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Context-dependent reproductive isolation mediated by floral scent and color

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Reproductive isolation due to pollinator behavior is considered a key mode of speciation in flowering plants. Although floral scent is thought to mediate pollinator behavior, little is known about its effects on pollinator attraction and floral visitation in the wild. We used field experiments with wild hawkmoths and laboratory experiments with naïve hawkmoths to investigate attraction to and probing of flowers in response to indole, a volatile emitted by *Ipomopsis tenuituba* but not its close relative *I. aggregata*, both alone and in combination with floral color differences. We demonstrated that indole attracts wild hawkmoths to flowers, but has little effect on the rate at which those attracted moths probe flowers. In contrast, white flower color did not influence hawkmoth attraction in the field, but caused more attracted moths to probe flowers. Thus, the moths require both scent and high visual contrast, in that order, to feed at flowers at dusk. Their preference for indole-scented flowers is innate, but species-specific preference is mitigated by previous experience and plant spatial patterning. This context-dependent behavior helps explain why these *Ipomopsis* species show geographical variation in the extent of hybridization and may potentially explain formation of hybrid bridges in other systems of hawkmoth-pollinated plants.

KEY WORDS: **Floral volatiles, hawkmoth, *Ipomopsis*, pollinator preference, reproductive isolation, speciation.**

Evolution of reproductive isolation is a critical component of speciation (Dobzhansky 1937). In flowering plants, pollinator behavior can cause reproductive isolation if related plant species receive flower visits from different animal pollinators (ethological isolation; Grant 1949). Although there is now evidence that color and morphology influence ethological isolation (Fulton and Hodges 1999; Bradshaw and Schemske 2003; Campbell and Aldridge 2006), the role of floral scent remains poorly understood (Raguso 2008). Recent studies demonstrate that differences in volatile organic compounds (VOCs) can restrict pollen movement between related species (Waelti et al. 2008), mediate a shift from wasp to fly pollination (Shuttleworth and Johnson 2010), and alter bumblebee and hawkmoth choices in laboratory settings (Klahre et al. 2011; Byers et al. 2014). However, the relative importance of floral scent in promoting ethological isolation in the wild remains unexplored outside of specialized orchids that use scent rewards or sexual deception to attract pollinators (Schiestl and Schlüter 2009). Scent could work in combination with color and other signals to influence ethological isolation in ways not easily predicted from laboratory manipulation of scent alone. First, floral traits perceived by a pollinator using different sensory modalities might influence sequential steps in floral visitation, such as attraction versus insertion of a proboscis into the flower. Although this concept has not been applied to ethological isolation in plants, signals of one sensory modality are known to alter responses to a subsequent signal (Hebets and Papaj 2005; Leonard et al. 2011). This process would resemble that observed in island flycatchers, for which sequential perception of divergent song elements and divergent plumage color patterns contributes to conspecific recognition (Uy and Safran 2013). Second, how insect pollinators respond to floral VOCs and generate species specificity may depend on the ecological context, including the coflowering

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species in the community and time of day when the pollinators forage. The composition of the community is particularly likely to matter if responses to floral VOCs depend on experience as well as innate preference (Dötterl et al. 2006; Riffell et al. 2008; Dötterl et al. 2011). Thus, it is important to measure behavioral responses in experienced and flower-naïve animals to understand how ecological context influences the degree of ethological isolation.

Here, we used field experiments with wild hawkmoths and flight cage experiments with naïve, laboratory-reared hawkmoths to investigate attraction to and probing of flowers in response to a species-specific floral volatile. Our approach allowed us to determine the relative importance of floral scent and color in enforcing pollinator specificity that generates partial reproductive isolation in the wild, and to separate innate and learned behavioral responses that contribute to explaining why reproductive isolation depends upon ecological context. In this article, we define innate preference as the behavioral response of flower-naïve adult moths, whether that response is genetically programmed or developmentally plastic.

We worked with plants in the genus *Ipomopsis* (Polemoniaceae), one of the original models for the concept of pollinator-driven speciation (Grant and Grant 1965). The extensive literature on this system allowed us to use our behavioral results to explain variation in hybridization in the field that results from ecological context (Campbell and Aldridge 2006; Aldridge and Campbell 2007, 2009). Flower color and shape contribute to reproductive isolation between the red-flowered *Ipomopsis aggregata* ssp. *aggregata* and the pale-flowered *I. tenuituba* ssp. *tenuituba* in the Rocky Mountains of Colorado in western North America (Campbell et al. 1997; Meléndez-Ackerman and Campbell 1998). Hummingbirds (*Selasphorus platycercus* and *S. rufus*) prefer to visit the wider tubed red flowers of *I. aggregata*, whereas hawkmoths (*Hyles lineata*) select for narrow-tubed flowers, leading to divergent selection on corolla width (Campbell et al. 1997). Experimentally eliminating the flower color difference between the species increases pollen transfer between them (Meléndez-Ackerman and Campbell 1998; Campbell 2004). Furthermore, geographical differences in pollinator specificity can explain variation in hybridization (Campbell and Aldridge 2006). At Grizzly Ridge (GR), Montrose County, Colorado, where the two plant species co-occur but rarely hybridize, foraging by hummingbirds and hawkmoths is species specific to *I. aggregata* and *I. tenuituba*, respectively, whereas near the natural hybrid zone at Poverty Gulch (PG), Gunnison County, Colorado, both types of pollinators visit both plant species and often transition between them (Aldridge and Campbell 2007). Transitions between the plant species result in pollen transfer (Campbell et al. 1998) and interspecies pollinations generate as many seed as conspecific pollinations (Campbell and Waser 2001), so behavioral differences do influence hybridization.

These two plant species also differ in floral VOCs. Flowers of both species emit large amounts of terpenoids, but only *I. tenuituba* emits indole (Bischoff et al. 2014), a nitrogenous VOC common to other hawkmoth-pollinated flowers (Knudsen and Tollsten 1993; Levin et al. 2001) and an antennal stimulant for *H. lineata* (Raguso et al. 1996). Indole is the compound that best separates the multivariate odor profile of the two species and is the sole nitrogenous VOC, whereas other compounds that differ between the species have related compounds in the scent mixture and usually differ only quantitatively between species (Bischoff et al. 2014). Indole is emitted at night (Bischoff et al. 2014), when hawkmoths actively forage for nectar at GR, but not during day, when these moths are active at the higher elevation site of PG. These results suggested that the timing of indole emissions and diel pattern of hawkmoth foraging activity might contribute to the variation in reproductive isolation.

