FLORAL EVOLUTION IN MILKWEEDS: EVIDENCE FOR SELECTION PAST AND PRESENT

By

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ABSTRACT

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Adaptation is an important process that allows species to utilize new habitats and to avoid extinction, contributing to the biodiversity we find on the planet. Many flowering plants rely on animals for pollination; the floral traits that are adaptive for pollination are those that influence attraction, rewards, or efficiency. Milkweeds (*Asclepias*) have unusual floral structures that consist of a gynostegium surrounded by five nectar-filled hoods. In many species, a horn develops from the inside base of each hood. Pollen grains are packaged into waxy packets (pollinia) and are positioned in the wall of the gynostegium. For fertilization, pollinia must be removed by pollinators and inserted into stigmatic openings in the wall of the gynostegium between adjacent hoods; pollination is carried out passively by a wide variety of pollinators that are almost entirely all insects. Milkweeds are hermaphroditic, so it is possible for the floral traits to be adaptive for male fitness, female fitness, or both.

The floral diversity across *Asclepias* is astounding. My dissertation investigates if and how the floral structures are adaptive, and if the variation among species is the result of natural selection. I used two complementary approaches. For the first approach, I used contemporary measures of selection and functional studies to focus on the process of adaptation in five species of North American *Asclepias*. Selection works on intraspecific variation within traits and the effect of that variation on fitness. I also utilized paternity analyses in two species to measure selection through male fitness. For the second approach, I used phylogenetic methods to find

signatures of past selection on traits across more than one hundred North American *Asclepias* species. Phylogenetic comparative methods focus on patterns of interspecific variation. I used tests of correlated evolution between pairs of traits, or traits and pollinators, to investigate functional relationships and possible selective agents. I also tested for convergent evolution, which can demonstrate adaptive evolution in response to a similar selective regime.

The six floral traits I studied had an effect on fitness, suggesting they are adaptive. I found that many of the floral traits were under significant selection through only one gender, but that the direction of selection was similar across genders, showing little conflict between male and female function or between male and female fitness. I predicted that the size of the hood and gynostegium would influence pollinator attraction, but they were instead more likely to influence the efficiency of pollination. I also found no significant link between female pollination success and female reproductive success in four of the five species, so traits that increased pollen receipt did not in turn affect female fitness, which is consistent with fruit production not being limited by pollen receipt. Using measures of viable seeds produced (annual female fitness) and viable seeds sired (annual male fitness), I determined that selection estimated using total fruit number is a good estimate of selection through viable seeds produced; however, total pollinia removed per plant, a common estimate of male function and fitness in milkweeds is not a good predictor of viable seeds sired. My studies of the gynostegia, hoods, and horns across the phylogeny showed that horn loss likely followed the closure of hoods, suggesting a possible loss of horn function. There were also three convergent floral phenotypes; each may have evolved in response to similar selective regimes. The convergent species provide an excellent starting point for future investigations of possible selective agents.

Copyright by RAFFICA JEANNE LA ROSA 2015 I dedicate this dissertation to my parents, Rosemary Warner and Mike LaRosa, who made the decision to leave the city for the rolling hills of southwest Wisconsin, and to Ben Gollmer, Sue Schmidt, and the rest of my family, near and far, for their love and support.

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CHAPTER 1

INTRODUCTION

Background

Adaptation is a fundamental process in biology that allows species to cope with their environment. Many flowering plants rely on animals for pollination; the floral traits that are adaptive for pollination are those that influence attraction (e.g., Conner and Rush 1996; Schemske and Bradshaw 1999; Johnson et al. 2003; Hansen et al. 2012), rewards (e.g., Real and Rathcke 1991; Silva and Dean 2000), or efficiency (e.g. Nilsson et al. 1987; Fulton and Hodges 1999; Conner et al. 2009). Adaptive traits can be studied utilizing complementary approaches including phylogenetic comparative methods (Larson and Losos 1996) to study patterns of interspecific variation to identify signatures of past selection (e.g., Mahler et al. 2013), and measures of contemporary selection that rely on intraspecific variation--a prerequisite for evolution. My dissertation combines these two approaches to study adaptive floral traits in North American milkweeds (*Asclepias*).

Milkweeds have highly unusual flowers that each consist of a fused male and female floral whorl that forms a cylindrical gynostegium surrounded by five hoods. The hoods contain nectar, and in many species, they also contain horns. Pollen grains are packaged into waxy packets called pollinia and are positioned in the wall of the gynostegium. For fertilization, pollinia must be removed by a pollinator and then inserted into a stigmatic opening between adjacent hoods. Milkweeds are hermaphroditic, so it is possible for the floral traits to be adaptive for male fitness, female fitness, or both.

My dissertation includes present-day studies of adaptation on six continuous floral traits: gynostegium width, hood length, hood height, horn reach, stigmatic slit length, and the gap width between adjacent hoods. I examined the function of these floral traits for male and female pollination success, and their effect on reproductive success in five species of *Asclepias*. I also used genetic paternity analyses to measure selection on these six traits through male fitness in two species. These results were paired with phylogenetic comparative methods that focused on patterns in the evolution of hood and gynostegium related traits, and predictive methods to find convergent phenotypes that are indicative of selection.

Organization of the dissertation

Chapter 2: Floral traits can interact with pollinators to affect both pollination success (pollen removal and deposition) and reproductive success (seed siring and production). In collaboration with Jeffrey Conner, I measured selection on six floral traits in naturally occurring populations of *Asclepias syriaca, A. viridiflora*, and *A. tuberosa* to understand if the unusual floral traits of *Asclepias* may be adaptive. To test the effects of these traits on male and female pollination success and female reproductive success, I used separate selection gradient analyses using the fitness components pollinia removed per flower, pollinia inserted per flower, and fruit number as dependent variables. I used path analyses to understand the sources of selection, including pollinator visitation and the relationships among the three fitness components. I found that most traits were under selection through only one gender, and many affected the efficiency of pollination rather than pollinator visitation. I also found no significant link between female pollination success and female reproductive success in any of the three species, so traits that

increased pollen receipt did not affect female fitness, which is consistent with fruit production not being limited by pollen receipt.

Chapter 3: In collaboration with Jeffrey Conner, I studied one naturally occurring population each of A. exaltata and A. incarnata over multiple years. I examined the function of six floral traits using estimates of pollinia inserted per flower (female function) and pollinia removed per flower (male function). I also measured natural selection on these traits through fruit number (female fitness) across multiple years. For a single year I genotyped all viable fruits and possible parental plants to assign paternity to all offspring in both populations. I then measured selection through seeds produced, seeds sired, and total seed number. Only slit length functioned to influence pollinia removals per flower in A. exaltata, and gynostegium width, hood length, slit length, and gap width all influenced pollinia insertions and/or removals per flower in A. incarnata. Gynostegium width was under selection through both genders, but hood height and gap width only affected female fitness. In a majority of cases, the direction of selection is the same between genders, showing little evidence for conflict. I found that selection estimated using fruit number is a good estimate of selection through seeds produced, but that total pollinia removed per plant, a common estimate of male function and fitness is not a good predictor of viable seeds sired.

Chapter 4: In collaboration with Jeffrey Conner and Mark Fishbein, I complemented the contemporary studies of selection from Chapters 1 and 2, with phylogenetic comparative methods to ask: (1) Was the evolution of floral traits correlated with other floral traits or pollinator composition? and (2) Is there convergence of floral traits suggesting a common selective environment? I mapped gynostegium, hood, and horn traits of 107 *Asclepias* species

onto a phylogeny of all 125 North American species. Results using Pagel's correlation method suggested horns have been lost a number of times following the closure of the hoods, and that flared hood openings are marginally correlated with high proportions of hymenopteran pollinators. I found that hood length and gynostegium width were positively correlated across species after correcting for phylogenetic relatedness, but that they were not correlated with the proportion of hymenopteran pollinators, and that 13 clades or species have converged towards three phenotypic optima (4-5 clades each), suggesting convergent evolution. Interspecific patterns of evolution suggest that *Asclepias* flowers are adaptive and likely evolved in response to selective agents.

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CHAPTER 2

SELECTION ON FLORAL TRAITS THROUGH MALE AND FEMALE POLLINATION SUCCESS AND FEMALE FITNESS IN THREE SPECIES OF MILKWEEDS (*ASCLEPIAS*) Introduction

Flowers of animal pollinated plants function to manipulate pollinators into transporting pollen to stigmas. The floral traits that are adaptive for pollination are those that influence attraction (e.g., Conner and Rush 1996; Schemske and Bradshaw 1999; Johnson et al. 2003; Hansen et al. 2012), rewards (e.g., Real and Rathcke 1991; Silva and Dean 2000), or efficiency (e.g., Nilsson et al. 1987; Fulton and Hodges 1999; Conner et al. 2009). If increased pollination success results in increased fitness, selection can act on the floral traits that influence pollinator behavior. Most flowering plants are hermaphroditic, so selection on floral traits can occur through both male and female fitness. Floral traits can interact with pollinators to increase effective pollen removal or receipt, or may influence fruit and seed production more directly through pollen germination or fertilization; uncovering such relationships helps us understand the functional role of individual floral traits.

Seed production (female fitness) is often limited more by abiotic resources such as carbon, nitrogen, and water than by pollen receipt, and Bateman (1948) predicted that this resource limitation will result in greater selection on floral traits through male than through female fitness. When pollinators are scarce and resources plentiful, seed production may be limited by pollen receipt rather than abiotic resources. In this case we expect a tighter relationship between pollen receipt (female pollination success) and seed production, translating into a greater possibility of selection on floral traits through female fitness (Wilson et al. 1994).

Most measures of selection on floral traits are solely through female fitness. Only a

handful of studies have measured selection in plants through male (seed siring) success (e.g., Meagher 1994; Morgan and Conner 2001; Hodgins and Barrett 2008; Sahli and Conner 2011; Kulbaba and Worley 2012). Milkweeds (*Asclepias* L.) have a long history of providing insight into male function in plants (Willson and Rathcke 1974; Willson and Price 1977; Queller 1983; Wilson et al. 1994; Wyatt and Broyles 1994; Fishbein and Venable 1996b), because male pollination success is easily estimated as the number of exported pollen packets (pollinia). To our knowledge, only two studies (Morgan and Schoen 1997; Caruso et al. 2005) have measured selection on floral morphology in milkweeds; both studied *Asclepias syriaca* L. and used pollinia removals as their estimate of male fitness. Several others have studied the relationship between milkweed reproductive traits, including total flower number and inflorescence size and number, and fitness components such as pollinia removal and insertion and female fruit (and seed) production (Willson and Rathcke 1974; Willson and Price 1977; Wyatt 1980; Shannon and Wyatt 1986; Broyles and Wyatt 1990).

Milkweed flowers have unusual floral traits (Fig. 1), including two features that evolved independently in the orchid family (Orchidaceae): pollen clustered into pollinia and the gynostegium. The latter is a cylindrical structure consisting of fused male tissue surrounding the two ovaries, with five stigmatic openings in the wall leading to the ovaries (Woodson 1941). The pollinia lay to either side of each stigmatic slit within the wall of the gynostegium. Each pair of pollinia are connected by a corpusculum, which has a small groove that catches the hairs or claws of pollinators, aiding in removal; a pollinium needs to be be inserted into another stigmatic slit for fertilization to occur (Wyatt 1978). Milkweeds also have a corona, consisting of a ring of hoods that surround the gynostegium. The hoods fill with nectar as a pollinator reward, but their size and volume vary between species. In many species, a horn forms within each hood, and

sometimes protrudes from the hood, arching over the gynostegium (Fig. 1A, C). Many of the milkweed species studied to date, including *A. syriaca* and *A. tuberosa* L., are pollinated by a host of generalist pollinators (Kephart 1983; Betz et al. 1994; Fishbein and Venable 1996a; Ollerton and Cranmer 2002; Ivey et al. 2003), including native bees, wasps, and butterflies. Non-native honey bees are also a common pollinator for some *Asclepias* species, but have only been present in the United States for a few hundred years (Brown 1898); thus, *Asclepias* floral traits may be maladapted to honey bee pollination and may be under stronger selection where this pollinator is common.

Our goal was to identify adaptive floral traits using selection gradient analysis (Lande and Arnold 1983) in three species of *Asclepias*—*A. syriaca, A. viridiflora* Raf., and *A. tuberosa*. Flowers of these species are diverse in size, shape, and color, yet populations can be found in close proximity and may overlap in their pollinator composition. We identified six floral traits that may affect male and female fitness components: gynostegium width, hood length, hood height, horn reach, slit length, and gap width (Fig. 1). We predicted that gynostegium width and the hood traits function to attract pollinators, because they affect the visibility of the flowers, and that horn reach, slit length, and gap width function to affect the efficiency of pollinators in removing and inserting pollinia. We tested these predictions using path analysis to complement the selection gradient analysis, as well as to determine the relationships among male and female pollination success and female reproductive success.

Methods

We studied one naturally occurring population of each of three perennial milkweed species in southwest Michigan; the maximum distance between the populations was 12.5 km. We sampled 45 *Asclepias syriaca* plants at the Pond Lab Facility of Michigan State University's

Kellogg Biological Station (42.410° N, 85.392° W). We selected individuals that were likely to be genetically distinct by only choosing one ramet (stalk) per cluster of likely clones; *Asclepias syriaca* has the ability to spread vegetatively by rhizomes, and Kabat (2010) found genets (genetic individuals) were made up of ramets that covered 1m² to 30m². We chose ramets from clusters that were separated by two or more meters and had floral traits that looked distinctively different, both in shape and color; ramets within a cluster typically had visibly similar floral characteristics (La Rosa pers. obs.). We sampled all 51 *A. tuberosa* plants in a clearing at Fort Custer Recreation Area (42.326° N, 85.331° W), and sampled 212 out of more than one thousand *A. viridiflora* plants at the Fort Custer Training Center (42.306° N, 85.333° W) by walking transects spaced 6m apart, and flagging the nearest plant within a 3m radius every 6m along each transect. Genets in these last two species are well-defined because they arise from a single root crown (Wilbur 1976). Data on *A. syriaca* and *A. tuberosa* were collected in 2008, and data on *A. viridiflora* in 2009.

For trait measurements we photographed 2-3 fresh flowers from each plant from the top and side (with 2.5 hoods removed as in Fig. 1) using a digital SLR camera with a 60mm macro lens. We first determined landmarks that were identifiable across the range of floral variability (Fig. 1), and then used these landmarks to make calibrated linear measurements from the digital photographs using ImageJ (Rasband 1997). Measurement error was quantified by measuring the traits on ten haphazardly chosen flowers from different plants, and then repeating the trait measurements on this same set of ten flowers for a total of three times. We calculated the variance of the three replicate measurements for each trait on each flower. For each trait, the mean of the ten variances (mean measurement variance within individual flowers) was divided by the variance in the ten means (variance among flowers) to estimate the measurement error as

a proportion of the variance among flowers from different plants. The measurement error of each trait was less than five percent, with the exception of gynostegium width in *A. tuberosa* (7%) and *A. syriaca* (11%), and slit length in *A. viridiflora* (41%).

Using the 2-3 flowers measured per plant in the full dataset, we estimated trait repeatability, the percent of the total variance in floral traits explained by the variance among plants, using ANOVA with plant as the random predictor variable. Among-plant variation was greater than within-plant variation for all traits except gap width in *A. tuberosa* and *A. viridiflora* (Table 1); note that gap width is the only trait measured that is a distance between two floral structures (i.e. adjacent hoods) rather than a dimension of a single floral structure. Also note that repeatability of slit length in *A. viridiflora* is almost 66% despite the high measurement error for this trait.

Annual fitness component estimates were taken from a single ramet per genet, because it is difficult to identify complete genets in *A. syriaca*, and we wanted sampling to be similar across species. *A. viridiflora* genets typically had only one ramet (mean = 1.19, s.d. = 0.43) and *A. tuberosa* often had several ramets in a single well-defined cluster (mean = 6.7, s.d. = 6.73, median = 5). Because flowers open asynchronously within umbels and remain continuously open for several days (Wyatt 1981; Kephart 1987), we counted pollinia insertions and removals on every flower from umbels that had all flowers open and with some flowers were beginning to senesce, maximizing the number of days flowers within the umbel were accessible to pollinators. To sample the greatest number of flowers per plant, we recorded pollinia insertions, pollinia removals, and display size of each ramet on a day when the maximum number of umbels were mature on that ramet. We sampled 100 (s.d. = 50.1) flowers per *A. syriaca* ramet, 52 (s.d. = 17.3) per *A. viridiflora* ramet, and 58 (s.d. = 36.8) per *A. tuberosa* ramet; thus, we sampled pollination

success for an average of 53% of all *A. syriaca* flowers and 28-32% of all flowers on the other two species (Table 2).

