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PLANT-ANIMAL INTERACTIONS - ORIGINAL PAPER

Additive effects of herbivory, nectar robbing and seed predation on male and female fitness estimates of the host plant *Ipomopsis aggregata*

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Abstract Many antagonistic species attack plants and consume specific plant parts. Understanding how these antagonists affect plant fitness individually and in combination is an important research focus in ecology and evolution. We examined the individual and combined effects of herbivory, nectar robbing, and pre-dispersal seed predation on male and female estimates of fitness in the host plant *Ipomopsis aggregata*. By examining the effects of antagonists on plant traits, we were able to tease apart the direct consumptive effects of antagonists versus the indirect effects mediated through changes in traits important to pollination. In a three-way factorial field experiment, we manipulated herbivory, nectar robbing, and seed predation. Herbivory and seed predation reduced some male and female fitness estimates, whereas plants tolerated the effects of robbing. The effects of herbivory, robbing, and seed predation were primarily additive, and we found little evidence for non-additive effects of multiple antagonists on plant reproduction. Herbivory affected plant reproduction through both direct consumptive effects and indirectly through changes in traits important to pollination (i.e., nectar and phenological traits). Conversely, seed predators primarily had direct consumptive effects on plants. Our results suggest that the effects of multiple antagonists on estimates of plant fitness can be additive, and investigating which traits respond to damage can provide insight into how antagonists shape plant performance.

Keywords Herbivory · *Ipomopsis aggregata* · Nectar robbing · Nectar · Phenology · Pollination · Pollen deposition · Seed predation · Trait-based approach

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Introduction

Species interact with myriad antagonists, both sequentially and simultaneously. A central goal in ecology is to understand and predict the outcomes of these complex species interactions (Morris et al. 2007). In some cases, the effects of multispecies interactions on host fitness will be additive and predictable from pairwise effects (Houle and Simard 1996; Maron 1998). However, in other cases, multispecies interactions are non-additive, and quantifying pairwise interactions is insufficient to predict the results of complex interactions (Wootton 1993). Whether the effects of species interactions are additive or not has important ecological and evolutionary implications (Strauss and Irwin 2004). For example, theory predicts that the evolutionary consequences of non-additive effects cannot be determined from pairwise selection gradients, and selection must instead be measured in a community context (Strauss et al. 2005). Our goal was to assess whether multispecies plant–animal interactions had additive or non-additive effects on male and female plant-fitness estimates and to examine if changes in plant and floral traits resulting from damage explained the responses observed.

Flowering plants experience damage by numerous antagonists (Strauss and Irwin 2004). Leaves are damaged

and consumed by herbivores and pathogens. Nectar is consumed by nectar robbers, which steal nectar through holes bitten in flowers, often without providing pollination service. Seeds are consumed by pre- and post-dispersal seed predators. Each of these pairwise plant–animal interactions can negatively affect plant performance (e.g., Crawley 1997; Irwin et al. 2001; Kolb et al. 2007). Yet, to our knowledge, no studies have examined the combined effects of simultaneous damage by herbivores, nectar robbers, and seed predators. One might expect non-additive outcomes when there are resource-based tradeoffs associated with damage, or if damage by one antagonist affects the survival of another. In a meta-analysis, Morris et al. (2007) suggest that, on average, the effects of multiple antagonists are additive. However, most of the studies included were of multiple herbivores. Whether this same pattern holds for antagonists that each consume different plant parts remains to be tested.

Changes in plant or reproductive characters following damage may explain, at least in part, how plants respond to damage from multiple antagonists. For example, plants that increase their relative growth rates after damage may simply re-grow following attack by one or more enemies, and incur few fitness costs of damage (Danckwerts 1993; Houle and Simard 1996). Or, if relative-growth rate or other resource-based traits are reduced after initial herbivore damage, the fitness costs of subsequent damage may be magnified (Hufbauer and Root 2002). Likewise, changes in pollination-related traits following damage may affect not only a plant's interaction with its pollinators and nectar robbers (Strauss et al. 1996; Adler et al. 2001; Suarez et al. 2009) but also the response to interactions that rely on pollination success, such as seed predation. Identifying the traits that change as a result of damage, and their subsequent effects on the outcome of other interactions, will provide a deeper mechanistic understanding of how multiple enemies affect host fitness.

Our aims were to assess plant response to damage from multiple antagonists and to explore if the plant responses were explained by changes in particular traits. We focused on the damage imposed by three antagonists—an ungulate herbivore, a nectar-robbing bumble bee, and a dipteran predispersal seed predator—that each feed on the hummingbird-pollinated host *Ipomopsis aggregata*. The effect of each of the antagonists on *I. aggregata* fitness has been studied independently (e.g., Paige and Whitham 1987; Brody 1992; Irwin and Brody 2000) or in two-way interactions (e.g., Juenger and Bergelson 1998; Sharaf and Price 2004; Brody et al. 2008). However, the combined effects of herbivores, nectar robbers, and seed predators on male and female function have remained unexplored. Because plant responses to damage are affected by pollination (Juenger and Bergelson 1997; Irwin and Brody 1998), we also examined how pollination and pollination-related floral traits were related to plant-antagonist outcomes. We addressed two questions. First, how do plant interactions with herbivores, nectar robbers, and seed predators individually and in combination affect male and female estimates of plant fitness? Because damage by these species may exert resource-based trade-offs on plants, and because herbivores and robbers may affect seed predator larval survival, we predicted that herbivores, robbers, and seed predators would exert non-additive effects on estimates of plant fitness. Second, do herbivory, nectar robbing, and seed predation affect the subsequent expression of plant and floral traits, and are changes in these traits associated with plant fitness estimates through pollination? We predicted that the outcome of herbivory, robbing, and seed predation on host fitness would be mediated, in part, by changes in pollination, given that some of the antagonists are known to alter pollination (Juenger and Bergelson 1997; Irwin and Brody 1998), and pollination is critical for *I. aggregata* reproduction.

