

Floral preference, flower constancy, and pollen transfer efficiency of the ruby-throated hummingbird (*Archilochus colubris*) in mixed arrays of *Iris nelsonii* and *Iris fulva*

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ABSTRACT

Background: *Iris nelsonii* is a homoploid hybrid species derived from three *Iris* species. (Homoploid hybrid species have the same number of chromosomes as their parent species.) *Iris nelsonii* shares a majority of its genome with one of its parents, *I. fulva*. The two species differ in floral colour and morphology. Pollinator isolation is a potential form of ecological divergence between a homoploid hybrid species and its parental species but the primary pollinator of both species is the ruby-throated hummingbird (*Archilochus colubris*).

Questions: Do hummingbirds prefer one of these two *Iris* species? Do hummingbirds exhibit flower constancy? Do hummingbirds transfer a pollen analogue between species?

Study system: The homoploid hybrid iris, *I. nelsonii*; its most closely related parental species, *I. fulva*; and the ruby-throated hummingbird.

Field site: Cypress Island Preserve, Breaux Bridge, Louisiana, USA.

Methods: We assayed hummingbird preference and constancy in a simple experimental array containing two flowers of each species. We assayed pollen analogue transfer efficiency in a separate experimental array containing two flowers: one dyed, the other not dyed.

Results: Hummingbirds did not show an initial preference for either flower when they entered multispecies arrays. But when a hummingbird first visited an *I. nelsonii* flower, it then visited another *I. nelsonii* flower significantly more than expected, revealing flower constancy that may result in reproductive isolation between these species of iris. Hummingbirds readily transferred pollen analogues both within and between species, so despite their morphological differences, mechanical isolation does not result in reproductive isolation of these species.

Keywords: pollinator isolation, floral isolation, mechanical isolation, ethological isolation, pollinator preference, homoploid hybrid speciation.

INTRODUCTION

Ecological isolation between species has recently received growing attention and has even been proposed as the underlying mechanism for most forms of reproductive isolation (Sobel *et al.*, 2010). Although ecological isolation is often described as the result of divergent selection acting on variation within an ancestral lineage, ecological isolation can also, and potentially more quickly, result from selection acting in a segregating hybrid population due to the large amount of genetic and morphological variation present in hybrid swarms. Ecological isolation is important for the stabilization of homoploid hybrid species (Buerkle *et al.*, 2000) and most homoploid hybrid lineages described to date are ecologically divergent from their parental species (Gross and Rieseberg, 2005). Although this ecological isolation is often seen in the form of habitat isolation, where the hybrid species is able to occupy a divergent habitat (Gross and Rieseberg, 2005), novel phenotypes available in hybrid populations could result in other forms of ecological isolation. For example, these hybrid phenotypes may be isolated from the parental species by pollinator isolation if the hybrid phenotype attracts a new pollinator guild (Straw, 1955) or is more attractive to pollinators than the parental species, thus increasing assortative mating in the hybrid lineage and reducing gene flow between the hybrid and its parental species [i.e. ethological pollinator isolation (Grant, 1994)]. Alternatively, a difference in floral morphology could result in differential pollen placement on the pollinator, thus reducing interspecific pollen deposition [i.e. mechanical pollinator isolation (Grant, 1994)]. A potential example of the evolution of pollinator isolation between a hybrid species and its parental species is present in the Louisiana Iris species complex.

Iris nelsonii Randolph, often cited as a classic example of homoploid hybrid speciation, was derived from hybridization between three widespread members of the Louisiana Iris group: *Iris fulva* Ker-Gawler, *Iris brevicaulis* Raf., and *Iris hexagona* Walt. (Randolph, 1966; Arnold, 1993). These species are all cross-compatible and hybrids produced between them have high fitness (Emms and Arnold, 1997; Johnston *et al.*, 2003; Taylor *et al.*, 2009), with pre-zygotic barriers likely most important in reducing gene flow between species. *Iris nelsonii*, like many other homoploid hybrid species (Gross and Rieseberg, 2005), occupies a novel habitat compared with the other species (Randolph, 1966) and responds differently to abiotic habitat conditions (Taylor *et al.*, 2011). Furthermore, pollinator isolation has been shown to reduce pollen flow between the red- and blue-flowered species in this complex, with hummingbirds primarily visiting the red flowers of *I. fulva* and *I. nelsonii* and bumble bees primarily visiting the blue flowers of *I. brevicaulis* and *I. hexagona* (Emms and Arnold, 2000; Martin *et al.*, 2008; Taylor *et al.*, 2012; J.P. Shaw *et al.*, unpublished).

Iris nelsonii is most genetically and morphologically similar to *I. fulva* (Randolph, 1966; Arnold *et al.*, 1990; Arnold, 1993) with which it largely overlaps in flowering phenology, and both species display floral morphologies characteristic of hummingbird pollination (Fig. 1). Despite many similarities in floral display between *I. nelsonii* and *I. fulva* (e.g. red colour, reflexed sepals), *I. nelsonii* flowers are much larger than those of *I. fulva*, have a closed stylar arm, and exhibit reduced variance in anther, petal, and sepal dimensions compared with populations of the progenitor species (Randolph, 1966) (Fig. 1). Such divergent morphology suggests that pollinator isolation may play an important role in reducing interspecific gene flow in sympatry.