We estimated male and female pollination success on a per-flower basis as the average number of pollinia removed per flower and average number of pollinia inserted per flower, respectively; these measures ranged from 0-5 because each flower had five pairs of pollinia and five stigmatic slits where pollinia could be deposited. We used a hand lens to count pollinia insertions and removals in the field. We measured display size as all of the flowers open on the ramet on that day, which included every flower sampled for pollinia insertions and removals, plus all open flowers from umbels that were not yet mature enough to be sampled; thus, display size was always equal to or greater than the number of flowers sampled.

We estimated total flower number by counting every umbel at the end of the season and multiplying by the mean number of flowers per sampled umbel for each individual. We estimated the total number of pollinia removed and inserted as the product of per-flower pollinia removals and insertions and total ramet flower number. We measured annual female reproductive success as the number of mature fruits on each focal ramet; we sub-sampled 203 fruits from 39 *A. syriaca* plants, to confirm that fruit and seed number were significantly correlated (r = 0.96, p < 0.001). Fruit number was counted in September, once the fruits had matured; a few plants from each species died or were damaged by deer or rodents before the fruits could be counted.

To relate floral traits to pollinator visitation, we collected pollinator data from each *A*. *syriaca* and *A. tuberosa* individual using Canon VIXIA HF10 high-definition digital video cameras. Ten or more minutes of video was taken of all open flowers on each focal ramet immediately after the pollinia insertions and removals had been counted. From the video we

recorded the number of pollinator visits, the duration of each visit, and pollinator taxon to order (subdivided further for Hymenoptera and Coleoptera). An insect taxon was considered to be a potential pollinator if at least one individual of that taxon had been captured or seen with pollinia on its body. Visitation to the *A. viridiflora* population was later observed over 3.5 hours in 2014 by walking through the population and recording all insect visitors that had pollinia on their bodies; results confirmed what we casually observed in 2009 when we collected the trait and fitness data.

Each of our selection gradient analyses regressed fitness component estimates (pollination success or reproductive success) onto floral traits and display size; the selection gradients for the floral traits were robust to the exclusion of display size in all of our models. Fitness component estimates were relativized by dividing by the mean, and the traits were standardized to a mean of zero and standard deviation of one. Each measure of pollination or reproductive success was regressed separately onto all of the floral traits and display size, totaling three linear multiple regressions per species. Selection gradient analyses were performed using JMP 10.0 (SAS Institute Inc. 2012).

To help interpret and integrate the results of the selection gradient analysis, we used structural equation models (SEMs) to visualize the relationships between the floral traits, pollinator visitation (*A. syriaca* and *A. tuberosa* only), male and female pollination success, and female reproductive success. The pollinator data for *A. syriaca* included several orders of insects combined, whereas *A. tuberosa* included only honey bees, as they were the dominant pollinator and the only one to visit the focal ramets in the videos. We fit hypothesized causal relationships through simple paths from each floral trait and display size to pollinia removed per flower (male pollination success) and pollinia inserted per flower (female pollination success), and through

compound paths leading to pollination success, but passing through pollinator visits and duration for *A. syriaca* and *A. tuberosa*. We hypothesized that display size influenced total flower number, and that flower number influenced the total number of pollinia removed and inserted on the ramet, as well as fruit number; additionally, we fit a compound path from flower number to fruit number passing through the total number of pollinia insertions.

Initially the model for each species had a significant chi-square goodness-of-fit value, indicating they were not acceptable fits to our data and that something was likely missing from our model. We used modification index values to identify influential relationships that were not included in our original model (Grace 2006). From these values, we chose to add a correlational (double-headed) arrow between the residual variation of total removals and insertions and causal (single-headed) arrows from gynostegium width, hood length, horn reach, and insertions per flower leading to fruit number. These were added to all three models, with the exception of horn reach in the *A. viridiflora* model. Two of the three final SEM models had non-significant chi-square goodness-of-fit values, indicating that they were acceptable fits to the data. The full model for *A. tuberosa* had a significant chi-square goodness-of-fit value (P < 0.001), but when we split the model into male-only and female-only models, both reduced models fit acceptably (p > 0.05). The coefficients and their significance did not qualitatively change between the full model and the within-gender models. All SEM analyses were performed in R v. 3.1.1 (R Core Team 2014) using the *lavaan* (Rosseel 2012) package.

Results

A. viridiflora and *A. tuberosa* were each visited almost exclusively by a single pollinator taxon, bumble bees (*Bombus* spp.) and honey bees (*Apis mellifera*) respectively, while the pollinators of *A. syriaca* were more diverse (Table 3). Still, two-thirds of the visitors to *A*.

syriaca were honey bees, with the next most common being soldier beetles (Cantharidae) at 13%. Both honey bees and bumble bees have been shown to insert pollinia in *A. tuberosa* in Arizona (Fishbein and Venable 1996a). Native bumble bee and non-native honey bee visitors to these *Asclepias* species consistently had pollinia on their bodies, perhaps due to their abundance of hairs and spines, as well as the frequency at which they gripped the flowers between the hoods as they foraged for nectar. The other pollinators with smoother appendages, such as flies and soldier beetles, tended to have fewer pollinia (La Rosa pers. obs.) that were only clipped to the ends of their tarsi, and never on their tibia or femur leg segments (unpub. data from *A. incarnata*).

Gynostegium width, hood height, and slit length were the least variable floral traits, with phenotypic coefficients of variation (CV_P) between three and nine, while hood length, horn reach, and gap width were more variable, with CV_P values from 13 to 23 (Table 2). *A. syriaca* had the largest flowers and *A. viridiflora* the smallest based on the geometric mean size of the floral traits (excluding gap width and horn reach). Correlations among the six floral traits were low and generally positive, except for some weakly negative correlations with gap width in *A. viridiflora* and *A. tuberosa* (Table 4). Display size was not significantly correlated with the six floral traits with one exception (positive correlation with hood height in *A. syriaca*).

Fruit number per flower was extremely low, as is common among milkweeds. Each species produced a mean of about 185 flowers per ramet, with each flower containing two ovaries for a potential of approximately 370 fruits, yet each ramet produced three to six fruits on average, less than two percent of their potential fruit production (Table 2). Fruit set did not appear to be limited by pollen receipt, as plants had an average of 14 to 24 pollinia inserted for every fruit they produced (Table 2), though the proportion of deposited incompatible self-pollen,

or perhaps improperly inserted pollinia, is unknown.

We found significant directional selection for increased hood length and hood height in *A. syriaca* and *A. viridiflora* (Table 5). Three of four significant selection gradients on the hood were through male pollination success (pollinia removed per flower), and the fourth was through female reproductive success (fruit number) in *A. viridiflora*. We hypothesized that the hoods affected fitness by attracting pollinators to the flowers, and the path analysis for *A. syriaca* (Fig. 2A) confirms that plants with larger hoods had more visits. However, increased visitation rate did not significantly increase pollinia removals; instead, the number of pollinia removed per flower was directly affected by hood height rather than through increased pollinator visitation, suggesting that hoods affect the efficiency of pollinia removal. The path analysis also suggests that the positive selection on hood length through fruit number in *A. viridiflora* was not mediated through pollinia insertions (Fig. 2B).

We found significant directional selection to increase stigmatic slit length through increased female pollination success (pollinia inserted per flower) in *A. viridiflora* and *A. tuberosa* (Table 5). We hypothesized that slit length was an efficiency trait and the path analyses confirmed that it directly influenced pollinia insertions per flower in both species, although this path was only marginally significant in *A. viridiflora*; furthermore, slit length did not affect honey bee visitation in *A. tuberosa* (Fig. 2C). The path analysis showed no relationship between pollinia insertions and fruit number in *A. tuberosa*, so the effect of slit length on insertions per flower and total insertions did not translate into increased female reproductive success, consistent with the selection gradient analysis based on fruit number (Table 5) and with fruit number not being limited by insertions (Table 2).

Horn reach, which we hypothesized would affect the efficiency of pollination, had no

effect on pollinia removed or inserted in either the selection gradient results (Table 5) or the path analyses (Fig. 2). Instead, it was under selection through fruit number in both species that have horns. In *A. syriaca,* where horns only extend over a portion of the gynostegium (see Fig. 1A), there was marginally significant selection for longer horns through fruit number (this was the strongest standardized selection gradient in our study), and a significant single causal path from horn reach to fruit number (Fig. 2A). Functionally, pollinators were attracted to smaller horns, and an increase in pollinator visitation increased the number of insertions per flower, consistent with the large negative (but not significant) selection gradients for insertions and removals. Because insertions did not affect fruit set (Fig. 2A), this negative functional relationship did not affect the direct positive effect of horn reach on fruit number. In *A. tuberosa,* where horns often overlap each other across the top of the gynostegium (see Fig. 1C), there was selection for shorter horns through fruit number, but the causal path from horn reach to fruits was only marginally significant (Fig. 2C).

There was significant directional selection to increase floral display size through fruit number in *A. syriaca* and *A. viridiflora*, and through pollinia removed per flower in *A. viridiflora* (Table 5). The selection through fruit number in both species was likely due to an effect of display size on total flower number, which in turn was a strong determinant of fruit number (Fig. 2). Because such a small percentage of flowers set fruit, these relationships may be due to overall ramet size. In *A. viridiflora* plants with larger displays had significantly more removals per flower, consistent with the selection gradient analysis, which translated into more total removals (Fig. 2B). In *A. tuberosa*, larger displays increased both the number and duration of honey bee visits, and longer visits caused increased pollinia removals per flower; this positive effect was partially counteracted by a significant negative effect of display size directly on removals per

flower, which may explain the lack of significant selection on display size through pollinia removed per flower (Table 5). Total flower number was the strongest determinant of both male and female fitness components, affecting male and female total pollination success in all three species, and female reproductive success in *A. syriaca* and *A. viridiflora*. However, across the three species the models explained only 28-48% of the variation in fruit number.

We tested for differences among the three species in the strength of selection on floral traits by comparing the magnitude of all 18-21 standardized selection gradients between pairs of species using three paired t-tests, where each selection gradient was paired with the selection gradient from the same fitness component and trait in the other species. *A. syriaca* and *A. tuberosa* each had significantly stronger average selection than *A. viridiflora* ($t_{17} = 3.17$, P = 0.003; $t_{17} = 5.03$, P < 0.0001, respectively), but average selection did not differ between *A. syriaca* and *A. tuberosa* ($t_{20} = 0.91$, P = 0.373). Thus, selection on floral traits was strongest in *A. syriaca* and *A. tuberosa*, species that were heavily visited by non-native honey bees. Note that these two species had much smaller sample sizes than *A. viridiflora*, raising the possibility that the stronger selection gradients were biased upward, akin to the "Beavis effect" (Beavis 1998); however, the proportion of individuals sampled in these two populations was much greater (~75-100%) than in *A. viridiflora* where, despite the large sample size, we sampled approximately 20% of the total population.

Discussion

Floral traits of hermaphroditic plants can affect male or female fitness exclusively or simultaneously; we found that only hood length had significant effects through both genders. Because selection in hermaphrodites can act through each gender separately, there is an opportunity for conflicting selection on individual traits through male and female fitness.

Nevertheless, we found no evidence of conflicting selection between genders on any trait within the three species, consistent with previous studies on other taxa (e.g., Sahli and Conner 2011; reviewed by Delph and Ashman 2006). Hood height influenced only pollinia removal by pollinators, while slit length and horn reach affected only female fitness components. Conflicting selection through the two genders also seems less likely, because pollinia removals per flower and insertions per flower were always positively correlated, albeit not significantly so in *A. tuberosa* (Table 2).

Our estimate of male pollination success (pollinia removed per flower) was most affected by hood dimensions, but not through pollinator visitation as predicted. Instead, hoods seemed to influence the effectiveness of pollinators in removing pollinia. Caruso et al. (2005) also found positive directional selection on hood length, but in the population of *A. syriaca* they studied, selection was through fruit initiation, their measure of female fitness. Milkweeds abort many of the fruits they initiate, so fruit initiation might better reflect female pollination success (pollinia insertions per flower) than reproductive success.

The only significant selection gradients through female pollination success were for increased slit length in *A. viridiflora* and *A. tuberosa*. The path analysis confirmed that slit length directly affected pollinia insertions, and it was not mediated through pollinator visitation in *A. tuberosa* (Fig 3C), supporting our prediction that this is an efficiency trait. A longer slit length may function to increase the chance that pollinator legs, claws, and hairs will slide between the adjacent anther wings that form the stigmatic opening, resulting in an inserted pollinium. We did not find selection on slit length in *A. syriaca*, but Morgan and Schoen (1997) found selection for longer slits through pollinia insertions in their study.

Selection through female reproductive success (fruit number) was for increased display

size in two of the species, horn reach in the two species with horns, and hood length in the one species without horns. The selection on horn reach was due to its effect on fruit production directly, without having an effect on pollinia insertions or pollinator visitation (Fig. 2). Selection on hood length in *A. viridiflora*, where the hood openings are extremely reduced and there are no horns, was positive and also affected fruit number without influencing the number of pollinia insertions; hood length in *A. viridiflora* may be acting similarly to horn reach in the other two species. By maneuvering pollinators, the horns and hoods may be altering the "quality" of an insertion, such that it is more or less likely to result in a fruit.

The strong selection on horn reach in populations dominated by honey bee pollinators suggests that horns may function to maneuver this relative newcomer into effectively depositing pollinia. The first recorded arrival of domesticated honey bees into what is now the United States was recorded in 1622 (Brown 1898), and different pollinator species can exert differential selection (Galen 1985; Schemske and Horvitz 1989; Conner et al. 2009; Sahli and Conner 2011); consequently, North American *Asclepias* species have had less time to adapt to honey bees relative to the native pollinators. This may also explain why *A. syriaca* and *A. tuberosa* are experiencing stronger selection than in *A. viridiflora* overall—they are not yet optimized for efficient pollination by honey bees. The population of *A. syriaca* that Caruso et al. (2005) studied had a mean horn reach very similar to our population, though they found significant selection for a shorter horn reach. Their study did not describe the pollinator composition, so the degree to which honey bees contributed to the selection they measured is unknown.

Alternatively, horns and hoods may be correlated with an unmeasured trait that facilitates fertilization once pollinia have been deposited. For example, hood length may be correlated with a trait such as nectar volume. Nectar is produced within the gynostegium, flows freely between

the gynostegium and hoods in *A. curassavica* (Galil and Zeroni 1965), and is required for pollen germination in *A. syriaca* (Kevan et al. 1989). Larger hoods may allow the plant to hold more nectar within the gynostegium to increase pollen germination. Future studies should examine the functional relationships between hood dimensions, nectar volume, and fruit number.

We never found selection on a trait through both insertions per flower and fruit number; in other words, floral traits influencing female pollination success were distinct from those influencing female reproductive success. In parallel with this, there were no significantly positive relationships between total insertions and fruit number for any of the species (Fig. 2); in *A. tuberosa,* the strong path between total insertions and fruit number had a large standard error and was therefore not statistically significant, and the significant path in *A. viridiflora* was actually negative. A disconnect between pollen received and fruit production suggests some combination of improper placement of pollinia by pollinators, self-incompatibility, and resource limitation of fruit production; we discuss each of these in turn, while noting that the our data cannot distinguish between these three mechanisms.

Pollinia that are improperly inserted (low quality insertions, above) may restrict pollen tubes from reaching the ovaries within the gynostegium. A pollinium has the advantage over loose pollen of potentially delivering a saturating amount of pollen in a single pollinator visit (e.g., Bookman 1984; Queller 1985). However, the pollen tubes grow from only one edge of the pollinium (Galil and Zeroni 1969; Wyatt 1976), so if the pollinium is inserted in reverse, and the edge where the pollen tubes emerge is not encased in nectar, the pollen grains within the pollinium will not germinate, preventing fertilization (Kevan et al. 1989; Sage and Williams 1995).

The inserted pollinia that we counted on A. syriaca and A. tuberosa may often have been

selfed due to the way honey bees forage. In A. syriaca, Howard and Barrows (2014) found that 88% of pollinia inserted by honey bees was selfed and Pleasants (1991) calculated that 71% of the pollinia that honey bees inserted came from within one meter of the recipient flower. Given that A. syriaca spreads vegetatively, ramets within one meter are often from the same genet. This level of geitonogamous selfing could be occurring in A. tuberosa populations as well, given that most ramets within a meter were from the same root crown. A possible mechanism that may reduce the occurrence of geitonogamous selfing is the rotation of pollinia upon removal; the arms connecting the pollinia to the corpusculum rotate the pollinia approximately 90 degrees over several minutes in A. sullivantii (Robertson 1886) and 107 seconds in A. exaltata (Queller 1983). The average duration of honey bee visits to the focal ramets of A. syriaca and A. tuberosa was 67 seconds and 40 seconds, respectively, but some honey bees remained on ramets for more than five minutes in both species, which would circumvent this mechanism. With very low levels of self-compatibility in A. tuberosa (Wyatt 1976), large numbers of pollinia inserted would not necessarily translate to high fruit set, and could even have a negative effect (as in A. viridiflora, Fig. 2B) by wasting resources on the initiation of fruits that are then aborted due to a late-acting self-incompatibility system (Gibbs 2014).