Methods

Study system

We studied the native montane herb, *Ipomopsis aggregata* (Polemoniaceae), near the Rocky Mountain Biological Laboratory (RMBL), Gothic, Gunnison County, Colorado, USA. In this region, *I. aggregata* is a monocarpic perennial. It grows as a vegetative rosette for 2–10 years, flowers once and dies (Waser and Price 1989). Thus, we could measure the lifetime reproduction of individuals in a single flowering season. Flowering plants typically produce a single stalk with numerous, red, trumpet-shaped flowers. The flowers are hermaphroditic, protandrous, and male- and female-phase flowers occur on the same plant at the same time. Plants bloom for 4–8 weeks (mid-June to mid-August). Flowers produce nectar at a constant rate of 1–5 μl of nectar per flower per day, with a sugar concentration of 20–25% (Pleasants 1983). Individual flowers abscise after 3–5 days. *Ipomopsis aggregata* is pollinated primarily by broad-tailed (*Selasphorus platycercus*) and rufous (*S. rufus*) hummingbirds (Waser 1978), and seed set is often pollinator limited (e.g., Campbell and Halama 1993).

Mule deer, *Odocoileus hemionus*, consume 50–70% of *I. aggregata* bolting stalks in a given year (Sharaf and Price 2004). Most browsing occurs early in the season, during the first 5–8 weeks after snowmelt. Browsing results in the release of apical dominance and the production of lateral meristems that subsequently flower. For *I. aggregata* growing around the RMBL, herbivory often decreases seed set (Freeman et al. 2003; Sharaf and Price 2004).

The nectar robber, *Bombus occidentalis*, uses its sharp, toothed mandibles to chew a hole through the side of the corolla near the basal nectaries from which it extracts all available nectar. The bees neither pollinate the plant nor damage the plant's reproductive or nectar-producing structures (Irwin and Brody 1998). High levels of robbing (>80% of flowers robbed) translate into a 50% reduction in both male (number of seeds sired) and female (number of seeds set) estimates of plant reproduction (Irwin and Brody 2000), an effect driven indirectly by hummingbird–pollinator avoidance of nectar-robbed plants and flowers (Irwin and Brody 1998).

The pre-dispersal seed predator *Hylemya* sp. (Diptera: Anthomyiidae) lays single eggs underneath the sepals of elongated buds or flowers (Zimmerman 1980). Upon hatching, larvae burrow into the ovaries and eat the developing seeds. All seeds of infested fruits are typically destroyed. *Hylemya* does not pollinate *I. aggregata*. Thus, *Hylemya* relies on hummingbird visits to flowers on which it lays eggs to cause fertilization and provide its larvae with a food resource. Seed predation can destroy as much as 80–90% of all seeds of a single plant (Brody 1991).

Field manipulations

To examine the combined effects of herbivory, nectar robbing, and seed predation, we haphazardly selected 264 bolting *I. aggregata* across two study sites in May 2003. Both sites were dry meadows facing south–west, separated by ~250 m. Plants were randomly assigned to one of eight treatments, representing a full-factorial design of two herbivory treatments (control vs. clipped) by two robbing treatments (low vs. high robbing) by two seed predation treatments (egg removal vs. no egg removal). There were 33 plants per treatment (20 plants per treatment at site 1 and 13 plants per treatment at site 2). The difference in the number of plants per treatment at the two sites was due to differences in the number of accessible and suitable *I. aggregata* (i.e., unbrowsed plants that were not growing on hillsides too steep to access). Plants assigned to the treatments did not differ in initial plant size, estimated using root–stalk diameter ($F_{7,256} = 0.53$, $P = 0.81$).

For the herbivory treatment, plants assigned to the "browsed" treatment were clipped with scissors to a height of ~5 cm to simulate ungulate herbivory. We clipped plants in early June (2–9 June 2003) when we first observed deer browsing *I. aggregata* at our study sites. At this time, *I. aggregata* were bolting, but none had produced buds or flowers. Several studies have established that clipping adequately mimics natural browsing by ungulates on *I. aggregata* (Paige and Whitham 1987; Paige 1992) and induces similar changes in plant architecture, growth, phenology, and reproduction (but see Freeman et al. 2003). To ensure that control and clipped plants were not naturally browsed, we tied plants to thin metal stakes. Deer avoided staked plants, but pollinators, robbers, and seed predators freely visited them.

In the nectar-robbing treatment, we assigned plants to receive either an artificially superimposed low (<10% of available flowers robbed) or high (>80% of available flowers robbed) robbing treatment. These low and high robbing levels represent low and high robbing levels commonly found in the field (Irwin and Brody 1998). To simulate robbing, we cut a hole, ca. 1 mm in diameter, in the side of the flower using dissecting scissors. We then removed all available nectar using a 10 µl microcapillary tube. Robbing treatments were performed at the whole-plant level approximately every other day throughout the flowering season. Naturally and artificially robbed flowers do not differ in pollen donation or receipt, fruit or seed set, or seed weight (Irwin and Brody 1998, 1999). Natural levels of nectar robbing were low in 2003; thus, we did not collar flowers to deter natural robbing.

To manipulate seed predation, we removed *Hylemya* eggs from under the sepals of flowers using fine-point forceps. Egg removals were performed at the whole-plant level approximately every other day throughout the flowering season, and we recorded the number of eggs removed. In the control (no-egg removal) treatment, we censused elongated buds and flowers for the number of eggs naturally oviposited by *Hylemya*. In this study, we relied on *Hylemya* to deposit eggs. Although adding eggs to flowers has been done previously (Brody and Waser 1995), doing so without damaging eggs and on a large number of plants was not practical.

How do herbivory, nectar robbing, and seed predation affect male and female plant fitness estimates?

