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Journal

Annals of Botany, 123(2)

ISSN

0305-7364

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Publication Date

2019-01-23

DOI

10.1093/aob/mcy143

Peer reviewed

PART OF A SPECIAL ISSUE ON ECOLOGY AND EVOLUTION OF PLANT REPRODUCTION

Experimental sympatry reveals geographic variation in floral isolation by hawkmoths

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Received: 28 February 2018 Returned for revision: 8 April 2018 Editorial decision: 2 July 2018 Accepted: 6 July 2018
Published electronically 18 July 2018

- **Background and Aims** Under a widely accepted model of pollinator-driven speciation, geographic variation in pollinator assemblage drives floral divergence and automatically causes reproductive isolation. Yet it is unclear whether divergent floral adaptation initially confers strong reproductive isolation, or whether that comes at later stages of speciation and requires other forms of reproductive isolation. This study uses a pair of recently diverged, interfertile and parapatric species in the genus *Clarkia* to determine whether adaptation to hawkmoths, a novel pollinator functional group, would automatically confer floral isolation upon sympatric contact.
- **Methods** First, genetically based differences in floral traits between *C. breweri* and *C. concinna* that would be maintained upon migration are quantified. Then scenarios of experimental sympatry are constructed in which arrays of flowers are exposed to the novel pollinator, the hawkmoth *Hyles lineata*, and pollinator preference and heterospecific pollen transfer are assessed. Source populations from across the ranges of each species are used to understand how geographic variation in floral traits within species may affect floral isolation.
- **Key Results** Although *H. lineata* has never been observed visiting *C. concinna* in the wild, it regularly moves between species in experimental floral arrays. Hawkmoth preference and heterospecific pollen transfer vary both among moths and among geographic source locations of *C. concinna*. The strength of floral isolation in this system is related to variation in flower size, especially hypanthium tube width, and nectar reward among *C. concinna* forms.
- **Conclusions** Although *C. breweri* has adopted a novel hawkmoth pollination system, both ethological and mechanical floral isolation by hawkmoths are incomplete and vary according to the specific phenotype of the *C. concinna* source population. The results suggest that strong floral isolation is not automatically conferred by a pollinator shift and may require additional evolution of deterrent floral traits and habitat isolation that reduces the immediate spatial co-occurrence of young species.

Key words: *Clarkia breweri*, *Clarkia concinna*, ethological isolation, floral isolation, *Hyles lineata*, mechanical isolation, pollination, reproductive isolation, speciation.

INTRODUCTION

Since Darwin (1877) first described the ‘various contrivances’ by which orchids are pollinated, biologists have linked floral adaptation to plant diversification, especially through the study of pollination syndromes, or suites of floral traits adapted to functional groups of pollinators with similar morphology and behaviour (Fenster *et al.*, 2004). The widely accepted Grant–Stebbins model for pollinator-driven speciation assumes that adaptation to the most effective pollinator across a geographic range drives floral divergence and automatically confers reproductive isolation (Grant and Grant, 1965; Stebbins, 1970, 1974). Floral divergence can have major consequences for assortative mating between species and the maintenance of species boundaries when closely related species come into geographic contact (reviewed in Kay and Sargent, 2009; Willmer, 2011; Van der Niet *et al.*, 2014). Yet the early evolutionary stages of novel pollination syndromes have rarely been examined empirically or in a way that tests their immediate contribution to reproductive isolation (but see Anderson *et al.*, 2010; Shuttlesworth and Johnson, 2011; Newman *et al.*, 2015).

Reproductive isolation caused by divergence of floral traits (hereafter ‘floral isolation’) is divided into two principle mechanisms: ethological isolation and mechanical isolation (Grant, 1994). Ethological isolation is conferred by divergent floral morphology, colour, scent and/or reward, resulting in differential pollinator preference (or, in some cases, constancy), whereas mechanical isolation results from reductions in pollen transfer efficiency because of the fit between flower and pollinator. If the same pollinator visits two species, differences in floral traits may reduce or prevent pollen transfer (Kay, 2006). Floral traits promoting precise pollen transfer by different pollinators (e.g. differences in stigma exertion or floral tube size) may act as ‘magic traits’ (*sensu* Servedio *et al.*, 2011), wherein divergence in the trait intrinsically leads to assortative mating (Haller *et al.*, 2014).

Floral isolation is well studied in the context of completed pollination syndrome shifts between partially sympatric species (e.g. Bradshaw *et al.*, 1998; Fulton and Hodges, 1999; Whittall *et al.*, 2006; Hoballah *et al.*, 2007; Dell’olivo *et al.*, 2011), but it

is unclear whether floral divergence takes a leading role in driving speciation. Late-stage systems typically show strong deterrent traits against ancestral pollinators and a variety of other strong forms of reproductive isolation, such as ecogeographic, habitat or post-zygotic isolation. There are many reasons why secondary sympatry may obscure early contributions of floral divergence to speciation: secondarily sympatric populations may undergo character displacement (Armbruster *et al.*, 1994; Smith and Rausher, 2008), reinforcement (Kay and Schemske, 2008; Hopkins and Rausher, 2012) or selection for deterrence of now ineffective ancestral pollinators in the current pollination environment (Thomson and Wilson, 2008), thus increasing floral divergence and causing the evolution of deterrent traits. For example, red colour in derived hummingbird-pollinated flowers deters ineffective ancestral bee pollinators (Schemske and Bradshaw, 1999; Castellanos *et al.*, 2004; Thomson and Wilson, 2008), and is not innately preferred by hummingbirds (Stiles, 1976; Melendez-Ackerman *et al.*, 1997). Despite the importance of understanding the early stages of speciation, the vast majority of studies examining floral isolation by different pollinator functional groups involve pairs of species at the endpoints of transitions between pollinators (but see Streisfeld and Kohn, 2007).