Even if pollinia are inserted properly and compatible, fruit production could be resourcelimited. Under conditions of resource limitation, variation in female pollination success should have little effect on fruit number. Without a link to reproductive success, the floral traits that function in female pollination success will not be under selection from pollinators. Caruso et al. (2005) supplemented water and fertilizer in *A. syriaca*; despite a 46% increase in the numbers of fruits initiated, demonstrating a reduction in resource limitation, there were actually fewer significant selection gradients through female fitness (fruits initiated) in the supplemented

treatment compared to the unmanipulated control. Additionally, despite evidence for resource limitation of female fitness in their unmanipulated control, there was not greater selection through male fitness compared to female fitness as predicted by Bateman's principle. However, it could be that full fruit production, not initiation, is where the impact of resource limitation on female fitness occurs. In our study, despite evidence of adequate pollinator availability that hints at possible resource limitation, we also did not find greater selection through male fitness. Instead, there were equal instances of significant selection through pollinia removed flower (male) and fruit number (female) across the three species (Table 5).

Milkweed flowers undoubtedly interact with pollinators to influence the movement of pollinia, but our data show that there is a disconnect between pollinia insertions and fruit number that we still do not fully understand. Some proportion of insertions are likely ineffective due to placement or incompatibility, and may be masking a positive relationship between effective pollinia insertions and fruit number. However, resource limitation will also undermine this relationship, reducing selection on floral traits through female fitness.

We also know very little about the relationship between pollinia removals and seeds sired, apart from one study in *A. exaltata*, where they were positively, but weakly, correlated ($R^2 = 22$; Broyles and Wyatt 1990). Because resource limitation is not expected to impact male fitness to the extent that it affects female fitness, the relative strength of selection on floral traits through male and female fitness could be impacted by the resource limitation of female fitness as predicted by Bateman (1948). Bateman's hypothesis has been invoked in milkweed studies (e.g., Queller 1983; Caruso et al. 2005) because of the relative ease of estimating male fitness through pollinia removed. However, without a clearer understanding of the relationship between pollinia removed and fruits sired, it is difficult to make a robust comparison of selection through male
and female reproductive success. We found that several floral traits function to increase pollen removal and deposition and are likely adaptive for pollination success in these species, but we have yet to uncover what factors strengthen the relationship between pollination success and reproductive success to cause adaptive evolution of floral traits in *Asclepias*.

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APPENDIX

	A. syriaca	A. viridiflora	A. tuberosa
Gynostegium width	66.1	81.2	59.2
Hood length	68.9	76.7	72.6
Hood height	83.1	81.8	78.4
Horn reach	70.2	-	52.8
Slit length	61.4	65.8	62.6
Gap width	82.8	36.5	32.8

Table 1. Trait repeatabilities The percent of total variation in floral traits explained by the difference among plants calculated from nested random-effects ANOVA.

Table 2. Descriptive statistics for the six traits (mm), display size, flower number, and fitness estimates. Means and phenotypic coefficients of variation (CV_P) were calculated for each species. The geometric mean of the floral traits (excluding gap width and horn reach) is an estimate of overall flower size. Relative size was calculated within each species by dividing each floral trait by the geometric mean. Flowers, pollinia removals, pollinia insertions, and fruits were counted on a single ramet. Pearson product-moment correlations are given between pollinia removals per flower and insertions per flower. Pollinia insertions per fruit were calculated by dividing the number of pollinia insertions per flower sampled by the number of fruits per flower over the entire ramet.

	<i>A. syriaca</i> (n = 42-45)			A. viridiflora	(n = 205-21	2)	<i>A. tuberosa</i> (n = 40-46)			
	Mean (SD)	Rel. size	CV_P	Mean (SD)	Rel. size	CV_P	Mean (SD)	Rel. size	CV_P	
Gynostegium width	2.19 (0.12)	0.76	5.4	2.74 (0.12)	1.67	4.3	1.70 (0.06)	0.64	3.5	
Hood length	3.24 (0.42)	1.12	12.9	0.40 (0.09)	0.24	23.1	2.58 (0.37)	0.98	14.1	
Hood height	5.08 (0.44)	1.76	8.9	4.74 (0.31)	2.89	6.5	5.93 (0.36)	2.25	6	
Horn reach	2.04 (0.34)	0.71	16.5	-	-	-	1.74 (0.25)	0.66	14.3	
Slit length	1.90 (0.12)	0.66	6.1	1.40 (0.09)	0.85	6.2	1.88 (0.08)	0.71	4.2	
Gap width	0.61 (0.12)	0.21	19.2	0.62 (0.08)	0.38	13.4	0.43 (0.09)	0.16	21	
Geometric mean	2.88			1.64			2.64			
Display size	109.51 (68.70)		62.7	110.46 (52.56)		47.6	66.04 (43.76)		66.3	
Total flower number	189.03 (111.21)		58.8	185.11 (107.51)		58	182.27 (179.52)		98.5	
Pollinia removals per flower	2.02 (1.21)		59.8	2.32 (0.75)		32.3	1.11 (0.67)		60.2	
Pollinia insertions per flower	0.42 (0.23)		56	0.30 (0.17)		57.2	0.18 (0.11)		62.5	
Removal-insertion correlation	r = 0.23*			r = 0.37*			r = 0.06			
Fruit number	5.95 (5.60)		94.2	3.42 (2.52)		73.6	3.18 (2.27)		71.6	
Pollinia insertions per fruit	24 (5.1)			20 (1.3)			14 (3.5)			

* P < 0.05.

Table 3. Pollinator visits to *Asclepias* **populations.** Visits to *A. tuberosa* were recorded from 8 hours and 10 minutes of video from 2008, and visits to *A. syriaca* were recorded from 9 hours and 29 minutes of video from 2008. Visitation to *A. viridiflora* was surveyed in the population in 2014 (see Methods). The mean (SEM) number of visits per minute and cumulative (total) duration per minute for *A. syriaca* and *A. tuberosa* is calculated from ten or more minutes of video of each focal ramet.

			Numbe	r of visits	(%)	
Pollinator	А.	syriaca	A. viri	idiflora	A. tu	uberosa
A. mellifera (honey bee)	86	(64.7%)			74	(93.7%)
Bombus spp.	5	(3.8%)	30	(100%)	1	(1.3%)
Diptera	6	(4.5%)			0	
Cantharidae sp.	17	(12.8%)			0	
Lepidoptera	6	(4.5%)			4	(5.1%)
Wasps	9	(6.8%)			0	
Hemiptera	4	(3.0%)			0	
Total number of visits	133		30		79	
Mean no. visits per min	0.25	(0.28)			0.17	(0.24) [†]
Mean total duration per min	0.28	(0.35)			0.19	(0.30) [†]

[†]Honey bee visits only.

Γable 4. Pearson product-moment correlations among six floral traits and display size in <i>A. syriaca</i> (n=45), <i>A. viridiflora</i>	
(n=212), and A. tuberosa (n=51).	

	A. syriaca				A. viridiflora				A. tuberosa								
	Gynost. width	Hood length	Hood height	Horn reach	Slit length	Gap width	Gynost . width	Hood length	Hood height	Slit length	Gap width	Gynost. width	Hood length	Hood height	Horn reach	Slit length	Gap width
Hood length	n 0.30*						0.13					0.20					
Hood heigh	t 0.28	0.42*					0.17*	0.15*				0.04	0.25				
Horn reach	0.23	0.63*	0.61*									0.17	0.18	0.22			
Slit length	ı 0.39*	0.26	0.34*	0.35*			0.24*	0.08	0.22*			0.26	0.14	0.28*	0.00		
Gap width	0.04	0.16	0.25	0.25	0.01		0.25*	-0.04	-0.05	0.01		0.31*	0.22	0.06	-0.26	0.08	
Display size	9 0.01	0.20	0.34*	0.29	0.07	-0.14	0.09	0.08	0.01	-0.05	-0.02	-0.26	-0.17	-0.02	-0.18	-0.05	-0.04

* *P* < 0.05.

		A. syriaca			A. viridiflora		A. tuberosa				
	Pollinia removed per flower	Pollinia inserted per flower	Fruit number	Pollinia removed per flower	Pollinia inserted per flower	Fruit number	Pollinia removed per flower	Pollinia inserted per flower	Fruit number		
Gynost. width	-0.10 (0.09)	-0.03 (0.10)	0.24 (0.14)	-0.01 (0.02)	-0.02 (0.04)	0.03 (0.05)	-0.09 (0.10)	-0.16 (0.10) [†]	-0.16 (0.11)		
Hood length	0.27 (0.10)*	0.18 (0.11)	-0.07 (0.17)	0.01 (0.02)	0.00 (0.04)	0.16 (0.05)**	0.14 (0.09)	0.12 (0.09)	0.07 (0.10)		
Hood height	0.28 (0.11)*	0.19 (0.12)	-0.04 (0.17)	0.05 (0.02)*	0.03 (0.04)	-0.01 (0.05)	-0.16 (0.09) [†]	-0.16 (0.09) [†]	0.10 (0.11)		
Horn reach	-0.20 (0.12)	-0.21 (0.13)	0.39 (0.20) [†]				0.06 (0.10)	-0.04 (0.09)	-0.23 (0.11)*		
Slit length	-0.09 (0.09)	-0.01 (0.10)	0.03 (0.14)	-0.01 (0.02)	0.09 (0.04)*	0.04 (0.05)	0.05 (0.09)	0.22 (0.09)*	-0.14 (0.10)		
Gap width	-0.14 (0.09)	-0.09 (0.09)	-0.11 (0.14)	-0.02 (0.02)	-0.06 (0.04)	-0.01 (0.05)	0.13 (0.10)	0.07 (0.09)	0.17 (0.10) [†]		
Display size	0.07 (0.09)	-0.01 (0.10)	0.34 (0.14)*	0.07 (0.02)**	0.05 (0.04)	0.19 (0.05)**	-0.06 (0.09)	0.13 (0.09)	0.17 (0.10)		
n	45	45	42	212	212	206	51	51	44		
Total R ²	0.35*	0.1	0.4*	0.08 **	0.05	0.13**	0.15	0.26 [†]	0.37*		

Table 5. Standardized selection gradient estimates (SE) for floral traits and total flower production in three Asclepias species.

** *P* < 0.01, * *P* < 0.05, [†] *P* < 0.10.

 R^2 is the percent variance in that fitness component explained by all the traits plus the significance of the whole model.



Figure 1. Top and side views of *A. syriaca* (A), *A. viridiflora* (B), and *A. tuberosa* (C) flowers showing the six floral traits: gynostegium width (gy), hood length (*hl*), hood height (*hh*), horn reach (*hr*), slit length (*sl*), gap width (gw). Note that hoods are greatly reduced and horns absent in *A. viridiflora*. Two and a half hoods were removed for the side views.



Figure 2. Path analysis relating traits, pollinator visitation over ten minutes, and fitness components to total yearly fruit production. Blue arrows are paths influencing male function (pollinia removals per flower), red arrows are paths influencing female function (pollinia insertions per flower) and fitness (fruit number), and black arrows affect both or show variation unexplained by the model when there is no trait at their origin. Blue, red, and black arrows are P < 0.10 and gray are P ≥ 0.10. Single-headed arrows are hypothesized causal relationships and double-headed arrows are correlations. Arrow thickness represents the magnitude of the path coefficient, and dashed lines represent negative coefficients. White boxes represent variables included in the selection gradient analyses. Correlations between residual variation of endogenous variables are not shown for simplicity. *P < 0.05, ** P < 0.01, *** P < 0.001.





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CHAPTER 3

FUNCTIONAL STUDIES AND ESTIMATES OF NATURAL SELECTION THROUGH MALE AND FEMALE FITNESS IN MILKWEEDS (ASCLEPIAS)

Introduction

Diversity in floral form has enticed biologists and non-biologists alike. Many flowering species rely on animals for pollination, which requires floral traits that can attract suitable pollinators and maneuver them efficiently. Some plant species have converged on suites of traits that may facilitate pollination by particular pollinator taxa (Fenster et al. 2004; Faegri and Van Der Pijl 2013), but many species appear to be more general in their pollination (Ollerton 1996; Waser et al. 1996; Zamora 2000), possibly because they experience selection from a dynamic network of pollinators. Regardless, the floral traits that directly increase the fitness of the plant are adaptive, and so identifying these adaptive traits and understanding how they function gives us insight into the diversity of floral forms.

Understanding these interspecific interactions can be achieved by studying how the flowers function to increase pollen receipt or removal—the effect of floral traits on pollination success (e.g. Galen and Stanton 1989; Murcia 1990; Young and Stanton 1990; Harder and Barrett 1993; Conner et al. 1995). Functional studies can then be followed by studies of natural selection showing which floral traits affect male and/or female fitness—the reproductive success of the plant (e.g. Johnston 1991; Conner et al. 1996; Caruso et al. 2003).

Available resources and pollen receipt are both important for plant fitness, and limitations in one or the other can have ecological and evolutionary consequences (Ashman et al. 2004; Knight et al. 2005). Female fitness is considered to often be resource limited; however, pollen

limitation cannot be overlooked, particularly in perennial outcrossing species (Charlesworth 1989) that rely on pollinators for adequate pollination (Burd 1994). Following Bateman (1948), male fitness is not as likely as female fitness to be limited by resources, and so pollinator visitation and effectiveness can strongly influence male fitness. These factors limiting female and male fitness can in turn affect selection on floral traits (Bateman 1948; Wilson et al. 1994; Ashman and Morgan 2004; Knight et al. 2005); in populations where female fitness is limited by resources, male fitness may be more variable, allowing for selection on traits affecting pollination, and in populations where both female fitness and male fitness are limited by pollinators, there may be selection through both (Wilson 1994).

Measures of selection in plants are often only through female fitness (Conner 2006), even though more than 90% of flowering species are hermaphrodites (Yampolsky and Yampolsky 1922). Male fitness is best estimated by the number of viable seeds sired. This estimate requires paternity analyses of offspring to identify likely sires. To date, there are but a handful of studies that have measured selection on plant traits using fruits sired or seeds sired as their measure of fitness (Meagher 1994; Morgan and Conner 2001; van Kleunen and Burczyk 2007; Hodgins and Barrett 2008; Sahli and Conner 2011; Kulbaba and Worley 2012).

For decades, *Asclepias* has been a common plant taxon for studying male function. The pollen packets, or pollinia, make it easy to estimate male pollination success, by scoring pollinia removals per flower or per plant. Much of this earlier work focused on investigating the very low fruit to flower ratio in *A. syriaca*, finding that only male function (estimated as the number of pollinia removed) suffered when flower number was reduced by half (Queller 1983), and that only male function was positively affected when flower number reached above a threshold

(Willson and Rathcke 1974; Willson and Price 1977). However, none of these studies addressed individual floral traits. Since then, two studies have measured selection on floral traits, including selection through male fitness components, using pollinia removals (Morgan and Schoen 1997; Caruso et al. 2005), and two studies have used paternity analysis or genotyped deposited pollinia in *Asclepias* to answer questions about seed siring within fruits, functional gender, and self-pollination rates (Broyles and Wyatt 1990; Howard and Barrows 2014), but none have used paternity analyses to measure selection through male fitness using seeds sired.

In addition to being an excellent system for asking questions related to male fitness, milkweeds are also known for their unusual floral structures. The flowers have a calyx of five sepals and a corolla of five petals, but are otherwise atypical (Fig. 3). The pistils are surrounded by a gynostegium that has five stigmatic slits leading to the central chamber. The gynostegium has ten pollen packets (pollinia) tucked into the wall; every two pollinia are attached by a small clip (corpusculum) that sits at the end of the stigmatic slit where it can catch onto pollinators. Surrounding the gynostegium is the corona, made up of five hoods that hold nectar, the only reward for pollinators. The hoods of many species contain horns, that arise from the inside base of the hood and extend up and out of the hood opening.

Our study focused on traits that we hypothesize affect pollinator attraction and efficiency. Traits such as gynostegium width, hood length, and hood height contribute to the overall size of the flower, making them likely to play a role in pollinator attraction, as well as helping position pollinator bodies, and legs in particular, between the hoods to contact the corpuscula and stigmatic slits. We also hypothesize that less conspicuous traits, such as slit length, horn reach, and the gap between hoods increase pollinator effectiveness. We predict that selection is more

likely to be directional (linear) rather than stabilizing (negative non-linear), because both species are generalists and are visited by pollinators of many shapes and sizes.

We investigated these traits from a functional perspective, evaluating their effect on pollination success (pollinia insertions and removals). We also examined their effect on reproductive success (fruit number, viable seeds produced, and viable seeds sired) in two milkweed species, *Asclepias incarnata* and *A. exaltata*. We asked: (1) Which floral traits function to increase the insertion and removal of pollinia and do they function similarly for both genders? (2) Which floral traits are under selection? Are they primarily adaptations for male fitness, female fitness, or both? and (3) Is there inter-annual variation in selection?