For all plants, we estimated correlates of male and female reproduction. Including multiple correlates of male and female plant function allowed us to understand the mechanisms that might be driving the effects of antagonists on plant response. We measured pollen production and pollen removal as male-fitness correlates. Pollen production provides a measure of the pool of pollen available for seed siring (Stanton et al. 1992), but see Ashman (1998). In *I. aggregata*, pollen removal is associated with pollen donation (Mitchell and Waser 1992), and pollen donation is associated with seeds sired (Irwin and Brody 1999, 2000). To estimate pollen production, we removed the anthers of 20% of the elongated buds (up to three buds) on each plant once per week. To estimate pollen removal each week, we paired additional elongated buds that had similar sizes and positions on the plants to those used to measure pollen production. We collected the anthers from the paired

flowers once they had been available to hummingbird pollinators for ~48 h. All anthers were collected in microcentrifuge tubes and allowed to air dry (one tube per bud or flower). Microcentrifuge tubes were filled with 1,500 µl of 90% ethanol and sonicated for 5 min to release all pollen from the anthers. For each sample, we then used a hemacytometer to count the pollen under a dissecting microscope (Kearns and Inouye 1993). We calculated three male fitness estimates for each plant: (1) pollen production per flower (averaged across the flowering season), (2) pollen production per plant (average pollen per flower multiplied by the number of flowers produced per plant; square-root transformed for analysis), and (3) pollen removal per flower (for the paired flowers, calculated as the amount of pollen produced per flower minus the amount of pollen remaining after flowers had opened, averaged across the flowering season).

To estimate female reproduction, for each plant we measured (1) total number of fruits, (2) proportion of fruit set, (3) mean seed set per fruit, and (4) total seeds per plant. When mature, all fruits were collected and recorded as having set seed or aborted. We then counted all of the seeds in each seed-bearing fruit. We also scored whether or not the fruits were damaged by *Hylemya* larvae; the larvae leave behind frass and partially eaten seeds. *Hylemya* usually destroy all of the seeds in attacked fruits. We scored attacked fruits as having set fruit, but attacked fruits were not included when calculating seed set per fruit. Proportion of fruit set per plant was calculated as the number of expanded fruits divided by the total number of fruits and aborts (arcsine square-root transformed). Seed set per fruit was the average number of seeds per successful fruit (square-root transformed). Total number of fruits per plant was log $(x + 1)$ transformed, and the total number of seeds per plant was square-root transformed prior to analysis.

Statistical analyses

We analyzed male and female estimates of plant reproduction separately, using MANOVA, to test how herbivory, nectar robbing, seed predation, site, and their interactions affected male and female plant function. A significant MANOVA was followed by univariate ANOVAs (Scheiner 1993). We included site as a fixed factor in the analyses (Collett 2003). None of the interactions with site were significant ($P > 0.05$ in all cases), so these interactions were removed from the final models (here and in analyses below). We left non-significant interactions among herbivory, robbing, and seed predation in the final models because the presence of these non-significant interactions was of interest in this study. For graphical display, we calculated responses to species interactions for male and female fitness estimates using the log ratio of the damage treatments divided by the undamaged treatment (no herbivory, low robbing, and seed-predator eggs removed), allowing for a visual comparison of the relative magnitudes of pairwise and multispecies interactions.

Because we allowed *Hylemya* seed predators to naturally oviposit eggs onto buds and flowers and then removed eggs in the egg-removal treatment, we also examined whether oviposition and fruit destruction by seed predators varied as a function of damage treatment, which could affect the interpretation of the effects of damage treatments on seed set. We used ANOVAs with herbivory, robbing, seed predation, site, and their interaction on the proportion of flowers oviposited on and the proportion of fruits destroyed (both arcsine square-root transformed). Statistical analyses were performed in JMP v. 8.0.

Do herbivory, nectar robbing, and seed predation affect plant and floral traits, and are trait changes associated with pollination and plant-fitness outcomes?

We measured traits associated with plant growth and investment in pollination. For plant traits, we measured plant height at the time of flowering to the nearest cm, the number of flowering stalks, and total plant biomass. We measured plant biomass after fruit collection by removing the above- and belowground biomass of each plant, drying it for 48 h at 60°C, and weighing it to the nearest 0.01 g.

For traits indicative of investment in pollination, we measured corolla length and width to the nearest 0.01 mm using digital calipers on three flowers per plant. We also measured the 48 h nectar-production rate and nectar-sugar concentration (using a hand-held refractometer) from flowers that hummingbird pollinators were excluded from and that did not receive nectar robbing for ca. 3 flowers per plant. Floral morphological and nectar measurements within plants are highly repeatable in *I. aggregata* (e.g., Wolf and Campbell 1995; Irwin et al. 2004). We averaged each of these floral measurements per plant. As an estimate of phenology, we measured the first flowering date for each plant. Finally, we counted the total number of flowers that plants produced on each date and over the entire season.

To understand whether changes in plant and floral traits associated with herbivory, robbing, and seed predation affected pollination, we emasculated flowers and used stigma–pollen loads to estimate hummingbird-pollinator visitation (Engel and Irwin 2003). We emasculated 20% of the elongated buds on each plant once per week throughout the flowering season. We then collected stigmas after the corollas had abscised. Collecting stigmas at this stage does not affect fruit or seed set (Waser and Price 1991). We stained the stigmas in basic fuchsin dye (Kearns and Inouye 1993) and counted the number of conspecific and heterospecific pollen grains under a compound microscope.

Very few heterospecific pollen grains were deposited on stigmas (mean ± SE: 11 ± 1 heterospecific pollen grains per stigma) and thus were excluded from further analysis.

Statistical analyses

To test how herbivory, robbing, and seed predation affected plant and floral traits, we used a MANOVA with herbivory, robbing, seed predation, their two- and three-way interactions, and site as fixed factors, and plant height, number of stalks, total biomass, corolla length and width, nectar-production rate, nectar-sugar concentration, first flowering date, and total number of flowers (square-root transformed) as response variables. A significant MANOVA was followed by univariate ANOVAs for each response variable.

