Reproductive ecology of a parasitic plant differs by host species: vector interactions and the maintenance of host races

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ABSTRACT

Parasitic plants often attack multiple host species with unique defenses, physiology, and ecology**.** Reproductive phenology and vectors of parasitic plant genes (pollinators and dispersers) can contribute to or erode reproductive isolation of populations infecting different host species. We asked whether desert mistletoe, *Phoradendron californicum* (Santalaceae tribe Visceae syn. Viscaceae), differs ecologically across its dominant leguminous hosts in ways affecting reproductive isolation. Parasite flowering phenology on one host species (velvet mesquite, *Prosopis velutina*) differed significantly from that on four others, and phenology was not predicted by host species phenology or host individual. Comparing mistletoe populations on mesquite and another common host species (catclaw acacia, *Senegalia greggii*) for which genetically distinct host races are known, we tested for differences in interactions with vectors by quantifying pollinator visitation, reward production, pollen receipt, and fruit consumption. Mistletoes on mesquite produced more pollinator rewards per flower (1.86 times the nectar and 1.92 times the pollen) and received ~ 2 more pollen grains per flower than those on acacia. Mistletoes on the two host species interacted with distinct but overlapping pollinator communities, and pollinator taxa differed in visitation according to host species. Yet, mistletoes of neither host showed uniformly greater reproductive success. Fruit set (0.70) did not differ by host, and the rates of fruit ripening and removal differed in contrasting ways. Altogether, we estimate strong but asymmetric pre-zygotic isolating barriers between mistletoes on the two hosts. These host-associated differences in reproduction have implications for interactions with mutualist vectors and population genetic structure.

Keywords: phenology, pollination, mistletoe, reproductive isolation, seed dispersal

INTRODUCTION

Parasitism is among the most successful modes of life. Transitions to parasitism are associated with increases in diversification rates (Wiens et al. 2015), and parasitism dominates interspecific links in food webs (Lafferty et al. 2006). Much of the diversity of parasites is thought to arise due to reproductive isolation following colonization of new host species (De Vienne et al. 2013). This reproductive isolation may occur as a consequence either of allopatry (the host species are allopatric or dispersal among hosts is not possible) or of traits that reduce the likelihood of gene flow among populations infecting different host species (Le Gac and Giraud 2004; Hopkins 2013). As even closely related host species may differ in defenses and ecology, parasite traits under positive selection on one host may be neutral or negative on another. Therefore, mating with an individual adapted to a different host species, or dispersing one's offspring to a different host species, can be costly. When selection reinforces traits that increase reproductive isolation, genetically differentiated host races will undergo reduction in gene flow, thereby increasing the likelihood of speciation (Drès and Mallet 2002).

Comprising over 4,400 species in c. 270 genera from more than 20 families, parasitic plants are a diverse but poorly studied group of angiosperms (Nickrent et al. 1998). Host affiliations vary widely, from extreme specialization [e.g., holoparasitic *Epifagus virginiana* (Orobanchaceae) almost exclusively parasitizes *Fagus grandifolia* (Li et al. 2010) and hemiparasitic mistletoe *Peraxilla colensoi* (Loranthaceae) almost exclusively parasitizes *Nothofagus menziesii* (Norton and De Lange 1999)] to extreme generalization [e.g., holoparasitic *Rhinanthus minor* (Orobanchaceae) parasitizes over 50 species from 18 families (Gibson and Watkinson 1989) and hemiparasitic *Ileostylus micranthus* (Loranthaceae) parasitizes over 200 species from 51 families (Norton and De Lange 1999)]. However, many parasitic species likely

46 contain vast cryptic diversity. Indeed, recent population genetic studies have uncovered evidence
47 of host-associated genetic differentiation at small geographic scales in both holoparasitic (De
48 Vega et al. 2008; Thurgood et al. 2008) and hemiparasitic (Jerome and Ford 2002; Zuber and
49 Widmer 2009; Yule et al. 2016) plant species. The mechanisms responsible for generating this
50 differentiation in parasitic plants are not well understood relative to those in other parasitic taxa.
51 Mistletoes, aerial hemiparasites from five families within the order Santalales, are among
52 the most diverse parasitic plants, comprising over 1500 species (Norton and Carpenter 1998,
53 Nickrent 2011). Mistletoe radiations have occurred in the Loranthaceae (c. 900 species), as well
54 as within *Phoradendron* (c. 230 species) and *Viscum* (c. 100 species) in the Santalaceae tribe
55 Visceae (syn. Viscaceae) (Nickrent 2002). Unlike mistletoes in the less speciose groups, species
56 in these three groups primarily rely on animal vectors for both pollination and dispersal
57 (Restrepo et al. 2002, Aukema 2003, Kahle-Zuber 2008). These mutualistic vectors will partially
58 control the extent of gene flow among parasite populations infecting different host species,
59 potentially impeding adaptation to any given host. Pollinators determine which individuals
60 exchange genetic material, and seed dispersers determine where offspring have the opportunity
61 to establish. For many better studied, directly transmitted parasites, divergence is facilitated by
62 mate choice, phenology, or sensitivity to host cues (Linn et al. 2003; Ferrari et al. 2006; Mattsson
63 et al. 2015). However, for vector transmitted parasites, vector feeding preferences can be the
64 dominant factor influencing host infections (Altizer et al. 1998; Simpson et al. 2012). Vector
65 transmitted parasitic plants can maintain host fidelity only indirectly, via traits that increase the
66 specialization or constancy of vectors to parasites growing on different host species.
67 Host associated divergence in reproductive phenology can be a powerful mechanism that
68 facilitates genetic differentiation. Many animal parasites are known to synchronize their

69 phenology with the physiological processes of the hosts. For example, the need for synchrony of
70 apple maggot fly (*Rhagoletis pomonella*) development to host fruit maturation creates strong
71 fitness tradeoffs across sympatric hosts with different phenologies (Feder and Filchak 1999). The
72 fitness of both ectoparasitic (van Dongen et al. 1997) and endoparasitic (Komatsu and Akimoto
73 1995) phytophagous insects can even be tightly linked to the phenology of host individuals. In
74 these and other systems, hosts constrain the possible parasite phenologies so that synchrony with
75 hosts can provide an automatic prezygotic isolating barrier when hosts have different
76 phenologies (Calero-Torralbo and Valera 2008; Kiss et al. 2011). The relationship between host
77 and parasite phenologies is not well understood for parasitic plants, however. While some
78 parasitic plants, such as those that parasitize annuals, may experience strong host-driven
79 constraints (Marquardt and Pennings 2010), others may be more labile in their phenology. For
80 parasitic plants that rely on vectors, phenology may also be constrained by mutualist activity or
81 competition with hosts for mutualist services.

