

DIRECT AND INDIRECT EFFECTS OF ALKALOIDS ON PLANT FITNESS VIA HERBIVORY AND POLLINATION

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Abstract. Herbivores and pollinators can simultaneously exert selective pressures on plant traits via direct and indirect effects. Net selection on plant traits, such as defensive chemistry, may be difficult to predict from studying either of these interactions in isolation. In this study, alkaloids were manipulated experimentally in the hemiparasitic annual plant *Castilleja indivisa* (Scrophulariaceae; Indian paintbrush) by growing these parasites with bitter (high-alkaloid) or sweet (low-alkaloid) near-isogenic lines of the host *Lupinus albus* (Fabaceae) in the field. To evaluate the effect of herbivores, half of the Indian paintbrush plants were randomly assigned to a reduced-herbivory treatment using insecticide, and the other half to a natural-herbivory treatment. Floral traits, bud and fruit herbivory, pollination, alkaloids, and plant performance were measured. These variables were used in a path analysis to dissect the direct and indirect effects of herbivory and pollination on lifetime seed set, and the direct and indirect effects of alkaloids on seed set via herbivory and pollination.

Bud herbivory and fruit herbivory directly decreased seed production, whereas pollination had a direct positive effect. In addition, bud herbivory had negative indirect effects on seed set by reducing the number of open flowers, which reduced pollinator visits. Alkaloids directly reduced bud herbivory but did not significantly affect pollination or fruit herbivory directly. However, because bud herbivory indirectly reduced seed set by reducing pollinator visits to flowers, alkaloids also had additional indirect benefits for plants by increasing pollination. Overall, the net benefit of alkaloid uptake was due to both reduction in herbivory and an increase in pollinator visits to flowers. This study demonstrates the importance of considering multiple interactions simultaneously when attempting to understand the mechanisms underlying correlations between plant traits and fitness.

Key words: alkaloids; *Castilleja indivisa*; direct effects; floral herbivory; fruit predation; hemiparasitic plants; Indian paintbrush; indirect effects; *Lupinus albus*; path analysis; pollinators; secondary compounds.

INTRODUCTION

Individuals frequently experience selective pressures simultaneously exerted by multiple interactions, including both mutualisms and antagonisms. For example, although plant–herbivore and plant–pollinator interactions are typically studied separately, most plants must attract pollinators while also escaping herbivores. Therefore, herbivores and pollinators can both exert selective pressures for plant traits via direct effects on plant fitness (Schemske and Horvitz 1988, Juenger and Bergelson 1997, Strauss and Armbruster 1997). In addition to direct effects on plant fitness, both herbivores and pollinators can have indirect effects on plant fitness by influencing interactions between the plant and other species. Herbivory can result in decreased pollination by reducing resources available for floral displays or rewards, or by damaging attractive tissues (Strauss

1997). Preference of pollinators for less damaged plants has been found in systems with floral herbivory (Karban and Strauss 1993, Lohman and Berenbaum 1996, Tennakoon and Pate 1996, Krupnick et al. 1999, Mothershead and Marquis 2000) and with leaf herbivory (Juenger and Bergelson 1997, Lehtila and Strauss 1997, Strauss et al. 1999, Mothershead and Marquis 2000). However, the relative importance of direct vs. indirect effects of herbivory on plant fitness has rarely been quantified (but see Schemske and Horvitz 1988, Krupnick et al. 1999).

Both herbivores and pollinators may exert selective pressures on plant traits involved in attraction and resistance. Floral structures that once functioned as defenses, such as triterpene resins, can be co-opted for pollinator rewards (Armbruster 1997, Armbruster et al. 1997), and pleiotropic effects of an allele determining floral pigmentation may influence vegetative resistance to herbivores (Simms and Bucher 1996). Because of these complex interactions, net selection on plant traits, such as resistance due to defensive compounds, is difficult to predict. If defensive compounds make plants

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PLATE 1. Inflorescence of an Indian paintbrush (*Castilleja indivisa*). Note the large, showy bracts and the smaller inconspicuous flowers. Photo taken by Darrell Morrison; courtesy of the Lady Bird Johnson Wildflower Center.

more attractive to pollinators by deterring herbivores, there may be positive, indirect selection through pollinators for greater production of secondary compounds. At the same time, if secondary compounds are present in floral tissues and deter pollinators, or if the cost of producing the compound results in less attractive floral structures, then pollinators may select against the production of these compounds (Detzel and Wink 1993, Strauss et al. 1999).

Hemiparasitic plants provide a unique opportunity to study chemically mediated interactions among plants, herbivores, and pollinators. Many hemiparasites take up secondary compounds from their host plants (Arslanian et al. 1990, Schneider and Stermitz 1990, Boros et al. 1991, Baeumel et al. 1992, Mead et al. 1992, Stermitz and Pomeroy 1992, Martin Cordero et al. 1993, Stermitz et al. 1993, Wink and Witte 1993). Thus, the presence of certain defensive chemicals varies within and among populations of parasitic plants, depending on the host association of individual parasites (Stermitz and Harris 1987). Parasitic plants are common members of every major ecosystem (Kuijt 1969), and the ecological consequences of secondary

compound uptake comprise a potentially important part of their ecology (Adler 2000). In addition, the ability of hemiparasites to take up host compounds provides a novel tool for manipulating secondary compounds in living plants and assessing the consequences of these compounds for both herbivory and pollination in the field.

We performed a field experiment to assess the effect of alkaloid uptake on herbivory, pollination, and plant fitness. Individuals of the annual hemiparasitic plant *Castilleja indivisa* (Scrophulariaceae), Indian paintbrush, from two populations were grown from seed with one of two hosts: sweet (low-alkaloid) or bitter (high-alkaloid) near-isogenic lines of the lupine *Lupinus albus* (Fabaceae). These lines are similar in morphology and in resource availability, but differ in the alkaloid content of their tissues (L. S. Adler and C. Huyghe, unpublished data). We measured pollination, herbivory, and alkaloid levels, and these quantitative field measures were used in path analysis to address the following questions:

- 1) How do bud herbivory, pollination, and fruit herbivory affect seed set in *C. indivisa*, and what is the relative importance of bud herbivory compared to fruit herbivory?
- 2) Does bud herbivory indirectly affect seed set by influencing pollinators?
- 3) Do alkaloids influence seed set via herbivory and/or pollination, and are these effects direct or indirect?