Methods

Field methods

We studied single naturally occurring populations of *A. exaltata* (poke milkweed) and *A. incarnata* (swamp milkweed) in southwest Michigan between 2007 and 2011. The population of *A. incarnata* was located at the Pond Lab Facility of Michigan State University's Kellogg Biological Station (42.410° N, 85.392° W) and flowered throughout July and early August. *A. incarnata* is a long-lived perennial with an unknown lifespan; this population was naturally established at some point after ponds were renovated in 2000, so was no more than six years old when our study began in 2007. Our study population of *A. exaltata* was located at the Fort Custer Training Center (42.298° N, 85.323° W). *A. exaltata* is also long-lived and flowers for two to three weeks in June; the age of plants in this population was unknown.

Asclepias exaltata and *A. incarnata* are not thought to spread from rhizomes (Wilbur 1976; but see Queller 1985), so we identified genetic individuals (genets) as clusters of ramets

that appeared to be growing from the same origin (i.e. growing from a single root crown). In 2007 and 2008 we identified genets by sight, and in 2010 (as well as 2009 for *A. exaltata*) we genotyped each ramet to check our assignments, especially for genets that were growing very close together; only about 4.5% of ramets had been wrongly assigned. In 2011, we used the assignments from 2010, and any new ramets were assigned to genets by sight. *A. exaltata* genets typically had just one ramet (mean = 1.14, s.d. = 0.40) and *A. incarnata* sometimes had two or more ramets per genet (mean = 1.89, s.d. = 1.42). *A. exaltata* is typically self-incompatible (Broyles and Wyatt 1993; Wyatt and Broyles 1994; but see Lipow 1999), and self-fertile plants suffer high inbreeding depression (Himes and Wyatt 2005). *A. incarnata* can produce hundreds to thousands of flowers and is sometimes self-compatible (Kephart 1981); however, despite the possibility of high rates of geitonogamy, populations typically have high rates of outcrossing (Ivey et al. 1999).

We collected trait and fitness data from the *A. incarnata* population for five years (2007-2011). In 2007 and 2008 we sampled the same 50 plants haphazardly chosen from around four ponds, and in 2009-2011 we sampled every flowering plant in the population; however, 40 of the 50 *A. incarnata* plants from 2007 and 2008, and a number of plants from 2009 were destroyed before 2010 due to pond renovations. We collected data from the *A. exaltata* population for three years (2009-2011), collecting data from every flowering plant in the population each of those years. In every year we measured floral traits, scored pollinia removals to assess male function of the floral traits, and counted fruits to measure selection through female fitness. In 2007-2009, we also scored pollinia insertions to assess female function. We collected trait, pollination, and fitness data from every ramet per plant, except in 2008, when we collected

these data from just one ramet per A. incarnata genet.

We measured floral traits by photographing two fresh flowers from each plant in 2007, three in 2008, and 1-4 (mean = 1.8-2.3) in each year between 2009 and 2011. We photographed each flower from the top and side (with 2.5 hoods removed to allow traits to be clearly seen) using a digital SLR camera with a 60mm macro lens. We first determined landmarks that were identifiable across the range of floral variability (Fig. 3), and then used these landmarks to make calibrated linear measurements from the digital photographs, that each included calipers set at 5mm, using ImageJ (Rasband 1997). The traits we measured were: gynostegium width, hood length, hood height, horn reach, slit length, and gap width. We did not measure gynostegium width or gap width in 2007, and gynostegium width was measured differently in 2011, because we changed to landmarks that could be identified more accurately (Fig. 3, *gr*) to estimate gynostegium width.

Using a subset of plants that had two or three flowers measured within a year, we calculated trait repeatabilities across plants within year as the percent of the total variance in floral traits in that year explained by the variance among plants using ANOVA with plant as the random predictor variable; we repeated this for each year in both species. Among-plant variation explained more than 60% of the variation for 20 of 28 traits across years in *A. incarnata*, and more than 70% of the variation for 16 of 18 traits across years in *A. exaltata*. Among-plant variation explained less than 50% of the variation in gap width in 2010 and 2011, and several traits in 2007—all in *A. incarnata* (Table 6); note that gap width is the only trait measured that is a distance between two floral structures (i.e. adjacent hoods) rather than a dimension of a single floral structure.

We estimated total flower number to account for effects of resources, age, and plant size on fitness. We treated flower number both as a predictor variable that may directly affect fitness, as well as a measure of plant condition that is correlated with environmental variables that could also affect fruit production (Scheiner et al. 2002; Stinchcombe et al. 2002). For *A. exaltata* we counted the total number of flowers on every plant. For *A. incarnata*, which can produce thousands of flowers, we estimated flower number in 2007 and 2011 as the total number of flowering branches across all ramets, in 2008 the flower number estimate was the number of ramets, and in 2009 and 2010 we estimated total flower number by counting all umbels and multiplying this by the mean number of flowers per umbel per plant. The correlations among ramet number, branch number, and umbel number ranged between 0.35 and 0.75. Resources are not likely to be affecting the individual floral traits as the magnitude of the correlation between flower number and the six floral traits was less than 0.35 in *A. incarnata* and less than 0.20 in *A. exaltata*.

To assess floral trait function, we scored pollinia removals and insertions. Using a hand lens in the field, we scored pollinia removed per flower, indicated by a missing corpusculum (arrow; Fig.1) (range: 0-5), and examined the five stigmatic slits for inserted pollinia (range: 0-5); we discontinued collecting data on pollinia insertions after 2009, because they were a poor predictor of fruit number (see results). Because flowers within an umbel open asynchronously, then remain continuously open for several days (Wyatt 1981; Kephart 1987), we scored pollinia removals and insertions on every flower only from umbels that had fully bloomed and were mature enough that some flowers were beginning to senesce; note that some flowers remained open for several days after sampling and likely continued to receive and donate pollinia. The

only exception was that in 2007 we sampled just three flowers from three mature umbels on each ramet. *A. incarnata* plants tended to have many umbels (in 2010: mean = 56.9, s.d. = 64.1), so we sampled 1-4 umbels per ramet across one to several days with the goal of sampling approximately 100 flowers per genet (Table 7). *A. exaltata* plants had fewer umbels (mean = 2.3, s.d. = 1.3), so we scored removals and insertions from nearly every flower from every umbel in the population in 2009-2010, so we also have estimates for total pollinia inserted and removed per plant; in 2011, we were unable to score pollinia removals and insertions from every plant before flowers senesced.

We conducted paternity analyses for offspring in 2010 using microsatellite markers to genotype parents and offspring. We collected leaf tissue from all flowering ramets in both populations and stored the leaf samples at -80 C. We collected tissue from a total of 216 *A*. *incarnata* ramets and 155 *A. exaltata* ramets. To genotype offspring, we allowed fruits to ripen on the plants, then collected every fruit prior to dehiscence, noting from which maternal plant and ramet it was collected; we collected 132 fruits from *A. exaltata* and 1971 fruits from *A. incarnata*.

Pollinator observations and pollen limitation

We conducted extensive pollinator observations over the duration of the *A. incarnata* flowering seasons in 2008 and 2010 and more limited observations late in 2009 that did not represent the full breadth of insect visitors. In 2008, we made video recordings of each of the 50 plants we sampled for at least ten minutes each for a total observation time of 9h. We used video so we could observe pollinators on the same day that we collected data on pollination success. We also wanted to document pollinators so their behavior, contact with corpuscula and stigmatic

slits, and pollinia loads could be observed on a frame by frame basis. In 2010, we observed 113 plants for a total of 9h 25m. In each year we calculated the percent pollinator composition by dividing the number of individuals in each pollinator group by the total number of pollinators observed. We also captured 119 insects, chilled and photographed them, scored the mean number of pollinia they carried, and divided that by the total pollinia carried by all pollinator groups to calculate the percent total pollinia. Pollinator importance was calculated by multiplying the mean pollinia carried for a taxon by the number of individuals of that taxon in a given year, and dividing by the grand total for all taxa (Table 8).

We attempted pollinator observations of the population of *A. exaltata*, including dusk and dawn observations, in addition to the many days we were in the population during peak flowering, but we saw fewer than five individuals carrying pollinia over three years. There is little published data on nocturnal pollinators in *A. exaltata*, but nectar is produced throughout the night and increases in sugar content throughout the day, reaching concentrations that are ideal for bee pollination (Wyatt and Shannon 1986). Bumble bees (*Bombus* sp.) and skippers (*Epargyreus* sp.) were the only two taxa that we saw with pollinia on their bodies; another study of *A. exaltata* recorded pollination by *Bombus* and *Apis* (Betz et al. 1994), though we never saw honey bees (*Apis* sp.) at this site. Additional visitors we recorded were: fritillary (*Speyeria* sp.), skipper (*Thorybes* sp.), and black swallowtail (*Papilio* sp.) butterflies, one ruby-throated hummingbird, and single Lepidopteran legs stuck in different corpuscula. Despite these other studies finding little to no nocturnal pollination, we cannot rule out the importance of nighttime pollinators in our study population given that the few pollinators we saw during the day cannot account for the number of fruits that were produced each year. For night flying insects, attraction, or the contrast

of flowers against their forest background, may be the most important factor influencing pollination.

In 2012, we conducted a pollen addition experiment to test for evidence of pollen limitation in the population of *A. exaltata* because of the scarcity of observed pollinators. We paired plants (n = 22 pairs) based on umbel number, flower number, and flowering time. We then randomly assigned one plant per pair to receive the treatment of one hand-inserted pollinium per flower from a mix of pollen donors. This was an average increase of 60 times the pollinia naturally received. We then recorded fruit initiation and final fruit number and compared the manipulated plants to the unmanipulated plants using a paired t-test.

Fitness measures

We estimated male and female fitness for both species. Because both of these species are perennial, we were unable to measure lifetime fitness, and instead estimated annual fitness across multiple years. We used total fruit number per plant as our estimate of annual female fitness; fruits per plant is highly correlated with total seeds per plant for both species (r > 0.97; Table 9). All fruits were counted in September once they had matured. In 2008, we counted fruits on just one ramet and then multiplied them by the total number of ramets. We estimated female and male fitness in 2010 using viable seeds produced and viable seeds sired (see below), and then summed these for a measure of total annual fitness.

Paternity

Milkweed fruits are commonly sired by a single father, because a pollinium has a sufficient number of pollen grains to fertilize all ovules within an ovary (Wyatt 1976; Kephart 1981). Flowers have five stigmatic openings; two openings lead to one ovary and three lead to

the other ovary, so there is the possibility of two or three sires if multiple pollinia are deposited in close succession. We measured the occurrence of multiple paternity for both populations by genotyping ten randomly chosen offspring per fruit for each of ten fruits in *A. exaltata*, and 8-19 randomly chosen offspring per fruit for each of 18 fruits in *A. incarnata*. We used GERUD2 (Jones 2001; 2005) to identify the combination of genotypes of likely fathers, given the maternal genotype. We found no evidence of multiple paternity in our population of *A. exaltata*; however, we found that one of the 18 *A. incarnata* fruits was sired by more than one father, yet the primary father sired up to 70% of the 19 seeds genotyped from that fruit. Given these results, we treated seeds within each fruit as full siblings for our paternity analyses.

We attempted to germinate 2-15 seeds per fruit for all fruits that contained at least one fully developed seed. Initially, 15 seeds from each fruit were nicked and sterilized in 10% bleach water, then were germinated in small petri plates between damp paper towel and transplanted into 2.25" pots. Later, 2-6 seeds were sown directly into moist soil in 72-cell trays without nicking or sterilization, then thinned to one seed per cell after germination. The latter technique had similar success germinating at least one seed per fruit (82% of fruits compared to 85% for the first technique). In both cases, seeds were kept moist and were cold-stratified for 4-5 weeks at 4-6C, then placed at ~27C to germinate where they received 14-16h daylight at around 24C for 4-6 weeks either in the greenhouse or a growth chamber. We collected tissue from one seedling per fruit for *A. incarnata* (1303 seedlings) and 1-3 seedlings per fruit for *A. exaltata*, (271 seedlings from 112 fruits).

We extracted DNA from 0.1g of parental and offspring tissue using FastDNA Kits from MP Biomedicals. We tested 41 microsatellite markers developed for *A. syriaca* (O'Quinn and

Fishbein 2009; Kabat et al. 2010; Straub et al. 2011) and nine of those amplified and had sufficient allelic variation in one or both of our populations. We genotyped *A. incarnata* individuals at six loci and *A. exaltata* individuals at five loci (Table 10). We genotyped each parent twice and each offspring once. The first set of parental PCR products was run on 5% acrylamide gels, visualized on an FMBIO; alleles were scored using FMBIO Analysis 8.0 (Hitachi Software Engineering 1991–1999) and placed in bins using Allelogram version 2.2 (Manaster 2002). The second set of the parents and all offspring were visualized using an Applied Biosystems 3730*xl* DNA Analyzer at Yale's DNA Analysis Facility and genotyped using Geneious version 8 (www.geneious.com; Kearse et al. 2012).

Analyses

We used maximum likelihood paternity analyses to assign fractional paternity to the seeds within each fruit in both population. Based on the population allele frequencies, the exclusion probability, or chance of excluding individuals that were not the true sire, given the maternal genotype, was 91% and 93% for *A. incarnata* and *A. exaltata*, respectively (Table 10). For *A. incarnata* we used Cervus (Marshall et al. 1998) to determine fractional paternity for all of the viable fruits. We assumed that all seeds within a fruit were full-siblings (see above), so possible sires were assigned seeds from that fruit proportional to their LOD scores for the single offspring from each fruit. Based on the exclusion probabilities and the number of parental plants, we expected an average of 8.9 sires per *A. exaltata* offspring and 12.5 sires per *A. incarnata* offspring. For each parent plant, their assigned seeds were summed across fruits to determine their total seeds sired in 2010, or annual male fitness.

Because we genotyped multiple seeds per fruit in 83% of A. exaltata fruits, we used

Colony2 (Wang 2004) to determine paternity. Similar to Cervus, Colony2 uses maximum likelihood to determine parent-offspring relationships and the output contains possible sires and their probability of paternity. Unlike Cervus, Colony2 can consider both paternal and maternal sibship among full siblings to increase the power to determine the paternal genotype of the unknown sire and exclude additional non-sires. The input file can be found in the supplementary materials.

We assigned paternity to 6,639 *A. exaltata* seeds and 65,941 *A. incarnata* seeds. We were able to assign a unique sire to only 3% of the *A. incarnata* fruits, and seeds were proportionally divided among fewer than 13 possible sires for 61% of the fruits (note that 12.5 was predicted average number of sires with positive LOD scores). On average, seeds within each fruit were divided among 12.5 (\pm 8.98) possible sires, exactly matching the exclusion probability. In contrast, we were able to assign a unique sire to 79% of the *A. exaltata* fruits, and the remaining 21% of fruits had five or fewer possible sires, which is much lower than the exclusion probability predicted; this was likely due to the inclusion of paternal sibship data.

Each of our selection gradient analyses (Lande and Arnold 1983) regressed male and female pollination success (pollinia inserted or removed per flower) or fitness estimates (fruit number, seeds produced, seeds sired, and total seeds) onto the six floral traits and flower number. We considered including ramet number as a covariate as an estimate of plant size, but its inclusion in the models had no effect on the outcome of the analyses for either species; ramets vary greatly in size (La Rosa pers. obs.) and so flower number may be a better estimate of the vegetative size of plants. Correlations between ramet number and flower number ranged from 0.35 - 0.65 (Table 11). All response variables were relativized by dividing by the mean, and all

predictor variables were standardized to a mean of zero and standard deviation of one. In total, we performed 2-5 linear multiple regressions per species per year. The distribution of all floral traits was normal in each year for both species. The residuals of some models were heteroscedastic; however, even though log transforming female fitness and flower number did improve the distribution of the residuals, it did not improve the fit of the models. Selection gradients, standard errors, and significance are thus from untransformed variables. Between 2009 and 2011 only 51 *A. exaltata* plants and 70 *A. incarnata* plants bloomed in all three years. In 2009, 41% of the *A. incarnata* ramets were destroyed by voles before the fruits had matured; these were excluded from analyses of selection through female fitness. This should not bias the selection gradients unless the vole damage was correlated with the floral traits. In all other years, fewer than 2% of plants died or were damaged by rodents before the fruits could be counted.

For the selection gradient analyses using seeds produced and seeds sired, the total number of seeds was summed from fruits that had at least one seed germinate (viable fruits), eliminating almost one third of the fruits, and the seeds they contained, from these analyses. The seeds assigned to possible sires also came from viable fruits, as those are the only fruits from which we could collect DNA and genotype to perform paternity analyses. However, there is a high correlation between total fruit number per plant (viable plus inviable fruits) and seed number from viable fruits; the correlation is 0.95 for *A. incarnata* and 0.87 for *A. exaltata* (Table 9). A majority of the fruits from eight plants were not genotyped and thus were not included in the paternity analysis for *A. incarnata*. We also found no negative relationship between total flower number and the proportion of the fruits that were viable that would have suggested that the inviable fruits were due to higher rates of geitonogamy and inbreeding depression on larger

plants. Using seeds produced and seeds sired, we also measured functional gender (Lloyd 1980) that ranges from 0 (male; plant fitness is entirely from seeds sired) to 1 (female; plant fitness is entirely from seeds produced). Measures of functional gender allow us to test the prediction that plants with more flowers function more as male. All selection gradient analyses were conducted using JMP 10.0 (SAS Institute Inc. 2012).

