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Reproductive biology of *Datura wrightii*: the benefits of a herbivorous pollinator

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• *Background and Aims* A deeper understanding of mutualism can be reached by studying systems with measurable costs and benefits. Most studies of this type focus on an unusual class of obligate, species-specific pollination mutualisms. The interaction between *Datura wrightii* (Solanaceae) and the hawkmoth *Manduca sexta* offers similar advantages but greater generality. Adult moths both nectar at and deposit eggs on the same plant; larvae are herbivorous. The antagonistic component of this interaction has been well studied. Here the role of *M. sexta* as a pollinator of *D. wrightii*, particularly in the context of this moth's frequent nectaring visits to the bat-pollinated plant *Agave palmeri*, is documented.

• *Methods* Hand-pollinations were used to determine breeding system and the reproductive consequences of mixed loads of *A. palmeri* and *D. wrightii* pollen. Plants and moths were caged overnight to assess whether nectaring visits led to fruit and seed set. Finally, pollen deposited on field-collected stigmas was identified, with a particular focus on documenting the presence of *D. wrightii* and *A. palmeri* grains.

• *Key Results Datura wrightii* is highly self-compatible, and a visit that deposits either outcross or self pollen almost doubles fruit and seed set compared with unvisited flowers. *Manduca sexta* transferred enough pollen to produce fruit and seed sets comparable to hand-pollination treatments. *Agave palmeri* did not interfere with *D. wrightii* success: in the field, stigmas received almost pure *D. wrightii* pollen, and hand-addition of large quantities of *A. palmeri* pollen had no measurable effect on fruit and seed set.

• *Conclusions* The floral visitation component of the *D. wrightii*–*M. sexta* interaction is indeed mutualistic. This finding is essential background to future development of this interaction as a model system for studying mutualism's costs and benefits. It is already proving valuable for dissecting third-species effects on the outcome of mutualism. Results indicate that *M. sexta*'s heavy visitation to *A. palmeri* has no negative effect on the benefits conferred to *D. wrightii*. However, it can be predicted to augment *M. sexta* populations to the point where the costs of the interaction begin to exceed its benefits.

Key words: *Datura wrightii*, *Agave palmeri*, pollination, herbivory, mutualism.

INTRODUCTION

A major advance in our growing understanding of mutualisms has been the recognition that these interactions not only confer benefits to their participants, but usually inflict costs as well. The costs of mutualism are unusually well documented in the case of biotically pollinated plants. Pollination requires investments into production of floral tissues, volatile attractants and nectar (Pyke, 1991; Galen *et al.*, 1999; Andersson 1999, 2000). Costs are also associated with the actions of antagonists attracted to flowers and floral rewards (Shykoff and Bucheli, 1995; Adler and Bronstein, 2004; McCall and Irwin, 2006). Pollination systems clearly evolve in the context of the ratio of benefits to costs. The evolution of floral morphology (Kobayashi *et al.*, 1999; Galen and Cuba, 2001), floral chemistry (Raguso, 2008), nectar traits (Thakar *et al.*, 2003; Dupont *et al.*, 2004), and even breeding systems (Johnson *et al.*, 2004; Tang and Huang, 2007) may only be interpretable in light of the costs of pollination investments (Bronstein *et al.*, 2006).

Weighing the costs and benefits of mutualisms can be challenging (Bronstein, 2001). It is difficult to express costs and benefits in the same currency, let alone to quantify their relative magnitudes. Some mutualisms offer unusual features that ameliorate these difficulties, however. In particular, the majority of empirical studies of the costs and benefits of mutualisms focus on pollinating seed parasite (sometimes termed nursery or brood site pollination) systems. In these interactions, the best known of which involve figs and yuccas, highly specialized insects both pollinate flowers and lay their eggs within plant reproductive structures (Holland and Fleming, 1999; Sakai, 2002; Dufaÿ and Anstett, 2003; Pellmyr, 2003; Herre *et al.*, 2008). The benefits of pollination in terms of seeds initiated can be compared with its costs, measured as the number of seeds lost to the pollinators' offspring (e.g. Pellmyr, 1989; Bronstein, 2001; Holland, 2002; Westerbergh, 2004). However, the highly unusual natural history of these pollination systems, including their extreme specificity, unusual floral attractants and rewards, and unique nature of the cost of mutualism, have constrained the generality of conclusions that can be drawn from studying them.

Datura wrightii (Solanaceae) and the hawkmoth *Manduca sexta* (Sphingidae) interact in a much more stereotypical pollination mutualism. The plant displays massive, white, sweet-smelling flowers that produce copious volumes of nectar highly attractive to these nocturnal insects, which make up the majority of its floral visitors; conversely, *M. sexta* adults

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rely heavily but not exclusively on *D. wrightii* for nectar (Alarcón *et al.*, 2008). Yet this interaction offers advantages similar to the more idiosyncratic pollinating seed parasite relationships for studying costs and benefits of mutualisms. After nectaring at *D. wrightii* flowers, *M. sexta* females lay eggs on the leaves. The larvae feed upon the tissue of their natal host plants. Thus, the benefits of pollination can potentially be weighed against the potentially high costs of herbivory by the pollinators' offspring (Bronstein *et al.*, 2007), generating an excellent test case of the conditions that promote persistence in the face of costs of mutualism.

