no fitness gain of increasing fruit output in the presence of herbivore attack). Considering that *R. nudiflora* is short-lived (life span: 2–3 yr), these patterns are strongly correlated with lifetime selection on reproductive output, in that we documented a third to half of total fruit output for the individuals sampled (Abdala-Roberts et al., 2014). We speculate that site differences in abiotic stress and resource availability could have influenced fruit output or fruit traits, which, in turn, influenced the seed predator’s functional response and ultimately led to site differences in selection. For example, lower maximum temperatures (i.e., less abiotic stress; Appendix S1) and higher values for soil variables correlated with soil fertility (e.g., cation exchange capacity; Appendix S1) at Vivero compared with Aldana could have resulted in greater fruit output at the former site. Consistent with this, previous work has shown that increased soil fertility boosts fruit output and weakens density-dependent recruitment by the seed predator (Abdala-Roberts et al., 2014).

Nonetheless, we have yet to perform multifactorial manipulations to determine the specific drivers of site differences in the seed predator’s functional response.

There were no detectable effects of parasitoids on seed predator selection on *R. nudiflora* fruit output. This result runs counter to previous work in this system showing that parasitoids dampen seed predator selection (Abdala-Roberts et al., 2014) and suggests that parasitoid effects on plant–seed predator evolution are labile and presumably also context-dependent. We speculate that parasitoid effects were weak because of the presence of an unidentified species of Pteromalidae, which was particularly abundant during this study. These wasps exerted a weaker seed-rescue effect than wasps in the genus *Bracon*, which were also abundant during this study but not as abundant as in previous studies (Abdala-Roberts and Mooney, 2013, 2014). Differences in parasitoid life-history traits may result in variation in the magnitude of top-down indirect effects on plant fitness. For example, *Bracon* species in our system are koinobionts, which prevent any further development of the host after parasitization, whereas other species attacking the seed predator are idiobionts, which allow the host to continue developing and feeding (Abdala-Roberts et al., 2010). Assuming that the Pteromalidae species recorded is an idiobiont (which we ignore), this would explain its weak indirect effects on seed output. These aspects emphasize the influence of differences in consumer species composition as a driver of spatial variation in species interactions and their evolutionary outcomes (Thompson, 2005; Gómez et al., 2009).

Although our findings indicate spatially divergent outcomes of selection from species interactions, the longer-term evolutionary implications of these patterns may be contingent on the degree of temporal variation in interaction strength within sites (Schemske and Horvitz, 1989; Caruso et al., 2003; Campbell and Powers, 2015). Previous work in this system suggests that, at least for some of the populations sampled, levels of seed predation and parasitism remain relatively consistent across years, and this includes sites that are near the seed sources and experimental sites used in the present study (Abdala-Roberts et al., 2010; Moreira et al., 2015). However, we have not previously measured functional responses in these nearby populations or at the experimental sites and thus lack sufficient information to predict long-term evolutionary outcomes from these interactions (Vanhoenacker et al., 2009). Further work conducted over multiple years and across a larger number of sites is needed to quantify spatial and temporal sources of variation in seed predator, as well as parasitoid functional responses and the potential for geographic mosaics of selection in this tri-trophic system.

CONCLUSIONS

Our findings emphasize the value in using functional responses to describe species interactions and associated changes in natural selection originating from such interactions (for analogous approaches, see Benkman, 2013; Vanhoenacker et al., 2013). Further work involving manipulations of factors influencing consumer functional responses (including multiple levels of plant density and other sources of plant population- or community-level variation) will advance our understanding of the ecological drivers and evolutionary consequences of variation in plant-associated species interactions. In this sense, a growing number of studies have measured

changes in selection from species interactions using factorial designs that manipulate biotic or abiotic factors (e.g., Sletvold et al., 2013; Abdala-Roberts et al., 2014) and/or have considered variation in such effects as a consequence of underlying ecological gradients (e.g., Lehdal and Ågren, 2015). Combining these approaches with explicit measurements of interaction strength will yield the greatest insight.

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