

Noncorrelated effects of seed predation and pollination on the perennial herb *Ruellia nudiflora* remain spatially consistent

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Received 9 July 2008; accepted for publication 19 August 2008

By simultaneously manipulating both seed predator and pollinator effects on the perennial herb *Ruellia nudiflora* at two sites in Yucatan (Mexico), the present study evaluated (1) whether a correlation (interaction) existed between seed predator and pollinator effects on *R. nudiflora* seed production and (2) whether such an interaction varied geographically. We used three populations per site, and a total of 20 plants per population ($N = 120$). Groups of five plants were randomly chosen at each population to simultaneously receive one of two seed predator and pollinator enclosure levels (present or excluded in each case). These two factors were fully crossed, resulting in each group being subjected to one of four possible combinations: pollinators excluded/herbivores present; herbivores excluded/pollinators present; herbivores excluded/pollinators excluded; or control (neither excluded). Response variables were the number of seeds produced per plant and the proportion of attacked fruits by seed predators per plant. Seed predators had a large impact on *R. nudiflora* seed production but did not show any preference for fruits from plants not excluded from pollinators. In addition, the pollination treatment was not significant, indicating no effect of pollinators on reproductive success. These findings resulted in a nonsignificant herbivory \times pollination interaction, which was consistent across sites, indicating lack of correlated selection of these two guilds on *R. nudiflora* seed production. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, 96, 800–807.

ADDITIONAL KEYWORDS: correlated selection – multiple interactions – pollinators – spatial variation.

INTRODUCTION

Studies on the ecology and evolution of biotic interactions involving plants have been traditionally biased towards investigating the isolated effect one guild has on a plant (e.g. plant–pollinator interactions), while ignoring the simultaneous effects of other guilds (e.g. seed predators). Such a pairwise interaction approach has presumably facilitated the understanding of the effects that one guild has on specific plant traits or fitness measures; nonetheless, it also represents an unrealistic view of the matrix of

interactions to which plants are exposed (Strauss, 1997; Herrera, 2000; Strauss & Irwin, 2004). Moreover, studying the isolated effect of one guild on a plant may lead to erroneous conclusions about the net effect this group has on plant ecology and evolution (Herrera, 2000).

During the last decade, there has been increasing interest in studying the simultaneous effect of two or more guilds on plant fitness measures (Strauss & Irwin, 2004). These studies have shown that these interacting groups commonly affect each other reciprocally, thus affecting the individual impact each one has on a plant. For example, in the case of herbivores and pollinators, the former may indirectly affect

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pollinator behaviour and efficiency by modifying floral traits (Strauss, Conner & Rush, 1996; Lehtilä & Strauss, 1997; Mothershead & Marquis, 2000), whereas pollinators might indirectly influence granivore or frugivore choices because these prefer larger fruits that result from pollinator service (Herrera, 2000; Cariveau *et al.*, 2004). Considering such shared indirect effects between guilds is of central importance for understanding the outcome or net effect of multiple interacting organisms on a given plant trait or fitness measure (Brody, 1992, 1997; Ehrlén, 1997; Herrera *et al.*, 2002). In addition, via experimental enclosures, some investigations have explicitly tested for individual and combined effects exerted by herbivores and pollinators on plant reproductive measures (Herrera, 2000; Herrera *et al.*, 2002; Gómez, 2005; Valdivia & Niemeyer, 2007), and the findings obtained in these studies suggest the frequent occurrence of correlated effects of antagonists and mutualists on plant fitness measures (Herrera *et al.*, 2002).

It is now recognized that plant reproductive fitness measures, as well as the evolutionary state of certain reproductive traits, are to some degree the result of opposing selective pressures exerted by antagonists and mutualists (Euler & Baldwin, 1996; Armbruster, 1997; Kudoh & Wingham, 1998; Herrera *et al.*, 2002; Gómez, 2008). Correlated selection by herbivores and pollinators on plant traits occurs when a significant interaction is found between the effects of these two guilds (Phillips & Arnold, 1989). Theory predicts that such condition is most likely to be detected when plant attraction signals (i.e. flower/fruit size and number) are used by both herbivores and pollinators, as well as when their selective impacts on the plant are intense (Herrera *et al.*, 2002).