We use a pair of recently diverged, interfertile and parapatric species of annual wildflowers in the genus *Clarkia* to ask whether adaptation to a novel pollinator functional group would automatically confer floral reproductive isolation if they were to come into geographic contact. The more derived species has adapted to novel hawkmoth pollinators, but is also visited by many of the same diurnal insects that pollinate its sister species, suggesting that it may be at an early or intermediate stage of a pollination syndrome shift. We first quantify floral morphology and nectar reward in a greenhouse common garden to establish genetically based differences in floral traits that would be maintained upon migration. We then construct scenarios of experimental sympatry in which we expose arrays of flowers to the novel hawkmoth pollinators. We use source populations from across the ranges of each species to understand how geographic variation in floral traits within species may affect floral isolation. We assess the potential for ethological isolation by quantifying pollinator preference in heterospecific arrays, and examine the effects of both ethological and mechanical floral isolation on heterospecific pollen transfer. Our study has implications for the role of plant–pollinator interactions in creating and maintaining angiosperm diversity.

MATERIALS AND METHODS

Study system

Clarkia concinna and *C. breweri* (Onagraceae) are parapatric and interfertile sister species of annual herbs, with *C. concinna* extending from the Central California coast ranges north to Humboldt County. *Clarkia concinna* meets *C. breweri* in the south-eastern part of its range, with *C. breweri*'s limited range extending further south (Lewis and Lewis, 1955). *Clarkia concinna* and *C. breweri* occupy different habitats, the former growing in the understorey of woodlands and evergreen forests, and the latter being restricted to steep, rocky exposed hillsides. Flowers of *C. breweri* have paler pink petals than *C. concinna*,

dehisce pollen in the evening and produce a sweet spicy floral scent (Raguso and Pichersky, 1995). *Clarkia breweri* also has wider, longer hypanthium tubes and more exerted anthers and stigmas than *C. concinna* (Fig. 1). *Clarkia concinna* is pollinated by long-tongued flies, a variety of pollen-collecting and nectaring bees, hummingbirds and butterflies. *Clarkia concinna* also shows striking differences in morphology, colour and scent among geographic regions, which may represent pollinator-driven ecotypic differentiation, although we have not yet tested the adaptive nature of these differences. *Clarkia breweri* is primarily pollinated by nocturnal hawkmoths, especially *Hyles lineata*, but is also visited by a variety of less effective pollinators, including hummingbirds, bees, flies and diurnal Lepidoptera (Miller *et al.*, 2014). *Clarkia breweri* is the only species of *Clarkia* pollinated by hawkmoths; pollen-collecting bees are ancestral pollinators in the Onagraceae and bees are dominant pollinators for all other outcrossing *Clarkia* species (MacSwain *et al.*, 1973; Raven, 1979; Moeller, 2006). Thus, hawkmoth pollination is a novel adaptation for *C. breweri*. Although *H. lineata* occur throughout the range of *C. concinna*, we have never observed any hawkmoth visitation to it. Yet our previous work shows that *H. lineata* will visit *C. concinna* in the presence of *C. breweri*, perhaps with the *C. breweri* scent acting as a feeding stimulus (Miller *et al.*, 2014). *Hyles lineata* is a much more efficient pollinator of *C. breweri* than of *C. concinna*, probably due to *C. breweri*'s more exerted stigma and anthers and wider hypanthium tube that accommodates insertion of the hawkmoth proboscis (Miller *et al.*, 2014). If a hawkmoth is unable to fit its proboscis deeply enough into the hypanthium tube, as we observed previously with *C. concinna*, it cannot make contact with the anthers and stigma, and should be prevented from transferring pollen.

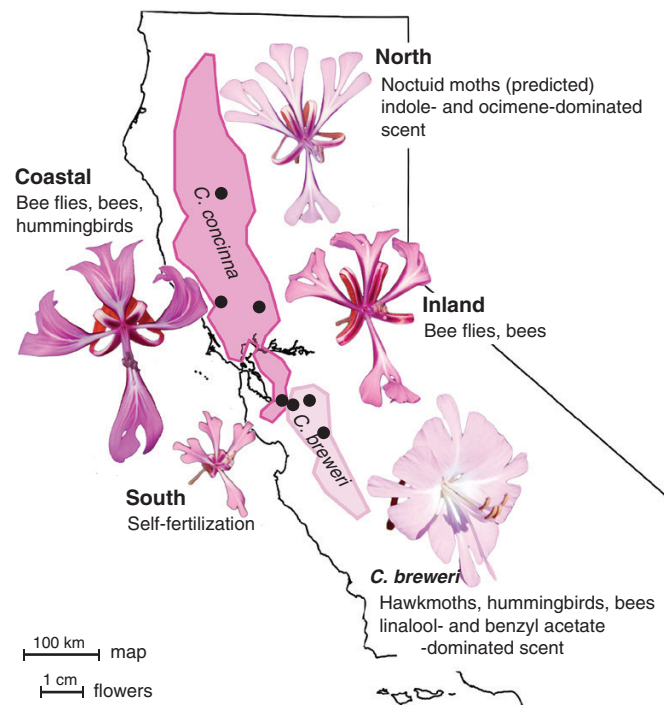


FIG. 1. Geographic ranges, floral phenotypes and flower visitors of focal *Clarkia* taxa. Images of *C. concinna* flowers (with scale indicated) were taken from the same photo with the background digitally removed.