82 Here we study the reproductive ecology of a biotically vectored parasitic plant in order to
83 characterize traits that will contribute to or erode reproductive isolation among populations
84 associated with different host species. Desert mistletoe (*Phoradendron californicum*) is a
85 dioecious hemiparasite that requires both pollinators and dispersers to reproduce and infect a
86 host. First, we examine whether the reproductive phenology of this mistletoe differs according to
87 host species, and if the phenology of host species themselves or host individual identity can
88 predict those differences. To answer this question, we describe the phenological distributions of
89 five host species and the desert mistletoes that infect them. Second, we ask whether desert
90 mistletoe's interactions with pollinators differ according to host species. For mistletoes of two
91 host species known to form distinct host races (Yule et al. 2016), we characterize pollen and

nectar production, as well as the pollinator community composition and degree to which pollinators specialize on a given host race. We use our phenological and pollinator data to estimate the strength of these pre-zygotic isolating barriers between the host races. Finally, we test whether differences in reproductive ecology between the host races influence mistletoe fitness by comparing components of female reproductive success (pollen receipt, fruit set, fruit ripening and fruit removal).

METHODS

Study system

Phoradendron californicum (subsequently termed "desert mistletoe") is a dioecious, xylem hemiparasite primarily of leguminous trees and shrubs in the lowland Sonoran and Mojave deserts of the southwestern US and northern Mexico. Mistletoes in the Santalaceae tribe Visceae (syn. Viscaceae) are unusual among biotically pollinated and dispersed plants and differ from the majority of mistletoes in the Loranthaceae (Ladley et al. 1997), in that they typically have more specialized seed dispersal than pollination. Desert mistletoe relies on generalist insect pollinators to vector pollen from male to female plants and a specialized bird, *Phainopepla nitens*, to vector its seeds to suitable establishment sites (Aukema 2003). Success depends on whether desert mistletoe is able to recruit onto the tree branch on which it is deposited and whether a nearby individual of the opposite sex is present, as females cannot produce seeds apomictically. As the sexes appear to accumulate at random on the hosts, large populations within hosts have roughly equal numbers of males and females, but smaller populations have variable sex ratios (Yule and Bronstein, *in revision*). Genetically differentiated host races of desert mistletoe are known to occur across small geographic scales on *Senegalia greggii* (catclaw acacia, hereafter "acacia") and *Prosopis velutina* (velvet mesquite, hereafter "mesquite") (Yule

et al. 2016) or *P. glandulosa* (Glazner et al. 1988). While dispersal can be the primary factor determining parasitic plant distribution across hosts in some systems (e.g. Roxburgh and Nicolson 2005), preferential dispersal to the host species of origin by the phainopepla does not appear to be a mechanism reducing gene flow between desert mistletoe host races. In fact, in one study, seed rain was most prevalent on a potential host species that was not infected at the site (Aukema 2002). Therefore, host compatibility at establishment is likely a stronger post-zygotic barrier contributing to host race maintenance, as evidenced by reciprocal transplant experiments showing local adaptation in the ability to establish on their host species of origin (Larson 1991, Overton 1997). The mechanisms underlying host compatibility in this mistletoe are not clear. *Phoradendron* spp. can have germination rates approaching 100% regardless of host and high mortality rates at establishment of the holdfast. For some species, this mortality is especially high on non-source host species, potentially due to differences in host branch growth rates and defenses (Clay et al. 1985, Larson 1991, Lichter and Berry 1991, Overton 1997). However, desert mistletoe dispersal between hosts and subsequent seedling establishment does happen about 5% of the time (Yule et al. 2016). The observation that flowering phenology is delayed on desert mistletoes growing on mesquite relative to those on acacia suggests that flowering phenology may also contribute to differentiation within this species through pre-zygotic mechanisms (Overton 1997; Yule et al. 2016). However, the consistency of host-associated phenological differences across time, space, and a broader range of hosts is not known for this long-lived plant.

Mistletoes flower in winter and spring, with each adult plant producing thousands of flowers. Each flower is about 1 mm in diameter and produces a minute amount of viscous nectar that coats the inside of the flower. Early in desert mistletoe's flowering phenology few other

pollen and nectar sources are available, and the flowers attract a diverse community of generalist small bees and flies (Wiesenborn 2016). However, it is not known whether desert mistletoes differ in their rewards to or visitation by pollinators in relation to their host species. Unripe fruits remain unripe through spring and summer. They mature the following October through March, overlapping with the next year's flowers. Infructescences contain 2-30 ripe fruits, each 0.5 cm in diameter, translucent, white to red in color, and single seeded.

Our research took place in semidesert grassland scrub communities below 1300 m elevation (Burgess 1995) at the Santa Rita Experimental Range (SR, about 65 km south of Tucson, AZ, USA), Tumamoc Hill (TH, within Tucson), Ironwood Forest National Monument (IF, about 55 km north of Tucson), and Catalina Regional Park (CRP, about 40 km northwest of Tucson). While host abundances and infection prevalence vary among the sites used for measuring phenology, mesquite is the dominant host at SR where the pollination portion of this study was conducted (Aukema 2004). Infection prevalence is highly structured spatially, ranging from 0 to 75% on mesquites within SR with an average intensity ranging from 0 to 10.5, likely due to the territoriality of the dispersers (Aukema 2004). In addition, prevalence varies by host species and is not predicted by seed rain or host density within a site (e.g. Aukema 2002 at IF).

Reproductive phenology of hosts and parasitic plants

Is parasite phenology related to host species identity? To determine whether desert mistletoe phenologies differ depending on the host species on which they grow, we recorded flowering and fruiting phenologies during censuses of tagged individuals from 2013-2015. We conducted weekly censuses from January 2013 to May 2013 at SR (n=10 on mesquite; n=10 on acacia) and TH (n=14 on mesquite; n=10 on acacia*)*. We conducted biweekly flowering and fruiting censuses from September 2013 to May 2014 at SR (n=30 on *Parkinsonia florida*, blue palo

161 verde; n=76 on mesquite; n=60 on acacia) and at IF (n=57 on *Olneya tesota*, desert ironwood;
162 n=29 on *Parkinsonia microphylla,* foothills palo verde). We conducted biweekly flowering
163 censuses January 2015 to May 2015 at SR (n=76 on mesquite; n=60 on acacia), TH (n=46 on
164 mesquite; n=43 on acacia), and CRP (n=62 on mesquite; n=57 on acacia). The host species
165 surveyed represent the most common hosts of desert mistletoe at those sites (K. Yule, unpubl.
166 data), and generally are the dominant large shrubs or trees at the site.
167 *Is parasite phenology explained by host individual?* To test the repeatability of the flowering
168 phenology of individual desert mistletoes and the effect of host individual on flowering
169 phenology, we recorded estimated first dates of flowering for tagged desert mistletoes on acacia
170 and mesquite at SR for both 2014 and 2015 (n above). We tagged up to three desert mistletoe
171 individuals / host individual.
172 *Is parasite phenology related to host species phenology?* To test whether differences in desert
173 mistletoe phenologies are consistent with differences in their hosts' phenologies, we examined
174 the flowering, fruiting, and leafing phenologies of the five desert mistletoe host species above in
175 California and Arizona from 01-Jan-2008 to 01-Jan-2015. Data were provided by the USA
176 National Phenology Network and the many participants who contribute to its *Nature's Notebook*
177 program (USA National Phenology Network 2015).
178 **Parasite interactions with pollinators**
179 *Does production of rewards for pollinators differ by host species?* To test host-associated
180 differences in the production of pollinator rewards, we quantified nectar and pollen production
181 by desert mistletoes infecting mesquite and acacia. We focus on reward production per flower
182 here, but note that desert mistletoes on mesquite are also larger and produce larger, more densely
183 packed inflorescences than desert mistletoes on acacia (detailed methods and results, Online

Resource S1; Table 1). We collected open flowers from male and female desert mistletoe individuals infecting sympatric individuals of mesquite and acacia at weekly intervals from January to May 2016 at SR. Flowers were collected from five plants of each sex growing on at least three different host individuals each week. To release the nectar, we submerged up to 20 flowers / individual in distilled water for 20 min. We measured the concentration of sugars in a sample of this solution in Brix (g Sucrose equivalent / 100 g solution) using a refractometer and converted it to the estimated μg of sugar / flower. To measure pollen production, we placed five flowers from each male plant individually in 70% EtOH to release mature pollen grains, then estimated pollen production / flower with two repeat counts using a hemocytometer.

