

Pollinator isolation in Louisiana iris: legitimacy and pollen transfer

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ABSTRACT

Background: *Iris brevicaulis* and *Iris fulva* can be found in sympatric populations in southern Louisiana. Ethological pollinator isolation is an important reproductive barrier, as the pollination syndromes of *Iris brevicaulis* flowers result in primarily bumblebee visits, while the pollination syndromes of *Iris fulva* primarily result in hummingbird visits. Other potentially important components of pollinator isolation – namely, ‘legitimacy of visitation’ (i.e. whether or not a floral visitor physically interacts with a flower in such a way that it can pick up pollen from an anther or transfer pollen to a stigma) and ‘pollen transfer success’ (i.e. whether or not a ‘legitimate’ floral visitor successfully transfers pollen from one flower to the stigma of another) – have not been explored.

Hypotheses: Hummingbirds, the primary floral visitors of *I. fulva*, are more likely to visit *I. fulva* pollination units legitimately than they are the pollination units of *I. brevicaulis*. Furthermore, hummingbirds are more likely to successfully transfer pollen in *I. fulva* intraspecific visitation bouts compared with either interspecific or *I. brevicaulis* intraspecific floral visits. Bumblebees, the primary floral visitors of *I. brevicaulis*, are more likely to visit *I. brevicaulis* pollination units legitimately than they are those of *I. fulva*. In addition, bumblebees are more likely to successfully transfer pollen in *I. brevicaulis* intraspecific visitation bouts compared with interspecific or *I. fulva* intraspecific floral visits.

Methods: We recorded the proportion of legitimate floral visits made by bumblebees and hummingbirds to both *I. fulva* and *I. brevicaulis* flowers. We also assayed intraspecific and interspecific pollen transfer effectiveness among bumblebees and hummingbirds visiting *I. fulva* and *I. brevicaulis* flowers using powdered fluorescent dye as a pollen analogue.

Results: The proportion of legitimate hummingbird visits was higher in *I. fulva* flowers than in *I. brevicaulis* flowers, and hummingbirds were more effective at transferring pollen among *I. fulva* flowers than between *I. fulva* and *I. brevicaulis* flowers or among *I. brevicaulis* flowers. Conversely, the proportion of bumblebee visits that were legitimate was higher in *I. brevicaulis* than in *I. fulva*, and bumblebees were more effective at transferring pollen among *I. brevicaulis* flowers than between *I. brevicaulis* and *I. fulva* flowers or among *I. fulva* flowers. Calculations of reproductive isolation (*RI*) were quite high such that reproductive isolation due to hummingbird visitation legitimacy was calculated as $RI = 0.892$, and that due to bumblebee visitation legitimacy as $RI = 0.557$. Isolation due to hummingbird pollen transfer efficiency was calculated as $RI = 0.747$, and that of bumblebees as $RI = 0.711$.

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INTRODUCTION

Speciation involves the evolution of reproductive barriers that impede gene flow between divergent lineages, with early-acting pre-zygotic barriers potentially contributing more to the total isolation observed between species than later-acting reproductive isolating barriers (Dobzhansky, 1940; Coyne and Orr, 2004; Rieseberg and Willis, 2007; Widmer *et al.*, 2009). Because many closely related angiosperm species differ in floral morphology, colour, scent, and reward, this can result in differential attraction among various pollinator functional groups (Fenster *et al.*, 2004), suggesting that ethological pollinator isolation (Grant, 1949; Coyne and Orr, 2004) may contribute substantially to reducing gene flow between closely related species in sympatry (e.g. Ramsey *et al.*, 2003; Kay, 2006; Martin *et al.*, 2008). While differences in floral colour, reward, and morphology may result in differential pollinator visitation, strict floral specificity resulting in completely different suites of pollinators is likely rare (Waser *et al.*, 1996), and interspecific pollinator bouts may occur, albeit infrequently, in sympatric populations. During floral visitation bouts that include more than one species of plant, differences in floral morphology may result in differences in rates of effective pollen transfer between visited flowers. Grant (1949, 1994) described this form of pollinator isolation as ‘mechanical pollinator isolation’, which results from the inability of pollinators to effectively transfer pollen from the anther of one flower to the stigmatic surface of a heterospecific flower.