Floral morphology and reward

Floral phenotypes show high variation among populations (K. M. Kay and R. A. Raguso, unpubl. res.); therefore, we grew field-collected seed in the UCSC greenhouse to assess genetically based differences in floral morphology and nectar reward. We collected seed from at least 20 maternal families from four geographic regions with visibly different flowers for *C. concinna*, hereafter called ‘south’, ‘coastal’, ‘inland’ and ‘north’ forms (Fig. 1; Supplementary Data Table S1). The south form is from the range edge that abuts *C. breweri*, has the smallest flowers, self-pollinates in the bud prior to flower opening but receives visitation from bees and flies upon opening (K. M. Kay, unpubl. res.). It is known taxonomically as *C. concinna* ssp. *automixa* (Bowman, 1987). All other forms of both species are highly protandrous with only delayed selfing after the flower wilts (Kay and Picklum, 2013). The coastal *C. concinna* form has the largest flowers and receives visits from bombyliid flies, a variety of nectaring and pollen-collecting bees, and hummingbirds (K. M. Kay, unpubl. res.). The inland form receives visits from bombyliid flies, a variety of nectaring and pollen-collecting bees, and butterflies (Groom, 1998; Miller et al., 2014). The north form has the palest flowers and is the only *C. concinna* that produces a noticeable scent. We have not thoroughly characterized the scent in these plants, but our preliminary gas chromatography–mass spectrometry (GC-MS) data show that it is dominated by indole and (*E*)- β -ocimene. Although indole is known to attract *H. lineata* (Bischoff et al., 2015), we observe that the hypanthium tube is too narrow to accommodate the *H. lineata* proboscis easily. We do not have pollination data for the north form, but predict that it attracts small noctuid moths based on its indole-scented, pale-coloured and highly dissected floral morphology. This combination of traits characterizes the flowers of *Phlox divaricata*, a moth-pollinated plant from eastern North America (Majetic et al., 2015). We also collected seed from at least 20 maternal families from three locations of *C. breweri*, spanning much of its small geographic range. *Clarkia breweri* locations did not show differences in flower morphology or nectar reward in the greenhouse common garden (see the Results), and thus we used the *C. breweri* from different populations interchangeably in trials with hawkmoths. Field-collected seed was stored at 4 °C in dry silica until the start of the experiment.

We planted two cohorts of *C. concinna* and *C. breweri* seeds in the UCSC greenhouse facility in autumn 2015 and 2016, staggering and repeating the plantings of each population to ensure flowering overlap. Seeds were planted in 3.8 cm diameter cone-tainers (Stuewe and Sons, Inc.) containing four parts Pro-Mix HP Mycorrhizae potting soil to one part perlite. Each cone-tainer received 3–6 seeds from a single family for a total of 20 families from each location. These seeds were germinated in Conviron E-15 growth chambers on a 15 °C, 10 h day and 10 °C, 14 h night schedule. The seeds and seedlings were watered with deionized water every other day. Once the seedlings had secondary leaves and were approx. 4 cm tall, we thinned the plants to one plant per cone-tainer. After thinning, we transferred the plants to the greenhouse, where they were kept between 13 and 25 °C with a 13.5 h day and daily overhead misting. Because *Clarkia* normally flower in the spring, we maintained day-length throughout the remainder of the

experiment by supplementing with 1000 W metal halide lights in the evenings. Plants began to flower at approx. 15 weeks, and floral measurements and hawkmoth trials began 1 week after, continuing for the following 10 weeks.

We measured hypanthium length, stigma and anther exsertion beyond the hypanthium tube opening, hypanthium width at its base and at its opening, overall flower height and width, and petal length and petal width with digital calipers to 0.1 mm accuracy. We avoided measuring the first two flowers on any plant and chose flowers at the beginning of the female phase with a freshly opened stigma in order to standardize the developmental stage of the plant and flower. Because nectar measurements often damaged the petals, we measured nectar on separate flowers from the morphology measurements. We quantified nectar volume with glass capillary tubes and sugar concentration with a refractometer. We used principal components analysis (PCA) in R (R Core Team, 2017) to investigate the covariance structure and visualize the overall dispersion of floral morphological traits by geographic location. For the PCA, we scaled the variables to unit variance and used singular value decomposition with the function ‘prcomp’. We log-transformed nectar volume to improve normality, and analysed nectar volume and concentration with one-way analyses of variance (ANOVAs) by geographic location followed by Tukey–Kramer post-hoc comparisons in JMP v. 13.

Floral arrays

We obtained hawkmoth pupae from a captive colony at Cornell University, originally established using eggs and larvae collected from Colorado Springs, CO (von Arx et al., 2013). Pupae were separated by sex and housed in two incubators for a 14 h/25 °C day 10 h/21 °C night cycle. After emerging, adult moths were moved to mesh cages based on emergence date in the same incubators. Adult moths were starved and introduced to floral arrays 3–4 d after emergence. Based on prior work with *H. lineata* (e.g. Miller et al. 2014; Bischoff et al., 2015), captive moths do not show reliable feeding motivation without a few days of post-emergence starvation, and providing this interval is common in hawkmoth behavioural studies (e.g. Haverkamp et al., 2016).