METHODS

The study system

Castilleja indivisa Engelm, Indian paintbrush (Plate 1), is an annual hemiparasite endemic to Texas that commonly grows with the native annual host *Lupinus texensis* Hook, Texas bluebonnet (Loughmiller and Loughmiller 1984). The term "hemiparasite" describes plants that contain chlorophyll and are photosynthetic, but obtain water and nutrients from host plants via haustorial connections, in this case through the roots (Kuijt 1969). *C. indivisa* is self-incompatible (L. S. Adler and C. Huyghe, unpublished data), with inconspicuous flowers that occur on terminal inflorescences with brightly colored bracts. *C. indivisa* takes up the alkaloids lupanine and isolupanine when parasitizing *L. texensis* (Stermitz and Pomeroy 1992). Alkaloids are found in the bracts, calices, and leaves of *C. indivisa* parasitizing *L. texensis* in the field, but not in the maturing gynoecium or in the nectar (Adler and Wink, in press).

"Sweet" varieties of many species of *Lupinus* have been developed that produce very low quantities of alkaloids (Hackbarth 1961). Sweet lupines are similar to bitter conspecifics in all aspects except alkaloid production (Wink 1993). Alkaloid content in sweet varieties is many-fold lower than in wild-type bitter lines, but the composition of alkaloids is similar, and sweet

lupines are readily consumed by many herbivores that avoid bitter lupines (Wink 1988, Aniszewski 1993, Saito et al. 1993). Because sweet varieties of the native lupine species *L. texensis* have not been developed, we used near-isogenic bitter and sweet lines of the annual *L. albus* L. (provided by Dr. C. Huyghe, Lusignan INRA, France), a species native to Europe, as hosts for *C. indivisa*. The alkaloid content of *L. albus* and *L. texensis* is similar, with lupanine being the principal constituent (L. S. Adler and C. Huyghe, unpublished data).

Advantages of a hemiparasite–host system

Because secondary compounds generally cannot be experimentally manipulated in living plants, it has been difficult to establish that they are causal agents of plant resistance. In recent years, approaches have been developed to experimentally manipulate plant resistance or specific compounds within plants, such as inducing responses to herbivore damage using the natural plant elicitor jasmonic acid (Thaler et al. 1996), creating transgenic plants (Verkerk et al. 1998), and breeding isogenic lines that vary in loci associated with resistance traits (Wink 1988). For all of these methods, costs of producing secondary compounds will vary between manipulated and unmanipulated plants. The use of a hemiparasite–host system presents a novel method for manipulating defensive compounds in living plants, and we would like to add this approach to the growing number of methods that can be used in studying the chemical ecology of plant–animal interactions. Because the hemiparasite does not manufacture host-obtained compounds, costs of producing compounds can be separated from ecological and autotoxic costs of possessing secondary compounds. In addition, by assigning hemiparasites from different maternal lines to host treatments, the effects of secondary compounds can be evaluated across varied genetic backgrounds, and provide a level of removal from pleiotropic or linked effects in the host plant. Finally, because uptake of secondary compounds from hosts is a common, natural aspect of hemiparasite ecology, the manipulation does not represent the introduction of a novel compound to naive herbivores and pollinators.

The field experiment

C. indivisa plants were grown from seed collected from two populations: the Stengl House Reserve in Bastrop, Texas, USA, operated by the University of Texas at Austin, and from a managed population that was the former site of the Lady Bird Johnson Wildflower Center (2600 FM 973 North ~5 km north of Highway 71). We randomly assigned *C. indivisa* seeds from these populations to sweet or bitter near-isogenic lines of the lupine *L. albus*. We planted two *L. albus* hosts with multiple *C. indivisa* per 10-cm pot in a greenhouse in January 1998. We used a 1:1 ratio of University of California at Davis soil mix:vermiculite

(Baker 1972, Evans 1998) and maintained a 16:8 day:night regime in the greenhouse with a 1000-W metal halide light. In March, plants were thinned to one parasite per pot and were transplanted to a fenced old field in the Lady Bird Johnson Wildflower Center, Austin, Texas, USA. Each of 15 blocks contained eight host–parasite pairs. Each parasite–host replicate was surrounded by a buried cylinder of nonwoven polypropylene fabric (Root Control, Oklahoma City, Oklahoma, USA) 22 cm in depth with a 25 cm diameter, to allow water penetration but prevent *C. indivisa* from parasitizing other wild plants. Indian paintbrush density was high, but well within the range of naturally occurring plants in the field (Loughmiller and Loughmiller 1984; L. S. Adler, personal observation).

To evaluate the effects of herbivores, half of the *C. indivisa* in each block were randomly assigned to a reduced herbivory treatment, and the other half to natural herbivory. Reduced-herbivory plants were sprayed twice weekly with *Bacillus thuringiensis*, a biodegradable, nonsystemic pesticide specific to Lepidoptera (Thuricide Concentrate, Bonham, Texas, USA), and control plants were sprayed with water. We did not spray host plants. In all, there were eight population–herbivory–host combinations (two levels of each in a factorial design) and 15 blocks, or replicates, of each combination, for a total of 120 plants.

Herbivory occurred principally on inflorescences; there was little foliar herbivory (L. S. Adler, personal observation). The most common herbivores were larvae of *Endothenia hebesana* Walker (Tortricidae), *Junonia coenia* Hubner (Nymphalidae), and *Plusia biloba* Stephens (Noctuidae). Inflorescences were pollinated primarily by the Black-chinned Hummingbird, *Archilochus alexandri*. We observed all plants simultaneously for 70 h during the field season, and counted the number of visits to flowers on each plant. We recorded floral display measures weekly, including the number of open flowers and the number of inflorescences per plant. At the end of the season, we recorded the fate of every flower (filled or unfilled fruit, and damaged or undamaged), and counted seeds for every filled fruit.