Since floral visitors are capable of recognizing differences in floral morphology and altering their foraging strategies accordingly (Goulson, 1999), categorizing components of pollinator isolation ‘neatly’ into separate ethological and mechanical components is potentially made difficult by the fact that the two forms of isolation may interact with one another. For example, while a potential pollinator may in fact be differentially *attracted* to alternative floral morphologies (a form of ethological isolation), the visitor may work the flowers in different ways such that it tends to ‘legitimately’ visit the flowers of one species while ‘illegitimately’ visiting the flowers of another. For instance, as a pollinator approaches a flower, the pollinator may access the reward (i.e. pollen or nectar) by legitimately entering the pollination unit in such a way that pollen could be deposited on the body of the pollinator (or transferred from the pollinator to the stigmatic surface). Alternatively, when a floral visitor approaches a flower, it may access the reward illegitimately without entering the pollination unit by accessing the nectary directly [termed ‘base working’ (Inouye, 1980)]. The legitimacy of floral visits (mediated by the behaviour of the floral visitor) may therefore depend on the floral morphology of the plant species attracting the floral visitor to its pollination unit (Inouye, 1980), which in turn may be an important barrier to interspecific hybridization. Thus, even if different species of plants attract a similar suite of floral visitors, reproductive isolation can result if visitors exhibit differential visitation legitimacy of the flowers of the two plant species. This form of ethological/mechanical isolation can actually be distinguished from a more easily categorized ‘pure’ form of mechanical isolation, where a floral visitor may legitimately visit the flowers of both species, yet interspecific bouts may reveal reduced pollen transfer success rates relative to intraspecific pollinator bouts. Many studies examining the effect of pollinators on reproductive isolation

have focused on ethological isolation as originally envisioned by Grant (1949), ‘owing to the constancy of the pollinating animals to one kind of flower’ (e.g. Ramsey *et al.*, 2003; Martin *et al.*, 2008), while the effect of mechanical pollinator isolation on reducing gene flow has been quantified in even fewer flowering plant systems (e.g. Kephart and Theiss, 2004; Kay, 2006). Fewer still have specifically examined the effects of ‘legitimacy’ (arguably an interaction among both ethological and mechanical isolating barriers) as an isolating barrier.

Natural hybridization of Louisiana irises has been investigated for almost a century (reviewed in Arnold, 1997, 2006; Arnold *et al.*, 2010). Hybrids formed between the species of Louisiana iris often do not suffer reduced fitness compared with the parents (Wesselingh and Arnold, 2000a, 2000b; Johnston *et al.*, 2001; Taylor *et al.*, 2009), indicating that pre-zygotic isolation is largely responsible for the total reproductive isolation observed in this study system. *Iris fulva* Ker-Gawler and *Iris brevicaulis* Raf. differ from one another in several floral morphological traits. *Iris brevicaulis* has a suite of traits often associated with a bee pollination syndrome such as blue flowers with marked yellow nectar guides and rigid sepals that serve as landing platforms for bees (Viosca, 1935; Bouck *et al.*, 2007), and bees visit *I. brevicaulis* flowers more often than *I. fulva* flowers in mixed-species arrays (Wesselingh and Arnold, 2000a; Martin *et al.*, 2008). *Iris fulva* flowers, on the other hand, have a number of traits that together are often associated with a hummingbird pollination syndrome, including red flowers without nectar guides, highly reflexed sepals, and protruding anthers (Viosca, 1935; Bouck *et al.*, 2007), and the primary visitor to *I. fulva* is the ruby throated hummingbird (Viosca, 1935; Wesselingh and Arnold, 2000a; Martin *et al.*, 2008). While these two species differ in their primary floral visitors, a majority of visits (80%) in mixed-species experimental arrays have been shown to be nearest-neighbour visits (Wesselingh and Arnold, 2000a), so ethological pollinator isolation is not a complete barrier between these species, and some amount of reproductive isolation is potentially generated by mechanical pollinator isolation – which has yet to be quantified in *Iris* to date.

Lowry *et al.* (2008) examined a number of known pre-zygotic isolating barriers between *I. fulva* and *I. brevicaulis* (immigrant inviability, flowering phenology, ethological pollinator isolation, and interspecific pollen competition) and calculated the total amount of pre-zygotic isolation due to these barriers as 0.752, which indicates that F1 hybrid formation should be relatively common (if only those barriers examined were acting to reduce hybridization). However, in naturally occurring sympatric populations of *I. brevicaulis* and *I. fulva*, the formation of new F1 hybrids has been shown to be exceedingly rare (Arnold *et al.*, 1993; Hodges *et al.*, 1996), likely owing to other forms of pre-zygotic isolation that have not yet been examined. Here, we consider two additional forms of pollinator isolation that have not been studied in Louisiana iris: pollinator legitimacy and pollinator effectiveness. In particular, we (1) demonstrate that hummingbirds and bumblebees differ in their ability to legitimately visit and transfer pollen among *I. brevicaulis* and *I. fulva*; (2) show that effective pollen transfer is significantly greater in intraspecific pollination bouts of both species than interspecific pollination bouts; and (3) calculate components of reproductive isolation separately for both legitimacy and pollen transfer success.