It is reasonable to predict that, given the costs that *D. wrightii* is likely to incur by attracting *M. sexta*, the pollination benefits that they confer in return must be extremely high. However, although *M. sexta* behaviour and neurobiology in response to *D. wrightii* floral traits have been well documented (Raguso *et al.*, 2003; Raguso and Willis, 2002, 2005; Guerenstein *et al.*, 2004; Thom *et al.*, 2004; Riffell *et al.*, 2008), the quality of *Manduca* as a pollinator is unknown. Determining that this interaction does in fact confer benefits to *D. wrightii* is of course essential before it can be considered further as a model system for the study of mutualism.

Here, the quality of *M. sexta* as a pollinator of *D. wrightii* in south-eastern Arizona, USA is assessed. (*a*) Experimental results documenting *D. wrightii* breeding system in south-eastern Arizona are presented. The magnitude of the difference in reproductive success between treatments that allow autonomous pollination only and treatments that involve active pollen transfer indicates the degree to which *D. wrightii* benefits from the services of pollinators. (*b*) Experiments in field cages designed to assess the ability of *M. sexta* to pick up and transfer pollen are described. If *D. wrightii* reproductive success resulting from *M. sexta* nectaring behaviour is comparable to the number produced from hand pollinations, this would indicate a clear pollination benefit from this moth. (*c*) Experimental results documenting reproductive consequences for *D. wrightii* of the deposition of *A. palmeri* pollen, the predominant pollen carried by *M. sexta* at the field sites, are presented. These studies indicate whether *M. sexta*'s reliance on *A. palmeri* constitutes one of the costs of this mutualism from the plant perspective. Finally, to investigate whether such a cost may be inflicted in nature, (*d*) the identity and number of pollen grains deposited on *D. wrightii* stigmas in the field are documented.

STUDY SYSTEM AND SITE

Datura wrightii Regel (formerly *D. meteloides*) is a common perennial herb found in sandy or gravelly microsites from western Texas to California and Mexico. In Arizona, *D. wrightii* occurs at 300–1980 m a.s.l. (Kearney and Peebles, 1960). Plants regrow each year to sizes that vary with season; when moisture is plentiful, they may grow to 1 m tall and >2 m in diameter. The primary growing season is during the hot summer monsoonal period. *Datura wrightii* blooms from mid-April to early November in this region, opening low numbers of exceptionally large (up to 25 cm), white, tubular flowers each night. Flowers are open for a single night and secrete copious ($65 \pm 10\ \mu L$) concentrated (25 % sucrose equivalents) nectar (Elle and Hare, 2002;

Raguso *et al.*, 2003; Riffell *et al.*, 2008). Fruits are spiny capsules containing up to several hundred seeds. Mature fruits split open and seeds drop to the ground. They possess an elaiosome, and are dispersed by ants (Ness and Bressmer, 2005).

Manduca sexta is a large, widespread North and Central American hawkmoth. Despite its broad geographic range, in any one region it has only a handful of larval host plants, almost exclusively Solanaceae. It also commonly feeds upon solanaceous crop plants, giving rise to its common name, the tobacco hornworm. In the south-western United States, where its agricultural hosts are not grown commercially, *M. sexta* oviposits only on *Datura* spp. and (more rarely) *Proboscidea* spp. (Martyniaceae) (Mechaber and Hildebrand, 2000; Mira and Bernays, 2002).

Before laying eggs on *D. wrightii*, a *M. sexta* female typically makes a nectaring visit to a flower on the same plant. Raguso *et al.* (2003) have calculated that the nectar within a single *D. wrightii* flower provides a 1·2 g *M. sexta* with 10–15 min of hovering capability; a short bout of five to ten flower visits provides a moth with enough energy to fly for 1 h or more. Yet, although over three-quarters of the visitors to *D. wrightii* flowers are *M. sexta* individuals and almost all moths examined carry at least some *D. wrightii* pollen, these moths clearly visit other plants for nectar as well. In particular, the majority of the pollen grains carried by the average *M. sexta* individual are *Agave palmeri* (Agavaceae; Alarcón *et al.*, 2008). This bat-pollinated species exhibits a radically different floral syndrome, but produces copious amounts of nectar (Slauson, 2000; Scott, 2004). It has been shown that while *M. sexta* is innately attracted to and prefers to forage upon *Datura*, it easily learns to incorporate *Agave* in its diet (Riffell *et al.*, 2008).

The benefit of *M. sexta* pollination, which is documented in the present paper, comes at the cost of herbivory. Female *M. sexta* can lay up to 400 eggs in their lifetime (Madden and Chamberlin, 1945) and up to 100 each night (G. Davidowitz, unpubl. res.). Provided that there is enough foliage, a larva will feed exclusively on its natal plant (primarily on leaves, but also on flowers and fruits). The few larvae that escape predation and parasitism grow to be so large that a single individual can completely defoliate its host by its last instar (McFadden, 1968). An individual *M. sexta* larva can process $1400–1900\ cm^2$ of leaves (Heinrich, 1971; Casey, 1976), which is greater than the size of many *D. wrightii* in natural settings. *Datura wrightii* rapidly regrows following defoliation, however, and the fitness cost of *M. sexta* herbivory is still unclear.

METHODS

Study site

The work reported here was carried out in, and/or using material collected at, the Santa Rita Experimental Range (SRER), located 45 km south of Tucson, Arizona, USA at the base of the Santa Rita Mountains (31·78°, W 110·82°, 1320 m. a.s.l.). The habitat is characterized by semi-arid grasslands with seasonally flowing washes, bordered by mesquite and oak woodlands (Medina, 2003). During the summer months (July–September) SRER receives an average of

29·2 cm of precipitation and has a mean maximum temperature of 31·7 °C (NOAA Western Regional Climate).