Does visitation by pollinators differ by host species? We conducted surveys of insect visitors to individual flowering desert mistletoe plants in 2015 at SR across 19 mesquite and 11 acacia hosts growing in sympatry. We made 20 min focal plant observations on 27 (10 female and 17 male) desert mistletoes on acacia across three dates (24-Jan-2015, 07-Feb-2015 and 14-Feb-2015) and 39 (17 female and 19 male) desert mistletoes on mesquite across four dates (01-Mar-2015, 08-Mar-2015, 14-Mar-2015, and 22-Mar-2015). We chose these dates to capture the peak flowering season of each host associated desert mistletoe population. We observed a single plant across multiple survey dates when flowering duration permitted, for a total of 108 20-min observations. We recorded the time spent foraging on desert mistletoe inflorescences and the taxon (order or lower taxonomic group for commonly recorded morphospecies) for each visitor. We recorded only insect taxa documented to carry desert mistletoe pollen grains (K. Yule, unpubl. data; Wiesenborn 2016); hereafter, they are referred to as pollinators. For measures of pollinator community composition, we considered all of the insects recorded in a 20 min observation a community, giving 90 total communities with at least one pollinator.

Strength of reproductive isolating barriers

What is the strength of reproductive isolation due to differences in flowering phenology and pollinator communities? We estimate the strength of reproductive isolation due to pre-zygotic barriers between mistletoes on acacia and mesquite caused by host-associated differences in flowering time and pollinator community following the methods of Sobel and Chen (2014). We outline the methods and assumptions for these calculations in Online Resource S5.

Reproductive success of parasites

Does pollen receipt differ by host species? To see whether pollen receipt differed between desert mistletoes on different host species, we collected open flowers from female desert mistletoe individuals infecting sympatric mesquite and acacia at weekly intervals from February to May 2016 at SR. Each week, we collected open flowers from five plants growing on at least three host individuals. We swabbed the stigmas of ten flowers / plant with fuchsin jelly to remove deposited pollen and counted stained pollen grains using light microscopy at 100x.

Does fruit set differ by host species? We censused reproductive success of female desert mistletoes infecting mesquite and acacia hosts at CRP (acacia: n=20; mesquite: n=20), SR (acacia: n=20; mesquite: n=20), and TH (acacia: n=11; mesquite: n=16) in December 2014, February 2015, and April 2015. The first census occurred before flowering of desert mistletoe commenced, the second around the peak of desert mistletoe flowering on acacia, and the third after flowering was finished on acacia and around the peak of flowering on mesquite. At two surveys / census, we assessed the state of fruits on an approximately 10 cm long marked section of branch / plant. When a consumer removes a fruit, the crater remains visible until the infructescence abscises, which occurs either when all fruits have been removed or the season has ended. Flowers that do not produce fruit also remain visible, as the flowers' perianths remain on

the infructescences. At the first survey, we counted the number of ripe, unripe and removed fruits. One to two weeks later, we collected the branch segment and counted ripe, unripe, removed, and failed fruits. We define relative fruit set as the proportion of flowers produced in 2014 (January-May) converted to fruit during the 2014-2015 censuses. We determined relative fruit set by counting the proportion of failed fruits (cavities containing only perianths) in the total possible fruits.

Does the rate of fruit ripening or removal differ by host species? We estimated fruit ripening rate from the above census data as the proportion of unripe fruit ripening between the two surveys divided by the duration of the census (days). We estimated fruit removal rate as the proportion of ripe fruits removed between the two surveys divided by census duration (days). This fruit removal rate provides an upper bound for the rate of dispersal and eventual establishment.

Statistical methodology

We described host and parasite phenological distributions with generalized epsilon-skew normal curves parameterized using maximum likelihood estimation, as discussed by Clark and Thompson (2011). We present the detailed methods and results of these models in Online Resource S2. We compared models of 2015 first flowering date using linear mixed models with the fixed effect of 2014 first flowering date both with and without host individual as a random effect. We modeled box-cox transformed pollen (grains) and nectar (μg) production with the fixed effects of host species, date, and sex (for nectar only). For pollinator visitation data, we tested whether the composition of these communities varied with host individual, host species, desert mistletoe sex, and observation date using distance-based redundancy analysis, a constrained ordination method modeling Bray-Curtis dissimilarities. We tested whether floral visitors more commonly visited desert mistletoes of a certain sex or host species using two-tailed

253 Fisher's exact tests of visit number. We modeled pollen receipt (grains) to female flowers with a
254 zero-inflated negative binomial regression with the fixed effects of host species and date. We
255 modeled fruit set as a binomial response variable with the fixed effect of host species. We
256 analyzed logit-transformed fruit ripening and removal rates with the fixed effects of host species
257 and census. We report details of model selection procedures, statistical packages used, and
258 random effects in Online Resource S3.

259 **RESULTS**

260 **Reproductive phenology of hosts and parasitic plants**

261 *Is parasite phenology related to host species identity?* Host species was the most important
262 factor influencing the flowering time of desert mistletoes. The estimated peak flowering day was
263 much later for desert mistletoe populations on mesquite (Julian day 82.5) than for populations on
264 other hosts (day 42.4) (Online Resource S2, Fig. S1; Fig. 1). Differences in the peak flowering
265 days of sympatric mesquite and acacia desert mistletoe populations ranged from 23.9 to 60.0
266 days (μ=37.8). In contrast, desert mistletoe fruiting phenology did not differ by host species
267 (Online Resource S2, Fig. S2). The best fitting model of flowering separately estimated
268 generalized epsilon skew normal parameters for each unique host species x year x site population
269 (Online Resources S2, Table S; Online Resource S3, Table S1), while fruiting phenology was
270 best fit separately only by site (Online Resource S3, Table S2).

271 *Is parasite phenology explained by host individual?* The timing of first flowering for desert
272 mistletoes was not significantly affected by host individual identity. Host individual identity did
273 not significantly affect the timing of first flowering date between years (Online Resource S3,
274 Table S3). However, first flowering date was consistent for desert mistletoe individuals between

years (acacia: R^2=0.27, slope= 0.59±0.14, t=4.22, p<0.001; mesquite: R^2=0.43, slope= 0.63±0.09, t=7.09, p<0.001) (Online Resource S2, Fig. S3).