In the field, we experimentally manipulated the presence of indole in flowers of both *Ipomopsis* species, in combination with flower color manipulations (Meléndez-Ackerman and Campbell 1998), and tested for attraction and flower probing by wild hawkmoths. We hypothesized that hawkmoths would more readily visit flowers with traits manipulated to resemble *I. tenuituba*, including indole emission. Because wild *Manduca sexta* hawkmoths required both visual and olfactory signals to probe *Datura* flowers at all (Raguso and Willis 2005), we hypothesized that white color and indole would show synergistic effects on foraging by *H. lineata* moths, with greater visitation to flowers presenting both signals than predicted from the additive effects of each signal alone. Our trait manipulations were done using wild, free flying moths in multiple ecological contexts: single species arrays of *I. aggregata* or *I. tenuituba* (which generates a larger scent plume of indole), or mixed arrays of both species with indole applied to either half or all of the plants. Comparison of results from these different settings allowed us to explore some effects of the ecological context, which could help explain disparate results from previous studies of moths foraging on related plant species with red or white flowers. In addition, we used laboratory-reared *H. lineata* to test whether field responses represent innate preference on the basis of either plant species or presence of indole. Finally, given the ability of many insect pollinators to learn floral signals (Riffell et al. 2008), we hypothesized that prior experience with the preferred plant species could modify innate preferences, and thereby impact ethological isolation. Prior experience with a rewarding plant could either increase specificity to that species in subsequent exposures, or alternatively could lead to generalized feeding on other plant species that share some cues with the preferred species. The latter possibility is predicted by the wide

foraging breadth sometimes observed for *H. lineata* (Raguso and Willis 2005; Alarcón et al. 2008).

Materials and Methods

STUDY SYSTEM

Ipomopsis aggregata ssp. *aggregata* (Pursh) V. Grant and *I. tenuituba* ssp. *tenuituba* (Rydb.) V. Grant are monocarpic perennials that are common throughout western United States and sometimes form natural hybrids in sympatry (Grant and Wilken 1988; Aldridge 2005). In *I. aggregata* ssp. *aggregata*, the tubular corollas are vividly red, shorter, and wider than the pale pink to white corollas of *I. tenuituba*. Flowers of *I. aggregata* produce more nectar of slightly lower sugar concentration than flowers of *I. tenuituba* (Meléndez-Ackerman 1997; Aldridge and Campbell 2007). In both species, the flowers remain open day and night for several days. The main pollinators are hummingbirds (*S. platycercus*, *S. rufus*) and hawkmoths (*H. lineata*; Aldridge and Campbell 2007). Two field sites were chosen for their night-time accessibility to abundant hawkmoths, and data were combined for analysis. The study sites are at GR on the north rim of the Black Canyon of the Gunnison, in Montrose County, Colorado (elevation 2375–2450 m.a.s.l.) where the two plant species co-occur but rarely hybridize, and at Spring Creek (SC) south of Mosca Campground, Gunnison National Forest, Gunnison County, Colorado (elevation 3048 m.a.s.l.). At the GR site, experiments were conducted at the *I. tenuituba* populations C, F, and I (Aldridge and Campbell 2009). At the SC site, arrays were set up adjacent to a natural population of *I. tenuituba* covering approximately 100 m^2 at the junction of County Routes 744 and 748. We added the second site of SC to boost replication after blooming ended at the GR site. *H. lineata* start foraging at both sites around dusk on dry, warm nights. All field observations were conducted between 20:00 and 23:00 h. Hawkmoth activity generally declined sharply after 22:00 h.

FLORAL TRAITS AND PHENOTYPIC MANIPULATIONS

Floral color manipulations were performed by painting the corolla of each flower with water-based acrylic paints, either titanium white (Custompaint Chemical Co., North Hollywood, CA) or naphthol red (Liquid-tex, England), to resemble the reflectance spectra of unmanipulated petals (Meléndez-Ackerman et al. 1997; Meléndez-Ackerman and Campbell 1998). These paints have shown no adverse effects on lepidopteran visitation (Pohl et al. 2011). Scent manipulations were performed by pipetting 1 μl of an indole solution (purity 99%, Sigma-Aldrich, CAS # 120–72–9) dissolved in paraffin oil (Sigma-Aldrich, CAS # 8012–95–1) onto each flower on the inflorescence. Paraffin oil was chosen as a carrier because it does not present an independent scent stimulus, it has a high viscosity that retards evaporation, and it acts as a nonpolar solvent for indole. We did not pipet onto the inner (adaxial) petal surface to prevent the indole treatment from running into the corolla tube, thus contaminating the nectar and potentially impacting nectar taste. Instead, the indole solution was applied to the outer (abaxial) surface of the corolla tube. We established the relationship between the quantity of indole in solution and in the headspace when applied to flowers with gas chromatography–mass spectrometry (GC–MS) sampling (Bischoff et al. 2014) and found that one order of magnitude is lost in transition between concentration in solution and in the headspace. We used two dosages, (1) 1 ng/μl, resulting in a headspace concentration of 100 pg indole augmented per flower (treatment low-indole), and (2) 100 ng/μl, resulting in a headspace concentration of 10 ng indole augmented per flower (treatment high indole). Indole-augmented flowers of *I. aggregata* emitted indole at concentrations similar to natural flowers of *I. tenuituba* 1 min after the experimental augmentation (Supporting Information Fig. S1), and the concentration of the indole treatment was within the range of natural quantities (mean and maximum night emission rates per flower = 3 and 17 ng/h) for *I. tenuituba* flowers in the field (Bischoff et al. 2014). Flowers were augmented with indole once each evening, and scent emissions decayed to control levels after 90 min (Supporting Information Fig. S1), by which time hawkmoth foraging had usually ceased. Experimental indole addition does not deter hawkmoths, and the impact of indole concentration on visitation by *H. lineata* is not dosage dependent at levels equal to or above the natural amount emitted by *I. tenuituba* when all plants nearby are of that species (Appendix1; Supporting Information Fig. S2).

DESIGN OF FIELD EXPERIMENTS

Field experiments utilized arrays of cut inflorescences in aqua picks, set up in proximity to *I. tenuituba* populations but at least 10 m away. Pilot studies showed that it was necessary to do experiments in proximity to *I. tenuituba* to observe any hawkmoths in the vicinity. Each array consisted of 12 inflorescences in a 3×4 matrix with 1 m between adjacent inflorescences (Supporting Information Fig. S3). Foraging hawkmoths can distinguish between stimuli presented >40 cm apart (Goyret et al. 2007). Inflorescences were chosen to present similar numbers (≥5) of open flowers, all containing nectar. Treatments were assigned randomly to positions in individual arrays.

In all arrays, we followed the sequence of inflorescences approached and flowers probed by each individual foraging *H. lineata* as it entered the array. Because our manipulations were at the level of plants (all with a single inflorescence), an inflorescence was treated as the independent unit of replication. We scored an inflorescence as attracting moths if one or more moths either approached the inflorescence with an attempt to visit, including searching with an extended proboscis (Supporting Information Fig. S3B), or visited successfully, probing a flower

with extended proboscis (Supporting Information Fig. S3C). We analyzed two variables that represented distinct steps in this behavioral sequence: whether an inflorescence attracted at least one moth (attraction), and whether at least one flower was probed given that the inflorescence was attractive (proportion probed). We also analyzed the overall impact for the plant as whether an inflorescence was visited (visitation = attraction × proportion probed), and the overall per-flower visitation rate (probes per flower per hour) to the inflorescence.