To test how herbivory, robbing, seed predation, site, and their interactions affected pollination across the entire flowering season, we used an ANOVA with mean pollen receipt per stigma per plant as the response. Because clipping has a strong effect on flowering phenology (Juenger and Bergelson 1998), we also examined pollen receipt per plant per week in clipped and unclipped plants using ANOVAs to determine whether any potential differences in pollination were due to pollinator preferences for or pollinator efficiency at plants of particular treatments or simply a phenological effect of clipping on pollen deposition (Sharaf and Price 2004). We used ANOVAs within weeks rather than a repeated-measures ANOVA because pollen receipt was only measured on plants that had buds of the appropriate size each week to emasculate; thus, we would have lost replicates from a repeated-measures ANOVA when plants did not have stigmas collected in all weeks. Although we performed multiple tests when we compared pollen receipt within weeks, we did not apply the sequential Bonferroni correction to significance levels because it greatly inflates the error rate, and instead report unadjusted significance values (Moran 2003). To assess how plant and floral traits were related to pollination, we used an ANCOVA. We included all of the traits that we measured except for plant biomass as covariates, given that all of the traits have been shown in this or other systems to affect pollinator-visitation rates or pollinator efficiency at depositing pollen onto flower stigmas. We included the clipping treatment and site as fixed effects. There were no significant interactions between clipping, site, and traits ($P > 0.05$ in all cases), so these interactions were removed from the final analysis. All variance inflation factors were less than 9, suggesting that multicollinearity did not strongly affect the results.

Last, because herbivory affected plant and flower traits, pollination, and female estimates of fitness (see "Results"), we wanted to tease apart the variation in plant reproduction caused by plant damage versus the indirect effects mediated through changes in pollination. To do so, we used two approaches. First, we used an ANCOVA with clipping and site as fixed factors, pollen receipt per stigma as a covariate, and seed set per plant as the response variable. A significant effect of clipping but not pollen receipt would suggest that herbivory, and not changes in pollination per se, were affecting plant response. Alternatively, a significant effect of pollen receipt but not clipping would suggest that the effects of damage on seed set were associated with the changes in pollination. Second, we used path analysis and structural equation modeling to quantitatively formalize the relationships among clipping, floral traits, pollination and seed set using an a posteriori approach (Petraitis et al. 1996). Because we included a categorical variable (clipping treatment) in the path analysis, we created a tetrachoric correlation matrix using the POLYCHOR macro in SAS and then ran PROC CALIS on the correlation matrix. We initially tested whether the fit of an independence model, which assumes no relationships among variables, provided a good fit to the data. Finding that the fit was poor (see "Results"), we then assessed the goodness-of-fit of the model in Fig. 1 and calculated standardized path coefficients and significance levels for each path. We ran the path analysis separately for the two sites and combined across sites; finding qualitatively similar results from both sets of analyses, we only show results from the analysis combined across sites.

Results

How do herbivory, nectar robbing, and seed predation affect male and female fitness estimates?

Species interactions affected some male (MANOVA whole-model: $\lambda = 0.71$, $F_{24,485} = 2.48$, $P = 0.0001$) and female (MANOVA whole-model: $\lambda = 0.67$, $F_{32,817} = 2.97$,

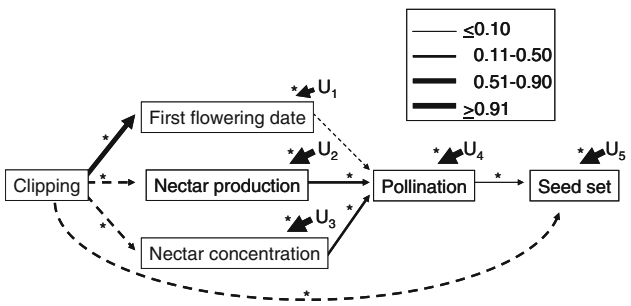

Fig. 1 Path model depicting the relationships among clipping, floral traits, pollination, and seed set. *Solid lines* indicate positive effects and *dashed lines* negative effects. The *width of the arrow* represents the magnitude of the standardized path coefficient. *U* represents unexplained variation in floral traits, pollination, and seed set. *Asterisks* indicate significant paths at $P \leq 0.05$. Correlations among first flowering date, nectar production, and nectar-sugar concentration are not shown for ease of viewing

$P < 0.0001$) estimates of plant reproduction. The effects were primarily additive, and we found little evidence for non-additive effects among antagonists on fitness estimates (Fig. 2). For male reproduction, pollen production per plant was 22% lower in clipped than unclipped plants ($F_{1,188} = 18.48$, $P < 0.0001$; Table 1, Fig. 2a). The effect of clipping on pollen production was driven by a reduction in flower number (Table 2) and not pollen per flower ($F_{1,188} = 2.21$, $P = 0.14$; Table 1). Higher levels of seed predation were also associated with lower pollen production per plant ($F_{1,188} = 1.28$, $P = 0.03$); plants from which *Hylemya* eggs were removed produced 19% more pollen than plants with eggs left intact (Fig. 2a). Egg-removal plants produced 15% more flowers than plants experiencing ambient levels of seed predation. Thus, the effect of seed predation on pollen production was driven by a marginal reduction in flower number (Table 2) and not by differences in pollen per flower ($F_{1,188} = 0.01, P = 0.93$). We found no effects of herbivory or egg removal on pollen removal per flower ($P > 0.05$ in all cases). We also found no effects of