Is parasite phenology related to host species phenology? Host species flowered later in the season than, but not in the same order as, their desert mistletoe parasites (Fig. 1). While desert mistletoe growing on mesquite flowered the latest in the year, mesquite peaked in flowering about 2.5 months before acacia. Host fruiting phenology was variable, but generally peaked around 100 days after the peak in host flowering (Online Resource S2, Fig. S4). These drought deciduous host species lost leaves in winter, primarily from mid-February to April, and varied in the degree to which individuals dropped leaves synchronously (Online Resource S2, Fig. S4).

Parasite interactions with pollinators

Does production of rewards for pollinators differ by host species? Desert mistletoe flowers each contained 0.004-0.368 μg sucrose equivalents in their nectar at the time of census. Nectar sugar production / flower for desert mistletoes on mesquite was 1.86 times that for those on acacia (Table 1; Fig. 2a). Male desert mistletoes produced more 1.41 times the nectar sugar / flower produced by females ($F_{1,144}$=9.96, p=0.002; Table 1). Nectar sugar production / flower increased with day of the year (slope: 0.002 ±0.0045 μg/day, $F_{1,144}$=12.64, p<0.001). Variation in nectar sugar production / flower was best explained by host species, desert mistletoe sex, date, and the interaction between sex and date (R^2 =0.34; Online Resource S3, Table S4).

Male flowers had produced 0 - >30000 pollen grains each by the time of census. Also more numerous and densely packed than those on desert mistletoe infecting acacia (Online Resource S1; Table 1), flowers of desert mistletoes on mesquite produced 1.92 times the pollen grains than did those on acacia (Table 1). Pollen production / flower on both host species decreased at later dates (slope=-0.15±0.11 grains / day, $F_{1,783.5}$=54.92, p<0.001) (Fig. 2b). The

best model to explain variation in pollen production / flower included the fixed effects of host species and date (R^2=0.22; Online Resource S3, Table S5).

Does visitation by pollinators differ by host species? Within 20 min focal observation periods, desert mistletoe individuals received 0-48 insect visits. A total of 839 insect individuals were observed. The most common pollinators were syrphid flies (n=308), *Lasioglossum* spp. bees (n=157), small flies (<2 mm in length, n=155), honeybees (*Apis mellifera* (n=132)), and non-syrphid Brachycera flies (n=48). Rare visitors (<5 visits / taxon) included lepidopterans, andrenid bees, vespid wasps, curculionid beetles, and crane flies.

The community of pollinators visiting desert mistletoe was strongly influenced by the timing of flowering and host species (Online Resource S4, Table S1). However, these two factors cannot be completely disentangled, as desert mistletoes on each host species were observed only during their respective periods of peak flowering, which are non-overlapping. The best model to explain variation in pollinator composition included the effects of host species, desert mistletoe sex, observation date, and host individual (Online Resource S3, Table S6). In general, desert mistletoe on acacia were visited more by syrphid flies, while those on mesquite were visited by more *Lasioglossum* bees, honeybees, and non-syrphid brachyceran flies (Table 2, Fig. 3). Additionally, within mistletoes of both host species, *Lasioglossum* spp. were more associated with later survey dates and syrphids were more associated with earlier survey dates (Fig. 3). Honeybees, syrphids, and *Lasioglossum* bees more commonly visited male desert mistletoes than females (Table 2). Males, which produced more nectar sugar (see above), generally received pollinator visits that were longer in duration (Online Resource S4, Fig. S1a). *Lasioglossum* spp. visited plants for longer periods than other taxa, while non-syrphid

320 brachyceran flies visited plants for shorter periods than other taxa (Online Resource S4, Fig.
321 S1b).

322 **Strength of reproductive isolating barriers**

323 *What is the strength of reproductive isolation due to differences in flowering phenology and*
324 *pollinator communities?* The overlap in male and female flowering phenology of desert
325 mistletoes on each host species (Online Resource S5, Table S1) translate to a strength of
326 reproductive isolation (sensu Sobel and Chen 2014) from 0.15 to 0.72 for mistletoes on acacia
327 and 0.76 to 1.0 for mistletoes on mesquite (Online Resource S5, Table S4). The mean similarity
328 in pollinator community composition for desert mistletoe females and males on each host species
329 (Online Resource S5, Table S2) corresponding to a strength of reproductive isolation of -0.02 for
330 mistletoes on acacia and 0.25 for mistletoes on mesquite (Online Resource S5, Table S4).
331 Overall, under the assumptions of our calculations, the strength of reproductive isolation is
332 asymmetric between the host races, with gene flow from the mesquite-associated host race to the
333 acacia-associated host race predicted to be higher than that in the reverse direction.

334 **Reproductive success of parasite**

335 *Does pollen receipt differ by host species?* Female flowers had received 0-387 desert mistletoe
336 pollen grains each at the time of census. Pollen receipt did not differ by host species overall (on
337 acacia: 2.49±0.32 grains; on mesquite: 4.45±0.62 grains; z-value=-0.14, p=0.89) (Fig. 4a).
338 However, pollen receipt decreased throughout the season for desert mistletoe flowers on both
339 host species (z-value=-3.61 $p<0.001$), and this decrease was significantly less pronounced for
340 desert mistletoe flowers on mesquite (-0.04±0.03 grains /day) than for those on acacia (-
341 0.10±0.02 grains /day; z-value=2.03, p=0.042). The model including the fixed effects of host

342 species, date, and the interaction between host and date best explained variation in pollen receipt
343 / flower (Online Resource S3, Table S8).
344 *Does fruit set differ by host species?* Relative fruit set varied from 0.047-1.0 / plant. Host species
345 did not affect fruit set (on acacia: 0.70±0.02; on mesquite: 0.70±0.02; $z=0.748$, $p=0.454$) (Fig.
346 4c), and the best model of variation in fruit set includes only random effects (Online Resource
347 S3, Table S9).
348 *Does the rate of fruit ripening or removal differ by host species?* The proportion of fruit ripening
349 / day on desert mistletoe plants varied from 0-0.143. Fruit ripening was 1.33 times as fast for
350 desert mistletoe on acacia (0.044 ± 0.004 / day) as for those on mesquite (0.033±0.003 / day;
351 $F_{1,202}=4.51$, $p=0.034$) (Fig. 4c). Variation in ripening rate was best explained by only the fixed
352 effect of host species ($R^2= 0.02$; Online Resource S3, Table S10).
353 The proportion of ripe fruit removed from female desert mistletoes varied from 0-0.167 /
354 day. Fruit removal rate on desert mistletoe on mesquite (0. 041 ±-0.003 / day) was 1.71 times
355 greater than for those on acacia (0. 024± 0.003 / day; $F_{1,246}=13.40$, $p<0.001$). The host species
356 effect was driven primarily by differences in removal rate during the first census (before
357 flowering commenced) (Fig 4d). The best model to describe variation in fruit removal rate
358 included the fixed effects of host species, census, and their interaction ($R^2= 0.09$; Online
359 Resource S3, Table S11).
360 **DISCUSSION**
361 In this study, we describe the reproductive ecology of parasitic plants infecting different
362 host species, and ask whether host-associated differences can contribute to or erode isolation of
363 genetically distinct host races. We found that desert mistletoes infecting one host species,
364 mesquite, flowered substantially later in the season than mistletoes on four other host species,