FIELD EXPERIMENT 1: ADDITION OF INDOLE TO TWO PLANT SPECIES IN THE FIELD

If indole helps to mediate species-specific foraging on *I. tenuituba*, addition of indole to plants of *I. aggregata* should make them more attractive to hawkmoths. We tested this prediction using arrays with four treatments: two scent treatments crossed with two species of inflorescences, *I. tenuituba* and *I. aggregata*. The scent treatments were 1 μl low-indole augmentation per flower and 1 μl paraffin control per flower. These mixed-species scent manipulation arrays were observed for a total of 11 h at GR in six separate arrays from 25 to 28 June 2011, recording a total of 27 foraging bouts. At SC, arrays were observed for a total of 7 h in four separate arrays recording a total of six foraging bouts. With 12 inflorescences per array, our total of 10 arrays yielded 30 replicate inflorescences for each of the four treatments. In two separate analyses, we analyzed attraction and proportion probed for an inflorescence, in each case using a generalized linear model with binomial response and fixed crossed factors of species and indole augmentation, with array as a blocking factor. We also analyzed the per-flower visitation rate using randomized block analysis of variance (ANOVA) and the same fixed factors. Analyses were performed with Proc Genmod and Proc GLM in SAS (ver. 9.2; SAS Institute, Inc., Cary, NC).

FIELD EXPERIMENT 2: FACTORIAL MANIPULATION OF FLOWER COLOR AND SCENT IN SINGLE PLANT SPECIES ARRAYS

To determine how color and scent act in combination and whether there are nonadditive effects, we manipulated them in factorial combination in single-species plant arrays. In each array, flowers on three inflorescences were painted white and received 1 μl high-indole augmentation per flower, three inflorescences were painted red with 1 μl high-indole augmentation per flower, three inflorescences were painted white with 1 μl paraffin control per flower, and three inflorescences were painted red with 1 μl paraffin control per flower (Supporting Information Fig. S3A and D). At the GR site, from 04 to 14 June 2012, we observed seven separate *I. tenuituba* arrays for a total of 10.5 h, recording a total of 22 foraging bouts, and three separate *I. aggregata* arrays for a total of 4.5 h, recording one foraging bout. Five additional *I. aggregata* arrays were observed for a total of 7.5 h at the SC site from 31 July to 01 August 2012, recording a total of five foraging bouts. Results for attraction, proportion probed, and per-flower visit rate were analyzed as for field experiment 1, except with fixed factors of color and indole augmentation. For per flower visitation rate, we assumed normally distributed errors as that distribution fit better than a zero-inflated negative binomial, as judged by Aikake information criterion (AIC), despite an excess of zeroes. For the *I. tenuituba* arrays, proportion probed was analyzed using a generalized linear model with binomial response and fixed crossed factors of color and indole augmentation, with array as a blocking factor. For the *I. aggregata* arrays, we used a Fisher's exact test comparing results for red- versus white-painted flowers because of the small number of moths attracted.

FIELD EXPERIMENT 3: MANIPULATION OF FLOWER COLOR FOR TWO SPECIES, ALL SUPPLEMENTED WITH INDOLE SCENT

Because our previous experiments indicated indole was required for hawkmoths to approach or probe flowers, we tested for additional effects of other floral cues, such as color, in its presence. All 12 inflorescences in each array had flowers augmented with 1 μl of high indole, and flower color (red vs. white) and species (*I. aggregata* and *I. tenuituba)* were manipulated in factorial combination. Five arrays were observed at SC from 30 July to 01 August 2012 for a total of 6.5 h, recording 18 foraging bouts. Results were analyzed as for experiment 2, except with fixed factors of color and species. In total, we made 53 h of after-dusk observations of hawkmoths to 408 inflorescences in 34 arrays for field experiments 1–3 and our high-dosage indole concentration test, along with an additional 47 h of nighttime observations to plant patches for our low-dosage indole concentration test (Appendix).

FLIGHT CAGE EXPERIMENT 1: RESPONSE OF NAÏVE HAWKMOTHS TO INDOLE AUGMENTATION

We performed no-choice foraging assays to test whether flower-naïve adult moths would visit *I. aggregata* flowers alone or augmented with indole, in the absence of floral stimuli from *I. tenuituba* or prior foraging experience. All flight cage experiments were carried out at Cornell University. *Hyles lineata* hawkmoths were reared in captivity as described by von Arx et al. (2012). *Ipomopsis* plants of both species were potted in the field in 2012, and were maintained in a greenhouse with a photoperiod based on the natural light cycle. *Ipomopsis tenuituba* plants emitted indole after 20:00 h, whereas *I. aggregata* did not, consistent with emissions in the wild (Bischoff et al. 2014). Hawkmoth behavior was recorded with a Sony Digital 8-TRV120 (New York, NY) video camera shooting in night mode and was scored for flower approaches and probes.

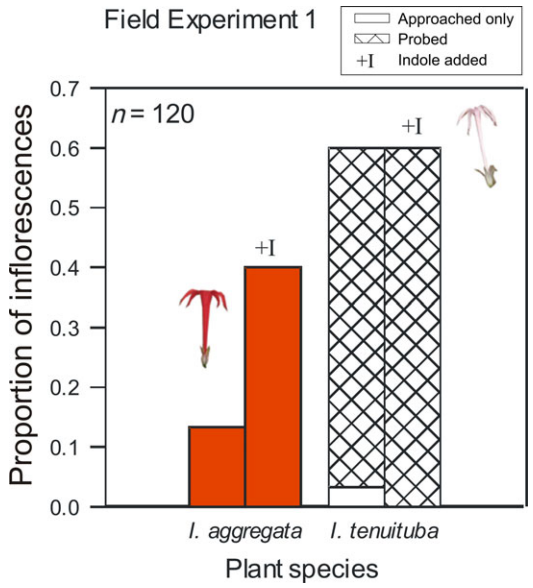

Figure 1. **Proportions of inflorescences that attracted hawkmoths (total bars) and received flower probes (hatched portions of bars) in field experiment 1. This experiment crossed the two plant species with addition of low-dosage (1 μl of 1 ng/μl) indole versus control (paraffin solvent only). Red shading indicates naturally red flowers. Experimental addition of indole is indicated by +I. *n*, total number of inflorescences. Low-dosage indole augmentation had a greater effect on attraction of moths to red flowers of *I. aggregata* than to white flowers of *Ipomopsis tenuituba* (likelihood ratio $X^2 = 3.79$, $P = 0.05$). For *I. aggregata*, the indole application tripled the attraction ($X^2 = 5.36$, $P = 0.0206$), however flowers on these plants were not probed.**