Alkaloid analysis

We determined the alkaloid content of Indian paintbrush inflorescences using gas chromatography. Inflorescences were collected at the end of the field season, dried at 50°C for 1 wk, and ground to pass through a 40-mesh screen using a Wiley Mill (Thomas Scientific, Swedesboro, New Jersey, USA). Alkaloids were extracted in 0.5 mol/L HCl with cinchonidine HCl added as an internal standard to 0.1% dry mass. The resulting extract was then made basic with addition of NH₄OH, and methylene chloride was added to extract alkaloids as free bases (Johnson et al. 1989). This methylene chloride extract was injected into a HP 5890A gas chromatograph (Hewlett Packard, Wilmington, Delaware,

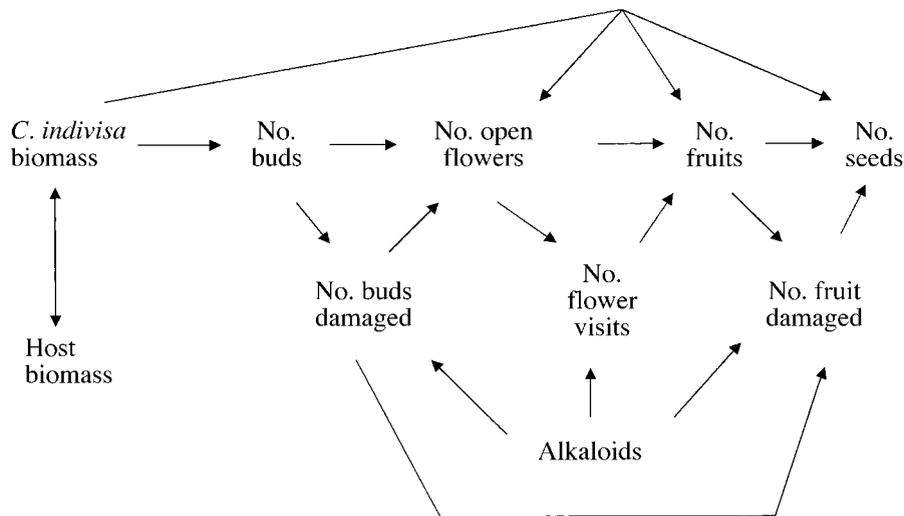


FIG. 1. Path diagram for the effects of alkaloids, herbivory, and pollination on seed set in *Castilleja indivisa*. Detailed descriptions of each variable are provided in *Methods: Path analysis*. Single-headed arrows depict path coefficients and point from the independent variable to the dependent variable. The double-headed arrow represents correlations between variables.

USA) with a DB-1 megapore capillary column 30 m long with 0.25 mm internal diameter and 0.25 μm film thickness (J&W Scientific, Folsom, California, USA).

Path analysis

Path analysis is a statistical modeling approach that can dissect correlations between variables into components due to different pathways via multiple linear regressions (Li 1975, Kingsolver and Schemske 1991, Mitchell 1993). This method lends itself particularly well to studies of herbivory and pollination (Schemske and Horvitz 1988, Mitchell 1993, Mothershead and Marquis 2000), in which many quantitative characters have the potential to affect plant fitness via multiple interactions. One must bear in mind, however, that path analysis is an analysis of correlations rather than causations, and is more useful for suggesting than for testing hypotheses.

We developed the path model (Fig. 1) based on the chronological order of interactions observed in the field. Buds develop into flowers and then fruit, which produce seeds. Plant resources, in this case measured as plant biomass, can be a correlated variable for each of these stages. In *C. indivisa*, one to many flowering stalks grow from a basal rosette, with a terminal inflorescence on each stalk. This architecture should result in a linear relationship between plant biomass and reproductive structures. The relationship between biomass and number of buds, flowers, fruits, and seeds had a large linear component, confirmed by regression incorporating both biomass and $(\text{biomass})^2$ as independent variables (the quadratic component was significant only for flower number, accounting for 28% of the variance explained). Including plant biomass as an independent variable for bud, flower, fruit, and seed number effectively standardizes each of these variables for

plant size, preventing the detection of strong positive path coefficients between variables that are due strictly to a correlation with plant size.

C. indivisa biomass may be related to host biomass in a complex fashion. Larger hosts may be able to support larger parasites, but larger parasites may be more detrimental to the growth of their hosts. Because it is difficult to predict the direction of this relationship, the path between *C. indivisa* biomass and host biomass was analyzed as a correlation rather than a regression.

As buds develop from flowers to fruits and seeds, each step is modified by plant-animal interactions. Inflorescence herbivores, particularly *Endothenia hebesana*, consume a significant portion of buds prior to their development into flowers. Flowers develop into fruit only if they are visited by pollinators. Fruits also suffer heavy herbivory from *E. hebesana*, which can reduce final seed production. Each of these interactions can have direct effects on seed set; e.g., bud herbivory can directly affect seed set by decreasing the number of flowers on a plant, which subsequently decreases fruit and seed set. Bud herbivory and pollination may also have indirect effects on seed set by influencing subsequent interactions; e.g., bud herbivory can reduce the number of open flowers that attract pollinators. A direct path from bud herbivory to fruit herbivory was also included because bud herbivores may remain on inflorescences and subsequently damage fruits (L. S. Adler, *personal observation*). Total alkaloid concentration of inflorescences may influence each of these interactions with animals; hence, paths from alkaloid concentration to number of buds eaten, number of flower visits, and number of fruit damaged were included in the model.