365 whereas mistletoe fruiting phenology did not differ by host species. Host species phenology was
366 not consistent with the phenology of the parasites, and host individual did not influence
367 flowering of the parasite. Compared to desert mistletoes on acacia, desert mistletoes on mesquite
368 were larger in size with larger, more densely packed flowers that produced more nectar and
369 pollen. While the observation date was strongly correlated to the pollinator community
370 composition, some pollinator taxa showed biases towards particular host races regardless of date.
371 We found that differences in both the flowering phenology and pollinator communities related to
372 male and female plants of the two host races provide isolating barriers that would reduce gene
373 flow from acacia-associated mistletoes to those on mesquite more strongly than in the reverse
374 direction. Previously identified host races infecting mesquite and acacia may in part be
375 maintained by a lack of opportunity for cross pollination, despite frequent dispersal between the
376 host species (Yule et al. 2016). Although they differ in reproductive ecology, neither host race
377 showed uniformly greater female reproductive success as neither host race had an advantage
378 across fruit set, fruit ripening rate, and fruit removal rate. Fruit set did not depend on host
379 species; fruit of acacia desert mistletoes ripened faster, but were removed more slowly than those
380 of mesquite desert mistletoes. Below, we discuss our results with respect to phenology,
381 interactions with vectors, and reproductive success and conclude by examining the consequences
382 for the reproductive isolation of desert mistletoe host races.

383 *Host and parasite reproductive phenology*

384 While we found that desert mistletoe flowering phenology is related to host species
385 identity, desert mistletoe and host phenologies do not reflect each other. That is, desert mistletoes
386 do not flower or fruit in the same order as their host species, nor are their phenologies
387 consistently synchronous or asynchronous with their hosts. Additionally, desert mistletoes

388 sharing a single host individual do not initiate flowering at more similar times than do other
389 desert mistletoes of the same host race. Together, these results show that desert mistletoe
390 phenology is not completely constrained by hosts, in contrast to several better known parasitic
391 animal systems involving host associated differentiation (van Dongen et al. 1997; Feder and
392 Filchak 1999; Kiss et al. 2011). While the benefits of disparate phenologies associated with
393 different hosts is clear in many animal parasite systems, the cause of the divergent flowering
394 phenology of desert mistletoes on mesquite is not yet understood. Our results and those of a
395 previous study (Yule et al. 2016) are, however, not consistent with plastic responses to host
396 physiology being primary drivers of this pattern.

397 While analogous to that for animal parasites, the relationships between host and parasitic
398 plant phenologies require additional consideration, especially when vectors, such as pollinators,
399 can interact directly with hosts. For example, overlap in flowering phenology with hosts could
400 have important consequences for pollination, especially if host and parasite share pollinators.
401 Mesquite and acacia can both be pollinated by common visitors to desert mistletoe, such as
402 *Volucella* spp. (Syrphidae), honeybees, and *Lasioglossum* spp. (Keys et al. 1995; Gaddis 2014).
403 Of the five host species we investigated, we found that only late-flowering mesquite desert
404 mistletoes overlap with their hosts in flowering time. Despite the potential benefit of an
405 increased floral display size, synchrony between host and parasitic plant flowering phenologies
406 has not been found to increase visitation and, rather, may increase competition for pollinators
407 (Ollerton et al. 2007; Candia et al. 2014). The relatively large display size and quantity of pollen
408 and nectar produced by desert mistletoes on mesquite may therefore have evolved in response to
409 competition with hosts for visitation. While attention has been placed on the selective pressure
410 that competitors, pollinators, herbivores, and seed predators can have on plant phenologies

411 (Rathcke 1983; Brody 1997), examination of selective forces exerted by host species will be
412 necessary to fully understand the evolution of parasitic plant flowering phenology.

413 In contrast to its flowering phenology, desert mistletoe fruiting phenology does not differ
414 according to host species. For other mistletoe species that share dispersers with their host
415 species, synchrony between host and parasite fruiting phenology can increase seed dispersal rates
416 for both species (van Ommeren and Whitham 2002; Candia et al. 2014). Host fruiting phenology
417 and attractiveness to dispersers can also have a large effect on mistletoe prevalence independent
418 of host-parasite compatibility (Caraballo-Ortiz et al. 2017). It should be noted, however, that
419 disperser activity need not be correlated with host or mistletoe fruiting depending on the
420 specifics of dispersers' diets (see Ladley and Kelly 1996 for activity related to host flowers and
421 honeydew producing insects). While many generalist bird and mammal species, such as
422 mockingbirds, western and mountain bluebirds, and Gila woodpeckers, do consume desert
423 mistletoe fruits, the specialist phainopepla disperses an order of magnitude more seeds per
424 female desert mistletoe than the next most common disperser (Larson 1996). Additionally, the
425 phainopepla harbors unique digestive adaptations that allow them to remove the exocarp of the
426 fruit while leaving the seed and much of the sticky viscin intact without grinding in the gizzard
427 (Walsberg 1975). Phainopepla population sizes are tightly correlated with desert mistletoe fruit
428 crops (Walsberg 1977), indicating that phenological mismatch between these mutualist partners
429 could be very costly for both species. Interestingly, length of the interval between flowering and
430 fruit ripening the following fall appears to be a labile trait among host races, as it is about two
431 months shorter for the later flowering desert mistletoes on mesquite. The length of time between
432 flowering and fruiting may also impact desert mistletoe fitness, especially if unripe fruit suffer
433 attacks from insects and pathogens.

Interactions with pollinators

Why mistletoes on mesquite have apparent advantages in size, pollinator reward production, and pollen receipt over mistletoes on acacia is not known, but several factors that differ between catclaw acacias and velvet mesquites may be important. For example, mistletoes may gain more resources from mesquite because these host trees are larger on average than the acacias, have large taproots that can reduce the impacts of drought, and differ in their propensity to form nodules with nitrogen-fixing bacteria (Schulze and Ehleringer 1984; Zitzer et al.1996; Overton 1997). Flowering in mid-spring, instead of winter, is also predicted to give mesquite-associated desert mistletoes access to more potential pollinators of more species. Consistent with this idea, we recorded longer visits by pollinators later in the season, especially to pollen-producing male plants growing on mesquite. Perhaps due to late emergence time, *Lasioglossum* bees and honeybees were much more frequent visitors to mesquite associated desert mistletoe, and we observed them carrying large amounts of desert mistletoe pollen (K. Yule, unpubl.). However, these species may be less effective pollinators than syrphid flies because they groom to collect pollen on their legs where it is less likely to contact desert mistletoe reproductive structures (Wiesenborn 2016) and because they strongly prefer foraging on male plants. In contrast, syrphid flies, especially *Copestylum* spp., are predicted to be among the most important pollinators of this desert mistletoe based on a previous study, due to both the amount and location of pollen found on their bodies (Wiesenborn 2016).The reduced visitation to mistletoes later in the season, especially those on mesquite, by syrphids may be the result of increased competition for their services from co-flowering plants.