MATERIALS AND METHODS

Plant material

Rhizomes of both *I. fulva* and *I. brevicaulis* were collected from multiple localities in southern Louisiana. These plants were maintained in pots at the Texas State University

greenhouse until flowering plants were used for this study. The experiments were conducted in the spring flowering seasons of 2009 and 2010 in the field in southern Louisiana (for hummingbird experiments) and in screened flight enclosures at Texas State University (for bumblebee experiments). Pollinator legitimacy and pollen transfer experiments were conducted simultaneously at each location by setting up a single two-flower array at a time – two of the same *Iris* species for measuring intraspecific pollen transfer, and two different *Iris* species for measuring intraspecific pollen transfer. The anthers of one ‘focal’ flower were dusted with fluorescent dye, while the other flower was not manipulated.

Legitimacy of visitation

We observed visitation by both bumblebees and hummingbirds to the flowers of *I. brevicaulis* and *I. fulva* in two separate experiments. The overlap in flowering time between *I. brevicaulis* and *I. fulva* varies in nature and can be quite narrow (Cruzan and Arnold, 1994), with no overlap in some years (Martin *et al.*, 2007). As such, we utilized greenhouse-maintained plants that had a greater overlap in flowering time as well as flowers collected from natural allopatric populations that were co-flowering. In order to maximize the number of visits to these plants by a native bee pollinator that exists throughout the sympatric ranges of *I. fulva* and *I. brevicaulis* (Bartholomew *et al.*, 2006), we conducted the bumblebee studies in an enclosure (~2 m wide by 2 m tall by 8 m long) with a purchased colony of *Bombus impatiens* (Biobest, Inc.) at the Texas State University-San Marcos greenhouse. *Bombus impatiens*, the commercially available and common Eastern bumblebee, is now utilized extensively for studies involving pollination of greenhouse crops and can be purchased from a number of biological supply and agricultural supply companies (e.g. Stubbs and Drummond, 2001). All two-flower arrays (*I. fulva* × *I. fulva*, *I. fulva* × *I. brevicaulis*, and *I. brevicaulis* × *I. brevicaulis*) were observed for bee visitors, and every time a bee directly entered a pollination unit of either flower, the visit was recorded as legitimate (Fig. 1A). For bees that landed in the centre of either flower and accessed overflowing nectar by base working without entering the pollination unit, the visits were recorded as illegitimate visits because pollen transfer was not possible (Fig. 1B).

Similarly, we observed hummingbird visitation to both *I. brevicaulis* and *I. fulva* in the lower Atchafalaya River Basin in central Louisiana. To increase the number of different hummingbird individuals that visited focal flowers in this study, hummingbirds were drawn to the study site with artificial feeders. Immediately before data collection, the feeders were removed so that the hummingbirds would primarily visit the study flowers. Again, all two-flower arrays (*I. fulva* × *I. fulva*, *I. fulva* × *I. brevicaulis*, and *I. brevicaulis* × *I. brevicaulis*) were observed for hummingbird visitors, and visits to either flower were classified in one of two ways: legitimate or illegitimate. A legitimate hummingbird visit was recorded as one in which the bird hovered outside of the flower and inserted its head and beak directly into a pollination unit (Fig. 2A). An illegitimate hummingbird visit was recorded as one in which the bird base-worked the flower by inserting its beak straight into the nectary through the calyx, thereby bypassing the pollination unit (Fig. 2B). Differences in legitimate visitation rates among bees and hummingbirds were assessed for both *Iris* species (see ‘Data analysis’ below).