Breeding system

Datura wrightii is known to be self-compatible. Self-pollination occurs when anthers and stigmas come into contact, both as the flowers open and as the corolla is shed the next day (Elle and Hare, 2002), as well as when visitors transfer pollen among flowers on the same plant. Hand-pollination experiments were used to quantify fruit and seed set resulting from outcross pollination, autonomous self-pollination (i.e. in unvisited flowers) and geitonogamy (i.e. due to active transfer of self pollen). Elle and Hare (2002) have presented some similar data for a moist-habitat Californian population. However, it was considered essential to document the breeding system at the same site at which moth pollination effectiveness was being studied, particularly since environmental factors are well documented to affect floral phenotype in this species (Elle and Hare, 2002).

Several hundred seeds were extracted from mature *D. wrightii* fruits collected at SRER in autumn of 2001. In late spring of 2002, 89 plants derived from the pooled seed collection were grown in the greenhouse in 8-in. standard pots, in a 3 : 2 : 1 Sungro Sunshine Mix #3–vermiculite–sand mixture. Plants were grown under natural daylight hours and watered three times weekly. They were fertilized once every 2 weeks, and transplanted to larger pots in late April. Plants began flowering in June and continued to open between none and five flowers each night at dusk through October. Expanded buds were tagged 2–3 d before they were expected to open and randomly assigned to one of three treatments, conducted when possible in rotating order on each plant. Flowers designated for the outcross hand-pollination treatment ($n = 45$ flowers) were emasculated at this point. Then, at dusk on the night each flower opened, anthers were clipped from a newly opened flower on a randomly chosen plant and swiped five times across the stigma. Two anthers were clipped from flowers assigned to the geitonogamous self-pollination (hereafter, hand self-pollination) treatment ($n = 108$ flowers) within several minutes of opening. These anthers were swiped five times each across the stigma of the same flower. Finally, in the autonomous self-pollination treatment, flowers were left unmanipulated ($n = 168$ flowers). The anther–stigma separation was measured in flowers assigned to the autonomous self-pollination treatments, as herkogamy is known to predict the rate of autonomous selfing in another *D. wrightii* population (Elle and Hare, 2002). Anther–stigma separation was measured as the height in millimetres from the top of the anthers to the tip of the stigma.

Tagged flowers were censused daily, and fruit initiation, development and abortion noted. Fruits were bagged with mesh netting approx. 5 weeks after the date of pollination. Once a fruit had dried, split open, and shed most of its seeds into the bag, approx. 7 weeks after pollination, the fruit and bag were removed. In the laboratory, fruits were allowed to fully dry in individual cups. They were then weighed. Seeds were extracted and weighed as a group. All seeds were then counted, noting any that were partially developed. Fruit set

was compared across treatments using G^2 tests, and seed set was compared across treatments using *t*-tests.

Pollen transfer by Manduca sexta

To determine whether *M. sexta* could function as a *D. wrightii* pollinator, during the summer of 2002, caged moths were allowed to freely visit flowering plants and the subsequent fruit and seed set measured. Moths had either been reared in the laboratory from field-collected larvae, or else were newly emerged moths from a laboratory-maintained colony (methods in Davidowitz *et al.*, 2003, 2004). Plants came from the same greenhouse-grown population described above.

Moths were placed in large ($2 \times 2 \times 2$ m) field cages with the plants overnight; no attempt was made to control the number of visits that individual flowers received. Two treatments were used. In the moth self-pollination treatment ($n = 31$ flowers from 23 plants), a single focal plant with between one and three open flowers was placed in the cage with one or two moths. Any pollen transferred onto the stigmas was therefore self pollen. Moths had not previously been exposed to flowers. Therefore, the only pollen they carried was that picked up in the course of the experiment. In the moth outcross pollination treatment ($n = 18$ flowers from 18 plants), the following were placed in the cage: (*a*) a focal plant bearing a single flower, which was emasculated prior to the experiment, using the protocol described above; (*b*) a second plant bearing one or two open flowers; and (*c*) between one and four moths. Fruit and seed set were recorded for the focal plant only, which could only have received outcross pollen. On the morning following all experiments, focal plants were returned to the greenhouse. Treatment flowers were labelled, and fruit and seed set subsequently recorded using the protocol described above. Fruit set and seed set per fruit were compared among treatments using G^2 tests and *t*-tests, respectively.

Consequences of foreign pollen deposition

Most *M. sexta* individuals captured in the field during 2004–2005 ($n = 148$ moths) carried *Agave palmeri* pollen. Indeed, 50–70 % of pollen grains on the average individual were *A. palmeri*; an additional 20–30 % were *D. wrightii*, with the remaining 10–20 % of pollen representing diverse other species (data shown in Alarcón *et al.*, 2008). In light of the high abundance of *A. palmeri* pollen on moths, consequences of its deposition for *D. wrightii* reproduction were experimentally investigated in 2005.

Four experimental treatments were designed to simulate possible pollination scenarios involving *A. palmeri* and *D. wrightii* in nature. These treatments used greenhouse-grown *D. wrightii* and field-collected *A. palmeri*. Each treatment was replicated 50 times. The first pair of treatments replicated the outcross hand-pollination treatment and the autonomous self-pollination treatment conducted in 2002. The same technique was used as in the earlier experiment; however, in the outcross treatment, pollen from flowers of multiple plants rather than a single plant was applied.