Reproductive success

While female flowers of desert mistletoe on mesquite received more pollen on average than those on acacia, potentially due to greater pollen production by the males or their higher density at the site (Aukema 2004), the success of pollination in terms of fruit set did not differ by host species. These results and our results on fruit ripening and removal provide no evidence that either host race is uniformly more fit, despite differences in reproductive traits and interactions with pollinators. We predict that reproductive traits most notably flowering phenology, are unlikely to be under uniform directional selection across host races. Rather, the differences in flowering phenology are consistent with reinforcement of reproductive isolation of host races in sympatry or character displacement (Hopkins 2013). If one host-associated flowering time uniformly increased fitness, the benefit of reducing hybridization could be outweighed by the cost of reduced mating opportunity in the individuals of the less fit host race. Alternatively, if reproduction was pollen limited, our results would be consistent with character displacement to reduce competition for pollination services (Hopkins 2013). However, the uniformly high fruit set (0.70) seen here is consistent with a lack of pollen limitation to desert mistletoe fruit production, in contrast to the results of pollen addition experiments for other mistletoes, such as bird-pollinated *Peraxilla* spp. (Robertson et al. 1999, Kelly et al. 2007).

Reproductive isolating barriers

The lack of pollen limitation does not mean that pre-zygotic mechanisms are unimportant to maintaining reproductive isolation of the host races in sympatry. Acting first and potentially reducing gametic waste, flowering time contributed the most to total reproductive isolation in most situations (Online Resource S5, Tables S1 and S4). The near lack of apparent F1 hybrids between host races in prior population genetic work (Yule et al. 2016) is consistent with the importance of pre-pollination barriers but could also be due to post-mating barriers, such as

479 pollen incompatibilities for which no data yet exist. While pre-pollination barriers are thought
480 generally to have the largest relative impact on total reproductive isolation, post-zygotic
481 mechanisms can also be important over the course of host race divergence (e.g. Craig et al.
482 1997). Although they are not direct tests of hybrid viability or fertility, reciprocal transplants and
483 population genetic structure can give us some indication of the strength of early-acting post-
484 zygotic barriers (see Online Resource S5, section on germination and establishment; Online
485 Resource S5, Table S3). Assuming that host compatibility has a genetic basis, these results
486 indicate that barriers to immigration and hybridization are much stronger at establishment than at
487 germination (Overton 1997; Online Resource S5, Tables S3 and S4). These estimates of absolute
488 and relative strengths of isolating barriers (Online Resource S5, Table S4) should be interpreted
489 with caution as they require a number of strong assumptions and data are missing concerning
490 several potential isolating barriers. While subject to these assumptions, all of the estimates are
491 consistent with stronger barriers to gene flow from the acacia-associated host race to the
492 mesquite-associated host race than in the reverse direction. Interestingly, we see the opposite
493 pattern in the distribution of adult desert mistletoe ancestry proportions across the host species
494 (Yule et al. 2016). The incongruence between the strength of barriers we measure and the
495 population genetic structure points to a need for testing whether unmeasured mechanisms, such
496 as post-mating pre-zygotic barriers, may be stronger for mistletoes on acacia than for those on
497 mesquite.

498 While our work focuses on host-associated differences affecting reproductive isolation of
499 pre-existing genetically-differentiated populations, these populations may not have evolved in
500 sympatry, nor need they be present or strictly associated with particular host species across the
501 geographic range. A recent study of desert mistletoe chloroplast haplotypes found that geological

factors, rather than host species, provide the best explanation of phylogeographic structure at a range-wide scale (Lira-Noriega et al. 2015). The disparities in the results of studies at different scales may indicate allopatric origins of host-associated differences that are now maintained in sympatry. Future studies of desert mistletoe population structure across a variety of host species are needed to determine the relative importance of flowering time and other aspects of reproductive ecology in the maintenance of isolation between sympatric host-associated populations.

Studies of parasite reproduction and population structure need to include a broader representation of the vast diversity of parasitic lifestyles. While the mechanisms of reproduction and dispersal that are responsible for promoting or inhibiting host associated differentiation in phytophagous insects have been well studied, our study focuses on a parasite with a life history that has thus far been underrepresented in the literature. Unlike most phytophagous insects, parasitic plants are commonly long-lived, sometimes similar in lifespan to their hosts, and rely on multiple biotic vectors to reproduce and infect new hosts. Here, we have shown that parasitic plant traits, particularly phenology and pollination, can provide strong isolating barriers among parasite host races. Together, interactions between hosts, vectors, and parasites play a critical role in determining whether divergence and eventual ecological speciation of parasite lineages will occur following colonization of a new host.

Data accessibility. Data are available on Dryad: http://dx.doi.org/XXX.

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676 ***Electronic Supplementary Material.***

677 **Online Resource S1.** Reproductive morphology of desert mistletoes (*Phoradendron*
678 *californicum*) infecting mesquite (*Prosopis velutina*) and acacia (*Senegalia greggii*).
679 **Online Resource S2.** Detailed methods and results for modeling phenological distributions of
680 desert mistletoes (*Phoradendron californicum*) and five common host species.
681 **Online Resource S3.** Details of model selection procedure results.
682 **Online Resource S4.** Methods and results for analysis of pollinator visit lengths to desert
683 mistletoes (*Phoradendron californicum*) and model results for pollinator community
684 ordination.
685 **Online Resource S5.** Methods and results for calculations of the strength of reproductive
686 isolating barriers between host races of desert mistletoe (*Phoradendron californicum*) host
687 races.

TABLES

Table 1. Morphology and pollinator reward production by desert mistletoe (*Phoradendron californicum*) females and males infecting acacia (*Senegalia greggii*) and mesquite (*Prosopis velutina*) hosts. All data are presented as mean ± standard error (sample size)

	On acacia		On mesquite		Host species effect	
	Females	Males	Females	Males	F	P
Branch length (cm)	21.95 ± 1.21 (55)	22.52 ± 1.34 (52)	39.23 ± 1.46 (90)	37.62 ± 1.94 (89)	89.86	<0.001
Flowers / inflorescence	6.67 ± 0.36 (55)	9.81 ± 0.47 (52)	12.87 ± 0.48 (90)	17.56 ± 0.75 (89)	129.16	<0.001
Inflorescence mass (mg)	0.73 ± 0.05 (55)	1.30 ± 0.10 (52)	2.05 ± 0.15 (90)	4.16 ± 0.56 (89)	30.84	<0.001
Inflorescences / branch length (cm)	4.05 ± 0.58 (55)	2.53 ± 0.38 (52)	4.18 ± 0.35 (90)	2.44 ± 0.28 (89)	<0.001	0.999
Nectar sucrose (μg) / flower	0.05 ± 0.01 (39)	0.09 ± 0.01 (38)	0.12 ± 0.01 (32)	0.14 ± 0.01 (40)	36.28	<0.001
Pollen grains / flower		3567 ± 162 (478)		6849 ± 323 (418)	9.16	0.014

Table 2. Fisher exact tests of differences in visitation by pollinator taxa to desert mistletoe (*Phoradendron californicum*) males and females infecting acacia (*Senegalia greggii*) and mesquite (*Prosopis velutina*). Full data are presented only for taxa with >5 visits. OR indicates the odds ratio