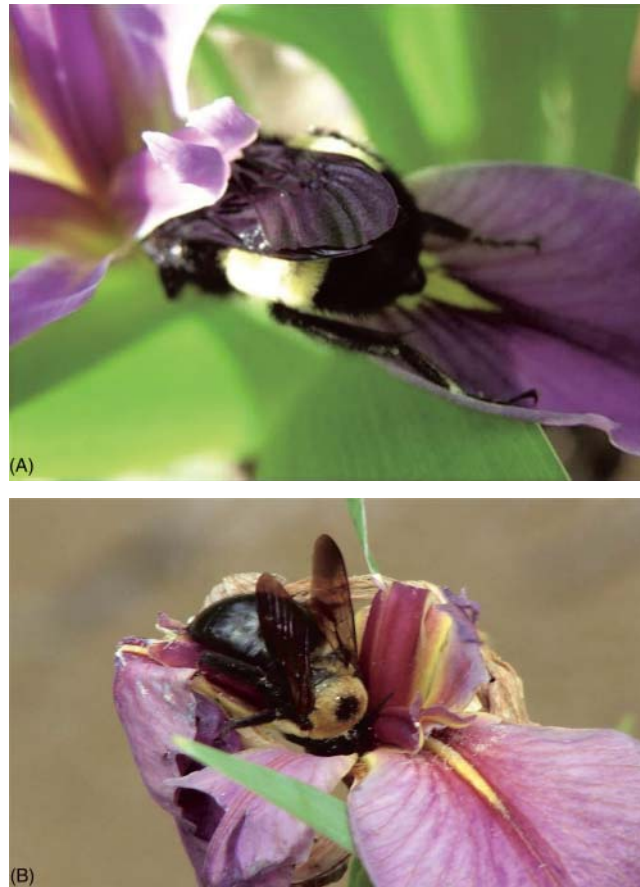


Fig. 1. Iris floral visitors: (A) legitimate bumblebee visitation and (B) illegitimate bumblebee visitation.

Pollen transfer success

Pollen transfer effectiveness was assayed in the two separate experimental designs described above. The pollen transfer effectiveness of bees was assayed using the captive colony at the Texas State University-San Marcos greenhouse, while hummingbird transfer effectiveness was assayed in southern Louisiana also using the same two-flower array study design described above.

Again, arrays included only two plants at a time: a focal flower and a recipient flower. The anthers of a focal flower were dusted with a fluorescent dye [commonly used as a pollen analogue because they are easily traceable (e.g. Shykoff and Bucheli, 1995; Goulson and Jerrim, 1997)] using a small paintbrush. The transfer of this pollen analogue was used as a proxy for determining the effectiveness of pollen transfer. While legitimacy data were collected on both flowers in the arrays, once a floral visitor (a bumblebee for the Texas State University experiment, a hummingbird for the southern Louisiana experiment) *legitimately* visited the dyed flower of the focal plant *and* subsequently visited a pollination unit of the other flower



Fig. 2. Iris floral visitors: (A) legitimate hummingbird visitation and (B) illegitimate hummingbird visitation.

in the array not dusted with dye, we immediately collected the non-dusted flower and saved it in sealed plastic bags, placed the bag on ice, and a new two-flower array with the anthers of a newly dusted focal flower was set up (alternating between intraspecific *I. fulva*, intraspecific *I. brevicaulis*, and interspecific arrays when sufficient experimental flowers were available). At the end of each day, the collected flowers were examined under ultraviolet light illumination of a dissecting scope for the presence of fluorescing dye on the stigmatic surface of the flower. If dye was located directly on or immediately adjacent to the stigma, the visit was recorded as an effective transfer. However, if the dye was not observed on the collected flowers or was not placed immediately on or around the stigma, the visit was recorded as an ineffective transfer. As the transfer rate of dye is higher than the transfer rate of pollen itself (Thompson *et al.*, 1986; Kearns and Inouye, 1993), we did not attempt to quantify dye concentration on the recipient stigma and simply analyse these data as presence/absence.

Intraspecific transfer success was examined for each class of visitor type (hummingbirds and bumblebees); however, only one interspecific transfer *direction* was tested for each visitor type. As bumblebees are preferentially attracted to *I. brevicaulis* (Martin *et al.*, 2008),

in the interspecific arrays, bees would almost always visit the *I. brevicaulis* flower first before either flying away or visiting the *I. fulva* flower. Likewise, because hummingbirds preferentially visit *I. fulva* (Martin *et al.*, 2008), hummingbirds almost always visited the *I. fulva* flower in the array first. As such, the ability of bumblebees to transfer pollen from *I. fulva* to *I. brevicaulis* and the ability of hummingbirds to transfer pollen in the opposite direction was not assayed – since an insufficient number of interspecific flights would have been recorded for analysis. No attempts were made to examine the pollen transfer success of illegitimate floral visitors because, once again, illegitimate visits do not result in contact between the floral visitor and the stigma/anther of the flower, and no dye would have been picked up by the visitor.