In flight cage experiment 1, we examined whether flower-naïve *H. lineata* would avoid feeding at unmanipulated *I. aggregata*, but would feed if indole was augmented. Behavioral assays were carried out with flower-naïve *H. lineata* in a 0.75 m × 0.75 m flight cage from 22 October to 13 November 2012 between the hours of 10:00 and 12:00. We used 75 male and 73 female *H. lineata*. Illumination was provided by a 30 W red bulb light source, and light levels for this experiment were at 15–18 lux. A single potted plant was placed in the flight cage, making the experiment a no-choice assay, which establishes baseline levels of attractiveness by measuring the proportion of moths showing a response (Goyret et al. 2008). Scent was added to red paper discs (diameter 5 mm) installed below the calyces of open *I. aggregata* flowers. One microliter of high-indole treatment or 1 μl of paraffin control was pipetted onto each disc at the start of each trial. Naïve *H. lineata* moths were introduced to the flight cage and given 5 min to take flight. If the moth took flight within that period, it was given another 5 min to approach the plant and feed from the flowers. For these experiments, a moth was the unit of independent replication. For moths that flew, we determined if the proportion feeding depended on indole by modeling feeding (yes or no) using a generalized linear model with a binomial distribution and fixed crossed factors of indole and moth sex.

FLIGHT CAGE EXPERIMENT 2: IMPACT OF PRIOR EXPERIENCE

A final experiment evaluated the impact of experience on hawkmoth foraging. Each trial consisted of a sequence of two consecutive nights per individual moth. We used 154 male and 142 female hawkmoths. This experiment was performed in a different flight room due to university renovations, resulting in slightly lower illuminance levels in the flight cage (<10 lux) than were measured in the previous experiment. However, both light levels are comparable to starlight conditions after dusk, as used in our previous experiments with *M. sexta* moths (13.5 lux; Kaczorowski et al. 2012). Hawkmoth behavior was recorded with a video camera as described above. The experiment was conducted from 23 August to 15 October 2012 between the hours of 20:00 and 23:00.

The four treatments consisted of all possible combinations of species on the first (*I. aggregata* or *I. tenuituba*) and second nights (*I. aggregata* or *I. tenuituba*). On each night, a moth was exposed to a single potted plant, making this experiment a no-choice assay as for flight cage experiment 1. All open flowers were augmented with 5 μl of a 10% sucrose solution to ensure consistent nectar rewards. On the first night of the trial, a starved flower-naïve *H. lineata* was introduced to the flight cage and given 5 min to take flight. If the hawkmoth took flight within that period, it was given another 5 min to approach the presented *Ipomopsis* plant and probe the flowers. We tested for innate preference and for sex differences in innate preference by modeling the incidence of probing on the first night using a generalized linear model with a binomial distribution and fixed crossed factors of plant species and moth sex. For individuals that experienced *I. tenuituba* on the first night, we then exposed all those that probed flowers to the experimental setup again the following night. Discarding the moths that did not probe is a common procedure in ethological experiments, in which moths, bees, or other insects are omitted from further trials when they fail a pretest for proboscis extension in response to sugar (Cunningham et al. 2004; Wright et al. 2007). Because almost none of the individuals presented with *I. aggregata* on the first night probed, we, however, tested all of those that flew the first night on the following night. If the moth failed to fly at all on the second night, it was removed from the dataset; otherwise it was scored for flower probing. Because we had different screening criteria for using a moth the second night depending on whether it experienced *I. aggregata* or *I. tenuituba* the first night, we analyzed those two sets of trials separately, in each case determining whether the proportion probing on the second night differed according to plant species presented, with a likelihood ratio X^2.

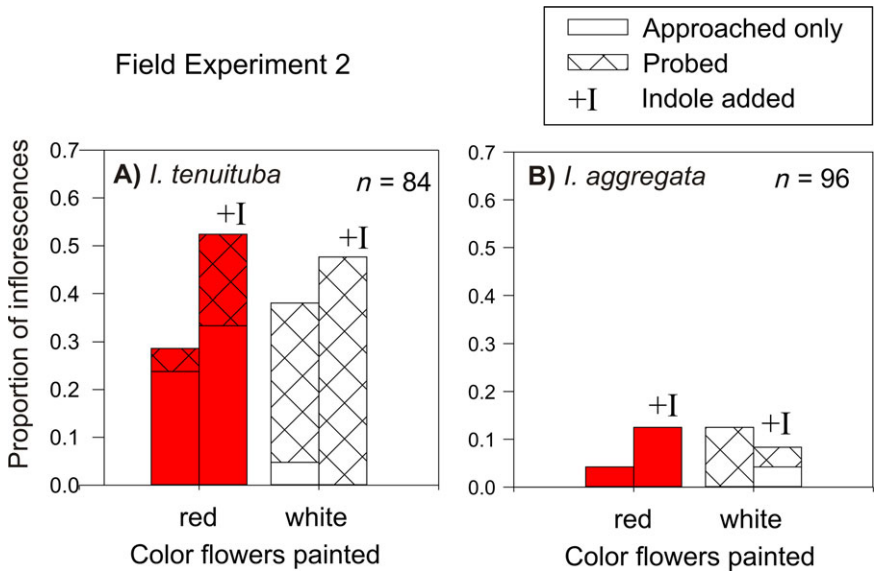

Figure 2. **Proportions of inflorescences that attracted hawkmoths (total bars) and received flower probes (hatched portions of bars) in field experiment 2. This experiment employed factorial manipulations of flower color and high-dosage (1 μl of 100 ng/μl) indole in single species arrays of (A) *Ipomopsis tenuituba* and (B) *I. aggregata*. Red (or gray) shading indicates flowers painted red. +I indicates indole added. The proportion of attractive inflorescences that were probed depended on color for both species (*tenuituba* $\chi^2 = 13.94$, $P < 0.001$; *aggregata* $\chi^2 = 4.67$, $P = 0.0308$). In (A) the overall proportion of inflorescences that were visited also depended on indole ($P = 0.0329$).**

Results

FIELD EXPERIMENT 1: ADDITION OF INDOLE TO TWO PLANT SPECIES IN THE FIELD

In experimental arrays in the field, low-dosage indole augmentation had a greater effect on attraction of moths to red flowers of *I. aggregata* (which do not normally emit indole) than to white flowers of *I. tenuituba* (interaction term in generalized linear model with binomial response, likelihood ratio $X^2 = 3.79$, $P = 0.05$). For *I. aggregata*, the indole application tripled the attraction ($X^2 = 5.36$, $P = 0.0206$). However, none of these moths probed any of the *I. aggregata* flowers (Fig. 1). The same treatment had no effect on the proportion of *I. tenuituba* inflorescences that attracted moths (Fig. 1; see also Supporting Information Fig. S2). Instead, a high proportion of *I. tenuituba* inflorescences (which emit indole naturally) attracted moths regardless of whether flowers were supplemented with low-dosage indole, and 35 of those 36 attractive inflorescences received probes. Thus, the per-flower visit rate was much higher for *I. tenuituba* than *I. aggregata* (two-way ANOVA with array as a blocking factor, $F_{1,107} = 53.95$, $P < 0.0001$).