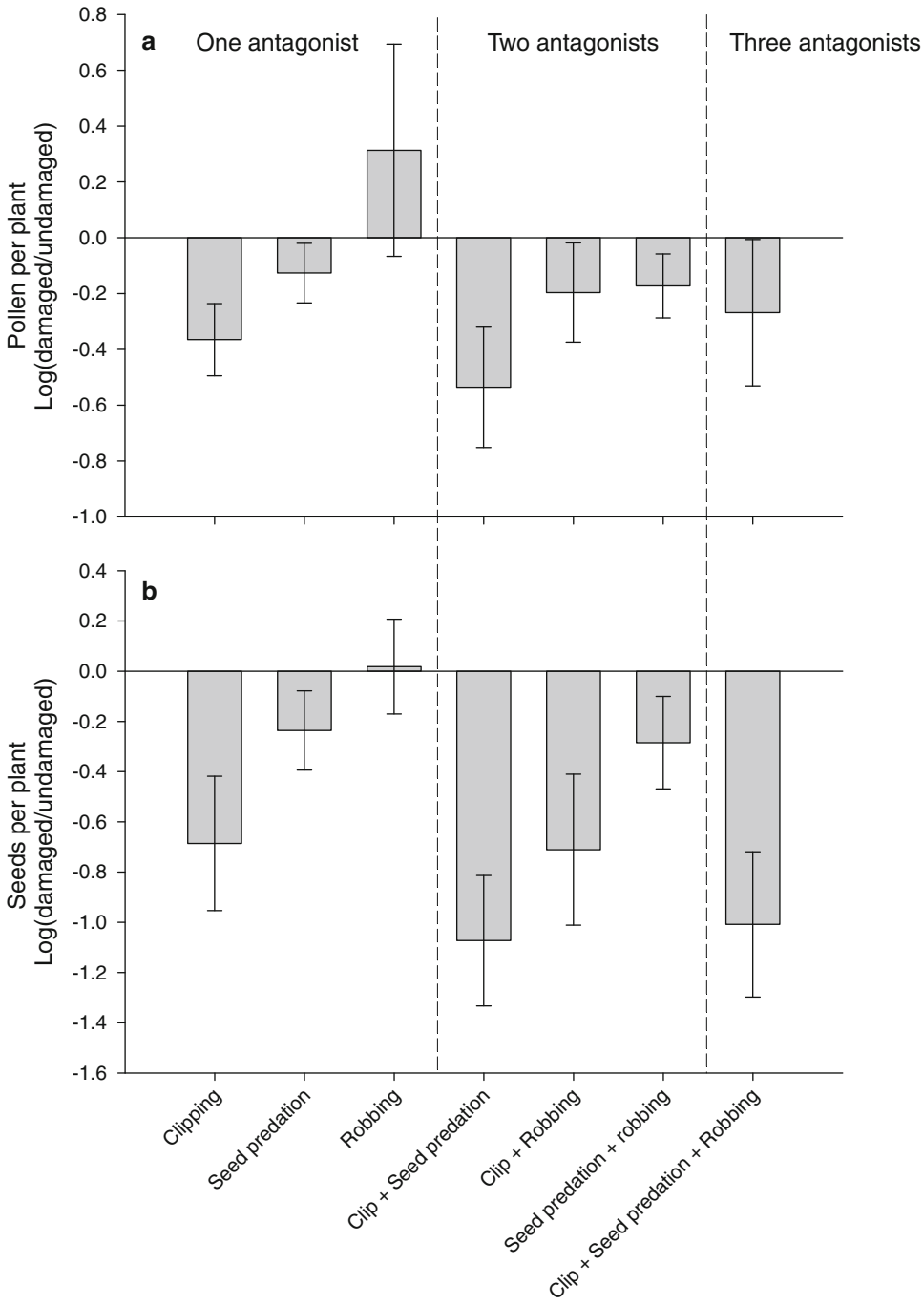

Fig. 2 A comparison of the relative magnitudes of the effects of herbivory, seed predation, and nectar robbing individually and in combination on **a** pollen production per plant and **b** seed production per plant. The *y* axis is the log ratio of the damage treatment divided by the undamaged treatment. The undamaged treatment includes plants with no clipping, low robbing, and seed-predator eggs removed. The *horizontal line* at zero represents the undamaged treatment, and the *bars* (mean ± SE) represent deviations between the damaged and undamaged treatments

Table 1 Plant reproduction, plant and floral traits, and pollen receipt in unclipped and clipped plants

Trait	Unclipped	Clipped
Pollen per plant	1,929.41 (133.77)	1,325.02 (122.99)
Pollen per flower	16.12 (0.52)	17.28 (0.70)
Pollen removal per flower	14.19 (0.64)	15.69 (0.73)
No. fruits	49.20 (3.59)	27.44 (3.00)
Prop. fruit set	0.43 (0.02)	0.36 (0.02)
Seed set per fruit	5.55 (0.42)	4.61 (0.19)
Seed set per plant	232.11 (18.71)	113.26 (13.73)
Prop. oviposition	0.20 (0.01)	0.13 (0.01)
Prop. fruits destroyed	0.10 (0.01)	0.08 (0.01)
Plant height (cm)	46.12 (0.83)	27.95 (0.86)
No. stalks	1.13 (0.18)	6.51 (0.19)
Biomass (g)	2.21 (0.11)	1.76 (0.11)
Corolla length (mm)	23.48 (0.25)	23.30 (0.26)
Corolla width (mm)	4.13 (0.06)	3.76 (0.06)
Nectar production (μl)	3.27 (0.16)	2.16 (0.17)
Nectar sugar (%)	28.30 (0.50)	23.84 (0.55)
No. flowers	105.83 (5.20)	63.65 (5.37)
First flowering date (days)	184.14 (0.43)	197.94 (0.44)
Pollen per stigma	138.41 (5.23)	119.57 (5.59)

Values are means with standard errors in parentheses. Pollen production and removal are reported in thousands of grains. First flowering date is reported in days after 1 January 2003

robbing on any male function estimate ($P > 0.05$ in all cases; Fig. 2a). Moreover, there were no significant two-way or three-way interactions for any estimate of male reproduction ($P > 0.05$ in all cases; Fig. 2a).