We reciprocally paired *C. breweri* with each of the four *C. concinna* forms and constructed monomorphic arrays of *C. breweri* for comparison. Floral arrays each contained six donor flowers with dehisced anthers and two emasculated recipient flowers with open, receptive stigmas, spread among 2–6 plants. Because we cannot reliably distinguish the pollen of these species, each floral array had only a single type of available pollen. Flowers of both species last several days, with the male phase lasting an average of 2 and 7 d prior to stigma receptivity for *C. breweri* and *C. concinna*, respectively (Kay and Picklum, 2013). The female phase lasts 3–5 d, during which time pollen remaining on the flower is still viable (K. M. Kay, unpubl. res.). We standardized the developmental stage of flowers by using donor flowers within 48 h of pollen dehiscence and recipient flowers within 48 h of stigma receptivity.

On any given day, the types of arrays that we constructed depended on flower availability, and available moths were randomly paired with available floral arrays. Flower position within the array was randomized as much as possible, given

that the flowers were attached to live plants, by assigning left to right positions along a cone-tainer rack, and we evened out flower height by stacking cone-tainers. Each trial consisted of introducing a single flower-naïve hawkmoth to a floral array in a $0.4 \times 0.4 \times 1$ m flight cage set inside a 2 m^3 flight cage in a windowless room with dim artificial lighting. Trials began between 14.00 and 15.00 h, approximating nightfall based on the incubator light/dark cycle. We maintained ambient humidity above 30 %, since humidity increases floral visitation by *H. lineata* moths (von Arx et al., 2012), and moderate to high humidity is common in hawkmoth behavioural studies (e.g. Haverkamp et al., 2016). Each moth was used for a single array only and then discarded. Sample sizes for arrays are detailed in Supplementary Data Table S2.

In order to assess preference, we recorded the number of visits to each flower type in each heterospecific array. We considered a moth to have begun visiting a flower upon its first attempt to insert its proboscis into a flower's hypanthium tube. Trials ended when the moth left the array for more than a minute and/or after the moth had visited all flowers in the array over the course of several minutes. To quantify preference, we used replicated G-tests in the R package RVAideMemoire to determine whether visitation fits the null expectation based on flower availability in heterospecific floral arrays. For each type of donor-recipient pairing, we conducted a G-test on goodness-of-fit to the expected proportions of 0.75 donor visits and 0.25 recipient visits. We conducted a G-test for each trial to determine whether each individual moth showed a preference. We then conducted a G-test for heterogeneity among trials of the same type to determine whether different moths vary in preferences for the same type of pairing. Finally, we conducted a pooled G-test for each type of trial to determine whether there is a deviation from the expected proportions across all trials of the same type.

In order to assess pollen transfer, we collected stigmas from recipient flowers immediately after each trial and stored them in a refrigerator at $4 \text{ }^\circ\text{C}$ until examination under a dissecting microscope. The number of pollen grains on each stigma was counted. When pollen loads were too large to count accurately on a whole, unstained stigma, stigmas were stained with fuchsin jelly and squashed on a glass slide. To analyse pollen deposition, we log-transformed counts of pollen grains to improve normality and used one-way ANOVAs of different pollen donors on *C. breweri* as the female-phase recipient and of different female-phase recipients with *C. breweri* as the pollen donor.

RESULTS

Floral traits

Clarkia breweri and *C. concinna* differ overall in morphology, with the former species having wider, shorter petals, longer hypanthia and more exerted anthers and stigmas (Fig. 2). The first two PCA axes account for 82 % of the variance in morphology and roughly distinguish flowers based on overall diameter and hypanthium tube size, respectively. *Clarkia concinna* forms also maintain the differences among geographic locations that we observed in the field. Whereas the 95 % confidence ellipses of the north ($n = 51$ flowers) and inland ($n = 7$) forms are almost perfectly overlapping, the coastal ($n = 34$) form has much larger

flowers, with longer and wider hypanthium tubes, and the south ($n = 21$) form has much smaller flowers. In contrast, different populations of *C. breweri* are highly overlapping in floral morphology in the greenhouse ($n = 11, 17$ and 19 flowers per population). *Clarkia breweri* ($n = 8, 8$ and 14 flowers per population) also produces significantly more nectar than *C. concinna*, and the *C. concinna* forms vary in nectar volume, with the coastal, north, inland and south forms producing decreasing amounts of nectar, respectively (Fig. 3; $F_{6,70} = 25.5, P < 0.0001$; $n = 11, 19, 8$ and 15 flowers). We find no significant differences in nectar concentration among floral forms ($F_{6,70} = 0.727, P = 0.63$), with an overall average concentration of 34 % sugar.