FIELD EXPERIMENT 2: FACTORIAL MANIPULATION OF FLOWER COLOR AND SCENT IN SINGLE PLANT SPECIES ARRAYS

In arrays of naturally indole-scented *I. tenuituba* in which we manipulated flower color and scent in all factorial combinations, inflorescences of all treatments were similarly effective at attracting hawkmoths ($P > 0.10$ for effects of color, scent, and the interaction; Fig. 2). The proportion of these that were probed depended on color ($X^2 = 13.94$, $P < 0.001$), but not on high-dosage indole augmentation or the color × scent interaction ($P > 0.10$). Red-painted flowers of *I. tenuituba* mostly attracted approaches from hawkmoths, whereas white-painted flowers had a higher proportion of inflorescences that were probed (Fig. 2A; $X^2 = 15.18$, $P = 0.0001$). In these mixed arrays, adding indole increased slightly the overall proportion of inflorescences visited ($X^2 = 4.55$, $P = 0.0329$). Visits per flower per hour increased more than sixfold by painting flowers white rather than red (two-way ANOVA with array as a blocking factor, $F_{1,74} = 13.94$, $P = 0.0004$; Fig. 3A). No interaction was detected between the effects of color and scent manipulation, contrary to our prediction ($F_{1,74} = 0.13$, $P = 0.72$; compare solid to dashed line in Fig. 3A).

In the *I. aggregata* arrays, all types of inflorescences were much less likely to attract hawkmoths than in the *I. tenuituba* arrays, and attraction did not depend on treatment (all $P > 0.10$; Fig. 2B). However, hawkmoths only probed flowers on the white inflorescences (four of five attractive inflorescences vs. zero of four for red inflorescences; Fisher's exact test, $P = 0.0476$). As a result, the proportion of inflorescences visited was higher if the flowers were painted white (color effect in generalized linear model, $X^2 = 4.67$, $P = 0.0308$; Fig. 2B). Visits per flower per hour were also higher for inflorescences with white-painted flowers ($F_{1,85} = 3.62$, $P < 0.05$ for one-tailed test of a priori prediction

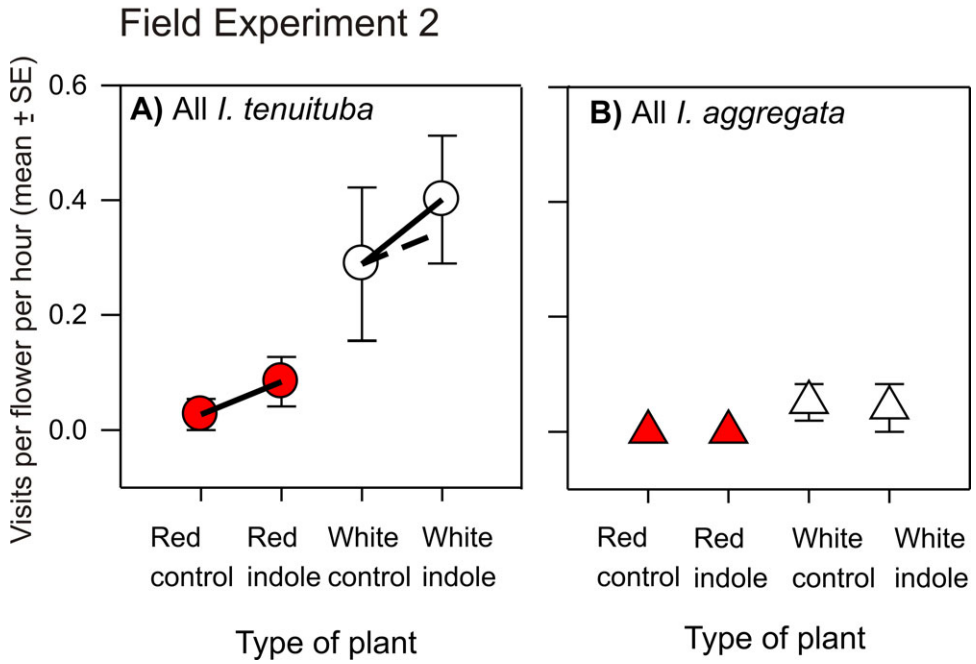

Figure 3. Per-flower visitation as a function of factorial manipulation of flower color and indole in single species arrays (field experiment 2). (A) *Ipomopsis tenuituba* arrays ($n = 84$). Solid lines indicate actual effects of supplementing with indole, whereas the dashed line indicates the expected effect of supplementing with indole for white flowers under the null hypothesis that it has the same effect as for the red flowers (additive effects of color and indole). (B) *I. aggregata* arrays ($n = 96$). Dashed and solid lines are not shown, as they would be on top of each other as the actual effect showed additivity.

that traits of *I. tenuituba* would increase visitation). Visit rate again did not show a detectable interaction between the effects of color and scent ($F_{1,85} = 0.04$, $P = 0.84$; Fig. 3B). For both plant species, flower color was a critical trait required for moths to probe flowers rather than just approach the inflorescence in these arrays.

FIELD EXPERIMENT 3: MANIPULATION OF FLOWER COLOR FOR TWO SPECIES, ALL SUPPLEMENTED WITH INDOLE SCENT

In mixed arrays with both plant species and high-dosage indole added to all flowers, painting flowers white rather than red had no detectable effect on the proportion of inflorescences attracting hawkmoths ($P = 0.99$) or the proportion of those events resulting in probing of flowers (Fig. 4). Inflorescences were visited in similarly high proportions regardless of plant species or flower color ($X^2 = 0.07$, $P = 0.7963$; Fig. 4). The difference between this result and the strong effect of color in the single-species arrays indicates that *H. lineata* will visit *I. aggregata* freely when *I. tenuituba* is intermixed and there is a large cloud of volatile indole. The amount of indole present was largest in this experiment because our augmentation produced a headspace concentration of 10 ng per flower, which is greater than the mean amount emitted by *I. tenuituba.* Thus, the ecological context influences visitation to *I. aggregata*. These results suggest that experience with nearby flowers of a preferred species may influence choice behavior by *H. lineata.* This hypothesis was tested with flight cage experiments.

FLIGHT CAGE EXPERIMENT 1: RESPONSE OF NAÏVE HAWKMOTHS TO INDOLE AUGMENTATION

When naïve *H. lineata* moths were presented with a single *I. aggregata* plant in a no-choice assay, of the 148 moths that engaged in flight behavior, a moth was more than 4 times as likely to probe flowers if augmented with indole than if the plant was left unmanipulated, so that no indole was present (Fig. 5A; main effect of indole, likelihood ratio $X^2 = 7.07$, $P = 0.0078$). The two sexes behaved similarly in response to indole (sex by indole interaction, $X^2 = 0.03$, $P = 0.86$).