Female fitness estimates revealed similar patterns to those of male estimates. Clipping reduced total fruits by 44%, percent fruit set by 16%, seed set per fruit by 17%, and seed set per plant by 52% relative to unclipped plants (Tables 1, 3; Fig. 2b). Not only did unclipped plants produce more seeds, they did so even though they experienced higher levels of oviposition ($F_{1,247} = 27.66, P < 0.0001$) and fruit destruction ($F_{1,219} = 6.96, P = 0.009$) by seed predators. In addition, plants that incurred seed predation produced 24% fewer total seeds than plants with eggs removed (Table 3). The effect of seed predation on total seed set was driven by seed predators destroying a higher proportion of fruits (twice as high) in the seed predation compared to the egg removal treatment ($F_{1,226} = 11.33, P = 0.0009$) as well as marginally higher total fruit production in the egg removal treatment (Table 3). We found no effect of robbing on any estimate of female reproduction (Table 3). The effects of clipping, robbing, and egg removal were primarily additive (Table 3; Fig. 2b). Only one interaction between treatments (clipping × robbing interaction for seed set per fruit, Table 3) was statistically significant—robbing magnified the negative effect of clipping on seed set per fruit.

Do herbivory, nectar robbing, and seed predation affect plant and floral traits, and are trait changes associated with pollination and plant-fitness outcomes?

Treatments affected plant and floral traits (MANOVA whole-model: $\lambda = 0.07$, $F_{7,21,255} = 9.38$, $P < 0.0001$). Clipping had the strongest effects on trait expression (Table 2). Clipped plants were 40% shorter at the time of flowering, produced five more stalks on average, and attained only 80% of the total biomass compared to that of unclipped plants (Tables 1, 2). The difference in plant biomass was driven by both lower root ($F_{1,247} = 13.39, P = 0.0003$) and shoot ($F_{1,247} = 6.69$, $P = 0.01$) biomass in the clipped plants. For floral traits, clipped plants produced flowers with 9% narrower corollas, a 34% lower nectar-production rate, and 16% less concentrated nectar sugar than unclipped plants (Tables 1, 2). Clipped plants also flowered 14 days later, on average, than unclipped plants (Tables 1, 2). There was no effect of clipping on corolla length (Table 2). Robbing and seed predation were only associated with one trait: first flowering date (Table 2). Plants with high robbing flowered 2 days later than plants with low robbing, and plants with eggs removed flowered one day later than plants without eggs removed; it is unlikely that these small differences in first flowering date are biologically significant. We found no significant two-way interactions among clipping, robbing, or seed predation for any traits (Table 2). There was one significant three-way interaction among clipping, robbing, and seed predation for nectar-production rate (Table 2); the strong negative effects of clipping on nectar-production rate outweighed the weak positive effects of nectar robbing and seed predation on nectar-production rate.

Clipping also had significant effects on pollination. Clipping decreased pollen per stigma by 14% relative to unclipped plants ($F_{1,233} = 6.85$, $P = 0.009$; Table 1). In addition, there was a marginally significant three-way interaction among clipping, robbing, and seed predation for pollen deposition ($F_{1,233} = 3.71$, $P = 0.056$); the combined effects of clipping, robbing, and seed predation were less than expected if the reduction in pollen deposition was purely additive. There were no effects of robbing or seed predation on pollen deposition, and no significant two-way interactions ($P > 0.05$ in all cases). The effect of clipping on pollen deposition was not simply driven by a phenological delay in clipping that reduced pollination, as mean pollen receipt per stigma per plant was lower in clipped than in unclipped plants in 5 weeks (Fig. 3).

Three floral traits affected by clipping were associated with pollination: first flowering date, nectar-production rate, and nectar sugar concentration (Table 4). Plants that bloomed earlier, had a higher nectar-production rate per flower, and more concentrated nectar received more pollen per stigma. In the analysis of pollen receipt which included

Table 2 Summary of ANOVAs testing the effects of herbivory, nectar robbing, seed predation, and their interactions and site on plant and floral traits

Source	Plant height			No. stalks			Total biomass			Corolla length		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Clipping	**1**	**266.76**	**<0.0001**	**1**	**421.02**	**<0.0001**	**1**	**9.30**	**0.003**	1	0.26	0.61
Robbing	1	1.40	0.24	1	0.56	0.46	1	1.69	0.19	1	0.46	0.50
Seed predation	1	0.58	0.45	1	1.63	0.20	1	3.20	0.07	1	1.20	0.27
Clip × rob	1	0.03	0.87	1	3.00	0.08	1	0.11	0.74	1	0.74	0.39
Clip × seed pred	1	1.47	0.23	1	3.66	0.06	1	0.09	0.76	1	0.01	0.97
Rob × seed pred	1	1.53	0.22	1	2.58	0.11	1	1.49	0.22	1	1.98	0.16
Clip × rob × seed pred	1	0.35	0.56	1	0.94	0.33	1	0.65	0.42	1	0.10	0.75
Site	**1**	**37.27**	**<0.0001**	1	0.01	0.93	**1**	**3.61**	**0.059**	**1**	**57.85**	**<0.0001**
Error	245			244			247			240		