Pollinator preference

Replicated G-tests for the fit of observed and expected visitation in the arrays show that *H. lineata* moths prefer *C. breweri* over *C. concinna*, but this varies by both flower type and individual moth (Fig. 4). Pooled G-tests show an overall preference for *C. breweri* when it is paired with either the south or inland *C. concinna* form, regardless of which flower is most common in the array, although moths in most of these array types show significant heterogeneity (Supplementary Data Table S2). Array types with the north and coastal *C. concinna* forms showed mixed results. With the coastal

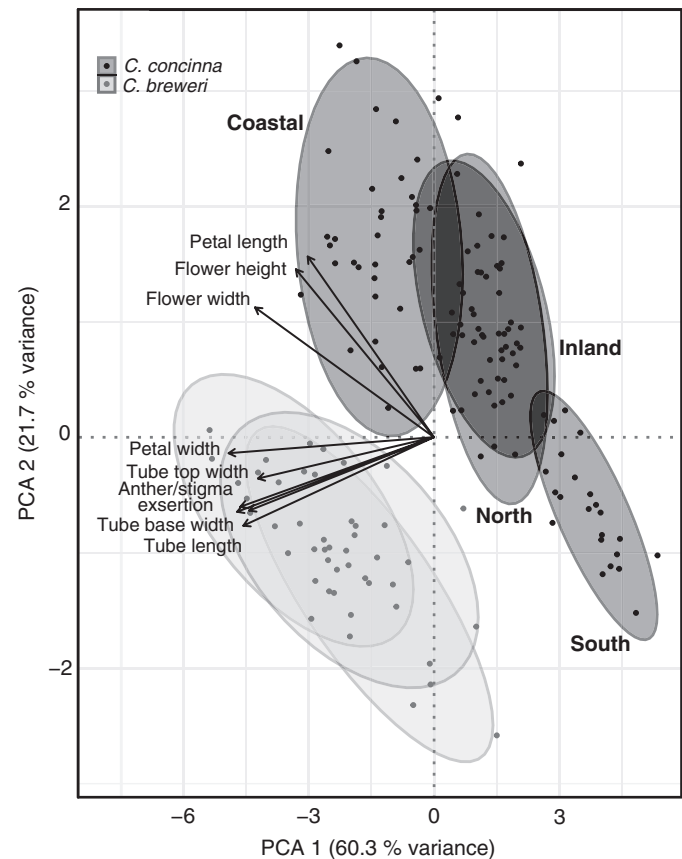


FIG. 2. PCA of floral morphology from a greenhouse common garden showing 95 % confidence ellipses and a biplot of trait loadings. *Clarkia breweri* sites show no significant differences for any trait and therefore were combined for this study.

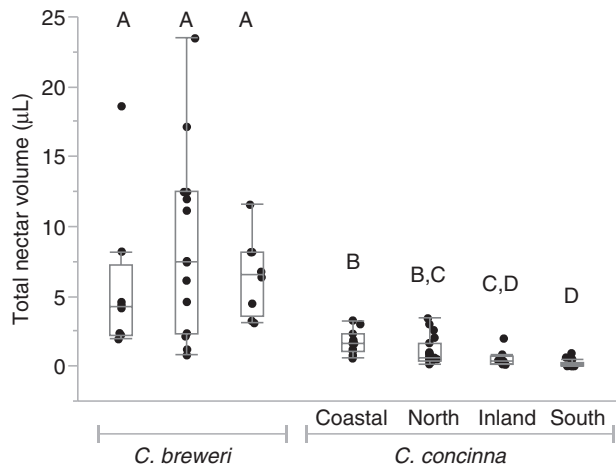


FIG. 3. Nectar volume varies by plant phenotype. Box plots indicate the minimum, maximum, median and quantiles, and letters indicate groupings in Tukey post-hoc comparisons. Log-transformed values are back-transformed for presentation, and overlapping points are jittered for clarity.

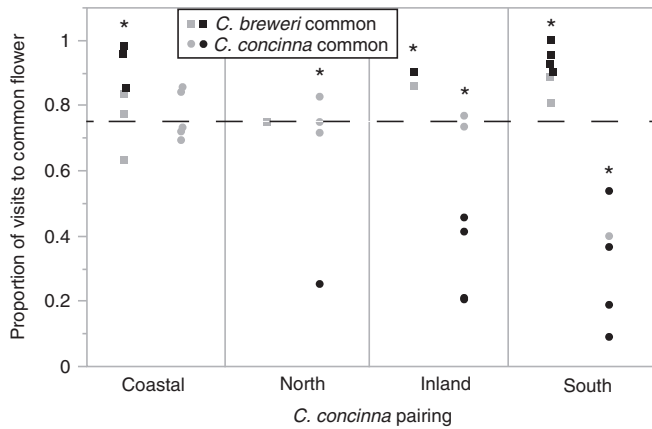


FIG. 4. Visitation preference of hawkmoths in floral arrays. Each point represents an individual hawkmoth trial. In each array, either *C. breweri* (squares) or *C. concinna* (circles) was the most common flower at a 6:2 ratio. The horizontal dashed line shows the null expectation for three-quarters of the visits to be to the common flower. Black symbols represent individual trials that deviate from the null expectation (G-tests), whereas grey symbols represent trials that do not. Asterisks denote sets of trials that deviate from the null expectation (pooled G-test). Overlapping points are jittered for clarity.

form, there was no overall preference when *C. concinna* was the common flower, but there was a bias towards *C. breweri* when it was more common. With the north form, we only recorded visit number in a single array with *C. breweri* as the common flower (with no preference), and three out of four arrays with the north form as the common flower showed no significant preference (although the pooled G-test is significant).