Results

The highest correlations among the six floral traits that we measured were between horn reach and hood height in *A. incarnata* (r = 0.52; Table 12a), and between horn reach and hood length in *A. exaltata* (r = 0.47; Table 12b). In both *A. exaltata* and *A. incarnata*, the trait means were similar across years and the phenotypic coefficient of variation for each trait remained relatively constant (Tables 13 and 14).

Functional effects of floral traits

The composition and quantity of hymenopteran pollinators visiting these two species differed, which may have resulted in differences in selection. *A. incarnata* had wasp and bee visitors of many sizes (Table 8), while we only observed large bees and lepidopteran pollinators visiting *A. exaltata*. Slit length was the only trait to affect pollinia insertions or removals per flower in *A. exaltata*; shorter slits increased pollinia removals in 2010 (Fig. 4b). In *A. incarnata*, longer stigmatic slits increased the number of pollinia inserted per flower in 2009 (Fig. 5a) and also increased the number of pollinia removed per flower in 2011 (Fig. 5b). We also found that increased gynostegium width (in 2 years), hood length (in 3 years), and gap width all significantly increased pollinia removals per flower in *A. incarnata*, the direction of the selection gradients matched between male and female function in 14 of 16

instances, suggesting very little conflict between genders; in *A. exaltata* they only matched two out of six times.

Finally, flower number in *A. exaltata* had a significant effect on both pollinia removals per flower and insertions per flower in 2009. This was not so in *A. incarnata*, where flower number was often 30 or more times that of *A. exaltata*.

Selection on floral traits

We detected selection on hoods and gynostegia (Figs. 6 and 7), which make up a majority of the flower. There was selection on hood height through fruit number in both species. Selection gradients for hood height were consistently negative across years in *A. exaltata*; in contrast, selection fluctuated between years in *A. incarnata*. Hood length was also under selection through fruit number, and selection was nearly always positive for both species, except in two years in *A. incarnata*. Selection on gynostegium width through fruit number in *A. incarnata*, was positive three out of four years.

There was selection on the less conspicuous trait slit length through fruit number in both species (Figs. 6 and 7). Selection on slit length was positive in all years but one in *A. incarnata*, and consistently positive in *A. exaltata*. There was also fluctuating selection on gap width in *A. exaltata*; there was selection to decrease it in 2009, then selection to increase it in 2010.

Comparing the results from pollinia inserted per flower (female pollination success) to the selection gradients for fruit number (female fitness), there was a very weak and negative correlation between the functional effects and fitness effects on the six floral traits (r < |-0.17|, p > 0.73 for both species). Pollinia insertions did not appear to affect fruit set, which could be due to a variety of mechanisms including resource limitation, genetic incompatibility, and improper

placement of pollinia.

To check for evidence of stabilizing or disruptive selection on the floral traits, we re-ran the selection models and included non-linear terms for each predictor variable in the selection gradient analyses. We found stabilizing selection on horn reach through fruit number ($\gamma = -0.18$, P < 0.05) in 2011 in *A. incarnata*; horn reach was the only trait that was never under significant directional selection in either species.

Flower number had the greatest effect on female fitness. Plants with more flowers, flowering branches, or ramets produced more fruits (Figs. 6 and 7). Recall that flower number did not affect floral function in terms of per flower removal and insertion rates, except for *A*. *exaltata* pollinia removals and insertions in 2009 (Fig. 4a, b).

Selection through seed production and seed siring success in 2010

In 2010, the only instance of selection through viable seeds produced was through gap width in *A. exaltata*—larger spaces between hoods results in higher male fitness. And gynostegium width was the only trait under selection through female fitness, but was under negative directional selection in both species. Overall, the direction of the selection through seeds produced and sired was never in conflict (Fig. 8).

In *A. exaltata*, the selection gradients for fruit number and viable seeds produced were consistently in the same direction, but were of different magnitudes for gynostegium width and hood height between the fitness estimates (Figs. 6 and 8a). There was significant selection on hood height through fruit number ($\beta = -0.23$, p = 0.01; Fig. 6), but there was not significant, or even marginally significant, selection through seeds produced ($\beta = -0.11$, p = 0.25; Fig. 8a). In *A. incarnata,* there was also significant selection on hood height through fruit number in 2010 ($\beta = -0.11$, $\beta = -0.21$).

0.18, p = 0.03; Fig. 7), but selection through seeds produced was identical, yet not significant (β = 0.18, p = 0.10; Fig. 8b). Significant selection on gap width in *A. exaltata* remained almost identical through fruits and seeds produced. The major difference between fruit number and seeds produced is the exclusion of the non-viable fruits.

The effect of flower number on fitness in 2010 was strong and significant for both species and through both genders (Fig. 8). We found that in *A. exaltata*, the effect of flower number was similar through male and female fitness, but in *A. incarnata* the effect was much stronger through female fitness than through male fitness. We suspect that the weaker effect on flower number through male fitness may be an artifact of fractional paternity, and that if we had more power to detect the single sire, the variance in male fitness would increase and the effect of flower number would be stronger, perhaps becoming similar to the effect through female fitness, as is the case for *A. exaltata*.

Flower number may play a role in the functional gender of individuals if additional flowers are in fact more important for male fitness. Our paternity analyses allowed us to quantify the functional gender of individuals and of each population (Fig. 9) by comparing identical fitness currencies for each gender. In the *A. exaltata* population, there were some plants that were exclusively female, (i.e., they produced seeds but did not sire seeds), but zero plants that were exclusively male. Of the 130 genets, 77 produced viable fruits, and 57 of those maternal genets sired all of the offspring. Thus, 53 plants had zero total fitness in 2010 and are not included in the functional gender plot. We found that plants within this population had a mean functional gender of 0.61 (Fig. 9a), making them more female than male on average. In *A. incarnata* plants were on average more male (functional gender = 0.35; Fig. 9b), but this is also likely an artifact of the

way paternity was assigned in *A. incarnata* that spread out the distribution of paternity. *Pollinators and Pollen limitation*

There were substantial differences in pollinator composition between 2008 and 2010 in *A*. *incarnata* (Table 8). In all years, pollinators appeared to be plentiful, and in the three years between 2007-2009 *A. incarnata* plants produced one fruit for every 34 pollinia inserted on average, suggesting their fitness was not limited by pollen. *A. exaltata*, on the other hand, produced on average one fruit for each pollinium that was inserted. Adding supplemental pollen to *A. exaltata* plants in 2012 had a strong effect that resulted in a fruit increase of more than 50%, which was a marginally significant increase in fruits per plant (0.86 ± 0.14 vs. 0.54 ± 0.15 , respectively; t = 1.69, df = 25, p = 0.0517), suggesting pollen limitation. If pollen was limited in 2009-2011 as well, we should find that there will be selection through female fitness on traits that interact with pollinators.

Discussion

In this study, we asked questions about the function of six floral traits for pollination and the effect of those traits on selection through both male and female components. We used pollinia inserted and removed per flower to find that slit length, gynostegium width, hood length, and gap width functioned to affected male and female pollination success. We then found that all six traits had some effect on fruit number, a common measure of female fitness in milkweeds (e.g., Willson and Rathcke 1974; Wyatt 1976; Morgan and Schoen 1997; Caruso et al. 2005), in one or both species. To quantify selection through male fitness, we calculated seeds sired, a measure that is more accurate than pollinia removed, but that is not often estimated in plants. We found that gynostegium width was important for male fitness in both species.

Function of floral traits

Four of the six floral traits functioned to increase the insertion and removal of pollinia on a per-flower basis, but only slit length had an effect in *A. exaltata*. Slit length is unlikely to affect pollination through attraction, so shorter slits increased pollinia removals per flower by possibly increasing contact with the corpusculum, the structure that attaches pollinia to pollinators. The end of the slit near the base of the gynostegium is raised away from the gynostegium and then tapers towards the corpusculum that is positioned at the upper end of the stigmatic slit near the top of the gynostegium. *A. exaltata* is one of the larger flowered *Asclepias* species in the region, and so individuals with longer slits may have more area for pollinators to grip the flower closer to the base, missing the region where the corpusculum is positioned, and reducing pollinia removals. This population of *A. exaltata* may also be visited by pollinators primarily at night; however, our lack of evidence of day pollinators, then floral display may be more important for attracting pollinators and only traits that contribute to pollinator efficiency, such as slit length, would have an effect on pollinia insertions and removals.

In *A. incarnata*, slit length and gynostegium width were important for male and female floral function. In almost every year, both traits acted to increase pollinia removals and insertions as they increased in size and length, so there was rarely any conflict between genders. Pollinators typically clasp the flowers between the hoods on either side of the gynostegium or across several flowers in this species, which results in their tarsi often contacting the corpuscula and the stigmatic slits (Macior 1965; La Rosa pers. obs.). If the width of the gynostegium correctly positioned pollinators, and the slit length in this small-flowered species allowed for increased
probability of pollinator legs sliding between the adjacent anther wings (the structures that form the stigmatic slit), then both male and female pollination success could be increased simultaneously. Two other traits, hood length and gap width functioned to increase pollinia removals per flower in *A. incarnata*, but had no effect on pollinia insertions per flower. Hood length showed the most consistent pattern of all the traits, having a significant positive effect on pollinia removals per flower for three consecutive years, but we do not know if this effect was do to an increase in pollinator visitation or an increase in efficient pollination.

Selection on floral traits

Hood height was consistently important for female fitness (fruit number) and was significant in 2010; however, selection on hood height was not significant through seeds produced in 2010. In that year, hood height contributed to total fruit production, but about one third of the fruits produced seeds that did not germinate. Fruits in milkweeds are known to be aborted after they have begun to develop, but typically this abortion happens while the fruits are still quite small, and all of the fruits that we collected were fully mature. Himes et al. (2005) found that in *A. exaltata*, plants that can mature fruits from self pollen, will have low seed germination; this could also be true of *A. incarnata*. The high inviability of seeds within mature fruits might indicate high levels of inbreeding depression due to geitonogamous selfing. Given that plants with large floral displays have been shown to have higher rates of geitonogamous selfing (Harder and Barrett 1995), we were surprised to find that plants with more total flowers did not have higher proportions of inviable fruits. Other factors, such as resource availability or disease, may have affected seeds as they matured.

Only hood height showed inter-annual variation in significant selection between 2009,

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when there was selection for shorter hoods, and 2010, when selection was for taller hoods. Our pollinator observations and others have shown that pollinator composition and importance can change drastically between years (Table 8) (Herrera 1988; Price et al. 2005; Wiggam and Ferguson 2005), although we did not have sufficient data to directly compare the pollinator composition in 2009 to the other two years. Pollinator composition can also change dramatically within a season (Herrera 1988; Ashman and Stanton 1991; Wiggam and Ferguson 2005). We do know that plants flowered 11-14 days later in 2009 than they did in 2010, and this may have caused a shift towards later season pollinators, which could affect fitness (Rafferty and Ives 2012). If pollinators were also very different in 2009, it may explain the fluctuations in significant selection in hood height, since we would expect the pollinator types to differ in the amount or direction of selection they impose as others have found in a generalist species (Schemske and Horvitz 1989; Sahli and Conner 2011).

Estimates of annual fitness for *Asclepias* that others have used in the past (fruit number and pollinia removals) differ in their ability to predict selection through viable seeds produced and sired. We found that measuring selection through fruit number is a good way to estimate the direction of selection, and in some cases the magnitude, but does not necessarily predict which traits are under significant selection through female fitness. By summing the pollinia removed per flower in *A. exaltata* and multiplying removals per flower by the estimated flower number in *A. incarnata* we estimated total pollinia removed and found that its correlation with seeds sired was 0.44 and 0.45 for these two species, which is nearly identical to the value calculated by Broyles and Wyatt (1990) for *A. exaltata*. Given that the R² is only 0.19, total removals are a rather poor predictor annual male fitness.

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Function, fitness, and limitations

Gynostegium width and slit length were the only traits to have a similar effect on female pollination success and reproductive success; otherwise, the overall disconnect between how the traits function to affect pollinia insertion and removal and how they affect female and male fitness may depend a lot on the availability of resources or pollinators. In populations where resources limit female fitness, individuals receive adequate pollen, so that the only differences in fitness are due entirely to available resources. Under such circumstances, we would not expect to find selection on a trait that differentially affects pollination success. Alternatively, if fitness is limited by the abundance or effectiveness of the pollinators, the traits that differentially affect pollinia insertions and removals may differentially affect female and male fitness, respectively.

The population of *A. incarnata* appeared to be resource limited due to its high number of pollinia insertions per fruit, yet we found traits that were similarly important for pollen receipt and fruit number in 2009. One possible explanation is that female fitness in the population of *A. incarnata* could have been limited by access to *effective* pollinators in 2009. It is pollinated by a variety of generalist pollinators, so it is likely that some pollinators are more effective than others and that the pollinia inserted into the stigmatic slits will not always result in fertilization. Studies have demonstrated that different insects are not equally effective at removing and inserting pollinia in *A. incarnata* (Ivey et al. 2003; Rafferty and Ives 2012) or in *A. exaltata* (Stoepler et al. 2012), and studies on other species have shown that different generalist pollinators may not be equally effective (Herrera 1988; Fishbein and Venable 1996; Sahli and Conner 2007; Rocca and Sazima 2012).

Flower number and fitness

Flower number appears to be overwhelmingly important for male and female fitness. Many people have studied milkweeds because of their puzzling low fruit to flower ratio and concluded that the large floral displays benefit male fitness components (Willson and Rathcke 1974; Willson and Price 1977; Chaplin and Walker 1982; Queller 1983). However, Broyles and Wyatt (1990) found that there was no negative correlation between functional gender and flower number in plants that had both male and female fitness (n = 17, r = 0.35) demonstrating that plants with more flowers did not tend to have greater male fitness relative to female fitness. We found this to be similarly true in both of our species. The correlation between functional gender and flower and flower number was 0.11 (n = 57) for *A. exaltata* and 0.54 (n = 104) for *A. incarnata*; positive correlations indicate that plants with more flowers tended to be more female than male.

Flower number appeared to have a strong positive effect on female fitness in both species, but it may be that flower number and fruit number are both correlated with resources, thus resources may actually be the primary cause of this strong relationship (Scheiner et al. 2002; Stinchcombe et al. 2002). Male fitness is not likely to be influenced by resources in the way that female fitness is (Bateman 1948), and so is interesting that the effect of flower number on male fitness in *A. exaltata* is nearly as strong as the effect on female fitness, showing again that total flower number in milkweed is indeed important for male fitness (Willson and Rathcke 1974; Willson and Price 1977; Chaplin and Walker 1982; Queller 1983). In *A. incarnata*, we found that the effect of flower number on male fitness is much weaker than on female fitness. We suspect that this to be an artifact from assigning fractional paternity to many sires, even though each seed had just one sire. By assigning paternity to many sires (mean = 12.5), we distributed seeds from

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each fruit to many possible sires across the population, and thus reduced the variation in male fitness. This reduction in variation potentially reduced the estimates of selection through male fitness. Additionally, we do not know if the difference in functional gender between the two species is biological or if they also differ as a result of the two fractional paternity methods. In the future, we will genotype additional seeds per fruit as we did for *A. exaltata* and rerun the selection analyses, using the additional paternal sibship information.

Conclusions

We found significant directional selection on all of the floral traits except horn reach, which we found to be under stabilizing selection for one year in *A. incarnata*. These results suggest that the floral traits are likely to be adaptive, and gynostegium width and slit length are adaptive by increasing pollinia insertions in *A. incarnata* in years when pollinators could be limiting. The population of *A. exaltata* was pollen limited, and so we did not expect more selection through male fitness (Bateman 1948), and our results from 2010 match this. We found that flower number was very important for total seeds produced and seeds sired; however, we do not know to what extent available resources contributed to female fitness or the production of flowers. Both species are visited by pollinators that differ in their behavior and effectiveness, and possibly the selection they impose. We have identified floral traits that are likely adaptive, but additional studies that separate the effects of different pollinators, remove external selective agents such as resources as Caruso et al. (2005) did in *A. syriaca*, while estimating male fitness using seeds sired, can help us further understand how the traits are adaptive in these species.

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APPENDIX A

Tables and Figures

Table 6. Trait repeatability. Among plant variation from plants with two or three photographed flowers. Plants sampled ranged from50-236.