	On acacia				On mesquite				Acacia vs	
	Prop. (No.) visits		F vs M		Prop. (No.) visits		F vs M		Mesquite	
Taxa	Female	Male	OR	P	Female	Male	OR	P	OR	P
Hymenoptera										
Apis mellifera	0 (0)	0.18 (49)	0	<0.001	0.12 (17)	0.23 (66)	0.51	0.02	0.66	0.03
Lasioglossum	0 (0)	0.00 (1)	0	1	0.26 (38)	0.41 (118)	0.63	0.03	0.01	<0.001
Diptera										
Syrphidae	0.69 (80)	0.58 (158)	0.83	0.28	0.19 (27)	0.14 (40)	1.3	0.33	4	<0.001
other Brachycera	0.04 (5)	0.03 (8)	1	1	0.20 (29)	0.02 (6)	9.4	<0.001	0.41	0.007
other <2 mm	0.24 (28)	0.18 (50)	0.92	0.8	0.20 (29)	0.17 (48)	1.2	0.52	1.1	0.54
Total (all taxa)	1.0 (116)	1.0 (272)			1.0 (146)	1.0 (286)				

688 **FIGURE LEGENDS**

689 **Fig. 1** Phenology of desert mistletoe and host in a, b) Ironwood Forest National
690 Monument (desert ironwood, *Olneya tesota*; foothills palo verde, *Parkinsonia microphylla*) and
691 c-d) Santa Rita Experimental Range (blue palo verde, *Parkinsonia florida*; catclaw acacia,
692 *Senegalia greggii*; velvet mesquite, *Prosopis velutina*). Curves represent the population level
693 generalized epsilon skew normal distributions parameterized with host data from the National
694 Phenology Network; parasite data from 2014-2015 censuses at each field site. Gray hatched
695 areas indicate the presence of ripe fruit on desert mistletoe

696 **Fig. 2** Temporal changes in a) nectar sugar (sucrose / flower) by females (F) and males (M) and
697 b) pollen production / flower by desert mistletoe (*Phoradendron californicum*) on acacia
698 (*Senegalia greggii*) and mesquite (*Prosopis velutina*). a) Nectar sugar production varies by host
699 species, sex, and date. Points are offset by 0.5 days to improve clarity. b) Pollen produced /
700 flower varies by host and date. Values are plant level means +/- SEM

701 **Fig. 3** Distance-based redundancy analysis of pollinator communities visiting desert mistletoe
702 (*Phoradendron californicum*) flowers on acacia (*Senegalia greggii*) and mesquite (*Prosopis*
703 *velutina*). The location in ordination space of the most common pollinators are indicated with
704 stars. The arrow indicates the influence of later survey dates on pollinator composition

705 **Fig. 4** Reproductive success of female desert mistletoes on acacia and mesquite. a) Pollen
706 receipt (grains / flower), b) relative fruit set, c) fruit ripening rate and d) fruit removal rate. Fruit
707 removal rate varies by host species and census. In d) data are given for three census dates. Points
708 are means +/- SEM; lowercase letters indicate significant differences ($p<0.05$) in model
709 coefficients

Figure 1

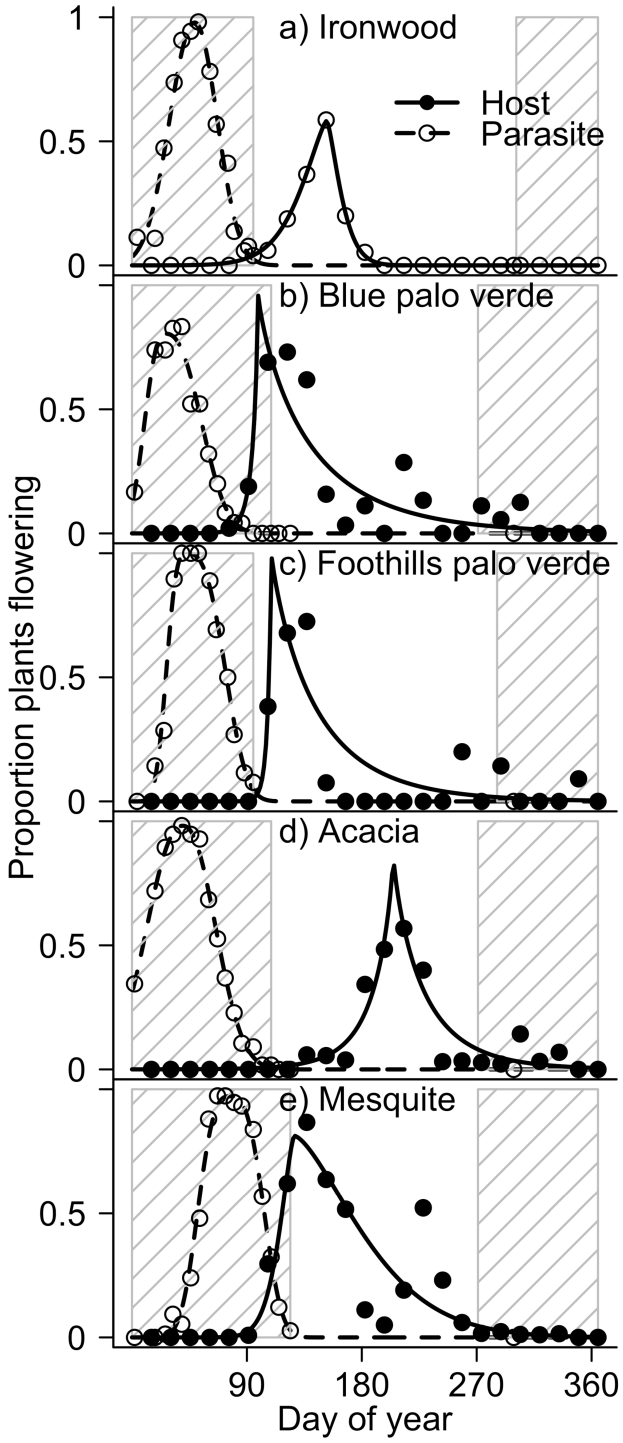

a) Ironwood
Host
Parasite
b) Blue palo verde
c) Foothills palo verde
d) Acacia
e) Mesquite
Proportion plants flowering
Day of year
0
0.5
1
90
180
270
360