Pollen deposition

Hyles lineata transfer high numbers of pollen grains in conspecific arrays of *C. breweri*, and generally lower numbers in heterospecific arrays, although we see differences among *C. concinna* forms and between donor/recipient directions (Fig. 5). Pollen deposition per stigma varied significantly

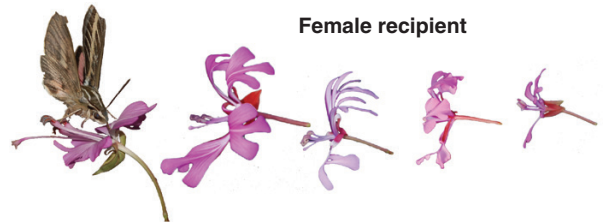
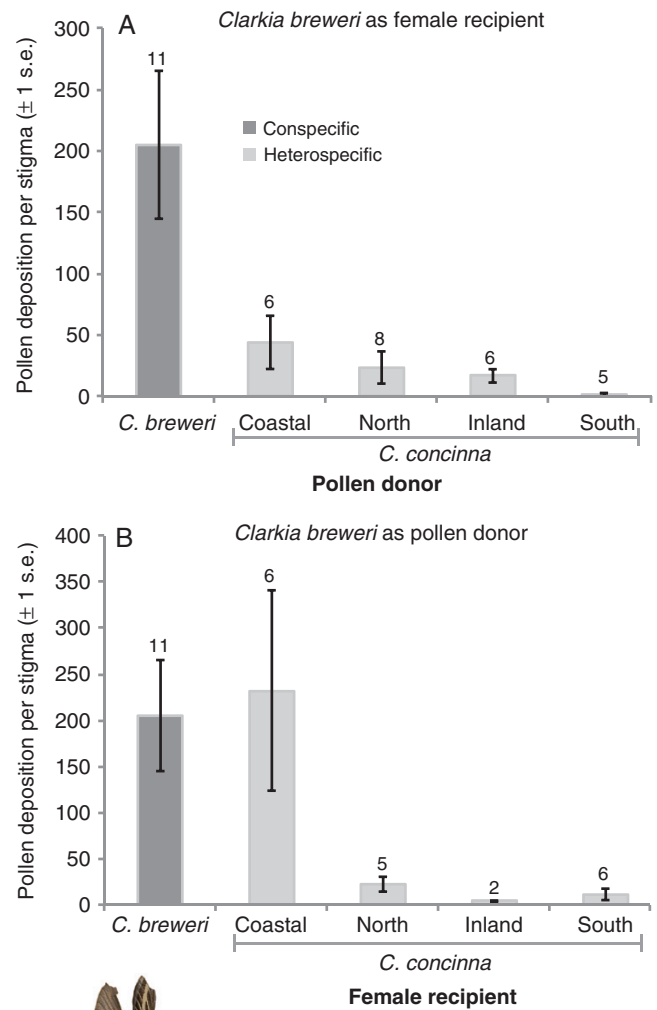


FIG. 5. Pollen deposition per stigma for different floral arrays with *C. breweri* as the female recipient (A) or the pollen donor (B) for the five types of flowers (pictured). Images of *C. concinna* flowers were taken from the same photo with the background digitally removed. *Clarkia breweri* is pictured with *Hyles lineata* with its proboscis fully inserted in the hypanthium tube. Log-transformed values are back-transformed for presentation. Numbers above bars are the number of arrays of each type.

with *C. breweri* as the female-phase recipient ($F_{4,65} = 6.61$, $P = 0.0002$) and with *C. breweri* as the pollen donor ($F_{4,51} = 3.95$, $P = 0.0072$). With *C. breweri* as the female-phase recipient, we see higher pollen deposition by *C. breweri* pollen donors compared with any of the *C. concinna* forms as pollen donors. Within *C. concinna* forms, the coastal form deposits the most pollen on *C. breweri*, with progressively lower amounts deposited by the north, inland and south forms, respectively. With *C. breweri* as the pollen donor, we see high and similar amounts of pollen deposited on *C. breweri* compared with the coastal *C. concinna* form, and lower amounts deposited on the north, inland and south *C. concinna* forms.

DISCUSSION

Our experiments show that adaptation to a novel pollinator type confers some floral isolation upon sympatric contact, but that it is not as strong as might be expected based on observations of geographically isolated populations. The variable and sometimes weak ethological isolation is especially striking. *Hyles lineata* moths occur throughout the range of *C. concinna*, but we have never seen them visit *C. concinna* despite hundreds of hours of observation across multiple sites (Miller et al., 2014). Yet when interspersed with *C. breweri* in experimental arrays, *C. concinna* is regularly visited. Some of the novel *C. breweri* floral traits, such as the pale colour and strong scent, probably function as long-distance attractants or elicitors of feeding behaviour (Raguso and Willis, 2005). Once hawkmoths are primed to visit flowers, they visit nearby flowers that do not fit expectations for a hawkmoth-attractive phenotype. Similar results have been found for *H. lineata* visiting *Ipomopsis* (Polemoniaceae), with scent bringing hawkmoths to mixed species floral arrays and colour mediating preference at small spatial scales (Bischoff et al., 2015). Thus strong ethological isolation cannot be inferred from a lack of hawkmoth visitation in the field.