		1	4. incarnate		A. exaltata			
Year	2007	2008	2009	2010	2011	2009	2010	2011
Gynostegium width	-	78.2	70	69.5	69.7	82.4	87	83.9
Hood height	80.9	81.9	81	67.5	82.1	85.8	82.9	90.1
Hood length	47	58.3	62.7	53.4	67.9	73.7	80.8	73.6
Horn reach	30.8	63.8	59.4	63.1	66.8	74.9	70.7	78.5
Slit length	43.6	70.7	63.1	69	60.1	58.7	79.2	76.3
Gap width	-	60	64.4	35.2	39	65.1	70.1	73.4
Plants sampled	50	50	236	127	132	95	144	127
Flowers photographed	2	3	2-3	2	2-4	2-3	2	2

		1	4. incarnata	A. exaltata				
Year	2007	2008	2009	2010	2011	2009	2010	2011
Mean flowers sampled per plant	12.1	100.7	53.3	92.1	117.3	22.6	36.3	30.3
Std. dev.	10.5	53.6	30	43.5	68.3	15.9	26	19.4
Median	9	92	53	85.5	88.5	19	36	25.5
Plants sampled	50	50	303	132	134	91	130	122

Table 7. Sampling effort in each year for A. incarnata and A. exaltata.

Table 8. Percent pollinator composition in the *A. incarnata* **population in 2008 and 2010.** Pollinia carried on pollinators gives the number of insects collected, the mean pollinia attached to each individual, and the standard error of the mean (SEM). The percent pollinia are the proportion of total pollinia carried by that pollinator type. Percent importance is calculated by multiplying the raw number of pollinators observed by the mean number of pollinia carried and dividing by the total of all pollinators.

	% Com	position	Pollinia carried on pollinators				% Importance	
	2008	2010	n	Mean pollinia	SEM	% pollinia	2008 poll. importance	2010 poll. importance
Butterflies	3	1	10	15.2	12.1	5.6	1.9	0.7
Flies	23	26	12	0.3	0.2	0.1	0.3	0.6
Bees (sm/med)	5	17	2	16.0	0.0	5.9	3.7	19.0
Honey bees	41	3	15	34.4	10.5	12.6	61.9	7.1
Bees (large)	5	1	11	43.8	16.7	16.0	10.0	4.0
Wasps (small)	14	38	28	11.0	3.7	4.0	7.0	28.4
Wasps (medium)	9	14	31	39.9	7.6	14.6	15.2	37.7
Wasps (large)	0	0	10	112.8	12.6	41.3	0.0	2.6
n	173	600						

Table 9. Pairwise correlation coefficients for female fitness measures in 2010 for *A*. *incarnata* (below the diagonal) and *A. exaltata* (above the diagonal and in italics). Seeds produced, seeds sired, and the sum of the two (p+s) come from viable fruits only. These two measures exclude fruits that could not be included in the paternity analysis because none of the seeds that were planted germinated. P < 0.0001 for all.

	Total fruits	Viable fruits	Total seeds	Seeds produced	Seeds sired	Seeds (p+s)
Total fruits	-	0.86	0.97	0.87	0.47	0.73
Viable fruits	0.94	-	0.82	0.96	0.53	0.81
Total seeds	0.99	0.95	-	0.89	0.47	0.74
Seeds produced	0.95	0.98	0.97	-	0.57	0.85
Seeds sired	0.37	0.36	0.38	0.38	-	0.91
Seeds (p+s)	0.93	0.95	0.95	0.97	0.58	-

Table 10. **Primer names, annealing temperature, exclusion probabilities and allele frequencies of microsatellite markers used on** *A. exaltata* **and** *A. incarnata*, **with genet sample sizes of n.** A106 is showing expected allele frequencies with a null allele; the expected null allele frequency is in parentheses. Primers were labeled with 6-FAM, HEX, or a HEX-labeled M13 tail to distinguish between loci with overlapping allele sizes.

						Alleles			
Locus	$T_{\mathbf{A}}$	P{excl.}	1	2	3	4	5	6	7
A. exaltata (n=129)		0.93							
Asyr-B121 _{HEX}	58	0.56	0.28	0.26	0.20	0.19	0.07	< 0.01	
Asyr-B2 _{HEX}	58	0.40	0.39	0.34	0.23	0.02	0.02		
Asyr-C102 _{HEX}	48	0.34	0.47	0.38	0.11	0.04			
Asyr-C103 _{HEX}	48	0.43	0.35	0.32	0.28	0.02	0.02	0.01	
As71725 _{M13-HEX}	48	0.25	0.70	0.17	0.13				
A. incarnata (n=142)		0.91							
Asyr-A106 _{FAM}	56	0.34	0.46	0.33	0.09	(0.08)	0.03	< 0.01	< 0.01
ASF9 _{HEX}	56	0.38	0.37	0.28	0.26	< 0.01	< 0.01		
Asyr-B102 _{FAM}	56	0.28	0.62	0.22	0.14				
Asyr-B121 _{HEX}	56	0.30	0.66	0.23	0.18				
Asyr-C124 _{M13-HEX}	56	0.35	0.49	0.34	0.11	0.03	< 0.01		
As71725 _{M13-HEX}	48	0.36	0.46	0.29	0.27	< 0.01			

Table 11. Pairwise correlation coefficients between estimated total flower number and ramet number for *A. incarnata* and *A. exaltata*.

		A		4. exaltate	a			
Year	2007	2008	2009	2010	2011	2009	2010	2011
r	0.64	-	0.64	35	0.75	0.36	0.65	0.35

(a) 1 analtata							
(a) A. exaliala	Gyn W	Hood H	Hood L	Horn R	Slit L	Gap W	Ramets
Hood H	-0.33						
Hood L	-0.29	0.32					
Horn R	-0.04	0.30	0.47				
Slit L	0.13	0.30	0.09	0.14			
Gap W	0.08	-0.03	0.00	-0.16	-0.00		
Ramets	-0.04	0.02	0.04	-0.04	-0.02	-0.01	
Flower number	-0.03	0.01	-0.20	-0.13	-0.11	-0.17	0.48

Table 12. Pairwise correlation coefficients for *A. exaltata* (a) and *A. incarnata* (b) combined across years. Bold is P < 0.05.

(b) A. incarnata

	Gyn W	Hood H	Hood L	Horn R	Slit L	Gap W	Ramets
Hood H	0.45						
Hood L	0.09	0.42					
Horn R	0.27	0.52	0.15				
Slit L	0.51	0.34	0.12	0.14			
Gap W	-0.01	0.1	0.1	0.03	0.09		
Ramets	0.08	0.07	0.02	0.06	-0.07	0.08	
Est. flower number	0.35	0.09	-0.01	0.06	0.27	0.11	0.41

Table 13. A. exaltata descriptive statistics for the six traits (cm), total flower number, and fitness estimates. Mean, standard deviation, and phenotypic coefficients of variation (CV_P) were calculated for each year. Pollinia removals, pollinia insertions, and fruits were measured on every flower, and fruits were counted on all ramets. Pearson product-moment correlations are given between total pollinia removals and total insertions in 2007-09, and between seeds produced and sired in 2010. Gynostegium width was measured differently in 2011.

	2009		2010		2011		
Aexa traits	mean (s.d.)	CV _P	mean (s.d.)	CVP	mean (s.d.)	CVP	
Gynostegium width	0.25 (0.02)	8.1	0.24 (0.01)	5.7	0.13 (0.01)	6.1	
Hood height	0.37 (0.04)	9.9	0.38 (0.03)	8.6	0.41 (0.03)	8.1	
Hood length	0.22 (0.03)	12.1	0.17 (0.02)	11.7	0.21 (0.02)	10.2	
Horn reach	0.25 (0.05)	20.1	0.20 (0.05)	26.1	0.23 (0.04)	18.5	
Slit length	0.25 (0.01)	5.5	0.24 (0.01)	4.9	0.24 (0.01)	4.6	
Gap width	0.06 (0.01)	22.1	0.06 (0.01)	18.6	0.06 (0.01)	20.1	
Estimated flower number	25.7 (18.4)	70.5	36.3 (26.0)	71.4	30.3 (19.4)	64.0	
Sample size	n = 91		n = 130		n = 153		
Insertions per flower	0.02 (0.03)	182.7					
Removals per flower	0.13 (0.15)	112.6	0.29 (0.15)	52.0	0.27 (0.19)	68.8	
Pollinia (male-female corr.)	$r = 0.60^{\circ}$	*					
Fruit quantity	0.73 (0.86)	117.1	1.02 (1.12)	110.4	1.01 (0.96)	95.0	
Seeds produced			51.1 (57.3)	112.2			
Seeds sired			51.1 (72.8)	142.6			
Total seeds			102.1 (115.40) 112.9			
Seeds (male-female corr.)			r = 0.57*	k			

Table 14. *A. incarnata* descriptive statistics for the six traits (cm), flower number (estimated as lateral branches in 2007, 2011; ramets in 2008; estimated flowers in 2009, and umbels in 2010), and fitness estimates. Mean, standard deviation, and phenotypic coefficients of variation (CV_P) were calculated for each year. Except in 2008, pollinia removals, pollinia insertions were sampled across genets, and fruits were counted on all ramets. Pearson product-moment correlations are given between total pollinia removals and total insertions in 2007-09, and between seeds produced and sired in 2010. Gynostegium width was measured differently in 2011.

(b) A. incarnata	ta 2007 2008 2009		2010		2011					
	mean (s.d.)	CV_{P}	mean (s.d.)	CV_{P}	mean (s.d.)	CV _P	mean (s.d.)	CV _P	mean (s.d.)	CV _P
Gynostegium width			0.14 (0.01)	4.4	0.14 (0.01)	5.1	0.13 (0.01)	4.4	0.07 (0.00)	4.9
Hood height	0.22 (0.02)	7.7	0.23 (0.02)	8.5	0.22 (0.02)	9.0	0.21 (0.02)	7.8	0.20 (0.02)	8.2
Hood length	0.14 (0.02)	12.8	0.14 (0.02)	10.7	0.14 (0.02)	12.3	0.13 (0.02)	11.0	0.14 (0.02)	11.2
Horn reach	0.10 (0.02)	16.9	0.09 (0.02)	22.2	0.09 (0.02)	21.5	0.08 (0.02)	23.0	0.07 (0.02)	20.4
Slit length	0.16 (0.01)	4.2	0.16 (0.01)	3.6	0.17 (0.01)	4.6	0.16 (0.00)	3.7	0.16 (0.01)	3.6
Gap width			0.04 (0.01)	15.0	0.05 (0.01)	22.7	0.05 (0.01)	15.8	0.05 (0.01)	11.8
Estimated flower number	9.9 (7.7)	77.7	3.1 (2.3)	72.6	818.2 (845.4)	103.3	1663.6 (2000.2)	120.2	9.4 (7.6)	80.6
Sample size	n = 50		n = 50		n = 305		n = 129		n = 134	
Insertions per flower	0.38 (0.26)	68.7	0.17 (0.10)	56.5	0.25 (0.17)	68.0				
Removals per flower	3.41 (0.73)	21.3	1.94 (0.64)	32.9	2.62 (0.73)	27.9	1.22 (0.48)	39.6	1.87 (0.71)	37.8
Pollinia (male-female corr.)	r = 0.16		r = 0.64*		r = 0.61*					
Fruit quantity	28.1 (24.3)	86.3	21 (24.5)	117.0	10.1 (11.8)	117.3	15.2 (18.6)	122.6	11.1 (11.7)	105.3
Seeds produced							556 (902)	162.2		
Seeds sired							511 (258)	50.5		
Total seeds							102 (115)	112.9		
Seeds (male-female corr.)							r = 0.38*			



Figure 3. Top and side views of *A. exaltata* (A) and *A. incarnata* (B) flowers showing the six floral traits and landmarks: gynostegium width (g; gr), hood length (hl), hood height (hh), horn reach (hr), slit length (sl), gap width (gw). The arrow points to the dark corpusculum, which is attached to a pair of pollinia within the wall of the gynostegium.



Figure 4. The effect of traits and flower number on female (a) andmale (b) floral function across three years in A. exaltata. Barsshow standardized selection gradients for the six floral traits andflower number estimates on pollinia inserted per flower (a) andpollinia removed per flower (b). Error bars represent two standard2010 (130)2011 (122)

A. incarnata



		n
Figure 5. The effect of traits and plant size on female (a) and	□2007	(50)
male (b) floral function across three years in A. incarnata. Bars	□2008	(50)
show standardized selection gradients for the six floral traits and	■2009	(292)
flower number estimates on pollinia inserted per flower (a) and	■2010	(126)
pollinia removed per flower (b). Error bars represent two standard	= 2011	(121)
errors. TP<0.10, *P<0.05, **P<0.01, ***P<0.001	2011	(131)

A. exaltata



Figure 6. Standardized selection gradient estimates for floral	
traits and flower number on female fitness (fruit number) across	n
three years in A. exaltata. Bars show standardized selection	■2009 (90)
gradients for the six floral traits and flower number estimates. Error	■2010 (130)
bars represent two standard errors. †P<0.10, *P<0.05, **P<0.01,	■2011 (121)
***P<0.001	

A. incarnata



Figure 7. Standardized selection gradient estimates for floral	□2007 (50)
traits and flower number on female fitness (fruit number) across	□2008 (49)
three years in <i>A. incarnata.</i> Bars show standardized selection gradients for the six floral traits and flower number estimates. Error bars represent two standard errors. †P<0.10, *P<0.05, **P<0.01, ***P<0.001	■2009 (180) ■2010 (126) ■2011 (131)











Figure 9. Distribution of functional gender (f/(m+f)) for individuals in a population of *A. exaltata* (a) and *A. incarnata* (b). Individuals with zero fitness are excluded.

APPENDIX B

Selection gradient analyses: effect sizes, standard errors, and model fit

Table 15. Selection through insertions per flower in *A. exaltata.* Standardized selection gradient estimates for floral traits and estimated flower number in 2009 in *A. exaltata.*

	2009
Gynostegium width	0.02 (0.25)
Hood height	0.14 (0.25)
Hood length	0.03 (0.23)
Horn reach	-0.04 (0.25)
Slit length	-0.17 (0.22)
Gap width	-0.08 (0.20)
Est. flower number	0.66 (0.21) *
Total R ²	0.14
Adj. R ²	0.07
Р	0.07
n	91

	2009	2010	2011
Gynostegium width	-0.07 (0.15)	-0.03 (0.05)	-0.08 (0.08)
Hood height	-0.05 (0.15)	0.04 (0.05)	-0.04 (0.08)
Hood length	0.12 (0.14)	0.02 (0.05)	-0.01 (0.07)
Horn reach	-0.04 (0.15)	-0.01 (0.05)	0.12 (0.07)
Slit length	-0.02 (0.14)	-0.14 (0.05) **	-0.04 (0.07)
Gap width	-0.17 (0.13)	-0.00 (0.05)	0.06 (0.07)
Est. flower number	0.30 (0.13) *	0.05 (0.05)	0.09 (0.07)
Total R ²	0.13	0.09	0.06
Adj. R ²	0.05	0.04	-0.001
Р	0.11	0.09 †	0.45
n	91	130	122

Table 16. Selection through removals per flower in *A. exaltata.*Standardized selectiongradient estimates for floral traits and estimated flower number across three years in *A. exaltata.*

Table 17. Selection through fruit number in *A. exaltata.* Standardized selection gradient estimates for floral traits and estimated flower number across three years in *A. exaltata.*

	2009		2010	2011
Gynostegium width	0.24 (0.13)	†	-0.02 (0.09)	0.01 (0.09)
Hood height	-0.20 (0.13)		-0.23 (0.09) *	-0.08 (0.10)
Hood length	0.27 (0.11)	*	0.13 (0.09)	0.10 (0.10)
Horn reach	-0.19 (0.13)		0.03 (0.09)	0.02 (0.10)
Slit length	0.19 (0.11)	1	0.06 (0.08)	0.05 (0.10)
Gap width	-0.19 (0.10)	Ť	0.24 (0.09) **	0.13 (0.09)
Est. flower number	0.74 (0.11)	***	0.66 (0.08) **	* 0.45 (0.09) ***
Total R ²	0.46		0.37	0.20
Adj. R ²	0.41		0.34	0.15
Р	< 0.0001	***	<0.0001 **	* 0.0005 ***
n	90		130	121

Table 18. Selection through viable seeds produced and sired, and total viable seeds in *A.exaltata.* Standardized selection gradient estimates for floral traits and estimated flower numberin 2010 in *A. exaltata.*

2010	Seeds set $\stackrel{\bigcirc}{\rightarrow}$	Seeds sired \mathcal{J}	Total fitness
Gynostegium width	-0.08 (0.09)	-0.25 (0.13) †	-0.16 (0.09) †
Hood height	-0.11 (0.09)	-0.02 (0.14)	-0.07 (0.10)
Hood length	0.13 (0.09)	0.15 (0.13)	0.14 (0.09)
Horn reach	0.01 (0.09)	0.05 (0.13)	0.03 (0.09)
Slit length	0.06 (0.08)	0.18 (0.12)	0.12 (0.09)
Gap width	0.22 (0.09) *	0.16 (0.12)	0.19 (0.09) *
Est. flower number	0.70 (0.08) ***	0.62 (0.12) ***	0.66 (0.09) ***
Total R ²	0.38	0.21	0.34
Adj. R ²	0.34	0.16	0.30
Р	<0.0001 ***	0.0002 ***	<0.0001 ***
n	130	130	130