Source	Corolla width			Nectar-production rate			Nectar-sugar conc.			First flowering date			Total flowers		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Clipping	**1**	**15.97**	**<0.0001**	**1**	**23.30**	**<0.0001**	**1**	**38.90**	**<0.0001**	**1**	**529.86**	**<0.0001**	**1**	**47.45**	**<0.0001**
Robbing	1	2.08	0.15	1	0.28	0.59	1	<0.01	0.99	**1**	**5.80**	**0.02**	1	0.79	0.37
Seed predation	1	0.36	0.55	1	0.02	0.90	1	1.05	0.31	**1**	**6.47**	**0.01**	1	3.28	0.07
Clip × rob	1	0.02	0.88	1	0.01	0.95	1	0.09	0.76	1	0.44	0.51	1	0.67	0.41
Clip × seed pred	1	0.01	0.97	1	0.18	0.67	1	2.86	0.09	1	2.27	0.13	1	<0.01	0.92
Rob × seed pred	1	1.00	0.32	1	0.33	0.56	1	1.33	0.25	1	0.24	0.63	1	0.02	0.88
Clip × rob × seed pred	1	0.04	0.83	**1**	**4.52**	**0.03**	1	0.02	0.88	1	2.60	0.11	1	0.07	0.79
Site	**1**	**4.22**	**0.04**	**1**	**10.22**	**0.002**	**1**	**29.87**	**<0.0001**	1	1.29	0.26	1	2.77	0.10
Error	240			239			217			247			247		

Statistical significance indicated in bold

Table 3 Summary of ANOVAs testing the effects of herbivory, nectar robbing, seed predation, and their interactions as well as site on estimates of female plant reproduction

Source	Total fruits			Prop. fruit set			Seeds per fruit			Seeds per plant		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Clipping	**1**	**37.63**	**<0.0001**	**1**	**9.05**	**0.003**	**1**	**6.77**	**0.01**	**1**	**39.64**	**<0.0001**
Robbing	1	0.03	0.86	1	0.28	0.60	1	2.59	0.11	1	0.04	0.84
Seed predation	**1**	**4.00**	**0.05**	1	0.82	0.37	1	<0.01	0.99	**1**	**6.05**	**0.01**
Clip × rob	1	3.13	0.08	1	2.60	0.11	**1**	**5.86**	**0.02**	1	0.47	0.49
Clip × seed pred	1	0.34	0.56	1	0.36	0.55	1	0.04	0.84	1	<0.01	0.98
Rob × seed pred	1	0.01	0.94	1	0.54	0.46	1	0.57	0.45	1	<0.01	0.96
Clip × rob × seed pred	1	0.09	0.76	1	1.74	0.19	1	0.65	0.42	1	0.04	0.84
Site	**1**	**4.87**	**0.03**	**1**	**4.11**	**0.04**	**1**	**12.61**	**0.0005**	**1**	**9.70**	**0.002**
Error	247			247			224			247		

Statistical significance is indicated in bold

floral traits and the clipping treatment, clipping was not significant (Table 4), suggesting that any effects of clipping on pollen receipt were driven by changes in floral and flowering traits and not other direct effects of clipping on pollination.

When we assessed whether clipping and pollen receipt were associated with changes in plant seed set, we found that both clipping ($F_{1,239} = 27.78$, $P < 0.0001$) and pollen receipt ($F_{1,239} = 5.33$, $P = 0.02$) significantly affected total seeds per plant, suggesting that both are important in affecting the seed-set response. Furthermore, the path analysis in general supported the relationships among clipping, floral traits, pollination, and seed set. The independence model did not provide a good fit to the data

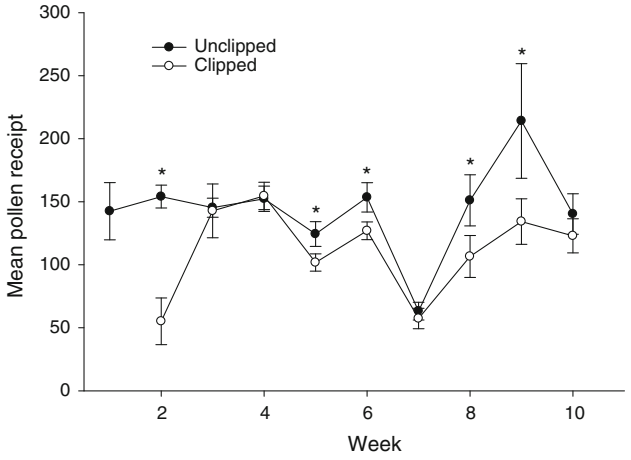

Fig. 3 Mean pollen receipt per stigma per plant across the flowering season. Pollen receipt was higher in unclipped plants (*black circles*) compared to clipped plants (*open circles*) in five different weeks (marked with *asterisks*)

Table 4 ANCOVA testing the effects of clipping, plant and floral traits, and site on pollen receipt per stigma

Source	*df*	*F*	*P*
Clipping	1	3.12	0.08
First flowering date	**1**	**5.45**	**0.02**
Nectar production	**1**	**3.66**	**0.05**
Nectar sugar (%)	**1**	**5.02**	**0.03**
Plant height	1	0.18	0.67
No. stalks	1	<0.01	0.97
Corolla length	1	0.37	0.54
Corolla width	1	1.89	0.17
No. flowers	1	0.11	0.74
Site	1	3.59	0.06
Error	208		

Statistical significance is indicated in bold

($\chi^2 = 398.59$, $df = 15$, $P < 0.01$), indicating that significant relationships existed among variables. The causal structure we proposed provided a reasonable fit to the data ($P > 0.05$), with both direct effects of clipping on seed set as well as indirect effects of clipping on seed set mediated through changes in floral traits and pollination (Fig. 1). The direct effects of clipping on seed set (direct path from clipping to seed set $= -0.31$) were stronger than the indirect effects mediated through changes in floral traits and pollination (combined indirect effects $= -0.016$).

Discussion

The damage inflicted by multiple antagonists has the potential to impact plant survival, growth, and reproduction (Crawley 1983; Marquis 1992). Here, herbivory, robbing, and seed predation had primarily additive effects on male and female fitness estimates. Herbivory (simulated by clipping) and pre-dispersal seed predation reduced both male and female estimates of reproduction, whereas robbing did not affect either male or female estimates in the year of this study. Herbivory directly affected the expression of plant and floral traits, including flower production and phenology, floral morphology, nectar-production rate, and nectar sugar concentration. In addition, herbivory indirectly affected female fitness estimates through changes in traits important to pollinators—the timing of flowering, nectar-production rate, and nectar sugar concentration. Our results concur with those of Morris et al. (2007) in demonstrating that multispecies interactions can have additive effects on plant reproduction. Moreover, by focusing on changes in traits important to pollination, we gained insight into the mechanisms of how antagonists shape plant performance.