FLIGHT CAGE EXPERIMENT 2: IMPACT OF PRIOR EXPERIENCE

In no-choice experiments with nectar rewards held constant, naïve *H. lineata* were presented with either blooming *I. aggregata* or *I. tenuituba* plants on night 1, and on night 2 those same moths, now with experience, were again presented with one of the two species. On night 1, 23% of the moths that flew within the first 5 min probed flowers if the plant was *I. tenuituba*, whereas only 1% did so if the plant was *I. aggregata* ($X^2 = 32.98$, $P < 0.0001$; Fig. 5B), indicating a strong innate preference for *I. tenuituba*,

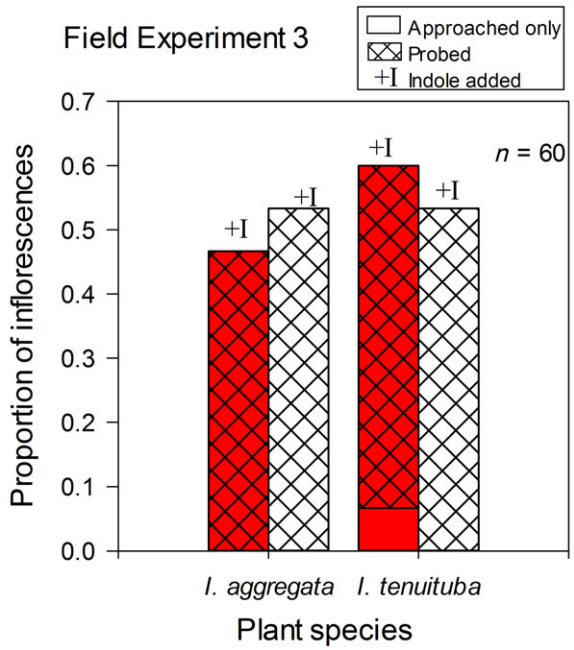

Figure 4. Proportions of inflorescences that attracted hawkmoths (total bars) and received flower probes (hatched portions of bars) in field experiment 3. This experiment crossed two plant species with manipulation of flower color, with high-dosage indole added to all flowers. Shading and symbols as in Figure 2. Inflorescences were visited in similarly high proportions regardless of plant species, flower color, or the interaction (all $P > 0.50$).

which the previous experiment showed was mediated in part by indole. This innate preference did not differ between the sexes of the moths (plant species by sex interaction, $X^2 = 0.00$, $P = 0.99$). Moths that had flown in the presence of *I. aggregata* on night 1 never visited *I. aggregata* on night 2, but 21% of such moths visited *I. tenuituba* (Fig. 5C left panel; $X^2 = 8.42$, $P = 0.0037$). Exposure to *I. aggregata* the night before did not affect the proportion of moths that visited *I. tenuituba* (compare Fig. 5B and 5C, left panel), even though the moths exposed to *I. aggregata* would have been hungrier and may have differed in motivation to feed. In contrast, moths that had experience with *I. tenuituba* on night 1 visited both species in similar proportions on night 2 (Fig. 5C right panel; $X^2 = 3.03$, $P = 0.0768$) even though without such experience they rarely visited *I. aggregata*. For unmanipulated plants, only prior experience with *I. tenuituba* led to more than incidental visitation to *I. aggregata* flowers in flight cage experiments.

Discussion

CONDITIONAL IMPACT OF FLORAL TRAITS ON ETHOLOGICAL ISOLATION

The relative importance of scent and other traits in mediating pollinator foraging and the consequences for plant reproductive isolation are frequently discussed (Fulton and Hodges 1999; Campbell 2004; Klahre et al. 2011; Van der Niet et al. 2014). Few studies, however, have addressed how different floral traits influence discrete steps in the behavioral sequence that begins with pollinator attraction and culminates in feeding, or how these mechanisms might vary with ecological conditions (Leonard et al. 2011). Here, we demonstrated that the floral volatile indole attracts wild hawkmoths to natural flowers of *Ipomopsis* plants, but has little effect on the rate at which those attracted moths probe flowers (Fig. 1). Application of indole to *I. aggregata* flowers influenced probing in flight cage experiments, but only in no-choice assays with starved, flower-naïve moths (Fig. 5). In contrast to indole, white flower color did not influence hawkmoth attraction in the field, but caused more of the attracted moths to probe flowers (Figs. 1–3). Thus, *H. lineata* moths require both floral scent (indole) and high visual contrast (white color), in that order, to find and feed from *I. tenuituba* flowers when foraging at dusk.

In the wild, *H. lineata* behavior varies between geographical sites. At GR, a lower elevation site in the Rocky Mountains, hawkmoth responses to floral scent and color lead to nearly exclusive visitation to *I. tenuituba*, despite lower nectar production in this species (Aldridge and Campbell 2007). Because hummingbirds strongly prefer *I. aggregata* at this site, ethological isolation is nearly complete, with only 4% of flight transitions between plants in balanced experimental arrays of the two species being interspecific (Aldridge and Campbell 2007). Reproductive isolation, however, breaks down between these *Ipomopsis* species at PG, a higher elevation site where plants in hybrid zones are visited by both hummingbirds and hawkmoths (Campbell et al. 1997), and 24% of flight transitions are interspecific in balanced arrays. Although hawkmoths visit hybrid swarms more frequently than *I. aggregata* populations at that site, they show weaker preferences within the hybrid zone, preferring narrower flowers but not probing preferentially on the basis of color (Campbell et al. 1997).

There are at least two possible explanations for this breakdown in pollinator fidelity, both related to the ecological context. First, the spatial arrangement of populations differs between contact sites. At GR, plants occur in a mosaic of discrete single-species patches, whereas at PG, the two species are at opposite ends of an elevational cline, with hybrid swarms in between. The presence of hybrids interspersed among parental phenotypes creates a mixed array of varying colors bathed in a cloud of scent. When we created mixed arrays, we observed increased visits by wild hawkmoths to naturally red or painted *Ipomopsis* flowers when a lot of scent was present, either because all plants were *I. tenuituba* (Fig. 2) or because all plants had indole added (Fig. 4). A white-painted, indole-scented plant in an array with all *I. aggregata* (Fig. 2B) may have received lower visitation than such a plant in an array with all *I. tenuituba* because of the additional indole signal provided naturally by that latter species