All of the variables used in the path analysis were quantified at the end of the season, with the exception of the number of open flowers and the number of flower

visits by pollinators. All variables refer to *C. indivisa*, except for host mass, and all were square-root(x) transformed to improve normality and skew of the residuals. Standardized path coefficients and significance were analyzed using the regression procedure of SAS. The STB option of the model statement provided standardized regression coefficients (Mitchell 1993). The variables are:

1) Host biomass: total combined dry mass of *Lupinus albus* individuals (typically two hosts per parasite) grown with each *C. indivisa* plant. Biomass includes the major tap roots but not fine roots.

2) *C. indivisa* biomass: total dry mass of *C. indivisa* individuals. Biomass includes the major tap roots but not fine roots.

3) Number of buds: total number of buds produced per plant.

4) Number of buds damaged: total number of buds per plant that did not produce flowers due to herbivory, evaluated by counting bud scars with herbivore damage.

5) Number of open flowers: the average number of open flowers per inflorescence, measured weekly on each plant, multiplied by the average number of inflorescences measured weekly per plant. Individual flowers are typically open for ~4–5 d, whereas inflorescences last several weeks (L. S. Adler, *personal observation*).

6) Number of flower visits: the total number of times flowers were visited per plant during the 70 h of observation. If the same flower was visited twice (on separate occasions or the same pollination bout), this counted as two flower visits.

7) Number of fruits: total number of fruits produced per plant.

8) Number of fruits damaged: total number of fruits damaged by herbivores per plant.

9) Number of seeds: total number of seeds per plant.

10) Alkaloids: the percentage dry mass of quinolizidine alkaloids present in inflorescences of *C. indivisa*.

In developing our model, we chose to use absolute rather than relative variables (i.e., the total number of flowers rather than the percentage of buds that became flowers; the total number of flower visits rather than the number of flower visits per open flower) for several reasons. Different questions are addressed by analyzing absolute compared to relative variables. To ask how an increase in open flowers affects total pollinator visits to whole plants, we should use absolute measures of floral visits. To examine how floral display affects per flower visitation rate, we should use relative measures. Because *C. indivisa* is an annual plant that has only one chance at reproduction, and that is also pollen limited in this study, the total number of flower visits to whole plants may be more relevant to plant female fitness than the per flower visitation rate. Developing the model for path analysis using proportional rather

than absolute variables created several relationships that were difficult to interpret. If 95% of buds are damaged prior to flowering, there may be an increase in the per flower visitation rate because so few flowers remain. However, it would be misleading to interpret this as herbivory increasing pollination success for the whole plant. For all of these reasons, we chose to analyze this model using absolute, rather than relative, variables.

Previous analyses found no significant interactions between the *C. indivisa* source population and any other factors (Adler 2000). Therefore, we combined data from both populations in the path analysis. In addition, pesticide spray reduced, but did not eliminate, herbivory in *C. indivisa*, and there were no significant interactions between herbivory treatment, host treatment, and/or *C. indivisa* source population on herbivore damage or seed set (Adler 2000). Therefore, both reduced- and natural-herbivory treatments were included in the path analysis to provide a greater range of herbivore damage.

Effect of resources on fruit set

Because there was a positive effect of plant mass on fruit production, independent of the number of open flowers (see *Results*), a separate multiple regression was performed to determine the effect of other floral display characters on flower visits. The variables used in the full model were calyx length, plant height, inflorescence length, date of first flower, and total days of flowering. Calyx length, plant height, and inflorescence length were measured weekly during the flowering season and were averaged over time. Calyx length was measured using digital calipers (Mitutoyo Corporation, Tokyo, Japan) for the longest open flower. Plant height was measured as the distance from the soil to the tip of the tallest inflorescence. Inflorescence length was measured as the length of inflorescence from the most basal calyx that had not senesced to the tip of the inflorescence; this was averaged over all inflorescences for each plant. Date of first flower is the beginning of the flowering season for each plant. All variables were square-root(x) transformed to improve normality and skew of the residuals, and were analyzed using the REG procedure of SAS (SAS Institute 1997). Because there were strong correlations between variables, a reduced model was selected on the basis of the standardized mean squared error of prediction C_p (a value of C_p less than p , the number of terms in the model, indicates collinearity, and values of C_p greater than p indicate biased estimation of parameters) and adjusted R^2 using the selection option of the REG procedure (Philippi 1993).

Relative magnitude of direct and indirect effects

In the terminology of path analysis, a direct effect refers to the path coefficient from an independent to a

TABLE 1. General statistics for morphology, chemistry, herbivory, and pollination of *Castilleja indivisa*.

Character	Bitter lupines			Sweet lupines		
	Mean	1 SE	Range	Mean	1 SE	Range
1) Host biomass (g)	1.92	0.093	0.24–4.47	1.56	0.08	0.72–3.71
2) <i>C. indivisa</i> biomass (g)	2.36	0.188	0.43–7.58	1.92	0.12	0.42–4.26
3) Number of buds	61.5	4.86	10–153	49.4	4.30	11–170
4) Number of buds damaged	21	2.27	0–73	21.7	2.01	0–76
5) Number of open flowers (mean)	12.9	1.03	3–34.2	13.0	1.05	1.5–33.6
6) Number of flower visits	1.89	0.45	0–14	1.17	0.32	0–9
7) Number of fruits	9.28	1.25	0–43	6.78	0.80	0–20
8) Number of fruits damaged	3.96	0.61	0–17	3.47	0.49	0–13
9) Number of seeds	610	114	0–3479	308	55.5	0–2024
10) Alkaloids (% dry mass)	0.028	0.004	0–0.125	0.0014	0.0007	0–0.022

Notes: For analysis, we used 57 *C. indivisa* parasitizing bitter lupines and 58 *C. indivisa* parasitizing sweet lupines. Untransformed data are presented here for ease of interpretation; all variables were square-root(x) transformed to improve normality and skew of the residuals for analysis. Detailed descriptions of each variable are provided in *Methods: Path analysis*.

dependent variable; an indirect effect refers to a path that passes through some other variable (Schemske and Horvitz 1988). However, in ecological interactions, a direct effect generally refers to an effect that one species has on another that is not mediated by a third species; an indirect effect refers to an effect that is mediated via a third species (Strauss 1991). Because the focus of this study is to dissect the direct and indirect effects of herbivores and pollinators on plant fitness in the ecological sense, the latter terminology will be used.