Despite the growing interest to evaluate the combined effect that two or more guilds have on plants, one aspect worthy of further attention is spatial variability in the effects of multiple interactions (Fedriani *et al.*, 2001; Herrera *et al.*, 2002; Rey *et al.*, 2006). The effect of multiple selective pressures on plant fitness measures may vary depending on the intensity or frequency of the effect caused by each guild, which may change as a function of each guild's species composition or abundance (Sánchez-Lafuente *et al.*, 1999; Gómez & Zamora, 2000). In this way, the net effect of two guilds on plant fitness measures may vary from site to site, thus favouring divergent ecological and evolutionary pathways for each plant population (*sensu* Thompson, 1994).

Ruellia nudiflora (Engelm. & Gray Urb.; Acanthaceae) is a self-compatible perennial herb distributed from Texas to southeast Mexico, and is usually found growing in disturbed areas (Tripp, 2007). In Yucatan (Mexico), its flowers are visited by several species of bees and butterflies, and its fruits are

attacked by larvae of a species of moth that feed on seeds before they are dispersed. Preliminary studies across different sites in Yucatan indicate that *R. nudiflora* presents spatial variation in floral traits (e.g. flower size, anther-stigma separation; D. Marrufo, unpubl. data), as well as in seed predation intensity (V. Parra-Tabla, unpubl. data), with both guilds potentially responding to the same plant traits (e.g. reproductive display size). By simultaneously manipulating both seed predator and pollinator effects for six populations of *R. nudiflora* in Yucatan, the present study at: (1) evaluating whether a correlated effect (interaction) existed between seed predator and pollinator impacts on *R. nudiflora* maternal fecundity (seed production) and (2) determining whether such interaction varied geographically.

MATERIAL AND METHODS

STUDY SPECIES

Ruellia nudiflora (Acanthaceae) is a perennial weed that typically measures 20–30 cm in height, and is distributed all the way from Texas to southeast Mexico. It is found growing mostly in disturbed open areas (Tripp, 2007) and has a wide distribution across the state of Yucatan (Mexico). It presents a mixed reproductive system, producing both flowers that open (chasmogamous; CH) and flowers that do not (cleistogamous; CL) and obligately self-pollinate. In the present study, we only worked with the former. Fruits on the other hand, are dry and dehiscent, each one normally producing between 10 and 12 seeds. *Ruellia nudiflora* is completely self-compatible, and CH flowers are visited by at least six bee species, such as *Apis mellifera* and *Trigona fulviventris*, and five butterfly species of which the most common is *Microtia elva*. This plant is assumed not to be pollen-limited (at least not strongly) because CH flowers frequently self-pollinate automatically during corolla dehiscence because stamens are fused to the corolla and anthers come into contact with the stigma when the corolla falls off the plant (V. Parra-Tabla, unpubl. data).

Ruellia nudiflora fruits are attacked by larvae of an unidentified species of moth (Lepidoptera: Noctuidae), which feed on the seeds prior to their dispersal; larvae attack anywhere from 15–45% of the fruits produced by each plant (V. Parra-Tabla, unpubl. data). Adult female moths ovoposit on recently formed fruits, and larvae develop inside the growing fruit, consuming all seeds within a fruit. Usually, only one larva is found per fruit and, before pupating, they excavate a tunnel which the adult uses to exit the fruit. In addition, seed-eating larvae are attacked by five to seven species of parasitoid wasps (including

Bracon sp. and *Chelonus* sp.) depending on the site; members of this trophic level are found in 30% of fruits attacked by the seed predator on average (V. Parra-Tabla, unpubl. data).

STUDY SITES

Two study sites were selected within the state of Yucatan (southeast Mexico). The first site (Xmatkuil: 20°48'N, 89°42'W) had environmental conditions characteristic of the central portion of the state. The second site (Hobonil: 20°00'N, 89°01'W) was located in the southern portion of the state. Three populations of *R. nudiflora* were identified at each site. Within each site, populations were separated by at least 100 m, whereas the distance between the two study sites was approximately 115 km.

Xmatkuil populations (central site) were growing at disturbed sites surrounded by low-height tropical deciduous forest of approximately 5 years of regeneration. Climate at this site is characterized by a mean annual rainfall of 750–1200 mm and a mean annual temperature of 26–27.6 °C (Duch, 1988). Populations at Hobonil were established along the edge of roads that were adjacent to medium-height subdeciduous forest. Mean annual rainfall at this site is of 1300–1500 mm, and mean annual temperature is 25.9–26.6 °C (Duch, 1988). Preliminary observations indicate that sites differ in *R. nudiflora* seed predation levels (V. Parra-Tabla, unpubl. data), as well as floral traits (i.e. greater flower size and anther-stigma separation in the south; D. Marrufo, unpubl. data), suggesting spatial differences in the selective regime imposed by herbivores and pollinators on the plant.