The second pair of treatments simulated mixed pollination. In both of these treatments, *A. palmeri* anthers were taken from mature umbels collected from SRER the previous day. In the *Agave + Datura* outcross hand-pollination treatment, a flower was emasculated, as in the *Datura* outcross hand-pollination treatment. *Agave palmeri* pollen was then applied by swiping a single mature anther across the surface of the *D. wrightii* stigma five times, leading to the deposition of a thick layer of pollen grains. Outcross *D. wrightii* pollen was then applied as in the *Datura* outcross hand-pollination treatment. The *Agave + Datura* autonomous self-pollination treatment involved addition of *A. palmeri* pollen to flowers allowed to self-pollinate without intervention.

Following the experimental pollination treatment, each flower was tagged and censused daily. Fruit initiation, development and abortion were noted as in the earlier breeding system experiment. Fruit collection and measurement of fruit and seed numbers and weights were recorded as in that experiment. Fruit set and seed set per fruit were compared among treatments using G^2 tests and *t*-tests.

Patterns of pollen deposition in the field

The identities and relative abundances of pollen that insects carry on their bodies do not necessarily reflect what those insects deposit on stigmas. Patterns of pollen deposition on *D. wrightii* stigmas were examined at SRER to determine if they were similar to the identities and proportions of pollen carried on the bodies of *M. sexta*, by far the most common visitor to these flowers at the study site (Alarcón *et al.*, 2008; Riffell *et al.*, 2008).

Stigmas were collected once weekly between 6 June and 21 July 2006 from 127 flowers that had been open the previous night on about 40 plants. Collections were made at two sites at SRER. One was located within 0·5 km of a large number of *A. palmeri* individuals, the other about 3 km distant, well within the flight range of an individual *M. sexta*. *Agave palmeri* was in flower on three of the collection dates, with its flowering peak on the final date (J. L. Bronstein *et al.*, unpubl. res.). *Datura wrightii* corollas had not yet wilted at the time stigmas were collected, suggesting that relatively little autonomous pollen had been deposited (Elle and Hare, 2002). Stigmas were placed in individual labelled glassine envelopes in a cooler, and returned to the laboratory. There, the stigmas were cut and placed on a microscope slide. They were stained with an aqueous fuschin stain, mixed with lightly dyed fuschin gel, heated over an alcohol lamp, and squashed with a coverslip. Slides were then examined under a compound microscope. All pollen grains were counted and identified to species to the greatest extent possible, using reference slides prepared in 2005 from all flowers located within 3 km of the study site whose flowers had been judged accessible to hawkmoths.

RESULTS

Datura wrightii was highly self-compatible (Fig. 1). Forty-seven per cent of all unmanipulated flowers (i.e. those in the autonomous self-pollination treatment) set fruit (Fig. 1A), with an average of 88·8 ± 69·0 seeds per fruit (Fig. 1B). Flowers in this treatment that did produce fruit had a significantly smaller
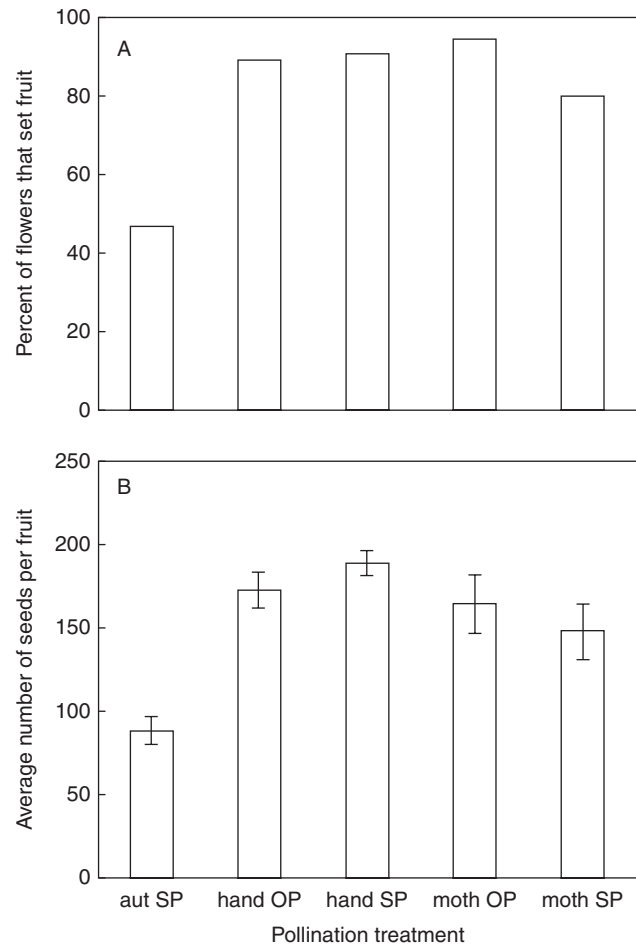

FIG. 1. Reproductive success from hand-pollination and caged moth pollination experiments. (A) Fruit set, i.e. the percentage of flowers in that pollination treatment that set fruit. Fruit set from autonomous self-pollination was significantly lower than other treatments, which did not differ significantly from each other (see text). (B) Mean (± s.e.m.) seed set per maturing fruit. Seed set from autonomous self-pollination was significantly lower than other treatments; moth hand-pollination resulted in lower seed set than did hand self-pollination, but other treatments did not differ significantly from each other (see text). Abbreviations: aut SP, autonomous self-pollination, from unmanipulated flowers; hand OP, hand outcross-pollination, i.e. from emasculated flowers on which pollen from other plants had been actively deposited; hand SP, hand self-pollination, i.e. from flowers on which pollen from other flowers the same plant had been actively deposited; moth OP, moth outcross-pollination, i.e. from emasculated flowers exposed overnight to *Manduca sexta* in a cage with flowers on other plants; moth SP, moth hand-pollination, i.e. from flowers exposed overnight to *M. sexta* in a cage with only that plant present.

anther–stigma separation than those that did not produce fruit ($t = 2·65$, $P = 0·009$; Table 1). In other words, flowers whose anthers were located much higher than the stigma were less likely to self-pollinate when the corolla slipped off compared with flowers in which the anthers and stigma were more similar in height (or in which the anthers were actually lower than the stigma; 1·7 % of 233 sampled flowers).