Table 19. Selection through insertions per flower in *A. incarnata.* Standardized selection gradient estimates for floral traits and estimated flower number across three years in *A. incarnata.*

	2007	2008	2009
Gynostegium width		0.11 (0.10)	0.21 (0.05) ***
Hood height	0.21 (0.12)	0.19 (0.11) †	-0.08 (0.05)
Hood length	-0.08 (0.11)	-0.10 (0.10)	0.01 (0.04)
Horn reach	-0.03 (0.10)	-0.15 (0.10)	0.02 (0.04)
Slit length	0.18 (0.10) †	0.07 (0.09)	0.08 (0.04) †
Gap width		-0.03 (0.08)	0.02 (0.04)
Est. flower number	-0.02 (0.11)	0.09 (0.08)	-0.06 (0.04)
Total R ²	0.15	0.15	0.14
Adj. R ²	0.05	0.01	0.12
Р	0.21	0.41	< 0.0001
n	50	50	292

Table 20. Selection through removals per flower in *A. incarnata.*Standardized selectiongradient estimates for floral traits and estimated flower number across five years in *A. incarnata.*

	2007	2008	2009	2010	2011
Gynostegium width		0.07 (0.06)	0.07 (0.02) ***	0.02 (0.04)	0.07 (0.03)*
Hood height	0.004 (0.04)	0.06 (0.07)	-0.01 (0.02)	0.05 (0.05)	-0.03 (0.04)
Hood length	-0.04 (0.04)	-0.02 (0.06)	0.05 (0.02) ***	0.09 (0.04) *	0.15 (0.03) ***
Horn reach	-0.02 (0.03)	-0.02 (0.06)	0.01 (0.02)	-0.01 (0.04)	-0.03 (0.03)
Slit length	0.05 (0.03)	-0.03 (0.05)	0.03 (0.02) †	-0.01 (0.04)	0.07 (0.03) *
Gap width		$^{-0.00}_{-4}$ (0.05)	0.03 (0.02)*	0.00 (0.04)	0.03 (0.03)
Est. flower number	0.02 (0.04)	0.04 (0.05)	-0.01 (0.02)	0.05 (0.04)	-0.04 (0.03)
Total R ²	0.09	0.08	0.17	0.11	0.31
Adj. R ²	-0.01	-0.07	0.15	0.08	0.27
Р	0.49	0.81	< 0.0001	0.05	< 0.0001
n	50	50	292	126	131

Table 21. Selection through fruit number in *A. incarnata.* Standardized selection gradient

 estimates for floral traits and estimated flower number across five years in *A. incarnata.*

	2007	2008	2009	2010	2011
Gynostegium width	-	0.15 (0.20)	0.16 (0.08)*	-0.05 (0.07)	0.04 (0.07)
Hood height	0.06 (0.09)	-0.11 (0.23)	-0.23 (0.08) **	0.18 (0.08) *	0.13 (0.09)
Hood length	0.11 (0.08)	-0.25 (0.21)	0.14 (0.07)*	0.09 (0.07)	-0.01 (0.08)
Horn reach	0.01 (0.08)	0.06 (0.21)	0.12 (0.07) †	-0.03 (0.07)	0.01 (0.08)
Slit length	0.15 (0.08) †	0.11 (0.17)	0.14 (0.07)†	-0.06 (0.07)	0.06 (0.07)
Gap width	-	0.18 (0.16)	-0.01 (0.06)	0.03 (0.07)	0.01 (0.07)
Est. flower number	0.79 (0.08) ***	0.46 (0.17) **	0.85 (0.06) ***	1.01 (0.06) ***	0.74 (0.07) ***
Total R ²	0.70	0.22	0.54	0.70	0.52
Adj. R ²	0.66	0.09	0.52	0.68	0.50
Р	< 0.0001	0.14	< 0.0001	< 0.0001	< 0.0001
n	50	49	180	126	131

Table 22. Selection through viable seeds produced and sired, and total viable seeds in A.
<i>incarnata.</i> Standardized selection gradient estimates for floral traits and estimated flower
number in 2010 in A. incarnata.

	Seeds produced \bigcirc	Seeds sired \mathcal{J}	Total fitness
Gynostegium width	-0.03 (0.09)	-0.08 (0.05) †	-0.05 (0.06)
Hood height	0.18 (0.11)	0.01 (0.05)	0.10 (0.07)
Hood length	0.14 (0.10)	-0.00 (0.05)	0.08 (0.06)
Horn reach	-0.08 (0.10)	-0.04 (0.05)	-0.06 (0.06)
Slit length	-0.07 (0.09)	0.00 (0.04)	-0.04 (0.05)
Gap width	0.02 (0.09)	0.05 (0.04)	0.04 (0.05)
Est. flower number	1.19 (0.09) ***	0.25 (0.04) ***	0.77 (0.05) ***
Total R ²	0.64	0.24	0.66
Adj. R ²	0.61	0.20	0.64
Р	< 0.0001	< 0.0001	< 0.0001
n	126	126	126

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LITERATURE CITED

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CHAPTER 4

PATTERNS OF ADAPTIVE TRAIT EVOLUTION ACROSS THE NORTH AMERICAN ASCLEPIAS

Introduction

Adaptation is a fundamental process in biology that allows species to cope with their biotic and abiotic environment. Studies of adaptation on extant species can identify which traits are currently adaptive (e.g. Andersson 1982; Conner 1988; Grant and Grant 2002), and phylogenetic comparative methods offer complementary approaches to build and test adaptive hypotheses by studying interspecific patterns using contemporary trait measurements (Doughty 1996). Two patterns that suggest evolution in response to selection are trait convergence and correlations with selective agents (Larson and Losos 1996). Convergence of a trait across clades suggests it evolved in response to a shared selective agent, or similar suite of agents—a selective regime (Coddington 1994; discussed by Pagel 1994b). A phylogenetically corrected correlation between traits suggests pairs of traits may be functionally related and correlations between a trait and a possible selective agent suggests the trait is an adaptation to that particular environment (Fenster et al. 2004; Weiblen 2004; Knudsen and Tollsten 2008).

The array of phylogenetic tests that are currently available complement each other to provide a multidimensional understanding of an adaptive trait—we can model the evolution of a trait and predict its ancestral form (ancestral state reconstruction), identify other traits that it evolved in conjunction with or selective agents it was responding to (correlated evolution), and predict the adaptive peaks it evolved towards (convergent evolution). For example, phylogenetic comparative methods using data from Caribbean *Anolis* lizards show that adaptive radiations on

four Caribbean islands resulted in phenotypic convergence of ecomorphs (Mahler et al. 2013).

Angiosperm diversity exploded 90 million years ago during the Cretaceous period. Prior to that time, plant reproduction was more dependent on abiotic factors (e.g., wind pollination), but a reliance on animals for reproduction introduced a new diversity of selective agents on floral traits. We expect that pollinators played an important role in the evolution of many floral traits (e.g. Gómez et al. 2015), yet it can be difficult to prove the link between an adaptive trait and its selective agent(s) (but see Bradshaw and Schemske 2003). In many cases we assume that the pollinators we see today are the pollinators that were important in the past, and for plants that are not specialized, those pollinators can be composed of an array of taxa.

The milkweed genus *Asclepias* has over 125 North American species (Fishbein et al. 2011), which all share the same floral structures that are diagnostic for the family, but show great diversity in their size and shape. In addition to five petals, the unusual *Asclepias* floral traits consist of a gynostegium housing two ovaries, the stigmatic chamber and pollinia (pollen packets), and a ring of five hoods that hold nectar (Fig. 10). Aside from the petals, the gynostegium and hoods together make up the bulk of the flower, with considerable variation in hood length and gynostegium width (5-6 standard deviations, see methods). The hoods also have discrete features; most of the 125 species have hoods with conspicuous openings rather than covered openings, and at least three quarters of the species have horns, which develop from the inside surface of the hoods (Kunze 1990) (Fig. 10). Further, *Asclepias* rely completely on animal pollination for sexual reproduction, because the pollen and stigmatic surface are isolated from each other within a flower. *Asclepias* tend to be generalists that are commonly pollinated by

insects such as bumble bees, honey bees, butterflies, and wasps (Woodson 1941; Pleasants 1991; Betz et al. 1994; Wyatt and Broyles 1994; Fishbein and Venable 1996; Ivey et al. 2003; Rafferty and Ives 2012).

Studies of contemporary selection have found that the width of the gynostegium, hood dimensions, and horn length can influence the fitness of several *Asclepias* species (Morgan and Schoen 1997; Caruso et al. 2005; Chapters 1 and 2), suggesting these floral traits may be currently adaptive. Gynostegium width had an effect on pollination success in *A. tuberosa* and *A. incarnata* (Chapters 1 and 2), longer hoods increased pollinia removals in two species and increased fruit production in three species (Caruso et al. 2005; Chapters 1 and 2), and the degree to which horns overlapped the gynostegium affected fruit number in *A. tuberosa* (Chapter 1).

We performed phylogenetic comparative analyses on hood, horn, and gynostegium traits to look for patterns of trait evolution that could indicate selection and complement the studies of contemporary selection (Morgan and Schoen 1997; Caruso et al. 2005; Chapters 1 and 2). We made several hypotheses regarding these three traits and their patterns of evolution across the genus. First, we hypothesized that if the hoods become closed, the horns cannot function. The function of the horns is unknown, yet they may interact with pollinators to guide their bodies between the hoods towards the reproductive structures or affect how pollinators approach the hoods to drink nectar. If the horns become enclosed by the hoods, they likely cannot do either of these proposed functions. Second, we hypothesized that different pollinator taxa are attracted to different hood shapes, as pollinators have been shown to preferentially visit flowers based on shape (Hegland and Totland 2005; Gómez et al. 2008). Third, different environments, biotic or abiotic, select for particular combinations of hood and gynostegium size. If the *Asclepias* hoods

and gynostegia are adaptive they likely evolved through natural selection to attract or to be effectively pollinated by particular pollinators. Some may have also evolved in response to harsh environments that can make it challenging to maintain nectar rewards for pollinators—many *Asclepias* species live in hot, dry, and sunny habitats (Woodson 1954).

Following these hypotheses, we made several predictions. We predicted that horn loss had evolved in conjunction with hood closure. We also predicted species with hoods that have a flared rim, giving the appearance of easy access to the nectar reward, were more attractive to hymenopteran pollinators and hoods that appear to have smaller and less conspicuous openings attracted more non-hymenopteran pollinators. We do not have specific predictions for the different phenotypes made from combinations of short and long hoods and wide and narrow gynostegia, but we do predict that particular environments select for similar phenotypes, and so we tested for convergent evolution of these two traits.

Methods

Traits

To study intraspecific patterns of trait evolution of the hoods, the horns that develop within the hoods, and gynostegia, we scored and measured floral traits on 107 of approximately 125 North American *Asclepias* species, two African species from the genus *Gomphocarpus*, and two more distantly related outgroups, *Pergularia daemia* and *Calotropis procera*, all from the tribe Asclepiadeae within Apocynaceae (Fishbein et al. 2011). Specimens were primarily collected by M. Fishbein, and supplemented with specimens from a number of other collectors between 1973 and 2010; all had been preserved in formalin-acetic acid (FAA) or ethanol. Using a Canon digital SLR with a 60mm macro lens, we photographed 1-3 flowers per species (mean =

1.6) from the top, side, and again from the side with 2.5 hoods removed, exposing the horn in order to identify horned species. For species that were sampled from more than one locale, we photographed one flower per locale and averaged the measurements within species.

We chose landmarks that would be visible across the diversity of floral morphology in the family, and took measurements using ImageJ (Rasband 1997). Our discrete variables were open (conspicuous) or closed (inconspicuous) hood openings, presence or absence of horns, and flared or domed hood openings—these are hoods that the opening does not flare out, but instead is straight or arcs inward (Fig. 11). The continuous traits we measured were hood length and gynostegium width, both measured from the top view of the flower (Fig. 10). Hood length was the distance from the outer most tip of the hood to its point of contact with the gynostegium (*h*, Fig. 10), and gynostegium width was estimated by taking the distance between adjacent corpuscula (*g*, Fig. 10) which can be divided by 0.588 to calculate the diameter. Using ANOVA with species as a random factor on a subset of 57 species that had 2-3 flowers measured, we found that 95% of the variation in hood length and 98% of the variation in gynostegium width was explained by differences between species. Variation explained changed to 97% for hood length and 98% for gynostegium width when we used only the 10 species for which we had three flowers measured.

Pollinators

We obtained pollinator data from ASCLEPOL (Ollerton and Liede 1997), a publicly available list of flower visitors to species in the former Asclepiadaceae family. ASCLEPOL is compiled from publications and personal communications. Visitors are coded to indicate if they were: (1) observed depositing pollinia into a stigmatic opening, (2) carrying pollinia, or (3)

contacting the flower; we considered all three categories to be possible pollinators. There was pollinator data for 27 North American *Asclepias* species plus the four outgroup species. The number of observed pollinators ranged from 1-157 (mean = 25) records per *Asclepias* species. We grouped insect genera and species by order (Hymenoptera, Lepidoptera, Diptera, Coleoptera, and Hemiptera) and calculated the proportion of total visits that were from hymenopterans. We also placed each species into the binary categories of less than 50% or greater than or equal to 50% Hymenoptera to allow comparisons to binary floral traits. Hymenopterans were by far the most common visitors, visiting 26 of the 27 *Asclepias* species and accounting for 57% of the 683 pollinators observed across all 27 species. Pollinator observations on five *Asclepias* species suggest that hymenopteran pollinators tend to fly between flowers (La Rosa pers. obs.). Fishbein and Venable (1996) and Ivey et al. (2003) found that hymenopterans consistently carry and transfer more pollinia than lepidopterans in two species of *Asclepias*.

Phylogeny

We used a maximum likelihood phylogram of 143 taxa made from three non-coding regions of the plastid genome (Fishbein et al. 2011). Branch lengths were proportional to substitutions and the tree was made ultrametric using the R package 'ape' (Paradis et al. 2004; Paradis 2012) in R (R Core Team 2014). We pruned species to produce a tree made of the 111 species (Tree111) for which we had floral trait data, which included four outgroup species from three genera. We pruned this tree down to the 31 species that we also had pollinator data for (Tree31). From Tree111, we pruned the two outgroup species leaving two species within the genus *Gomphocarpus* to produce a tree with 109 species (Tree109). Next we pruned all

outgroups from Tree109 and Tree31, as their clades are underrepresented and they may influence adaptive models of trait evolution; this resulted in trees with 107 species (Tree107) and 27 species (Tree27).

Analyses

Reconstructing the ancestral history allowed us to make predictions of the trait value of the ancestor to the *Asclepias* genus, which establishes the direction of evolution, and what the new possibly adaptive phenotype is. We identified the ancestral form for each discrete trait (hoods open/closed and horns present/absent) using maximum likelihood (ML) (Pagel 1994a) with equal rates of change using 'ape,' which provides likelihoods of the trait character at all nodes across Tree111. For the continuous traits, we also used ML to estimate ancestral states (Felsenstein 1985) using the R package 'phytools' (Revell 2012), which displays trait values as a heat map ranging from small trait values (red) to large trait values (blue) across Tree109.

We assessed the pattern of trait evolution to determine if there was strong phylogenetic signal (evolving through random Brownian motion (BM)) or if traits had likely evolved towards optima. We estimated phylogenetic signal of discrete traits using Purvis and Fritz's D (Fritz and Purvis 2010) using the phylo.d function in the R package 'caper' (Orme et al. 2013). D ranges from zero to one; values near zero indicate a phylogenetic signal. For continuous traits, we used the phylosig function in the R package 'phytools' (Revell 2012) to calculate Pagel's lambda (λ) as an estimate of phylogenetic signal (Pagel 1999). Lambda ranges from zero to one; values near one indicate a phylogenetic a lack of signal.

We tested two predictions of correlated trait evolution. We predicted that horns only have a function when the hoods are open and they can contact the pollinators. Depending on the

ancestral state of hoods and horns, we predicted either a gain in horns would be correlated with a shift to open hoods, or a loss of horns with a shift to closed hoods. We used Pagel's correlation method (Pagel 1994a) in Mesquite (Maddison and Maddison 2011), which tests for correlation between two discrete traits and takes branch lengths into account. Next we tested our prediction of a positive correlation between gynostegium width and hood length, two major size components of the flower. We tested for correlations between phylogenetic independent contrasts (PIC) (Felsenstein 1985) of hood length and gynostegium width using the R package 'ape' (Paradis et al. 2004).