EXCLOSURE EXPERIMENT

In May 2007, a total of 20 plants were selected from each population. Plants were matched in terms of both reproductive (flower/fruit display) and vegetative size (number of leaves, height). Each plant was randomly chosen to receive one of two seed predator exclusion levels (herbivory factor: excluded, not excluded), and one of two pollinator exclusion levels (pollinator factor: excluded, not excluded). Following Herrera (2000), these two factors were fully crossed which resulted in each plant being subject to one of four possible combinations: pollinators excluded/herbivores present, herbivores excluded/pollinators present, herbivores excluded/pollinators excluded, or control (neither group excluded). Each particular combination of treatment levels was assigned to five plants (20 per population; total $N = 120$). This experimental design allowed to test for both individual as well as combined effects (interaction effect or corre-

lated response) of pollinators and herbivores on plant fitness measures (Herrera, 2000).

Seed predators were excluded by using a wide-spectrum systemic insecticide (Resifum, Agroquímica Tridente, Mexico) which was applied to the basal portion (away from the inflorescence) of plants selected for this treatment once a week at each population over a 3-week period. Insecticide application took place at dawn, before pollinator activity started, aiming to minimize its effects on pollinator visit rates. Once this 3-week period had been concluded, each population was then visited and eight to 12 mature flower buds (depending on availability), which were one day from opening, were marked on each experimental plant. Once flower buds were marked, we immediately proceeded to place pollinator enclosures for selected plants, which consisted of cylindrical cages made of steel wire and mesh that covered the entire plant (diameter 20 cm, height 40 cm). Flowers last for only a day, so pollinator enclosures were removed the next morning, once corollas of marked flowers had fallen off the plant. One week after the pollinator treatment had been conducted, fruits produced by all marked flowers were collected from each plant.

RESPONSE VARIABLES

For each experimental plant, we calculated the total number of seeds produced per plant and the proportion of attacked fruits by seed predators. The number of seeds per plant was calculated by opening each collected fruit and counting the number of enclosed seeds (which had escaped predation). The proportion of attacked fruits per plant was calculated as the number of fruits which showed evidence of seed predator presence (i.e. faeces, larvae) divided by the total number of fruits collected per plant (damaged and undamaged). Results with respect to this latter variable would allow us to confirm herbivore preference for fruits from one of the pollinator treatment levels (presumably they would prefer fruits from plants which were the result of outcrossing, and not excluded from pollinators).

STATISTICAL ANALYSIS

All analyses were conducted using SAS statistical software, version 9.1 (SAS Institute). PROC GLIMMIX was used to evaluate the effects of site (fixed, two levels), population (nested within site; random, six levels), herbivory (fixed, two levels) and pollination (fixed, two levels), as well as their interactions on the number of seeds and the proportion of attacked fruits per plant. Herbivory and pollination effects were fully crossed, which allowed us to evalu-

Table 1. Main effect and interaction results for number of seeds per plant and proportion of fruits attacked for *Ruellia nudiflora*

Source of variation	Number of seeds			Proportion of attacked fruits		
	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>
Site (S)	1, 4	6.10	0.06	1, 4	0.21	0.67
Herbivory (H)	1, 97	42.79	< 0.0001	1, 97	133.35	< 0.0001
Pollination (P)	1, 97	0.60	0.44	1, 97	0.62	0.43
H × P	1, 97	0.73	0.39	1, 97	0.33	0.56
S × H	1, 97	2.57	0.11	1, 97	8.85	0.003
S × P	1, 97	0.30	0.58	1, 97	1.60	0.20
S × H × P	1, 97	0.03	0.85	1, 97	5.83	0.01
Fruit number	1, 97	195.31	< 0.0001	–	–	–
Reproductive display size	–	–	–	1, 97	3.30	0.07

Herbivory, seed predator treatment (excluded or present); pollination, pollinator treatment (excluded or present). Reproductive display size and fruit number were used as covariates.

ate the herbivory × pollination effect on seed production. Finally, by including the effect of site, it was possible to evaluate spatial variation in the herbivory × pollination interaction via the site × herbivory × pollination interaction.