Flowers on which self pollen was hand-deposited experienced higher fruit set (91 %; $G^2 = 62·03$, $P < 0·0001$) and produced significantly more seeds per fruit ($t = 8·90$, $P < 0·0001$) than those allowed to self-pollinate autonomously.

TABLE 1. *Anther–stigma separation and fruit set in autonomously self-pollinated flowers*

	n	Mean	s.e.	Range
Autonomously pollinated flowers that did set fruit	48	11·5	7·1	−10–25
Autonomously pollinated flowers that did not set fruit	62	14·9	6·2	5–31

Anther–stigma separation predicted the likelihood of fruit set: flowers that did not set fruit had anthers significantly more distant from (higher than) the stigma than in flowers that did set fruit (*t*-test, $t = 2·65$, $P = 0·009$). Values are shown in mm as either positive (anthers above stigmas) or negative (anthers below stigmas).

Hand-deposition of self versus outcross pollen resulted in very similar fruit sets ($G^2 = 0·104$, $P = 0·75$; Fig. 1A) and seed sets ($t = 1·19$, $P = 0·24$; Fig. 1B). Hand-outcrossed fruits produced significantly more seeds ($t = 6·15$, $P < 0·0001$) than those resulting from autonomous self-pollination, and fruit set was significantly higher as well ($G^2 = 79·57$, $P < 0·0001$).

Flowers visited by caged *M. sexta* that received only self pollen experienced fruit set (80 %) similar to flowers in the hand self-pollination treatment ($G^2 = 2·41$, $P = 0·12$, Fig. 1A), although seed set per fruit was significantly lower ($t = 2·37$, $P = 0·02$, Fig. 1B). Flowers visited by caged *M. sexta* in which the moths deposited outcross pollen experienced fruit sets (94 %, $G^2 = 0·47$, $P = 0·49$) and seed sets ($164·9 ± 70·5$, $t = 0·41$, $P = 0·68$) similar to those seen in the hand outcross pollination treatment (Fig. 1).

Breeding system results from the 2005 experiment designed to look at the effects of *A. palmeri* pollen deposition on *D. wrightii* reproductive success were qualitatively similar to those reported above from 2002. As in the earlier experiment, fruit set was about 40 % higher in the hand-outcross treatment ($G^2 = 25·397$, $P < 0·0001$; Fig. 2A). Fruits from the hand-outcross treatment set significantly more seeds per fruit than those from the autonomous self-pollination treatment ($t = 8·485$, $P < 0·0001$; Fig. 2B).

At the study site, *M. sexta* carries large amounts of *A. palmeri* pollen (Alarcón *et al.*, 2008; Riffell *et al.*, 2008). What would be the consequences if this pollen were deposited on *D. wrightii* stigmas? In fact, hand-deposition of large amounts of *A. palmeri* pollen had no significant effect on *D. wrightii* reproductive output (Fig. 2). The number of seeds per fruit between the *Datura* outcross hand-pollination and *Agave* + *Datura* hand-pollination outcross treatments did not differ ($t = 1·069$, $P = 0·2879$). Nor was there a significant difference in seed numbers per fruit between the *Datura* autonomous self-pollination and *Agave* + *Datura* autonomous self-pollination treatments ($t = 0·401$, $P = 0·6897$). Fruit set of outcrossed flowers with and without *A. palmeri* pollen was indistinguishable (98 %, $G^2 = 0·005$, $P = 0·9412$), as was fruit set of selfed flowers with and without *A. palmeri* (62 % vs. 55 %, $G^2 = 0·544$, $P = 0·4608$).

Data on stigmatic pollen loads in the field (Table 2) suggest that, in spite of the large amounts of *A. palmeri* pollen carried by floral visitors, very little of it is deposited on *D. wrightii* stigmas. Stigmas received a median of 408 pollen grains, of which 95 % was *D. wrightii*. Only 0·2 % of all observed pollen grains were *A. palmeri*. All of this pollen was deposited during
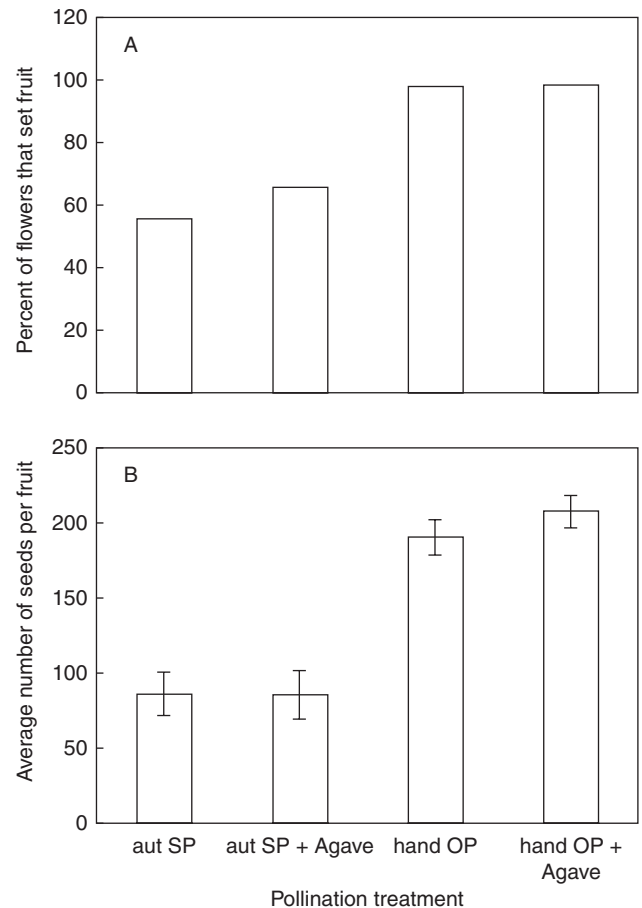