Additionally, we see incomplete and sometimes weak mechanical isolation, despite the large differences in flower morphology between the species. When *H. lineata* visits *C. breweri*, it easily finds the hypanthium tube opening, inserts its proboscis fully in the tube and typically makes anther/stigma contact with its legs or abdomen (Supplementary Data Video S1). In contrast, moths often behave erratically while probing *C. concinna*. Inexperienced moths were observed probing the back of the hypanthium, repeatedly missing the hypanthium opening and probing in between the dissected petals alongside the tube, or clasp their legs around the entirety of a flower during visitation, perhaps in an effort to gain better purchase for nectaring. Moreover, moths were typically unable to insert their proboscis fully in the narrow tube, positioning their bodies away from the flowers as they hovered (Supplementary Data Video S2). The variety of approaches and persistence of the moths during visits resulted in inconsistent anther/stigma contact with different body parts including legs, abdomen and tongue. The *Pedicularis* type of mechanical isolation proposed by Grant (1994) assumes that pollinators behave consistently and precisely when removing and depositing pollen, but this runs counter to our observations of *H. lineata*. Similar imprecision of pollen placement resulting in heterospecific pollen transfer has even been found in *Pedicularis* (Armbruster et al., 2014). This study of three sympatric bumblebee-pollinated *Pedicularis* species showed that pollen of each species was dusted over the entirety of the bees' bodies and was transferred between species indiscriminately. Strong floral mechanical isolation may be more likely in systems with pollen packaging, such as in orchids that precisely attach pollinaria (Brantjes, 1982; Manning and Linder, 1992; Maad and Nilsson, 2004; Ramirez et al., 2011) or in systems with larger pollinators and more body area for segregating pollen placement (Temeles and Kress, 2003; Kay, 2006; Muchhala and Potts, 2007).

The floral isolation we measured varies greatly according to the specific phenotypes of the populations involved. Concerning ethological isolation, *H. lineata* moves much more readily to the large-flowered, highly rewarding coastal form

and discriminates most strongly against the small-flowered highly selfing and unrewarding south form of *C. concinna*. Interestingly, we see more discrimination against the inland form than the north form of *C. concinna*, although their morphology is essentially identical. We hypothesize that the paler colour, indole-laden scent and marginally higher nectar reward are responsible for this pattern. Mechanical isolation varies in a similar way to preference across *C. concinna* forms. The coastal form of *C. concinna* is the only form with a hypanthium tube wide enough consistently to accommodate the moth proboscis, ensuring anther/stigma contact and high pollen transfer, especially from *C. breweri* to *C. concinna*. In the other forms, moths typically probe the hypanthium opening where nectar wells up, but cannot fully insert their probosces. These differences in the tube are small, ranging from 1.10 mm in the south form, to 1.69 mm in the coastal form, to 1.85 mm in *C. breweri*, yet have strong effects on pollen transfer, as has been demonstrated for other hawkmoth-pollinated plants (More et al., 2007). We posit that the coastal form may be adapted for hummingbird pollination, at least in part. Compared with other forms of *C. concinna*, it has expanded bright red sepals and high nectar volume, and is the only form to which we have observed hummingbird visitation, albeit rarely. In general, hummingbird and hawkmoth flowers share many commonalities, which may account for the prevalence of transitions between hummingbird and hawkmoth pollination syndromes (Rosas-Guerrero et al., 2014) and also for the several observed hybrid zones between close relatives that differ in these syndromes (Aldridge, 2005; Aldridge and Campbell, 2007; Noutsos et al., 2014). The strongest isolation we observe in our arrays is with the highly selfing (south) form of *C. concinna*, and this is likely to be even stronger in the field where it self-pollinates in the bud prior to any opportunity for heterospecific pollen transfer. The selfing syndrome is recognized in many systems as providing strong reproductive isolation (Fishman and Wyatt, 1999; Martin and Willis, 2007), although it may also lead to isolation among conspecifics and a loss of evolutionary potential (Stebbins, 1970; Goldberg et al., 2010).

In *Clarkia*, floral isolation probably acts synergistically with other forms of reproductive isolation, as has been found in other systems (Grant, 1952; Angert and Schemske, 2005). Our prior work with *C. breweri* and the inland form of *C. concinna*, in which we interspersed patches of each species, did not find any evidence of ethological isolation (Miller et al., 2014). Taken together with the current study, these results suggest that the spatial arrangement of plants strongly affects ethological isolation, and we see evidence for striking habitat differences between our focal species that would be likely to reduce their opportunity for immediately co-occurring if migration or range shifts were to occur. In fact, at the only site in which they occur within sight of each other at the parapatric range edge, *C. breweri* is found on an open, rocky and south-facing slope of a ravine, whereas *C. concinna* is found on the opposing shady, wooded and north-facing slope. The species also differ in flowering time, with *C. breweri* cycling faster, even when grown in common greenhouse conditions, although their flowering times are partially overlapping in the field (K. M. Kay, unpubl. res.). In fact, it may be that strong ecogeographic isolation drove the adaptation to hawkmoth pollination in *C. breweri*,

since the flies and bees that pollinate *C. concinna* are much less common visitors in the geographic range and local habitat of *C. breweri*, even to standardized *C. concinna* floral arrays (Miller et al., 2014).