Figure 2

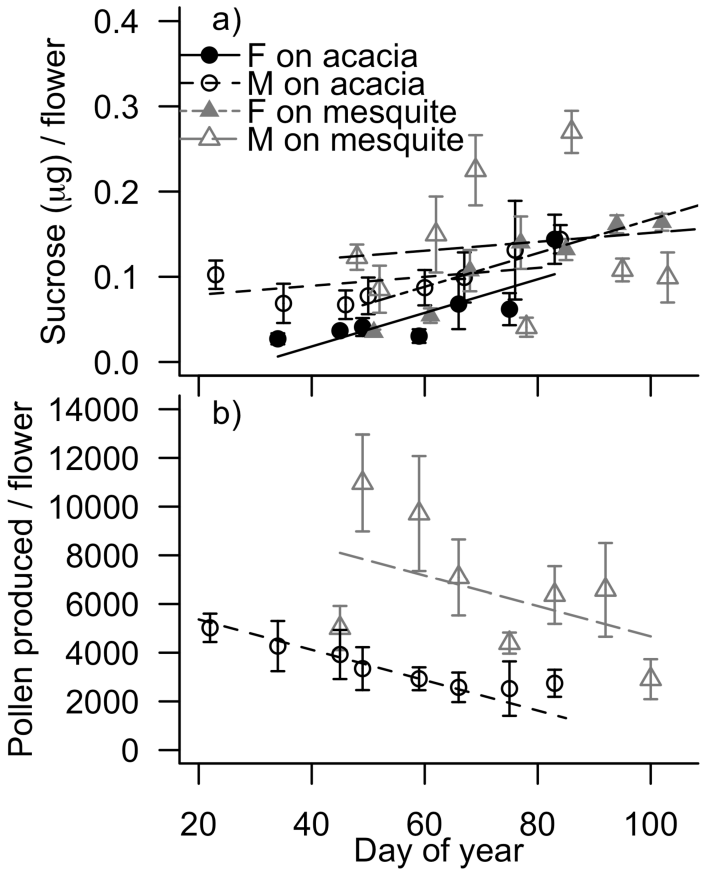

a)
F on acacia
M on acacia
F on mesquite
M on mesquite
Sucrose (μg) / flower
0.0
0.1
0.2
0.3
0.4
b)
Pollen produced / flower
0
2000
4000
6000
8000
10000
12000
14000
20
40
60
80
100
Day of year

Figure 3

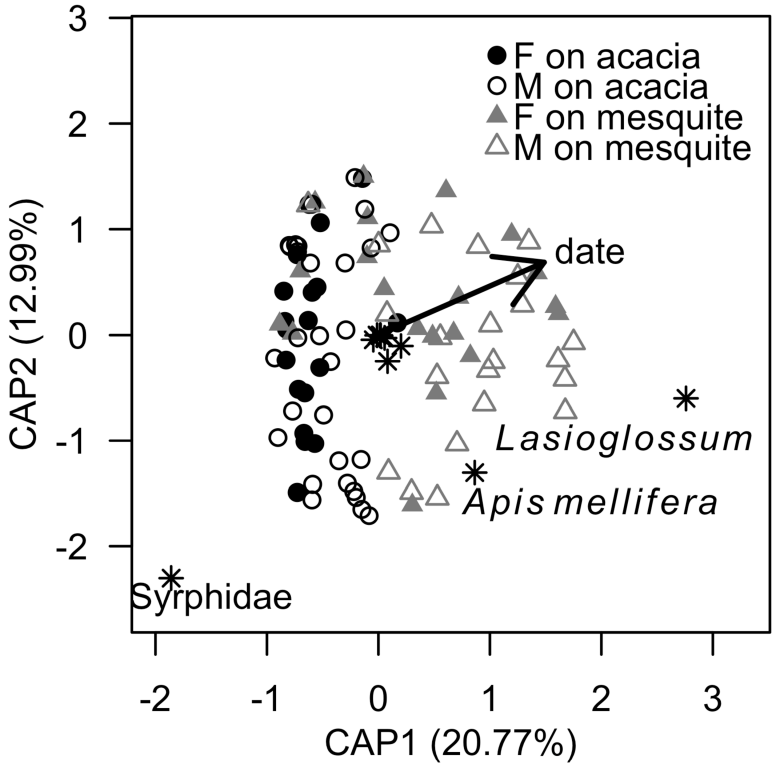
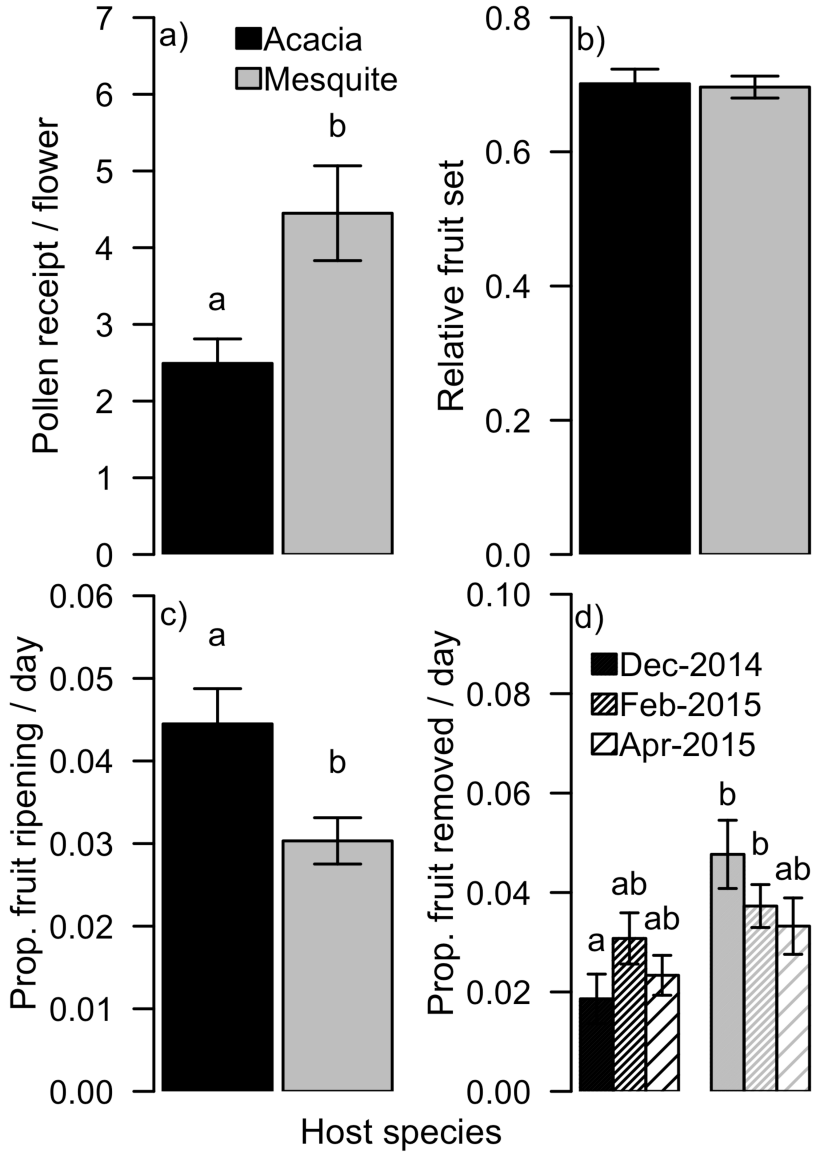

F on acacia
M on acacia
F on mesquite
M on mesquite
date
Lasioglossum
Apis mellifera
Syrphidae
CAP2 (12.99%)
CAP1 (20.77%)
3
2
1
0
-1
-2
-2
-1
0
1
2
3

Figure 4


a)
Acacia
Mesquite
Pollen receipt / flower
0
1
2
3
4
5
6
7
a
b
b)
Relative fruit set
0.0
0.2
0.4
0.6
0.8
c)
Prop. fruit ripening / day
0.00
0.01
0.02
0.03
0.04
0.05
0.06
a
b
d)
Prop. fruit removed / day
0.00
0.02
0.04
0.06
0.08
0.10
Dec-2014
Feb-2015
Apr-2015
a
ab
ab
b
b
ab
Host species