FIG. 2. Reproductive success of hand-pollinated flowers that were and were not augmented with *Agave palmeri* pollen. Addition of *A. palmeri* pollen had no effect on fruit set (A) or mean (± s.e.m.) seed set per fruit (B). In contrast, as in the earlier experiment whose results are shown in Fig. 1, flowers receiving outcross pollen set significantly more fruits and seeds per fruit than those that were autonomously pollinated. Abbreviations: aut SP, autonomous self-pollination, from unmanipulated flowers; hand OP, hand outcross-pollination, i.e. from flowers on which pollen from other plants had been actively deposited; aut SP + *Agave*, flowers on which *A. palmeri* pollen alone was actively deposited; hand OP + *Agave*, emasculated flowers to which both outcross *D. wrightii* and *A. palmeri* pollen were actively deposited.

the period in which the *A. palmeri* population nearest to the study sites was flowering. The most common identifiable foreign pollen was *Argemone* sp. (Papaveraceae), the California prickly poppy, which was common at the study site and in flower during the study. *Argemone* pollen was never identified on the body of any local hawkmoth (Alarcón *et al.*, 2008). It may have been imported by honey bees, which were occasional visitors to *D. wrightii* flowers and frequently observed on *Argemone* (J. L. Bronstein *et al.*, unpubl. obs.).

DISCUSSION

In the south-western United States, the life history of *Manduca sexta* is intertwined with that of *Datura wrightii* at two life-history stages: adults partially subsist on *D. wrightii* nectar, while larvae consume *D. wrightii* nearly exclusively (Bronstein *et al.*, 2007). This linkage emerges because

Table 2. *Pollens represented on 127* Datura wrightii *stigmas sampled in the field from approx. 40 plants from 6 June to 21 July 2006*

Species	% stigmas	% of all pollen deposited	Range deposited
Datura wrightii	100	94·7	8–1950
Argemone sp.	69·3	2·7	1–149
Misc. unidentified*	59·0	2·4	1–289
Agave palmeri	3·9	0·2	1–26
Pinaceae	3·9	≪0·01	1
Compositae[†]	4·7	≪0·01	1–3
Onagraceae	3·1	≪0·01	1–2

Flowers open at dusk for a single night; stigmas were collected the following morning.

*The 'Miscellaneous unidentified' class includes a large number of unidentified species.

[†]'Compositae' are species in this family other than *Argemone* spp.

M. sexta females characteristically lay their eggs on *D. wrightii* leaves during nectaring visits to these plants. Floral visits have the potential to result in pollen transfer.

The linkage in this system between pollination by adults and consumption by offspring creates a marked ecological resemblance to the so-called pollinating seed parasite or nursery pollination systems, well studied interactions that have helped shape our current understanding of plant/animal mutualisms (Holland and Fleming, 1999; Sakai, 2002; Dufaÿ and Anstett, 2003; Pellmyr, 2003; Herre *et al.*, 2008). In these associations, the best-known of which involve figs and yuccas, a highly specialized insect is obligately associated with (most commonly) a single plant species. It pollinates the plant associate, in many cases through deliberate actions, and then deposits its eggs on or within the reproductive tissue; pollinator offspring destroy or develop at the expense of some of the seeds. These plants have little or no ability to self-fertilize. Hence, whatever level of reproductive success achieved by these plants is due to the actions of their obligate, specific seed consumers (Bronstein, 2001).

The *M. sexta*−*D. wrightii* association is considerably more open and generalized than these pollinating seed parasite relationships. Consequently, it may be more useful as a general model for exploring how costs and benefits mediate mutualism dynamics. First, as in most plant/pollinator interactions, *M. sexta* visits flowers in order to obtain nectar. In contrast, most pollinating seed parasites are not seeking this reward, and their plant associates commonly produce little or no nectar (e.g. Fleming and Holland, 1998). Some, including fig wasps, lack feeding mouthparts. Since nectar production is perhaps the best-understood cost of pollination to plants (e.g. Pyke, 1991) and one of the most thoroughly studied costs of mutualism overall, it is fitting that a model system should involve it. Secondly, the pollinators' offspring feed primarily on leaves (e.g. Bernays and Woods, 2000; Kester *et al.*, 2002), rather than seeds or gall tissue as in pollinating seed parasite mutualisms. The extensive knowledge that has accumulated in the past century on the ecology, evolution and physiology of herbivory can therefore be taken advantage of to interpret the cost of this interaction. Furthermore, *M. sexta*