What might happen if *C. breweri* and *C. concinna* were to establish in sympatry? If there are fitness costs associated with inefficient pollination and heterospecific pollen transfer, we might expect the evolution of deterrent traits or floral filters that are commonly found in sympatric relatives differing in syndrome (Schemske and Bradshaw, 1999; Thomson and Wilson, 2008; Burger et al., 2017). For *C. concinna*, there might be selection for more narrow tubes, lower nectar or increased selfing, and our study shows that there is abundant genetic variation in these traits on which selection could act. For *C. breweri*, there might be selection for daytime flower closure or loss of floral anthocyanins, both common traits in other independently derived hawkmoth-pollinated Onagraceae (Gregory, 1963; Raven, 1979). The outcome is likely to depend on the specific phenotypes that come into contact and the ecological context of the local pollinator assemblage, the co-flowering plant community and the fine-scale spatial arrangement of plants. In *Ipomopsis aggregata* and *I. tenuituba*, one of the few systems with in-depth studies of floral isolation at multiple sites, natural hybridization rates vary depending on the ecological context of particular contact sites, and the spatial arrangement of plants determines the strength of floral isolation in laboratory experiments (Aldridge, 2005; Aldridge and Campbell, 2007; Bischoff et al., 2015). Alternatively, if pollinator sharing is not costly, sympatric taxa may experience selection for decreased floral divergence to facilitate pollination through shared display or resource supplementation (Thomson, 1981; Moeller, 2006), and hybridization could facilitate the collapse of the young species. We know that hybrids between *C. breweri* and *C. concinna* are easily made with hand pollinations in the greenhouse and that they are at least partially fertile through the F_3 and backcross generations (Raguso and Pichersky, 1999), but we do not know their relative fitness in the field.

Our study sheds light on what might happen if *C. breweri* and *C. concinna* were to come into geographic contact, but has several limitations. We only examined potential cross-pollination contributed by hawkmoths, although both species are visited during the day by a mix of bees, flies, butterflies and hummingbirds, with visitation frequencies varying by geographic location (Miller et al., 2014). Although these diurnal visitors are ineffective pollinators of *C. breweri*, rarely contacting the highly exerted stigma, they may pick up pollen from *C. breweri* that could be deposited on *C. concinna* stigmas. We were unable to work with these other pollinators in captivity, and were not allowed to place non-native pollen-bearing plants in the wild, but hypothesize that these pollinators would increase the potential for pollen transfer from *C. breweri* to *C. concinna*. In addition, since we cannot reliably distinguish the pollen of the two species, we were unable to observe pollen transfer patterns in arrays with both species in male phase. If the species were to co-occur in nature, there would probably be mixed pollen loads, and conspecific pollen precedence could lead to stronger reproductive isolation than we infer from our arrays (Howard, 1999). Finally, our results may be specific to the size of our arrays and the naiveté of our pollinators. In the wild, if the species were to co-occur, there might be more flowers dispersed

across a larger area, and pollinators might arrive experienced at handling the more locally common flower. In a previous study with *C. breweri* and the inland form of *C. concinna*, Miller et al. (2014) presented larger arrays to captive hawkmoths and found no significant preference, but in that study the flowers were clumped by species within each array. It may be that hawkmoths show more discrimination when different floral phenotypes are presented side by side than when phenotypes are clumped. Experienced moths may also differ in preference and handling time from naïve moths in unpredictable ways, either increasing or decreasing floral isolation. Nevertheless, although these limitations may affect our quantitative estimates of floral isolation, our qualitative result of incomplete floral isolation varying by *C. concinna* population is unlikely to be affected.

Counter to the Grant–Stebbins model, adoption of a novel pollination syndrome on the part of *C. breweri*, with its concomitant changes in morphology, reward, colour and scent, has not automatically resulted in reproductive isolation strong enough to cause speciation. In our study, both ethological and mechanical isolation by hawkmoths are variable and, at times, weak. In many systems, floral isolation needs to act in concert with other forms of isolation, and the idea of a single magic trait, even a multivariate one such as a pollination syndrome, causing speciation may be too simplistic (Kay and Sargent, 2009). Although we only examined floral isolation contributed by a single pollinator species, *H. lineata* is nearly cosmopolitan and the most common hawkmoth pollinator in western North America, and it has driven independent evolutionary shifts to hawkmoth pollination syndromes across many angiosperm lineages (Grant, 1983, 1985). Perhaps the best evidence for pollinator adaptation driving speciation comes from highly specialized pseudocopulation or perfume collection interactions between orchids and male bees in which simple changes in scent chemistry cause strong ethological isolation (e.g. Xu et al., 2011; Whitehead and Peakall, 2014; Hetherington-Rauth and Ramírez, 2016). Nevertheless, in those systems, it is unclear how new phenotypes establish since mating partners would be initially rare, whether there are intermediate stages that attract multiple pollinators or how well those examples apply to more common food rewarding systems. It will be important in the future to examine the immediate consequences of many types of pollination shifts for reproductive isolation in order to understand the generality of our results.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Video S1: a captive and flower-naïve *Hyles lineata* visits *Clarkia breweri* in a flight cage at UCSC. Video S2: a captive and flower-naïve *Hyles lineata* visits the inland form of *Clarkia concinna* in a flight cage at UCSC. Table S1: source locations for field-collected seeds. Table S2: replicated G-tests of moth flower preference in heterospecific floral arrays.

ACKNOWLEDGEMENTS

We thank J. Velzy and M. Dillingham for expert plant and moth care, D. Tataru and M. Vergara for greenhouse and flight cage

assistance, and T. Miller for seed collections. Special thanks to S. Topp and D. Jacobsen for *Hyles lineata* colony maintenance at Cornell. This work was supported by the National Science Foundation [DEB1342792 to R.A.R.].

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