Data analysis

All data were analysed using separate χ^2 tests using 2×2 contingency tables. For the legitimacy data, we hypothesized that the pattern of pollinator visitation (legitimate or illegitimate) was dependent upon the plant species/pollinator species combination. Thus, a χ^2 test was performed to determine whether the number of legitimate and illegitimate visits (across all experimental days and across all interspecific and intraspecific arrays) differed between plant species for both bumblebees and hummingbirds.

For the pollen transfer experiments, we hypothesized that the proportion of effective pollen transfer was dependent upon the plant species/pollinator species combination. As bumblebees primarily visit *I. brevicaulis* (Martin *et al.*, 2008), we used a χ^2 test to determine whether there was a difference in the ability of bumblebees to transfer pollen from *I. brevicaulis* to *I. brevicaulis* and from *I. fulva* to *I. fulva*. We then used a χ^2 test to determine whether there was a difference in the ability of bumblebees to transfer pollen from *I. brevicaulis* to *I. brevicaulis* and from *I. brevicaulis* to *I. fulva*. Similarly, as hummingbirds primarily visit *I. fulva* (Wesselingh and Arnold, 2000a; Martin *et al.*, 2008), we used a χ^2 test to determine whether hummingbirds differed in their ability to transfer pollen between *I. fulva* flowers and between *I. brevicaulis* flowers. We then used a χ^2 test to determine whether hummingbirds differed in their ability to transfer pollen from *I. fulva* to *I. fulva* or from *I. fulva* to *I. brevicaulis*.

Because visitation legitimacy and visitation efficiency differ among hummingbirds and bumblebees, we calculated measures of reproductive isolation (*RI*) for legitimacy and for efficiency separately for the two pollinator classes. Martin and Willis (2007) devised methods for calculating measures of *RI* that reflect the degree to which each barrier reduces the chance of F1 hybrid formation relative to that of pure-species formation.

Reproductive isolation due to hummingbird visitation legitimacy was calculated as

$$RI = 1 - \frac{\text{proportion of legitimate visits to } I. \text{ brevicaulis}}{\text{proportion of legitimate visits to } I. \text{ fulva}},$$

while that due to bumblebee visitation legitimacy was calculated as

$$RI = 1 - \frac{\text{proportion of legitimate visits to } I. \text{ fulva}}{\text{proportion of legitimate visits to } I. \text{ brevicaulis}}.$$

Reproductive isolation based on the pollen transfer efficiency of hummingbirds was calculated as

$$RI = 1 - \frac{\text{proportion of } I. \textit{fulva} \text{ to } I. \textit{brevicaulis} \text{ dye transfer events}}{\text{proportion of } I. \textit{fulva} \text{ to } I. \textit{fulva} \text{ dye transfer events}},$$

while that based on the pollen transfer efficiency of bumblebees was calculated as

$$RI = 1 - \frac{\text{proportion of } I. \textit{brevicaulis} \text{ to } I. \textit{fulva} \text{ successful dye transfer events}}{\text{proportion of } I. \textit{brevicaulis} \text{ to } I. \textit{brevicaulis} \text{ successful dye transfer events}}.$$

In all four cases, measures of reproductive isolation range from 0 to 1, with 0 representing no reproductive isolation and 1 indicating that the barrier acts such that it completely prevents F1 hybrid formation. These measures of pre-zygotic isolation are analogous and comparable to measures of post-zygotic isolation in which $RI = 1 - (\text{hybrid fitness}/\text{pure-species fitness})$ (Ramsey *et al.*, 2003; Coyne and Orr, 2004; Husband and Sabara, 2004).

RESULTS

Legitimacy of visitation

Bumblebees, which preferentially visit *I. brevicaulis* over *I. fulva* (Martin *et al.*, 2008), also *legitimately* visited *I. brevicaulis* at significantly higher rates than flowers of *I. fulva* (74.3% vs. 32.9% respectively, Fig. 3A; $\chi^2 = 33.5$, d.f. = 1, $P < 0.0001$). Hummingbirds, which preferentially visit *I. fulva* flowers over those of *I. brevicaulis* (Martin *et al.*, 2008), also *legitimately* visited *I. fulva* flowers at significantly higher rates than those of *I. brevicaulis* (94.4% vs. 10.3% respectively, Fig. 3B; $\chi^2 = 80.1$, d.f. = 1, $P < 0.0001$).