is nearly unique among insects in having the neurobiological basis of its foraging choices under intensive study (e.g. Guerenstein *et al.*, 2004; Riffell *et al.*, 2008). Thirdly, the *M. sexta*−*D. wrightii* interaction does not exhibit the extreme species-specificity characteristic of pollinating seed parasite systems. Pollen loads indicate that *M. sexta* adults at the study site regularly visit flowers of at least one other plant species, *Agave palmeri*, for nectar (Alarcón *et al.*, 2008; Riffell *et al.*, 2008). Conversely, pollen loads and field observations reveal that *D. wrightii* receives visits from at least two other hawkmoth species, as well as from honey bees (Alarcón *et al.*, 2008; Riffell *et al.*, 2008; J. L. Bronstein *et al.*, unpubl. obs.). Although it is not known if these visits can result in pollen transfer, this seems likely, as it has been found that almost any jostling of the flower leads some pollen to be shed from the anthers, which are continuously distributed but typically located well above the stigma (Fig. 2). The interaction is also not species-specific at the larval consumption phase. Across its range, *M. sexta* consumes a range of solanaceous host plants, including both native and domesticated *Nicotiana* species, tomato, *Capsicum* and other *Datura* species (e.g. Madden, 1945; Kester *et al.*, 2002; Mira and Bernays, 2002). It has been documented to use a single genus from a different plant family, *Proboscidea* (Martyniaceae) (Mechaber and Hildebrand, 2000). Conversely, *D. wrightii* has other herbivores (cf. Hare and Elle, 2002), although they consume considerably less tissue than does *M. sexta*. Finally, unlike pollinating seed parasite systems but like the majority of plant/pollinator interactions, the interaction (while close) is not obligate for either partner. A few *M. sexta* adults carry no *D. wrightii* pollen, and in fact for part of the summer appear to forage almost exclusively on *A. palmeri* (Alarcón *et al.*, 2008; Riffell *et al.*, 2008); late in the summer, *Proboscidea parviflora* is available as an alternative host plant (Mechaber and Hildebrand, 2000; Mira and Bernays, 2002; J. L. Bronstein *et al*., unpubl. res.). Furthermore, as has been shown here and which is consistent with other studies, *D. wrightii* is highly self-compatible (Figs 1 and 3). About half of all unvisited flowers set fruit, producing about 88 seeds per fruit. When self pollen is actively deposited, seed set and fruit set are identical to outcrossed flowers (Fig. 1). It has been found that selfed seeds germinate as readily as do seeds produced by outcross pollination, although the seedlings perform poorly when subjected to water stress (A. Tyler, University of Arizona, unpubl. res.). Thus, the easily identified and quantified antagonistic component of this relatively open association makes it a valuable general model for weighing the benefits versus costs of mutualism.

Of course, the *M. sexta*−*D. wrightii* association can only be a useful model system in this regard if it is in fact a mutualism. Surprisingly, although its antagonistic component has been relatively well studied, almost no information has been available on its potentially mutualistic component. Previous authors have documented *M. sexta* nectaring visits to *D. wrightii* (Grant and Grant, 1983; Adler and Bronstein, 2004; Guerenstein *et al.*, 2004; Thom *et al.*, 2004; Raguso and Willis, 2005), and its floral and nectar traits are well described (Grant and Grant, 1983; Elle and Hare, 2002; Raguso *et al.*, 2003; Guerenstein *et al.*, 2004). However, the

reproductive consequences of these visits have not been examined previously. Here, it has been shown that this moth is a highly effective pollinator of *D. wrightii*. Visits from moths caged overnight with flowers led to fruit and seed sets typical of those produced in hand-pollination experiments (Fig. 1). Although these experiments may have led to the deposition of unnaturally high pollen loads, given that few flowers were available to the caged moths relative to the numbers these large-bodied moths may visit in the field (Raguso *et al.*, 2003), field-collected stigmas themselves received a median of 388 *D. wrightii* pollen grains. Furthermore, almost every field-collected stigma bore at least some *D. wrightii* pollen, and the deposited loads were remarkably pure (Table 2). In combination, these data point to *M. sexta* as a highly reliable, if not exclusive visitor to *D. wrightii* that regularly deposits large quantities of pollen that can lead to high fruit and seed set. The degree to which *M. sexta* deposits self versus outcross pollen is not known. Nor is it known what the proportion of self pollen on stigmas of *M. sexta*-visited flowers that arrived from other flowers on the plant is relative to pollen that fell passively from the anthers during moth visits. However, in light of the high degree of self-compatibility and elevated reproduction of visited flowers regardless of pollen source, its nectaring visits clearly benefit *D. wrightii*.

The high quality of *M. sexta* as a *D. wrightii* pollinator is particularly intriguing in light of the moths' heavy reliance on *A. palmeri* as a nectar source. *Agave palmeri* is locally abundant in parts of the study site. Individuals bear several large panicles of flowers, each of which contains about 600 μL of nectar. In contrast, *D. wrightii* plants typically bear only 0–30 flowers at a time. Its flowering season stretches from April to November, but even at its midsummer flowering peak it is relatively uncommon: it made up <0·3 % of all flowers in a community-level study conducted in 2005 (Alarcón *et al.*, 2008). Overall, the nectar standing crop of *A. palmeri* offers about a 6-fold higher energy content than *D. wrightii* and can sustain *M. sexta* flight for much longer durations (Riffell *et al.*, 2008). *Manduca sexta* has a strong, innate preference for *D. wrightii* over *A. palmeri*, presumably linked to females' requirement to locate *D. wrightii* as a host plant; however, it can easily learn to incorporate *A. palmeri* into its diet (Riffell *et al.*, 2008).