One of the advantages of path analysis is that the relative strength of different pathways can be calculated using the path coefficients. The total correlation between two variables can be decomposed by tracing all of the paths from a dependent to an independent variable (see Schemske and Horvitz 1988, Mitchell 1993); for example, in the model presented here (Fig. 1):

$$r_{\text{open flowers, buds}} = p_{\text{open flowers, buds}} + p_{\text{open flowers, bud damage}} \times p_{\text{bud damage, buds}}$$

where r_{AB} represents the correlation between variables *A* and *B*, and p_{AB} represents the path coefficient from independent variable *B* to dependent variable *A* (Schemske and Horvitz 1988). Using this approach, calculation of the relative importance of direct and indirect effects, in an ecological sense, is straightforward.

RESULTS

Magnitude of herbivory and pollination

Floral herbivory for *Castilleja indivisa* was substantial (Table 1); on average, nearly 40% of the buds were consumed before producing flowers. Observed pollinator visits were extremely low, and ultimately only 14% of the original buds (23.5% of undamaged buds) produced fruit. Of these, nearly half were then damaged by fruit herbivores, which consumed a portion or all of the seeds within a capsule (L. S. Adler, *personal observation*). The sum of these interactions resulted in

a wide range of final seed production, from no seeds to nearly 3500 seeds.

Direct effects of herbivory and pollination

Both bud and fruit herbivory had direct negative effects on seed set (Fig. 2, Table 2). Thus, plants were unable to fully compensate for herbivory on reproductive structures. Bud damage reduced seed number directly by reducing the number of open flowers, which reduced fruit and seed production. The number of fruits damaged also significantly reduced seed production. In addition, bud herbivory was positively related to fruit herbivory (Fig. 2, Table 2), probably because herbivores that established on inflorescences consumed buds, remained, and subsequently damaged fruits (L. S. Adler, *personal observation*). Therefore, bud herbivory also reduced seed production by increasing fruit herbivory; this would be considered a direct effect because it does not involve a third species. Overall, the direct effect of fruit herbivory on seed set was much larger than that of bud herbivory; the path coefficient between fruit herbivory and total number of seeds was -0.360 , compared to -0.113 for the summed direct effects of bud herbivory (Table 3).

The number of flower visits positively affected the number of fruits, indicating that plants were pollen limited and set more fruit with increased flower visits. Thus, pollination directly influenced seed set by increasing fruit production; the positive direct effect, 0.336 , was almost as large as the negative direct effect of fruit herbivory, -0.360 , and was much larger than the direct effect of bud herbivory, -0.113 (Table 3). Although the extent of pollen limitation may be overestimated in this study because of artificially dense planting, *C. indivisa* generally flowers densely in fields (Loughmiller and Loughmiller 1984) and is self-incompatible (L. S. Adler and C. Huyghe, *unpublished data*). Thus, *C. indivisa* is likely to be pollen limited in nature as well as in this study.

Indirect effects of herbivory and pollination

Bud herbivory reduced the number of open flowers per plant (Fig. 2, Table 2). Because more open flowers

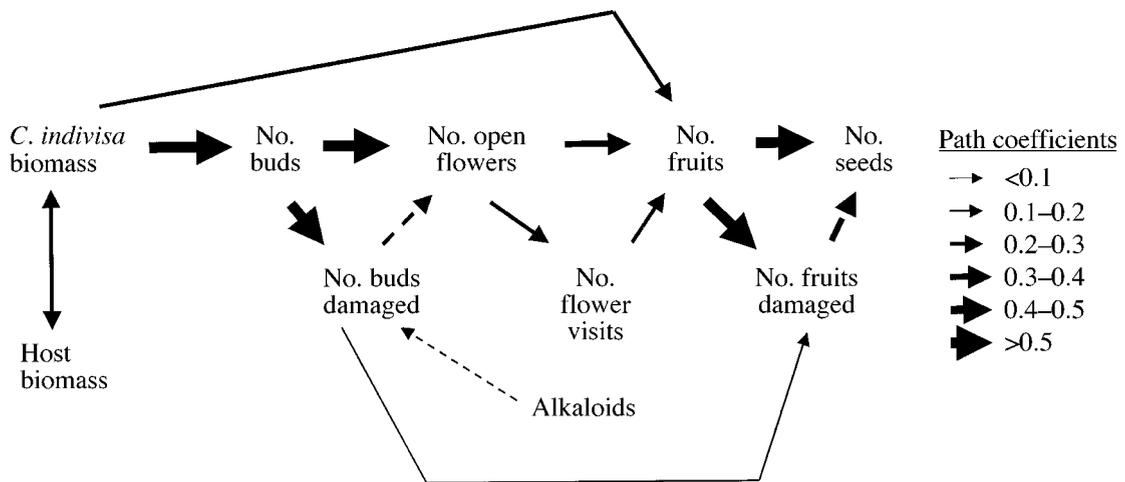


FIG. 2. Results of the path analysis for the effects of alkaloids, herbivory, and pollination on seed set in *Castilleja indivisa*. Only paths that were statistically significant were included ($P < 0.05$). The thickness of the arrows represents the magnitude of the standardized path coefficient. Dashed arrows represent negative path coefficients. See Table 2 for actual values, levels of significance, and the magnitude of unanalyzed causes.

per plant translated to more flower visits per plant, but herbivory reduced seed set indirectly by reducing pollination. The magnitude of this indirect effect (-0.017) was small relative to the total effect of bud herbivory on seed set (-0.126).