Additive effects of multiple enemies on plant performance

Like our study, Juenger and Bergelson (1998) found additive effects of ungulate herbivores and pre-dispersal seed predators on female reproduction. However, they found that the effects of pre-dispersal seed predators and florivores were non-additive. Brody et al. (2008) found primarily additive effects of robbing and seed predation on the female function of *I. aggregata*, despite finding some reduction of pre-dispersal seed predation on plants that incurred high levels of robbing, but only robbing and not seed predation was manipulated. These results underscore that diverse species interactions can combine with different outcomes, although findings of additive effects are more common than non-additive effects for multispecies interactions with *I. aggregata*. It is important to note that our failure to detect non-additive effects was not merely due to low power, as we had fairly high power to detect effects for some of the response variables (range from a power analysis: 0.62–0.97).

One surprising result from our study was that plants fully compensated for the negative effects of robbing, despite previous studies to the contrary (Irwin and Brody 1999). In prior studies, robbers of *I. aggregata* primarily had indirect effects on plant reproduction mediated through pollinator avoidance of nectar-robbed plants and flowers (Irwin and Brody 1998). It is interesting to note in this study that clipping reduced pollination (likely through a change in nectar traits), which resulted in reduced seed set. Why robbing, which also results in a reduction in nectar volume, did not result in a change in pollen deposition and plant reproduction is unknown. One possibility is that high

levels of pollination, or pollinators that did not discriminate between robbed and unrobbed flowers (Burkle et al. 2007; Zhang et al. 2009), resulted in the lack of a robbing effect on plant reproduction in this year of study. To our knowledge, only one other study has examined how pollinators respond to flowers from clipped versus robbed plants; Torres et al. (2008) found that changes in nectar availability affected pollinator behavior for plants with simulated herbivory and robbing. Whether robbers would have additive or non-additive effects in combination with herbivory and seed predation in *I. aggregata* in years when we don't see full compensation for robbing warrants further attention.

Although this study was designed to test for additive versus non-additive effects of multiple antagonists, it is important to note that the effects of antagonists and mutualists, such as pollinators, can also be non-additive (Morris et al. 2007). Because we did not manipulate pollination, we do not know the nature of antagonist–pollinator effects in the year of this study beyond clipping affecting pollen deposition. However, Juenger and Bergelson (1997) manipulated pollination and clipping and found that pollination did not fully rescue plants from the negative effects of clipping.

A trait-based approach to studying multispecies interactions

Understanding trait changes induced by antagonists can provide powerful insight into the mechanisms driving ecological patterns of species interactions. As in previous studies of *I. aggregata*, we found that simulated herbivory delayed flowering, increased branch production, reduced the height of flowering plants, decreased flower and fruit production, and decreased corolla width (e.g., Bergelson and Crawley 1992; Gronemeyer et al. 1997; Juenger and Bergelson 2000). Our work also revealed that herbivory affected nectar traits important for pollination, which is heretofore undocumented in *I. aggregata*. Clipping reduced nectar production and nectar sugar concentration, two traits important in pollinator visitation. In other systems, although the effects of herbivory on flower morphology have been well documented (e.g., Mothershead and Marquis 2000; Gómez 2003), studies examining the effects of herbivore damage on nectar traits are rare, but growing (e.g., Strauss et al. 1996; Torres et al. 2008). Given the fundamental importance of nectar to plant-pollinator interactions, nectar should be considered an important trait that could influence the effects of damage on plant-pollinator interactions.

Teasing apart the mechanisms of antagonist effects revealed both direct and indirect effects of antagonists on plants. For the herbivory treatment, clipping reduced seed set both directly and indirectly (by altering pollination likely via changes in nectar and phenological traits). Prior studies of *I. aggregata* have suggested that unbrowsed plants that flower earlier in the summer often enjoy higher pollination and fruit set success than their browsed neighbors (Freeman et al. 2003). Our results suggest that the reduction in pollination success in clipped plants is not merely driven by a delay in flowering. Here, even within some weeks across the flowering season, unclipped plants garnered higher pollen loads, indicative of visitation, relative to clipped plants. Hummingbird pollinators of *I. aggregata* have been shown to preferentially visit plants and flowers with more nectar or sugar (Mitchell 1993; Irwin et al. 2008). Thus, the difference in pollen loads between clipped and unclipped plants may be driven by clipping-induced changes in hummingbird behavior as a function of nectar production. Increased hummingbird visitation to *I. aggregata* often results in increased seed set in years when plants are pollen limited. The path analysis provided a quantitative framework for the relationships among some of the variables and revealed that the direct effects of clipping on seed set were stronger then the indirect effects via changes in floral traits and pollination. Due to the multiplicative nature of path analysis, the weak indirect effect of clipping on seed set was driven, in part, by the weak positive relationship between pollen deposition and seed production (also see Irwin 2006). The path analysis also revealed a large amount of unexplained variation in seed set (and other variables), suggesting that other factors besides those measured in this study or included in the path analysis drive variation in *I. aggregata* reproduction.

For the seed predation treatment, seed predators primarily had a direct negative effect on seed set through the consumption of seeds, and we observed no strong effects of seed predators on traits important for pollinator attraction or on pollination. One caveat is that high levels of seed predation had a marginal negative effect on flower number, which drove a reduction in male function through whole-plant pollen production. While we found no evidence in this study that pollination was affected by flower number, studies in this and other systems suggest that pollinator visitation (or pollination) is strongly affected by the number of flowers that plants produce (e.g., Brody and Mitchell 1997; Gómez et al. 2009). The mechanisms underlying the effects of seed predation on flower number remain a mystery and warrant further study.

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