Pollen transfer

As expected considering the floral morphologies, bumblebees were much more effective at transferring fluorescent dyes between *I. brevicaulis* flowers than between *I. fulva* flowers, with 72.2% of *I. brevicaulis*–*I. brevicaulis* intraspecific flights resulting in effective dye transfer, while only 7.69% of *I. fulva*–*I. fulva* flights resulted in effective dye transfer. These differences were highly significant ($\chi^2 = 7.4$, d.f. = 1, $P = 0.007$; Fig. 4A). Hummingbirds

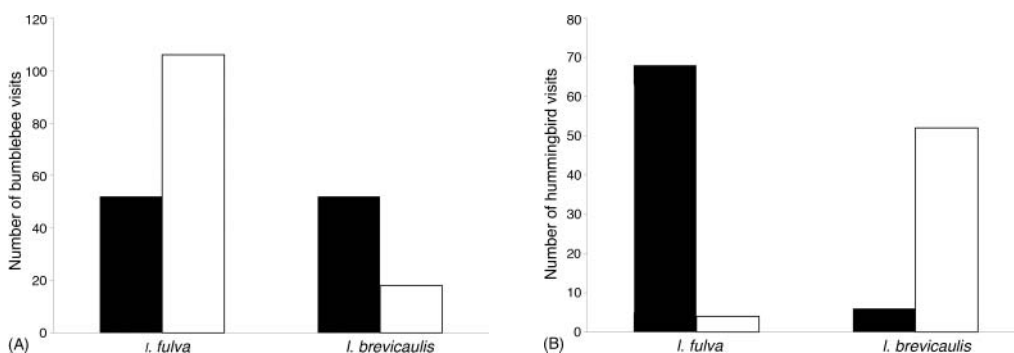


Fig. 3. The number of legitimate and illegitimate visits of (A) bumblebees and (B) hummingbirds to *I. fulva* and *I. brevicaulis*. Solid bars indicate the number of legitimate visits and the open bars indicate the number of illegitimate visits.

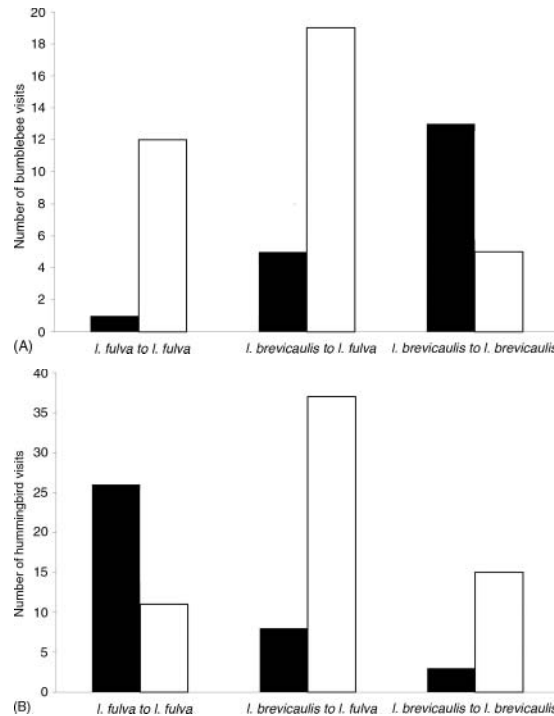


Fig. 4. The number of legitimate visits that were effective and ineffective in transferring pollen between flowers by (A) bumblebees and (B) hummingbirds. Solid bars indicate the number of visits that resulted in pollen transfer and the open bars indicate the number of visits that did not result in pollen transfer.

were much more effective at transferring fluorescent dye between *I. fulva* flowers than they were at transferring dye between *I. brevicaulis* flowers, with 70.3% (Fig. 4B) of *I. fulva*–*I. fulva* intraspecific flights resulting in effective dye transfer, and only 16.7% (Fig. 4B) of *I. brevicaulis*–*I. brevicaulis* observations resulting in pollen transfer. This difference was also highly significant ($\chi^2 = 9.83$, d.f. = 1, $P = 0.002$; Fig. 4B).