Increased flower visits led to increased numbers of fruit per plant (Fig. 2, Table 2). However, plants with more fruit had higher numbers of fruit damaged by herbivores. Therefore, there was a small negative effect of pollination on seed production by increasing fruit damage. This effect (-0.081) was $\sim 25\%$ as large as the positive effect of pollination on seed set (0.336).

Overall, the total effect of fruit herbivory had the greatest impact on seed production; this path coefficient (-0.36) was larger than the combined direct and indirect effects of pollination (0.255) or bud herbivory (-0.126) on seed production (Table 3). However, herbivory was experimentally manipulated in this study with an insecticide treatment, whereas pollination was not manipulated. Therefore, the relative importance of herbivory vs. pollination on seed production may be overestimated.

Effect of alkaloids

The alkaloid content of inflorescences negatively affected bud herbivory, but had no statistically significant direct effect on flower visits or fruit herbivory (Fig. 2, Table 2). However, bud herbivory increased fruit herbivory, probably because the same herbivores that damaged buds also damaged fruit. Therefore, alkaloids had an additional direct positive effect on seed set by reducing fruit herbivory. Also, because bud herbivory decreased pollination, alkaloids had an indirect positive effect on seed set by increasing pollination. The overall effect of alkaloids on seed production was calculated by summing all possible paths between these two var-

iables (Table 3). Effects through bud herbivory were the largest, making up 43% of the total benefit of alkaloid uptake. Reducing fruit herbivory accounted for 33% of the benefit of alkaloids, and the benefit via increased pollination was $\sim 24\%$ of the total. Most of the benefit was via direct effects of herbivores or pollinators on seed production; indirect effects constituted less than $<5\%$ of the total effect of alkaloids on seed set.

Effect of resources

C. indivisa biomass had a strong positive effect on the number of buds. The number of buds was positively correlated with the number of open flowers, which was positively correlated with number of fruits produced, which was positively correlated with seed production (Fig. 2, Table 2). Aside from this developmental pathway, plant biomass did not have other significant effects, with the exception of a positive effect on fruit production that was independent of flower number. This could be due to resource limitation for fruit set, although the positive effect of flower visits on fruit set indicates that plants are also pollen limited. Alternatively, this path could indicate that plant biomass affects variables, other than the number of open flowers, that are important for pollinator attraction.

To test the latter possibility, a multiple regression was performed to determine the effect of many floral display characters on flower visits. Because of multicollinearity between variables, a reduced model was selected using total days of flowering, plant height, and calyx length as independent variables. Both days of flowering and plant height significantly affected flower visits, but calyx length did not (Table 4). Both days of flowering and plant height were significantly affected by plant biomass (Table 4), suggesting that these fac-

TABLE 2. Standardized path coefficients and the proportion of variance explained (R^2) by each multiple regression model used to build the path analysis.

Dependent variable	Independent variables	Path coefficient	R^2	$p_{u, \text{variable}}^\dagger$
<i>C. indivisa</i> biomass	host biomass (correlation)	0.28**	0.08	0.96
No. buds	<i>C. indivisa</i> biomass	0.70****	0.50	0.71
No. buds damaged	no. buds alkaloids	0.54**** -0.19*	0.31	0.83
No. open flowers	no. buds no. buds damaged <i>C. indivisa</i> biomass	0.86**** -0.20** -0.01	0.59	0.64
No. flower visits	no. open flowers alkaloids	0.25** 0.05	0.06	0.97
No. fruits	no. open flowers no. flower visits <i>C. indivisa</i> biomass	0.34**** 0.29*** 0.29***	0.49	0.71
No. fruits damaged	no. fruits no. buds damaged alkaloids	0.78**** 0.15** -0.05	0.68	0.57
No. seeds	no. fruits no. fruits damaged <i>C. indivisa</i> biomass	1.16**** -0.36**** -0.04	0.76	0.49

Note: We used 115 *C. indivisa* individuals for analysis.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

† U represents unmeasured factors affecting a given dependent variable; $p_{u, \text{variable}}$ the magnitude of the influence of unanalyzed causes, is calculated as $\sqrt{1 - R^2}$ for each variable.

tors could be responsible for the positive effect of plant biomass on fruit set. The ideal analysis would include plant biomass in this multiple regression to determine if biomass still affects flower visitation when these floral characters are included. Unfortunately, plant biomass is so strongly correlated with these floral characters ($P < 0.0001$ for both plant height and total days of flowering) that these variables cannot be meaningfully separated using multiple regression. Thus, it is not possible in this study to conclude that the effect of biomass on fruit production is due either to increased resource availability or to increased attraction of pollinators. To do this, it would be necessary in a future study to experimentally manipulate floral characters independently of plant biomass to dissect the contribution of floral characters to fruit set when biomass is controlled.

Host plant effects

Although it was not the main goal of this study, our results also show that alkaloids may benefit hemiparasites by protecting their host plants from herbivores. At the end of the experiment, bitter-lupine host plants were larger than sweet-lupine hosts (Table 1), suggesting that alkaloids benefited host plants as well as hemiparasites. In the absence of herbivores, bitter lupines were not larger than sweet lupines (L. S. Adler and C. Huyghe, unpublished data). Therefore, we attribute the larger size of bitter hosts in the field to the defensive properties of alkaloids against herbivores. There was a positive correlation between host plant biomass and hemiparasite biomass, and strong positive

effects of hemiparasite biomass on reproduction (Fig. 2). Thus, alkaloids contributed to hemiparasite lifetime seed production not only by reducing herbivory and increasing pollinator visits, but also by improving resource availability in the host.

DISCUSSION

Many other studies have found that both herbivory and pollination play important roles in plant fitness (see reviews in Marquis 1992, Burd 1994, Delph et al. 1997). In several studies, the cause of low seed set, especially in rare plants, has been either a severe lack of pollinators or excessive seed loss due to herbivory (Tremblay 1994, Armstrong and Marsh 1997, Borba and Semir 1998). However, very few studies have assessed the relative magnitude of direct vs. indirect effects of herbivores and pollinators on correlates of plant fitness (but see Schemske and Horvitz 1988, Krupnick et al. 1999). In this research, bud herbivory, pollination, and fruit herbivory each had direct effects on seed set, but bud herbivory also had indirect effects by influencing subsequent plant-animal interactions. Although indirect effects of herbivores on pollinators were detected, the relative magnitude of these effects were not large (Table 3), consistent with general observations that direct effects tend to be stronger than indirect effects (Schoener 1993).