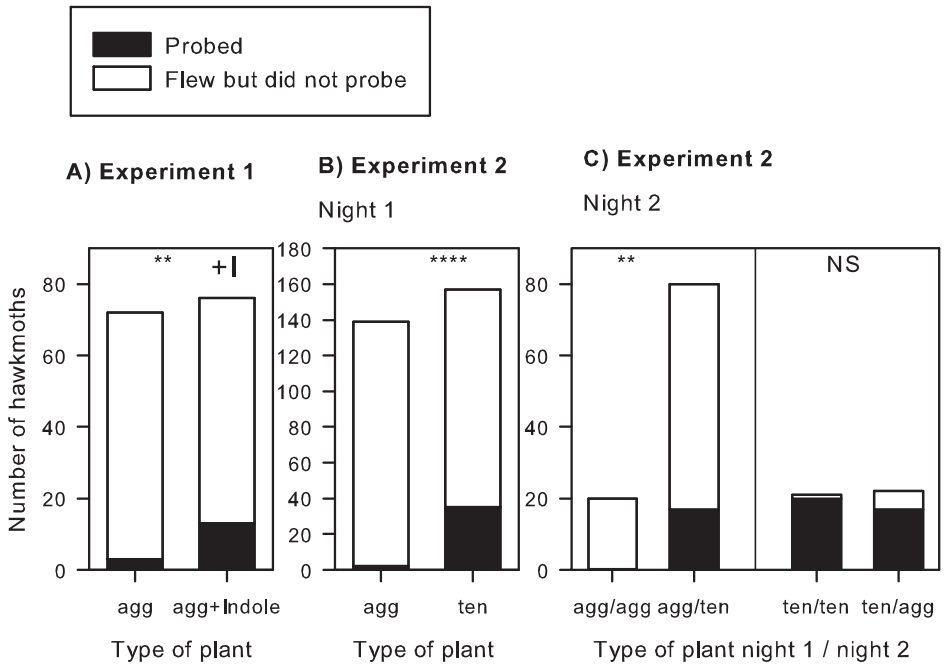

Figure 5. Numbers of *Hyles lineata* moths probing flowers out of total moths flying in flight cage experiments. (A) Experiment 1: naive moth presented with *Ipomopsis aggregata* alone (agg) or *I. aggregata* with indole augmentation (agg + Indole). (B) Experiment 2: naive moth presented with *I. aggregata* (agg) or *I. tenuituba* (ten) on night 1. (C) Experiment 2: response of moth on night 2 as a function of the type of plant offered on night 1 and on night 2. Treatments are indicated as X/Y where X indicates the type of plant on night 1, and Y indicates the type of plant on night 2. agg, *I. aggregata* and ten, *I. tenuituba.* Proportion probing two indole treatments (A) or two plant species (B and C) was compared with likelihood ratio X^2. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

(which could affect moth attraction from a distance). It is, however, possible that the low visitation to the all *I. aggregata* arrays also reflects other phenotypic differences between the species in flower shape or alpha-pinene emission (Bischoff et al. 2014). Laboratory assays showed that moths with experience visiting flowers of *I. tenuituba* were more likely to visit *I. aggregata* the following evening (Fig. 5C), whereas naïve moths never do. Thus, spatial proximity of color morphs within a scented local environment, and an apparent priming effect of foraging on scented flowers may reduce the specificity of floral choice by *H. lineata*.

The second aspect of ecological context is that the two contact sites differ in timing of hawkmoth foraging; *H. lineata* forages only after dark at GR but is often diurnal at PG (Campbell et al. 1997). Both visual perception and olfactory signals would differ between day and night. Hawkmoths in the current experiments after dark were more likely to probe the white flowers, whereas diurnally foraging *H. lineata* at PG do not preferentially visit naturally paler *Ipomopsis* flowers (Campbell et al. 1997) and often visit flowers of other colors, such as the blue-flowered *Delphinium barbeyi* (Campbell, pers. obsv.). In addition, *I. tenuituba* emits indole only at night (Bischoff et al. 2014), potentially explaining the fidelity of hawkmoths to *I. tenuituba* only at GR. Whether due to cold evenings (Heinrich 1971) or other factors, diurnally foraging *H. lineata* may rely less on scent and more on other traits to find flowers. Because the contact sites GR and PG differ not only in the level of ethological isolation, but also in the rate of hybridization as measured using molecular markers (Aldridge and Campbell 2009), the pollinator responses to floral scent and color not only contribute to ethological isolation, but also help to explain geographical variation in the extent of hybridization. Thus, the ecological context alters prezygotic reproductive isolation. Similar phenomena may occur in other systems, such as cichlid fishes for which female responses to male coloration differ between fish from turbid versus clear water lakes (Maan et al. 2010).

BEHAVIORAL RESPONSES OF NAÏVE AND EXPERIENCED HAWKMOTHS TO SCENT AND COLOR

As predicted, hawkmoth responses to scent and color led to greater visitation in the field for traits characteristic of *I. tenuituba*. Wild, experienced *H. lineata* were attracted to flowers by indole, and showed increased probing at white flowers, evidence for an intersignal interaction (Hebets and Papaj 2005). Previous studies of another hawkmoth, *M. sexta* (Raguso and Willis 2005; Goyret et al. 2007) also led us to predict a synergistic interaction, in which indole provides the context for recognition and feeding at white flowers. Instead, our results suggest that indole plays an alerting function (increasing floral encounters; Fig. 1) for

H. lineata, comparable to animals for which sequential attraction from a distance (olfactory or acoustic signals) gives way to close range (visual or contact chemical) signals in courtship (Leonard and Hedrick 2010; Uy and Safran 2013). Rather than synergistic, interactions between floral scent and color in our study appear additive when plants are in mixed arrays (Fig. 3). *Hyles lineata* moths will feed at flowers that present only indole or only white color under certain conditions (Figs. 2 and 4). This greater behavioral flexibility is consistent with the wide foraging breadth of *H. lineata* in comparison with the more narrowly restricted *M. sexta* (Raguso and Willis 2005).

Flight cage experiments with flower-naïve moths revealed an innate preference of *H. lineata* for *I. tenuituba* over *I. aggregata*, and specifically for flowers emitting indole (Fig. 5A and 5B). These responses contribute to species-specific feeding when moths are offered a mixture of *I. tenuituba* and *I. aggregata*, but can be mitigated by prior experience (Fig. 5C). Although small quantities of indole influence hawkmoth visitation at *Ipomopsis* flowers, a suite of additional VOCs are emitted (Bischoff et al. 2014), including other known antennal stimulants for hawkmoths (Raguso et al. 1996). We did not explicitly test whether indole is sufficient to attract hawkmoths, or whether indole is effective only in the presence of other VOCs emitted by flowers of both *Ipomopsis* species (Bischoff et al. 2014). Flowers of the two *Ipomopsis* species also differ in other VOCs, and the roles of those other compounds remain to be tested. Indole is a common scent component of white, hawkmoth-pollinated flowers worldwide (Knudsen and Tollsten 1993; Levin et al. 2001), including the wild ancestors of perfume sources, such as jasmine, gardenia, and jonquils (Kaiser 1993; Dobson et al. 1997), but it is not the only volatile attractant for hawkmoths (Raguso et al. 1996). This VOC plays diverse roles in other pollination systems, including the attraction of male euglossine bees to diurnal orchids (Williams and Whitten 1983) and female flies to brood-site deceptive flowers (Kite and Hetterscheid 1997), and can also influence herbivory (Andrews et al. 2007).