For F1 hybridization to occur between these two *Iris* species, a pollinator must visit a flower legitimately so that it can take up pollen, and subsequently make a legitimate visit to the flower of a heterospecific plant species. Of those heterospecific flights examined, bumblebees were significantly more effective at transferring pollen between two *I. brevicaulis* flowers (72.2%) than they were between *I. brevicaulis* and *I. fulva* flowers (20.8%; $\chi^2 = 11.1$, d.f. = 1, $P = 0.001$; Fig. 2). Hummingbirds were significantly more effective at transferring pollen between *I. fulva* flowers (70.3%) than between *I. fulva* and *I. brevicaulis* flowers (17.8%; $\chi^2 = 23.1$, d.f. = 1, $P < 0.001$; Fig. 2).

Measures of reproductive isolation

Calculations of reproductive isolation were quite high for all four measures. Reproductive isolation due to hummingbird visitation legitimacy was calculated as $RI = 0.892$, while that of bumblebee visitation legitimacy was $RI = 0.557$. Isolation due to pollen transfer

efficiency of hummingbirds was calculated as $RI = 0.747$, while that of bumblebees was calculated as $RI = 0.711$.

DISCUSSION

It is well known that pollinator isolation is an important barrier to gene flow between *I. fulva* and *I. brevicaulis*, although most studies to date examining pollinator isolation in this species pair have focused on ethological isolation, i.e. pollinator choice (Viosca, 1935; Martin *et al.*, 2008). In the present study, two other forms of pollinator isolation are described between *I. fulva* and *I. brevicaulis*. First, the primary floral visitors of these two species also differ in the proportion of legitimate and illegitimate visits made to each species. Hummingbirds, which visit *I. fulva* much more often than they do *I. brevicaulis* in sympatry (Martin *et al.*, 2008), also legitimately visited *I. fulva* more often than *I. brevicaulis* (Fig. 3), while bumblebees, which prefer to visit *I. brevicaulis* (Martin *et al.*, 2008), legitimately visited *I. brevicaulis* more so than they did *I. fulva* (Fig. 3). The difference in legitimate visitation is likely due to differences in floral morphology of the two species (Inouye, 1980). The reflexed sepals of *I. fulva* allow hummingbirds easy access to the nectar source while legitimately visiting the pollination unit, but bumblebees are unable to easily land on the reflexed sepals of *I. fulva* flowers and primarily visit *I. fulva* flowers illegitimately by base working (observations made during the present study). The stiff, upright sepals of *I. brevicaulis*, on the other hand, allow bumblebees to land on the sepal and access the nectar legitimately. However, this same sepal arrangement appears to hamper the ability of hummingbirds to hover outside of the pollination unit and directly access the nectar legitimately. This suggests that divergence in floral architecture results not only in differences in pollinator visitation rates, but also in a reduced potential for interspecific pollen transfer owing to the fact that hummingbirds and bumblebees, if they do visit *I. brevicaulis* or *I. fulva* respectively, do so illegitimately with no ability to pick up pollen.

In addition to differences in legitimate visitation rates, pollinators also differed in their ability to effectively transfer the fluorescent dye between flowers of the same species. Fluorescent dyes have been successfully used to examine pollen transfer in a variety of plant species with a diverse array of floral visitors, and the transfer of dyes has generally been shown to be positively correlated with pollen transfer (Fenster *et al.*, 1996; Rademaker *et al.*, 1997; Adler and Irwin, 2005). Waser (1988) examined the movement of both fluorescent dyes and pollen in hummingbirds and bees that visited *Delphinium nelsonii* flowers and found that while dyes closely tracked the movement of pollen in bees, for hummingbirds there was a slightly larger amount of pollen deposition and pollen carryover than that of dye – especially during long multi-flower foraging bouts. In the current study, only a single two-flower array was set up at a time, and floral visitors spent little time flying between the dye-dusted and non-dusted flowers. No grooming behaviour was noted during these brief inter-floral flights, and thus the fluorescent dyes used in this study are likely highly correlated with pollen transfer. As such, and as was perhaps predicted by the *Iris* pollination syndromes, hummingbirds showed significantly greater effectiveness in transferring the pollen analogue from *I. fulva* to *I. fulva* than from *I. brevicaulis* to *I. brevicaulis*, while bumblebees had greater effectiveness in transferring the pollen analogue from *I. brevicaulis* to *I. brevicaulis* than from *I. fulva* to *I. fulva*. As hummingbirds primarily visit *I. fulva* in arrays and only rarely legitimately visit *I. brevicaulis* (Martin *et al.*, 2008), we measured pollen transfer by hummingbirds from an *I. fulva* flower to an *I. brevicaulis* flower.