Alkaloids reduced bud herbivory but did not reduce visitation by pollinators; this led to a direct benefit of alkaloid uptake. Because bud herbivory reduced pollination and was correlated with increased fruit herbivory, alkaloid uptake had the additional benefit of

TABLE 3. Magnitude of direct and indirect effects of bud and fruit herbivory, pollination, and alkaloids on seed set.

Type of effect	Pathway	Magnitude
Bud herbivory		
DE	$P_{\text{flowers, buds damaged}} \times P_{\text{fruits, flowers}} \times P_{\text{seeds, fruits}}$	-0.078
DE via fruit herbivory	$P_{\text{flowers, buds damaged}} \times P_{\text{fruits, flowers}} \times P_{\text{fruits damaged, fruits}} \times P_{\text{seeds, fruits damaged}}$	0.019
DE via fruit herbivory	$P_{\text{fruits damaged, buds damaged}} \times P_{\text{seeds, fruits damaged}}$	-0.054
IE via pollination	$P_{\text{flowers, buds damaged}} \times P_{\text{flower visits, flowers}} \times P_{\text{fruits, flower visits}} \times P_{\text{seeds, fruits}}$	-0.017
IE via pollination and fruit herbivory	$P_{\text{flowers, buds damaged}} \times P_{\text{flower visits, flowers}} \times P_{\text{fruits, flower visits}} \times P_{\text{fruits damaged, fruits}} \times P_{\text{seeds, fruits damaged}}$	0.004
Total effect of bud herbivory		-0.126
Pollination		
DE	$P_{\text{fruits, flower visits}} \times P_{\text{seeds, fruits}}$	0.336
IE via fruit herbivory	$P_{\text{fruits, flower visits}} \times P_{\text{fruits damaged, fruits}} \times P_{\text{seeds, fruits damaged}}$	-0.081
Total effect of pollination		0.255
Fruit herbivory		
DE	$P_{\text{seeds, fruits damaged}}$	-0.360
Total effect of fruit herbivory		-0.360
Alkaloids		
DE via bud herbivory	$P_{\text{buds damaged, alkaloids}} \times P_{\text{flowers, buds damaged}} \times P_{\text{fruits, flowers}} \times P_{\text{seeds, fruits}}$	0.015
DE via bud and fruit herbivory	$P_{\text{buds damaged, alkaloids}} \times P_{\text{flowers, buds damaged}} \times P_{\text{fruits, flowers}} \times P_{\text{fruits damaged, fruits}} \times P_{\text{seeds, fruits damaged}}$	-0.004
DE via bud and fruit herbivory	$P_{\text{buds damaged, alkaloids}} \times P_{\text{fruits damaged, buds damaged}} \times P_{\text{seeds, fruits damaged}}$	0.010
IE via bud herbivory and pollination	$P_{\text{buds damaged, alkaloids}} \times P_{\text{flowers, buds damaged}} \times P_{\text{flower visits, flowers}} \times P_{\text{fruits, flower visits}} \times P_{\text{seeds, fruits}}$	0.003
IE via bud herbivory, pollination and fruit herbivory	$P_{\text{buds damaged, alkaloids}} \times P_{\text{flowers, buds damaged}} \times P_{\text{flower visits, flowers}} \times P_{\text{fruits, flower visits}} \times P_{\text{fruits damaged, fruits}} \times P_{\text{seeds, fruits damaged}}$	-0.0008
Total effect of alkaloids via bud herbivory		0.0232
DE via pollination	$P_{\text{flower visits, alkaloids}} \times P_{\text{fruits, flower visits}} \times P_{\text{seeds, fruits}}$	0.017
IE via pollination and fruit herbivory	$P_{\text{flower visits, alkaloids}} \times P_{\text{fruits, flower visits}} \times P_{\text{fruits damaged, fruits}} \times P_{\text{seeds, fruits damaged}}$	-0.004
Total effect of alkaloids via pollination		0.013
DE via fruit herbivory	$P_{\text{fruits damaged, alkaloids}} \times P_{\text{seeds, fruits damaged}}$	0.018
Total effect of alkaloids via fruit herbivory		0.018
Total effect of alkaloids		0.0542

Note: In this study, direct effect (DE) refers to an effect of one species on another that is not mediated by a third species, and indirect effect (IE) refers to an effect of one species on another that is mediated by a third species.

increasing pollinator flower visits and reducing fruit herbivory. The direct effect of alkaloid uptake on bud herbivory, but not pollination or fruit herbivory, may be due to differential incorporation of these alkaloids into plant tissues. Alkaloids from lupine hosts are present in the calyx and bracts, but not in nectar or ma-

turing fruit of *C. indivisa* (Adler and Wink, *in press*); thus, they would be encountered by bud herbivores but not pollinators or seed predators.

Bud herbivory had indirect, as well as direct, effects on lifetime seed set by reducing pollination, and pollination had indirect effects by increasing subsequent

TABLE 4. Multiple regression of flower visits on floral characteristics, and floral characteristics on *C. indivisa* biomass.

Dependent variable	Independent variable	Parameter estimate	R ²
Flower visits	days of flowering	0.43**	0.18
	plant height	0.44*	
	calyx length	-0.31	
Days of flowering	<i>C. indivisa</i> biomass	0.99****	0.29
Plant height	<i>C. indivisa</i> biomass	0.66****	0.23
Calyx length	<i>C. indivisa</i> biomass	0.02	0.00

Note: All variables were square-root transformed for analysis.
 * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

fruit herbivory (Table 3). Although these indirect effects were not large relative to direct effects, a more clear understanding of plant–animal interactions is gained by considering both indirect and direct selective pressures (Wootton 1994) on plant traits such as secondary chemistry. If pollination and fruit herbivory had not been measured in this study, the direct importance of bud herbivory on seed set would have been overestimated because it would have included the indirect effects of bud herbivory on pollination. By the same token, if only bud herbivory were manipulated in a study that controlled pollination, the importance of bud herbivores (and the effect of alkaloids on seed set via bud herbivory) would be underestimated because the study would have measured only direct effects.