Data for number of seeds showed a normal distribution and homogeneity of variances after conducting a square root transformation ($P > 0.1$, Kolmogorov–Smirnov test; satisfactory kurtosis and bias values). Thus, for the analysis of this variable, we used the normal distribution and the identity as link function. The number of fruits collected per plant was used as a covariate to account for differences in seed production between plants. On the other hand, we used the binomial distribution and logit link function to analyse data for proportion of attacked fruits per plant; data for this variable were not found to be overdispersed (Littell *et al.*, 1996). In this case, reproductive display size (number of flower buds + open flowers + number of fruits) per plant was used as a covariate to account for possible differences between plants in the potential to attract herbivores. Finally, pre-planned contrasts were conducted using the LSMEANS statement to test for differences between treatment levels for significant main effects; when interaction terms were significant the SLICE option was included in this statement to test for statistical significance of a given factor at different treatment levels of another factor (Littell *et al.*, 1996). *P*-values from multiple comparisons were corrected based on the Bonferroni adjustment.

RESULTS

NUMBER OF SEEDS

Significant effects of herbivory and number of fruits (covariate) were observed for the number of seeds per

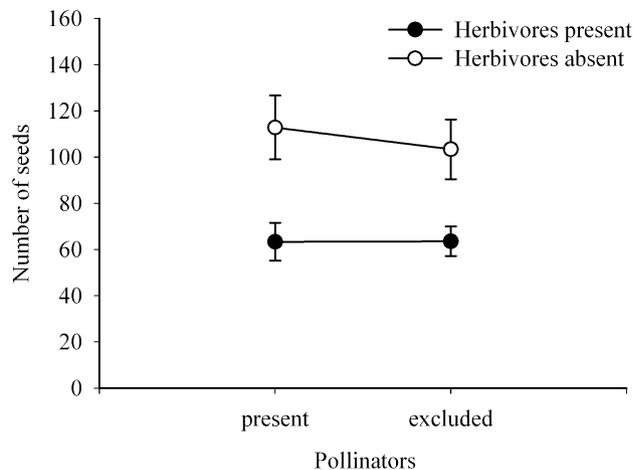


Figure 1. Interaction graph for herbivory (seed predator) and pollinator treatment effects on the number of seeds per plant of *Ruellia nudiflora*. Data are presented as the mean ± SE.

plant (Table 1). All other main effects and interactions were not significant (although the site effect was marginally significant). With respect to the herbivory effect, plants that were excluded from seed predators had 70% more seeds compared to those that were not (herbivores excluded: 108.3 ± 9.5 ; herbivores present: 63.5 ± 5.2 ; Fig. 1). The insecticide treatment was highly effective at reducing seeds lost to seed predation, and showed that seed predators had a large impact on seed production for *R. nudiflora* for the study populations (in many cases, close to half of the seed production per control plant was consumed). On the other hand, pollinators had no effect on the number of seeds per plant because flowers self-pollinated automatically in most cases. The lack of a