These data suggest that by subsidizing the flight of *M. sexta* when *D. wrightii* flowers are rare, *A. palmeri*, a bat-pollinated species, is facilitating the success of the *M. sexta*–*D. wrightii* pollination interaction. However, for this interpretation to be valid, it must be shown that deposition of *A. palmeri* pollen does not interfere with *D. wrightii* reproductive success. If it did, then *Agave*'s role would likely be negative.

It has been shown here that *A. palmeri* pollen interferes in no detectable way with the quality of *M. sexta* visits to *D. wrightii* flowers. Remarkably little *A. palmeri* pollen is deposited by the moths on *D. wrightii* stigmas (Table 2). There is no direct evidence that the moths that visited the flowers from which stigmas were collected were in fact carrying *A. palmeri*. However, it has been documented in other years that the majority of *M. sexta* individuals do carry *A. palmeri* pollen; during weeks in which *A. palmeri* is abundant and *D. wrightii* scarce, pollen loads are strongly *Agave*-biased (Alarcón *et al.*, 2008; Riffell *et al.*, 2008). Furthermore, phenological censuses during this study

indicated that the nearest *A. palmeri* stands were in full flower on the last three dates on which *Datura* stigmas were sampled. Thus, it is highly likely that moth visitors did indeed carry large loads of *A. palmeri*. In the present pollen transport studies, pollen from moth prosboces has been recorded. Elsewhere it has been demonstrated that pollen loads on moths' ventral surfaces are no different (Alarcón *et al.*, 2008). However, the precise location on the proboscis occupied by pollens of different species has not been documented. It is possible that *A. palmeri* and *D. wrightii* pollen are deposited on different parts of the proboscis, such that when moths visit subsequent *Datura* flowers, only *Datura* grains come in contact with stigmas. Similar phenomena are well documented in studies of plant species that share pollinators (Morales and Traveset, 2008); in the present case, two plant species share floral visitors but visits result in pollination for only one of them. Alternatively, *A. palmeri* pollen might adhere to the proboscis in a way that resists deposition. This species shows clear adaptations for pollination by bats (Slauson, 2000; Scott, 2004; Riffell *et al.*, 2008), so it might not be surprising if the moth proboscis proved to be a poor mediator of pollen transfer. Further experiments will be necessary to distinguish among these possibilities.

Even if *A. palmeri* pollen did land on *D. wrightii* stigmas, the present experiments indicate that it would not interfere with reproductive success, a not uncommon result in studies of this type (Morales and Traveset, 2008). Adding *A. palmeri* pollen, even in large quantities, did not produce *D. wrightii* fruit sets or seed sets any different from what would have been experienced upon receipt of *D. wrightii* pollen alone (Fig. 2). The experimental protocol was intended to maximize the likelihood of finding pollen interference: negative effects have been more commonly found in hand-pollination experiments such as ours in which heterospecific pollen is deposited first (Morales and Traveset, 2008). These data further support the interpretation that *A. palmeri* facilitates the success of the *M. sexta*–*D. wrightii* pollination interaction.

It is nevertheless possible that *Agave*'s overall role is negative from the perspective of *Datura*. *Manduca sexta* is not only a highly effective pollinator, but a specialized herbivore of *D. wrightii*. A single larva can consume the entire aboveground biomass of an average-sized plant (McFadden, 1968). *Agave palmeri*'s subsidization of flight for feeding and reproductive activities potentially increases *M. sexta*'s local population size, as well as that of the many other hawkmoths that appear to rely on it in this habitat (Alarcón *et al.*, 2008). While this should lead to an increase in *D. wrightii* pollinator abundance at the local scale, plant reproductive success should plateau (or even decrease; Young and Young, 1992) above some visitation level. The cost of the association, however, should continue to rise with increasing visits by *M. sexta* females. Individual eggs have a very low likelihood of survival to pupation (about 1 % in studies of Mira and Bernays, 2002). The more eggs that are deposited, the greater the chance that *D. wrightii* will be completely defoliated by the pollinators' offspring. The fitness cost of defoliation to these perennial herbs has not yet been fully measured. However, the potential for *Agave* to affect the cost as much or more than the benefit of the *Datura*–*Manduca* association raises the possibility that the net effect of the association might be negative, not positive,

when *A. palmeri* is locally abundant. Such context-dependency seems likely to be a geographically variable phenomenon. Not all south-western *D. wrightii* populations grow within *M. sexta*'s flight range from *A. palmeri* (or similar agaves). Future work will test whether rates of pollination and herbivory in this system are more widely dependent upon the presence of additional nectar resources, be it *A. palmeri* or other species.

Third-species effects on the outcomes of mutualism are increasingly well documented in a wide range of mutualisms, particularly ones that are not strictly species-specific (Bronstein, 1994; Bronstein and Barbosa, 2002). Outcomes of pollination mutualisms, and in some cases their evolutionary trajectories, can be altered by nectar-robbers, predators, herbivores, seed predators and competitors (e.g. Herrera, 2000; Dedej and Delaplane, 2004; Schatz *et al.*, 2006; Irwin and Adler, 2006; McCall and Irwin, 2006; Ferrière *et al.*, 2007; Muñoz and Cavieres, 2008). The *D. wrightii–M. sexta* association will provide an ideal opportunity to document such effects in depth, due to the ability to identify, separate and measure effects of a third species on both the benefits and the costs of the interaction. This example highlights the potential value of this association as a model system for the study of mutualism.

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