The effects of herbivory on plant fitness can vary for different stages of plant development. For example, damage by the same herbivore can occur on both buds and fruit. Although both types of damage can be detrimental to plant fitness (Louda 1982, Louda and Potvin 1995), bud herbivory has the potential to affect pollination by decreasing floral display, whereas fruit herbivory would not be expected to influence pollinator choice. In systems in which plants are pollen limited, it might be beneficial for plants to allocate limited defenses to floral structures that will decrease bud, rather than fruit, herbivory. On the other hand, the direct effect of fruit herbivory on seed set was still much greater than the combined direct and indirect effects of bud herbivory (Table 3), a result consistent with another study (Cunningham 2000). Maturing fruits also require a larger investment of plant resources than do flowers. Therefore, we might expect selection for fruit defense to be greater than selection for floral defense, and for fruits to be highly defended in the absence of other constraints. The high alkaloid content of seeds in many species (Zangerl and Bazzaz 1992) suggests that, in many systems, if plants are allocating defenses optimally to protect valuable tissues (McKey 1979, Zangerl and Rutledge 1996), fruit predation may be more detrimental to fitness than bud herbivory.

This research contributes to the growing number of studies that have demonstrated that pollinators can distinguish between plants on the basis of prior herbivory, and prefer to visit less damaged plants (Miao et al. 1991, Karban and Strauss 1993, Lohman and Berenbaum 1996, Strauss et al. 1996, 1999, Juenger and Bergelson 1997, Krupnick et al. 1999, Mothershead and Marquis 2000). Over the flowering season, pollinators visited a greater percentage of *Castilleja indivisa* parasitizing bitter (high-alkaloid) hosts than sweet (low-alkaloid) hosts, and also visited more *C. indivisa* that had been sprayed with insecticide compared to natural-herbivory controls, regardless of alkaloid content (Adler 2000). Path analysis indicated that the benefit of alkaloids was due to their effect on herbivory, and that there were no direct effects of alkaloids on pollinator attraction. Preference of pollinators for less damaged

plants has been found in systems with floral herbivory (Karban and Strauss 1993, Lohman and Berenbaum 1996, Tennakoon and Pate 1996, Krupnick et al. 1999, Mothershead and Marquis 2000) and with early damage to vegetative structures (Juenger and Bergelson 1997, Lehtila and Strauss 1997, Strauss et al. 1999, Mothershead and Marquis 2000). In the latter studies, vegetative damage often resulted in smaller floral displays, smaller pollen size, and/or smaller number of pollen grains, which were correlated with the number or quality of pollinator visits (for effects of herbivory on floral and pollen characters where pollination was not observed, see also Hendrix and Trapp 1981, McKone 1989, Allison 1990, Frazee and Marquis 1994, Quesada et al. 1995, Mariano and Dirzo 1996, Mutikainen and Delph 1996, Delph et al. 1997, Gronemeyer et al. 1997, Thompson 1997). Thus, herbivores and pollinators may commonly affect each other through their shared interactions with plants.

Results from this study are relevant to the ongoing debate about whether costs of defense exist in plants. Theoretical models have attempted to explain why plant defenses are variable both within and between species (Rhoades and Cates 1976, Coley et al. 1985, Herms and Mattson 1992). Many of these models assume that there is a cost of defense (e.g., Simms and Rausher 1987), either through physiological (Bergelson and Purrington 1996) or ecological mechanisms (Simms 1992). One type of ecological cost of resistance could be decreased pollination in resistant plants (Detzel and Wink 1993, Strauss et al. 1999). In this study, there was no evidence for an ecological cost of defense via reduced pollination. Rather, increased pollinator visits to alkaloid-containing plants would be expected to increase the directional selection for defensive chemical uptake and/or production.

Host plant effects on herbivores of a hemiparasite have been examined in only a few systems. In all previous studies, different host species were compared, rather than lines of the same species. The hemiparasite *Castilleja wightii* suffered more herbivory from aphids when parasitizing the host *Lupinus arboreus* than when parasitizing nonleguminous hosts (Marvier 1996). This effect was attributed to the high nitrogen uptake from *L. arboreus*, which may be more important than alkaloid content for sucking insects such as aphids. Aphid performance on the holoparasite *Cuscuta campestris* was also influenced by host plant, and was particularly reduced with onion hosts (Harvey 1966). The generalist herbivore *Trichoplusia ni* and the specialist *Euphydryas anicia* both had decreased performance on *Castilleja sulphurea* containing alkaloids from the host *Delphinium occidentale* compared to *Castilleja* parasitizing hosts without alkaloids (Marko et al. 1995). However, adult oviposition and larval performance in the specialist herbivore *Euphydryas editha* on the hemiparasite *Pedicularis semibarbata* was unaffected by parasitism of the alkaloid-containing host *Lupinus ful-*

cratus (Stermitz et al. 1989). In the present study, alkaloid content was manipulated within the context of one host species. This approach allows the effect of alkaloids on herbivores and pollinators to be isolated from other differences that may exist between host species.

In conclusion, herbivory and pollination had both direct and indirect effects on lifetime seed set of a parasitic plant, *Castilleja indivisa*. Alkaloid uptake from host plants had a direct effect on seed set by reducing bud herbivory; in addition, there were indirect benefits of alkaloid uptake by increasing pollination. Thus, the combined direct and indirect benefits of alkaloids should result in even stronger selection for alkaloids than would be predicted by studying only direct effects.

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