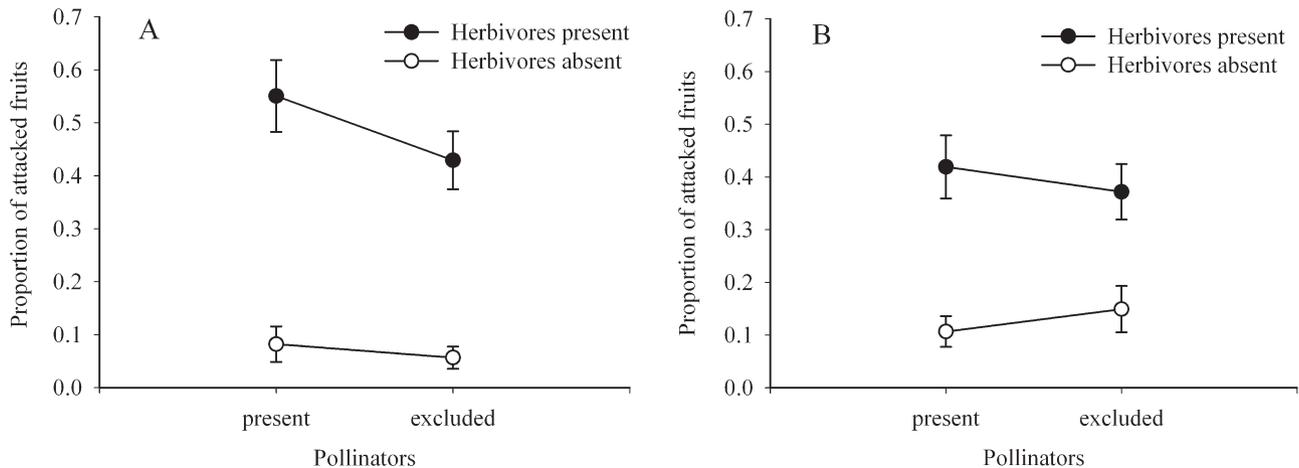


Figure 2. Interaction graphs for herbivory (seed predator) and pollinator treatment effects on the proportion of attacked fruits per plant of *Ruellia nudiflora* at Xmatkuil (A) and Hobonil (B) sites. Data are presented as the mean \pm SE.

significant herbivory \times pollination interaction indicated that the effects these two guilds had on seed production in *R. nudiflora* were independent of each other (i.e. were not correlated; Fig. 1). In addition, the nonsignificant site \times herbivory \times pollination interaction indicated that the lack of interaction between the effects of seed predators and pollinators remained consistent across sites (Table 1).

PROPORTION OF ATTACKED FRUITS

Significant herbivory, site \times herbivory, and site \times herbivory \times pollination effects were observed on the proportion of attacked fruits by seed predators (Table 1). All other main effects and interactions including the covariate were not significant (although the covariate effect was marginal; Table 1). Plants that were not excluded from seed predators had an average proportion of attacked fruits five times greater than those for which predators were excluded (herbivore excluded: 9% of fruits attacked \pm 0.12; herbivores present: 44 \pm 0.23%). This confirmed the effectiveness of the insecticide treatment in excluding seed predators and the large impact seed predators had on *R. nudiflora* reproductive output. Because a larva inside a fruit in most cases ate all the seeds, this resulted in an average loss of 40% of the seeds produced per unprotected plant.

The site \times herbivory interaction resulted from a difference in magnitude of the herbivore exclusion treatment effect across sites; nonetheless, at both sites, this effect was strong and in the same direction. In addition, the site \times herbivory \times pollination interaction was also significant and this result may have occurred because at Xmatkuil herbivore present/pollinator present plants had almost 12% more attacked fruits compared to herbivore present/

pollinator absent plants at this site (Fig. 2A); this pattern however, was not observed at Hobonil (Fig. 2B). Although this result suggests herbivore preference for fruits that were potentially subject to outcrossing at Xmatkuil, the difference between these two treatment level combinations was not significant at this site ($t = 0.11$, $P = 0.91$; SLICE), and more importantly it did not translate to a similar pattern in terms of seeds production.

DISCUSSION

Although previous studies have found an interaction between the effect of herbivores and pollinators on plant fitness measures (Herrera, 2000; Gómez, 2005), we did not find evidence of such condition for *R. nudiflora* seed production (Valdivia & Niemeyer, 2007). Moreover, such a lack of correlated effects was spatially consistent because the observed herbivory \times pollination pattern was quite similar between sites. These results are in contrast to the findings reported by Herrera *et al.* (2002), who found spatially consistent correlated effects of herbivores and pollinators on recruitment for *Helloborus foetidus*. In addition, Gómez (2005) also found a correlated effect of herbivores and pollinators on *Erysimum mediohispanicum*, but did not evaluate whether this condition varied geographically. For both these studies, a strong reduction in fitness was observed in the absence of pollinators, as well as strong and biased impact of herbivores towards insect-pollinated fruits. By contrast, our results suggest that, although herbivores had a large impact on seed production of *R. nudiflora*, they did not show a clear preference for fruits from open pollination (see below). In addition, pollinators did not affect *R. nudiflora* seed production (i.e. lack of