ETHOLOGICAL REPRODUCTIVE ISOLATION IN PARALLEL SYSTEMS

The repeated evolution of night-blooming, pale-colored, and scented hawkmoth-pollinated plants across the world (Haber and Frankie 1989; Martins and Johnson 2007) suggests broad applicability of our findings: first that wild hawkmoths preferentially visit on the basis of scent, contributing to ethological isolation, and second that they show reduced discrimination of other traits in the presence of large amounts of a preferred scent in close proximity. In western North America, species of *Aquilegia* provide a parallel system to *Ipomopsis*, with *Aquilegia formosa* pollinated mainly by hummingbirds and *A. pubescens* by hawkmoths (Grant 1992). Manipulative experiments demonstrated floral isolation due to flower orientation and spur length (Fulton and Hodges 1999; Hodges et al. 2002), however hybrid swarms also occur between these species and are visited at high frequencies by *H. lineata* (Fulton and Hodges 1999). Although the role of scent has not been investigated in this system, volatiles could play an additional role in the extent of reproductive isolation, just as indole contributes to the effects of flower color and shape in *Ipomopsis* (Campbell 2004).

Wind tunnel experiments have demonstrated responses of *M. sexta* moths to scent (methyl benzoate) in *Petunia*, with a preference for wild-type moth-pollinated *Petunia axillaris* over a white, unscented recombinant inbred line (RIL), and red-flowered, scented *P. exserta* like RIL over wild-type (unscented) hummingbird-pollinated *P. exserta* (Klahre et al. 2011). Although *M. sexta* did not distinguish between the trait-swapped RILs (red, scented vs. white, unscented) in binary choice experiments, the assays were performed in a wind tunnel, in which the moths flew upwind in the odor plume of the scented RILs before making a choice. Interestingly, when reciprocal RILs were constructed between *P. axillaris* and *P. inflata*, a bee-pollinated species (Dell'Olivo and Kuhlemeier 2013), *M. sexta* showed no preference between wild-type *P. axillaris* and an RIL expressing the purple allele from *P. inflata*, both of which emitted methyl benzoate, but strongly preferred a white flowered RIL of *P. inflata* over the purple flowered wild type. As for *H. lineata* foraging at *Ipomopsis*, these results suggest that hawkmoths show reduced color preference when foraging within a plume or cloud of an innately preferred scent. This phenomenon could also explain why hawkmoths in Brazil made more interspecific movements in plots of white, long-tubed *Nicotiana alata* and red, short-tubed *N. forgetiana* that included F_1 hybrids than they did in plots containing only the parental species, creating a hybrid bridge for gene flow between the species (Ippolito et al. 2004). If the F_1 plants emit intermediate amounts of scents, the arrays with hybrids added would have produced a more intense scent plume. Finally, hawkmoths in Japan did not preferentially visit plants with stronger total scent emissions in arrays containing hybrids between scentless and scented daylilies of the genus *Hemerocallis* (Hirota et al. 2012), which might be explained by a short interplant distance generating a strong scent cloud over most plants, particularly when the more strongly scented F_1 hybrids were used. In summary, floral scent contributes to ethological isolation via attraction in hawkmoth-pollinated plants, but close spatial proximity of unscented plants to scented plants, along with moth experience, can also lead to breakdown of this reproductive isolation.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.650sb. Data archival location is dryad (datadryad.org).

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Appendix: Impacts of Indole Augmentation in Plants Naturally Emitting Indole

HIGH-DOSAGE INDOLE TEST

Before examining the effects of indole in flowers that do not normally emit it, we first tested the effects of indole augmentation, using all *Ipomopsis tenuituba* inflorescences that emit the compound naturally. Both low- and high-dosage indole were tested in separate experiments. The high-dosage test utilized arrays of 12 inflorescences in a 3×4 matrix with 1 m between adjacent inflorescences, as for our main tests of indole and color manipulation (Supporting Information Fig. S3). Three treatments were assigned to positions in the individual arrays at random: 1 μl high-indole augmentation per flower, 1 μl paraffin control per flower, and natural untreated flowers. Four indole concentration arrays were observed for a total of 6 h on four nights from 29 May to 02 June 2012 at site GR, recording a total of 29 foraging bouts by *Hyles lineata*. In these arrays, all plants that attracted hawkmoths were also visited. We analyzed whether an inflorescence was visited by a hawkmoth using a generalized linear model with a binomial response and a fixed factor of indole treatment, along with array as a blocking factor. We also analyzed the per-flower visitation rate using randomized block ANOVA.

The proportion of inflorescences receiving visits (here = attraction) was similar whether each flower on the inflorescence received a high-dosage (1 μl of 100 ng/μl) augmentation of indole in paraffin, paraffin oil alone as a solvent control, or was left unmanipulated (generalized linear model with binomial response, $X_2^2 = 1.21$, $P = 0.546$). Mean visits per flower per hour was also similar across treatments (1.07, 1.07, and 1.05, respectively; randomized block ANOVA, $F_{2,42} = 0.01$, $P = 0.995$).

LOW-DOSAGE INDOLE TEST

In a second test of indole augmentation, we examined the impact of adding 1 μl of low-indole augmentation per flower to whole natural patches of *Ipomopsis tenuituba*, adding 1 μl paraffin per flower as a control to other patches. The experiment utilized a randomized block design, with each of 14 blocks containing 1 augmented and 1 control patch of *I. tenuituba* with approximately equal numbers of open flowers (mean = 49 flowers) and within a distance of 10 m. Two observers simultaneously recorded per-flower visitation rate during a minimum observation period of 90 min between 20:00 and 23:00 h during 12 June to 26 June in 2011. We found no difference in the per-flower visitation

rate of *Hyles lineata* between augmented and control patches of *I. tenuituba* flowers (Supporting Information Fig. S2; $F_{1,14} = 0.02$, $P = 0.89$). The two indole augmentation experiments together show that experimental indole addition is not a deterrent to hawkmoths, and the impact of indole concentration on visitation by *H. lineata* is not dosage dependent at levels equal to and above the natural amount emitted by *I. tenuituba* when all plants nearby are of that species.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Quantification of the high-dosage indole augmentation treatment applied to *Ipomopsis* flowers.
Figure S2. Mean and standard error for per-flower visitation rate of *Hyles lineata* to whole patches of *I. tenuituba* with and without low dosage indole added to the already scented flowers.
Figure S3. Layout of a sample array used in the field experiments and behaviors of *Hyles lineata* scored in each array.