Hummingbirds were significantly less effective at interspecific dye transfer than intraspecific dye transfer. Fifteen of the interspecific bouts made by hummingbirds resulted in dye deposition on the sepal of the *I. brevicaulis* plant instead of the stigmatic surface, while absolutely no dye was found on the flowers for the other 22 ineffective visits. This suggests that at least 40% of the failed transfers were due to the inability of hummingbirds to deposit dye on the stigmatic surface of *I. brevicaulis*, not an inability of the hummingbird to pick up pollen from the *I. fulva* flower. Conversely, as bumblebees primarily visit *I. brevicaulis* in arrays and only rarely legitimately visit *I. fulva* (Wesselingh and Arnold, 2000a; Martin *et al.*, 2008), we measured pollen transfer by bumblebees from *I. brevicaulis* to *I. fulva*. Bumblebees were also significantly less effective at interspecific pollen transfer than intraspecific pollen transfer. When bumblebees performed interspecific pollination bouts, the majority of the visits resulted in deposition of fluorescent dye on the sepal of the recipient flower instead of on or near the stigmatic surface. This indicates that while the bees were potentially effective at picking up dye from the *I. brevicaulis* flower, they were likely unsuccessful at depositing the dye on the stigmatic surface of *I. fulva* due to the extreme differences in floral morphology.

Pre-zygotic isolating barriers have the potential to be the most important barriers at preventing current gene exchange between recently diverged hybridizing taxa for two major reasons. First, because reproductive barriers act in sequential fashion, the early-acting pre-zygotic barriers, simply because they act first, can effect more reproductive isolation than later-acting barriers (Schemske, 2000; Ramsey *et al.*, 2003; Martin and Willis, 2007). Second, measures of pre-zygotic isolation calculated for individual barriers, and across multiple flowering plant systems, have been shown to be about twice as strong as later-acting post-zygotic barriers (reviewed by Lowry *et al.*, 2008). Pollinator isolation, in fact, has been shown to be a strong barrier to gene flow in many disparate plant taxa, with measures of reproductive isolation caused by this early-acting barrier averaging about $RI = 0.65$ (from figure 1 in Lowry *et al.*, 2008). Interestingly, only ethological isolation has been included in those measures of pollinator isolation, and mechanical pollinator isolation is only rarely included in calculations of reproductive isolation (e.g. Kay, 2006). Although rarely quantified, mechanical pollinator isolation is potentially an important isolating barrier, especially between species that utilize the same pollinators (Kay, 2006).

The measures of reproductive isolation calculated in this study for these two components of mechanical pollinator isolation are not insubstantial and range from $RI = 0.557$ to $RI = 0.892$. Lowry *et al.* (2008) examined 19 hybridizing species pairs of flowering plants and found that the total reproductive isolation (RI_{total} = a multiplicative function of a number of identified pre-zygotic and post-zygotic isolating barriers) was greater than 0.95 for 15 of the 19 taxa. Interestingly, the total isolation that was calculated for *I. fulva* and *I. brevicaulis* was revealed to have the lowest value among all species pairs at $RI_{\text{total}} = 0.240$ (Lowry *et al.*, 2008). By incorporating reproductive isolation due to both forms of mechanical isolation examined in this study, hummingbird legitimacy and transfer patterns result in RI_{total} (using the methods of Lowry *et al.*, 2008 and Ramsey *et al.*, 2003) now being calculated as $0.97923 (1 - [(1 - 0.240) \times (1 - 0.892) \times (1 - 0.747)])$, and that of bumblebees as $RI_{\text{total}} = 0.784 (1 - [(1 - 0.240) \times (1 - 0.557) \times (1 - 0.711)])$. Thus, these previously unexamined reproductive isolating barriers have the potential to effect large amounts of pre-zygotic isolation between *I. fulva* and *I. brevicaulis*, potentially helping to explain the rarity at which F1 hybrids are observed in naturally occurring sympatric populations (Arnold, 1994; Arnold *et al.*, 1993; Hodges *et al.*, 1996). Mechanical isolation is rarely measured (but see Kay, 2006). Indeed, Lowry *et al.* (2008)

made no attempt to incorporate either form of mechanical isolation in their examinations of reproductive isolation in flowering plants. Considering the impact that these forms of reproductive isolation have on the total isolation calculated between *I. fulva* and *I. brevicaulis*, it is likely that this is an overlooked yet important reproductive barrier in other flowering plant systems also.

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