pollination effect), which precluded any chance of correlated selection occurring. Preliminary data show that, in Yucatan, *R. nudiflora* exhibits spatial variation in floral traits that could presumably be linked to pollinator selection. For example, southern populations have larger flowers (corolla width and length) and greater anther–stigma separation relative to central populations, and the latter condition has been shown to promote outcrossing (Barrett, 2003). Nonetheless, in the present study, seed production by *R. nudiflora* was not affected by excluding pollinators, and this was true at both sites. Such a result is likely due to the fact that, regardless of pollinator visitation, *R. nudiflora* self-pollinates automatically as the anthers and stigma come into contact when the corolla falls of the plant, suggesting that *R. nudiflora* will not be significantly pollen-limited in most cases. Based on the fact that pollen limitation intensifies selection on floral characteristics and the pollination system (Knight *et al.*, 2005; Fishman & Willis, 2008), the results obtained in the present study suggest weak pollinator selection on plant reproduction for *R. nudiflora*, and this condition will consequently limit the potential for correlated selection of pollinators and herbivores on *R. nudiflora* reproduction. However, pollinator selection on *R. nudiflora* floral traits should not be ruled out in the sense that pollinators may be needed to produce seeds and progeny of better quality (Knight *et al.*, 2005), and fruits that result from outcrossing may have a lower chance of being aborted as has been shown for self-compatible and partially self-incompatible species (Stephenson, 1981; Vaughton & Carthew, 1993; Kenta *et al.*, 2002). These issues, however, remain to be addressed for *R. nudiflora* (*sensu* Herrera *et al.*, 2002).

Seed predators on the other hand, had a large impact on seed production and this has shown to be a typical condition for many herb species (Herrera *et al.*, 2002). Plants unprotected from seed predators had close to 44% of their fruits attacked on average, which resulted in practically all seeds from these fruits being consumed (except for a small proportion of seeds saved by parasitoids; see below). The insecticide proved to be highly effective because plants that received it had only 9% of their fruits attacked on average and this resulted in them having 70% more seeds compared to plants that did not have insecticide. Although no clear evidence was found of seed predator preference for fruits potentially produced from pollinator visitation (Herrera, 2000; Cariveau *et al.*, 2004), plants at Xmatkuil with herbivores/with pollinators had 12% more fruits attacked than plants with herbivores/without pollinators (pattern not observed at Hobonil; significant three-way interaction). However, this argument remains speculative because

these two treatment level combinations were not significantly different at this site and, more importantly, this result did not affect results for number of seeds. In any case, the opportunity for seed predators to constrain pollinator selection on reproductive traits can be ruled out for the study populations because, as mentioned earlier, pollinators apparently do not have a selective impact on *R. nudiflora* maternal fecundity to start with. Moreover, seed predator attack rate and reproductive display size were only weakly related (marginally significant covariate effect), suggesting that seed predator selection on reproductive display was not strong, at least for the study season (Kolb, Ehrlén & Eriksson, 2007). Overall, both weak pollinator selection on floral traits and a strong but unbiased impact of seed predators on fruits most likely limits the potential for correlated selection of pollinators and herbivores on the reproductive traits of *R. nudiflora*.

Interestingly, seed-eating larvae of *R. nudiflora* are attacked by five to seven species of parasitoid wasps, which are found inside 15–45% of herbivore-attacked fruits per plant depending on the year and site. A small proportion of seeds is saved by these wasps, which kill their host larva or slow down their consumption rate (V. Parra-Tabla, unpubl. data). Based on this, and given the large impact imposed by seed predators on the plant, we suggest that, in some cases, the third trophic level may have an important impact on seed production in *R. nudiflora*. Thus, although we did not control for the effect of the third trophic level on the correlated effects of herbivores and pollinators on *R. nudiflora* reproductive measures, future studies should aim to evaluate how the third trophic level influences the correlated selection of insect herbivores and pollinators on plant fitness.

To summarize, the results obtained in the present study show that, although correlated selection between herbivores and pollinators is expected to occur frequently in herbs (Herrera *et al.*, 2002), *R. nudiflora* seed production did not show any evidence of such pattern for the studied populations. Such finding is likely due to: (1) the pollination system of *R. nudiflora* (i.e. the ability to self-pollinate automatically), which weakens pollinator selection on this species' floral traits and reproduction, and (2) the lack of seed predator preference for fruits produced by outcrossing. We consider that further studies should continue to evaluate spatial variation in the correlated effects of multiple interactions on plant traits, by explicitly comparing species or populations with varying levels of pollinator dependence or seed predator foraging behaviour. Such investigations will help to determine the ubiquity of such correlated effects and corroborate predictions based on system-specific characteristics.

ACKNOWLEDGEMENTS

We thank Jorge Navarro for statistical advice and Dennis Marrufo for field work assistance. This study was financially supported by CONACYT as part of a grant given to VPT (SEP-2004-CO1-4658A/A1).

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