To test for pollinator-mediated selection on the traits, we first tested the association between the discrete values for the shape of the hood opening and two pollinator categories: less than 50% hymenopteran pollinators and greater than or equal to 50% hymenopteran pollinators; a distribution of the proportion of hymenopterans can be seen in Figure 12. We again used Pagel's (1994a) correlation method in Mesquite (Maddison and Maddison 2011) and our 31species tree (Tree31). We also used the subset of 27 *Asclepias* species to test for correlations between the phylogenetically corrected continuous traits hood length and gynostegium width and the proportion of recorded visitors that were hymenopterans using PIC (Felsenstein 1985).

Expanding out to our larger tree and using our full trait data from 107 species, we tested our prediction of selection towards two adaptive peaks using the two hypothesized pollinator categories and each of the continuous traits, hood length and gynostegium width. We conducted these analyses using the R package 'ouch' (Butler and King 2004, King and Butler 2009). OUCH allowed us to compare models including a null Brownian-motion model (representing no adaptive peaks), an Ornstein-Uhlenbeck (OU) model with just a single adaptive peak, an OU

model with two adaptive peaks representing our two pollinator categories with their ancestral nodes having been reconstructed using maximum likelihood, and an OU model with three adaptive peaks representing an unknown ancestral group and the two pollinator groups of the extant species.

We then used a reverse method; rather than testing hypotheses of a predicted number of selective agents each selecting for an optimum phenotype, we used trait values of the 107 species to predict the number of convergent adaptive optima, or peaks—the interpretation being that each adaptive optimum was the result of a similar selective regime. We used the R package 'surface' (Ingram and Mahler 2013) to conduct this analysis. SURFACE is a two-phase analysis that uses stepwise Akaike information criterion (AIC) to find convergent phenotypes. In the forward phase it used gynostegium width and hood length together, accounting for relatedness, to find trait combinations of individuals or clades that were divergent from close relatives. Then in the reverse phase of the analysis it compared the locally divergent species and clades across the phylogeny to find ones that converged on the same phenotype. The outcome was the number of likely convergent adaptive optima of the trait values—each optimum corresponded to a potentially similar selective regime. An advantage to this method is that we can utilize data from the larger phylogeny (Tree107) to identify convergent phenotypes, allowing us to make predictions of selective regimes that can later be tested.

Results

We found that there was variation in horn presence, hood characters, hood length, and gynostegium width across the phylogeny. Of the 107 extant *Asclepias* species we sampled, 85% of them had horns and 70% of them had open hoods (Fig. 13). The base of the *Asclepias* clade

shows a very high likelihood that the ancestral state had open hoods containing horns (solid blue nodes; Fig. 13). Over half of the 107 species (56%) had flared hoods, but the hood shape of the ancestor of *Asclepias* was not clear (Fig 5A); of the subset of 27 species for which we had pollinator data, 63% had flared hoods. Hood length and the estimated gynostegium width were variable and ranged from 0.33mm to 7.35mm and from 0.74mm to 3.14mm, respectively. *Correlated evolution of traits*

The closure of hoods and the loss of horns was highly correlated (P < 0.01), and hood closure likely proceeded horn loss as demonstrated by the shift in likelihood shown by the pie charts at shallower nodes in Figure 13A. Hood openings have closed at least four times, and were often followed by horn loss in at least one species; horns have been independently lost at least six times (Fig. 13A). This has left 16 extant *Asclepias* species without horns and 32 without conspicuous hood openings. Note that there are also some instances of species with open hoods with no horns, and vice versa.

We found that hood length had a weak phylogenetic signal ($\lambda = 0.14$; Table 15), indicating that it has evolved more than we would expect based on its phylogenetic history (Fig. 15). Gynostegium width had a much higher phylogenetic signal ($\lambda = 0.72$; Table 15); its evolution more closely resembled a BM model of evolution (Fig. 16). Despite the difference in phylogenetic signal, gynostegium width and hood length were positively correlated after correcting for phylogenetic non-independence using PIC (r = 0.56, P < 0.001; Fig. 17).

Correlated evolution of traits and environment

Hood shape (flared vs. domed) appeared to be rather labile. Species with flared hoods are scattered across the phylogeny, so it is not surprising that hood opening shape shows very little

phylogenetic signal (D = 0.74, Table 15). This evolutionary lability may be caused by pollinatormediated selection, as species in which the majority of pollinators are hymenopterans were marginally more likely to have hood openings that were flared (Fig. 14B). As this association was only marginal, we also looked for associations between the pollinator categories and our continuous variables to bolster our finding.

By comparing OU models across the 27 *Asclepias* species, we were unable to find additional support for evolution in response to the two pollinator categories above for either of our continuous traits, hood length or gynostegium width (Table 16). Our two-optimum OU model, where hood length evolved towards the two pollinator categories, did not fit significantly better than models of one or three optima or random BM trait evolution (Table 16). The same was true when we compared OU models of gynostegium width evolution. We also found no significant correlation between the evolution of hood length or gynostegium width and the continuous proportion of hymenopterans after correcting for phylogenetic non-independence using PIC (Figs. 18 and 19, respectively) These analyses tested adaptive hypotheses, but could only be conducted on the reduced phylogenetic tree as we only had pollinator data for 27 species. We followed these analyses with tests that could use all 107 species to form additional testable adaptive hypotheses.

Convergent evolution of floral traits

When we modeled hood length and gynostegium width together, we found that they predicted evolution towards multiple adaptive optima. Our results from the forward phase of the analysis in SURFACE found that there were 15 clades or species that had phenotypes that diverged from their closest relatives (Fig. 20A). The reverse phase of the analysis found that

those 15 clades/species clustered into four phenotypic groups based on gynostegium width and hood length together (Fig. 20). Three of these phenotypic groups each consisted of 4-5 convergent clades or individual species (Fig. 20B). The hood length and gynostegium width of each species is plotted in Figure 20C, and the species mostly fell along a size axis. The clusters also mainly separated along a size axis for these two traits, with the gray (non-convergent) clade having the smallest phenotype, the 71 species that were neither convergent nor divergent (black) having larger hoods and gynostegia, and the ten species that make up the blue clades having even larger hoods and gynostegia. The red and green clades diverged from the size axis, with the red clades having exceptionally long hoods relative to their gynostegium width, and those in the green clades having hoods that are shorter relative to the gynostegium; four representative species from the convergent clusters are shown in Figure 20D. Keep in mind that the position of the species in Figure 20C is due solely to their trait values, while the colors of the dots incorporate phylogenetic relatedness.

Unfortunately the pollinator data in ASCLEPOL only contains a few of the species from the convergent clusters, and collection locations and species distributions did not strongly suggest that species from each of the convergent clusters live in divergent habitats. The four species in the red regime live in a wide range of habitats; one species grows in Florida wetlands, two others in mountains in the southwest United States, and the fourth lives in a broad range of habitats that vary in moisture. Species in the green and blue regimes live in deserts in the southwestern US and Mexico.

Discussion

Studies of contemporary selection have found evidence for selection on hood traits and

gynostegium width in several North American *Asclepias* species (Caruso et al. 2005; Chapters 1 and 2) suggesting these traits are currently adaptive. Interspecific patterns of trait evolution that are non-random are suggestive as signatures of selection, keeping in mind that non-random evolution of traits does not necessarily refute the presence of selection. In this present study we found evidence that the hoods in *Asclepias* are likely to be adaptive, because their evolution does not follow their phylogenetic relatedness, they are correlated with the evolution of other traits, and a subset of species have phenotypes that show associations, albeit weakly, with hymenopteran. Our analyses of convergent evolution suggest that the hoods and the gynostegia are evolving in response to a common selective regime and provide new adaptive hypotheses that require further investigation.

Correlated evolution of traits

Hoods have independently evolved a closed phenotype multiple times. Open hoods, the ancestral state, would have been easily accessible by pollinators and non-pollinating insects alike. The vulnerability of losing nectar to thieves, inefficient pollinators, or evaporation may have resulted in selection for the closure of hoods in some environments. Changes in pollinators can cause changes in multiple traits (e.g. Castellanos et al. 2004). The ancestor likely had horns, and with the closure of hoods, horns likely lost their function, removing or relaxing selection (Lahti et al. 2009) to keep them, leading to their loss. Armbruster et al. (2002) found that the evolution of larger flowers in *Collinsia* reduced precision of selfing—an example of a change in a floral trait that corresponded with a loss of function of another trait.

Comparisons of flowers with open hoods and closed hoods will be needed to understand why hood closure evolved. Of the five species with closed hoods and no horns for which we also

have pollinator data, four are associated with prairie or grassland habitat, are light green, and are predominantly visited by bumble bees (*Bombus* spp.). Evaporation may have been a strong selective force, but closing hoods to reduce visits by inefficient pollinators may also be a possible explanation for the phenotype. Determining if other species with closed hoods and no horns are also visited primarily by bumble bees, if they generally have fewer pollinator taxa than species with open hoods, and if they have lower nectar production could explain some of the consequences of hood closure and horn loss, and shed light on the selective regime that lead to this current phenotype.

Correlated and convergent evolution of traits in response to their environment

We have some evidence that a pollinator fauna that is more than half hymenopteran corresponds with flared hoods, yet we found no evidence that the two pollinator regimes or the range of proportion of hymenopterans had an effect on hood length or gynostegium width. Whether or not hymenopterans represent the majority of pollinator visitors does not make for distinct selective regimes that select for two phenotypic optima of our other hood and gynostegium traits. This two-optimum scenario is not the best fit to explain the evolution of hood length and gynostegium separately, but in combination, these traits fit a 4-optimum scenario (three convergent optima and one non-convergent optimum) produced by our analysis in SURFACE. Pollinator data on most of the *Asclepias* species is incomplete, and additional observations would improve our ability to test these hypotheses of evolution towards the three adaptive optima. The three convergent clusters are excellent starting points to look for environmental similarities, as they offer three replicate scenarios, involving multiple independent

origins, where we would expect the pollinators and/or habitats to be more similar to each other than to the pollinators and habitats of the other *Asclepias* species.

The convergence of species towards three distinct adaptive optima for hood length and gynostegium width is very suggestive that these traits are evolving in response to selective agents and that they are not evolving randomly. Our use of a large phylogeny that demonstrates independent evolution toward a particular phenotype multiple times strengthens our assertion that these floral traits are adaptive (Doughty 1996).

Studies have used comparative methods utilizing floral traits as a way to understand shifts in pollinators (e.g. Perez et al. 2006); however, our focus is on understanding the reasons for shifts in the floral traits (e.g. Friedman and Barrett 2008), which we predict is mostly in response to pollinators, and is more indicative of the direction that selection occurs. Our study has the advantage of having trait data for nearly all of the North American *Asclepias* species, a rather large clade of 125 species, yet we still lack sufficient pollinator data for most of the species. A good starting point for future data collection will be the 25 species in the three convergent clusters. Much like the significance of wings in birds, insects, and mammals, there is power in the repeated independent evolution of a phenotype to support adaptive hypotheses. In our phylogeny of over one hundred *Asclepias* species, the three repeated phenotypes, each with four or more independent origins, likely evolved in response to common selective regimes. The next step of acquiring data on the possible selective agents, including the pollinators that visit these species and their abiotic environment, will be important to confirm these adaptive hypotheses.

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Table 23. Phylogenetic signal of continuous and discrete traits. Phylogenetic signal for continuous traits is measured by λ ; values near 0 have no phylogenetic signal are may have evolved in response to selection and values near 1 have a phylogenetic signal representative of Brownian-motion. For discrete traits the scale is reversed; when D is near 0 there is high BM phylogenetic signal and when D is 1 there is no phylogenetic signal. Sigma squared indicates the rate of evolution.

Continuous/Discrete	Trait	λ	logL	σ^2	D	H ₀ : $D = 0$	H ₀ : $D = 1$
Continuous	Gynostegium width	0.72	-64.9	604			
Continuous	Hood length	0.14	-170.3	490			
Discrete	Horn presence				0.71	P = 0.025	P = 0.069
Discrete	Hood open				0.55	P = 0.035	P = 0.012
Discrete	Hood opening shape				0.74	P = 0.004	P = 0.067

Table 24. Model comparisons of trait evolution towards different adaptive optima using AIC.c (a size corrected AIC for small sample sizes). Brownian motion (BM) models a random walk with no adaptive optima; OU1 models a single optimum; OUanc models two adaptive optima (majority and minority Hymenopteran) with ancestral state reconstruction; OU3 models three optima (majority and minority Hymenopteran, and a third unknown ancestral optimum).

Model/Trait	BM	OU1	OUanc	OU3	
Hood length	109.8	106.0	107.7	110.2	
Gynostegium width	41.1	42.7	45.1	43.7	



Figure 10. A photograph of an Asclepias exaltata flower from the side and top.

Asclepias floral traits include five petals (P), a gynostegium (G), five hoods (Hd), and the hoods of some species each contain a horn (Hn). Pollinia are housed within the wall of the gynostegium and pairs are connected by a dark clip-like corpusculum (C) that is located at the top of the stigmatic slit (S) where the pollinia are deposited for fertilization. Our measured traits were hood length (h) and gynostegium width estimated from the distance between adjacent corpuscula (g).



Figure 11. Discrete binary floral traits: (a) horns present, (b) horns absent, (c) hoods conspicuously open, (d) hoods closed, (e) hood rim flared, (f) hood rim domed.



Figure 12. Distribution of the proportion of hymenopteran out of the total number of visitors across 27 *Asclepias* species.



Figure 13. Maximum likelihood ancestral state reconstruction of character traits: hood presence/absence (a) and conspicuous hood opening (b) mapped onto a mirrored phylogram of 107 *Asclepias* species. The black circles represent the ancestral node to *Asclepias*. Boxes represent the character state of each species and the pie charts represent the maximum likelihood character reconstruction at each node. There was a significant correlation between these two traits (p < 0.01) using Pagel's correlation method.

(a)





Hood flaredHood domed

Figure 14. Correlated evolution between hood shape and hymenopteran pollinators. (a) A phylogram of the maximum likelihood ancestral state reconstruction of hood opening shape. (b) A phylogram of 31 species with hood opening shape (domed or flared; green and blue) and pollinator composition ($< \text{ or } \ge 50\%$) hymenopteran; light green and light blue, respectively) mapped to the tips. Boxes represent the character state of each species and the pie charts represent the maximum likelihood character reconstruction at each node There was a marginally significant association between hood opening shape and pollinator composition (P = 0.087).







Figure 15. Evolution of hood length. Phylogram using a continuous color gradient to show maximum likelihood ancestral trait reconstruction for hood length (see inset). The black circle represents the ancestral node to *Asclepias*. The rate of evolution (σ 2) is 490.5 and the phylogenetic signal (λ) is 0.14.



Figure 16. Evolution of gynostegium width. Phylogram using a continuous color gradient to show maximum likelihood ancestral trait reconstruction for gynostegium width (estimated by the distance between adjacent corpuscula; see inset). The black circle represents the ancestral node to *Asclepias*. The rate of evolution (σ 2) of *Asclepias* species is 604.3 and the phylogenetic signal (λ) is 0.72.



Figure 17. Correlation between phylogenetic independent contrasts for gynostegium width and hood length.

(a)



Figure 18. Test of correlated evolution of hood length and the proportion of pollinators that are hymenopterans. (a) Predicted evolution of hood length (right) and corresponding changes in proportion hymenopteran (left) visualized using a continuous color gradient to show maximum likelihood ancestral reconstruction. (b) Correlatin between phylogenetic independent contrasts for hood length and percent hymenopteran (r = 0.06, P = 0.77).



(a) Afascicularis Averticillata subverticillata Apulchra Aincarnata Acurassavica Aparishii Acryptoceras Ahypoleuca Aerosa Atomentosa Aviridiflora Ahirtella Asyriaca Ainterior2816 Aexaltata Aquadrifolia Ahallii Aspeciosa Alongifolia Aamplexicaulis Apurpurascens Aviridis Asullivantii Alatifolia Asolanoana Asubulata 0.783 trait value 3.142 0 trait value Proportion Hymenopteran Est. Gynostegium Width

Figure 19. Test of correlated evolution of the estimated gynostegium width and the proportion of pollinators that are hymenopterans. (a) Predicted evolution of gynostegium width (right) and corresponding changes in proportion hymenopteran (left) visualized using a continuous color gradient to show maximum likelihood ancestral reconstruction. (b) Correlation between phylogenetic independent contrasts for gynostegium width and percent hymenopteran (r = 0.22, P = 0.29).



(a)

Figure 20. Results of a **SURFACE** analysis of gynostegium and hood evolution supporting four adaptive optima of these traits in the North American Asclepias. (a) Phylogenetic tree with divergent clades and species in gray. (b) Phylogenetic tree with convergent (colored) and non-convergent (grey) clades painted onto the branches; Black clades were not placed in any phenotypic optima. Numbers in circles represent transitions to the phenotypic optima. (c) Trait values for each species (small circles) and estimated optima (large circles) calculated as the phenotypic mean of the species within each regime, with regime colors matching those in the tree. (d) Photos of representative species of each regime. Colored frames on each box match the regime color; gray is on the left, blue in the center, green on the right, and red on top.





Figure 20 (cont'd)



Est. Gynostegium Width (mm)
Figure 20 (cont'd)









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