Both *I. nelsonii* and *I. fulva* are most often visited (and pollinated) by ruby-throated hummingbirds (Wesselingh and Arnold, 2000a; Martin *et al.*, 2008; Taylor *et al.*, 2012; J.P. Shaw *et al.*, unpublished). However, in other systems pollinators have been shown to have a preference for larger



Fig. 1. Comparison of *Iris* floral phenotypes. *Iris fulva* flowers (A) are small, a copper red in colour, and have an open stylar arm (photograph courtesy of Steve Shepard). *Iris nelsonii* flowers (B) are large, a deep red in colour, and have a closed stylar arm. Photos were not taken at the same scale.

flowers (e.g. Galen and Newport, 1987; Stanton and Preston, 1988; Martin, 2004; Fenster *et al.*, 2006), so it is possible that hummingbirds differentially visit these two species because of the hummingbirds' preferences for larger floral phenotypes (Campbell *et al.*, 1997; Fenster *et al.*, 2006). Specifically, we hypothesized that the much larger floral size of *I. nelsonii* may be more attractive to hummingbirds than the much smaller flowers of *I. fulva*. In addition, we hypothesized that the divergent floral morphologies of these species could result in mechanical pollinator isolation. Here, we assayed floral preference, flower constancy, and mechanical isolation between *I. nelsonii* and *I. fulva* in experimental arrays.

MATERIALS AND METHODS

Components of pollinator isolation were assayed in arrays set up in southern Louisiana on 12–13 April near the peak of the 2013 flowering season. As these species are cross-compatible and pollen carryover cannot be controlled in natural populations, arrays with cut flowers were set up away from nearby populations of these species outside The Nature Conservancy's visitor centre at the Cypress Island Preserve at Lake Martin near Breau Bridge, Louisiana. Populations of *I. fulva*, *I. hexagona*, and interspecific hybrids are located on the preserve property, so hummingbirds are able to recognize and appropriately manipulate the plants. As *I. nelsonii* was derived from hybridization between at least these two species and has a floral phenotype composed of characteristics from each of these species – with only fruit morphology seemingly derived from *I. brevicaulis* (Randolph, 1966) – this property presents an opportunity to observe pollinator behaviour perhaps as it was when *I. nelsonii* was first derived.

Floral preference

Hummingbird visitation patterns were assessed in experimental arrays on 13 April 2013. For the duration of this experiment, only hummingbirds visited the experimental arrays. The arrays were composed of four cut iris flowers, two of *I. nelsonii* and two of *I. fulva*, with each flower placed into a clear plastic vase and arranged in the four corners of a 1-metre square. While individual iris stalks may have up to three flowers blooming at any one time, only stalks with single flowers at the terminus were utilized in the current study, and all flower stalks were cut to the same height. Iris flowers have three pollination units (see Fig. 1), and only legitimate visits (i.e. those where the hummingbird physically probed at least one pollination unit of a flower) were recorded. Whenever an individual hummingbird entered the array, the number of pollination units visited at each flower, the sequence of visits among the flowers, and the identity of each species of flower visited were recorded. After each foraging bout was completed and the hummingbird exited the array, all flowers were replaced with four new flowers. The location of each species in the array was changed after each foraging bout such that all six possible arrangements were presented to the hummingbird community in sequential order. These data, collected from 41 separate visitation bouts, were utilized to assess hummingbird preference and constancy.

Because *I. nelsonii* flowers are larger and showier than those of *I. fulva*, we hypothesized that hummingbirds might show a preference for these larger flowers. Three separate methods were used to test this hypothesis using the data generated from the above array observations. First, a non-parametric sign test was used to determine whether there was a significant difference with respect to the species identity of the first flower approached by the hummingbirds (41 bouts in total). Second, separate paired *t*-tests were employed to determine (a) whether the number of flowers and (b) the total number of pollination units visited per bout differed between the two *Iris* species. No transformations to correct for deviations from normality were conducted, as *t*-tests are robust to such deviations.

Flower constancy

Foraging bouts in which two or more different flowers in the array were visited by an individual hummingbird were examined to determine whether hummingbirds exhibited flower constancy, thereby reducing the chance for interspecific pollen flow. Only the first two visits to independent flowers were used to examine flower constancy. In the four-flower experimental arrays, once a hummingbird visits a flower of any particular species, it has a one-third probability of visiting a flower of the same species if it is foraging at random, and thus binomial tests were used to determine whether hummingbird foraging behaviour deviated from these random expectations. Three separate binomial tests were performed: one test examined the proportions of all intraspecific and interspecific foraging flights, and two additional tests examined those birds that initiated their foraging bouts on *I. fulva* and *I. nelsonii* flowers separately.

Following Martin and Willis (2007), a measure of ethological pollinator isolation ($RI_{\text{ethological}}$) due to flower constancy was calculated as:

$$RI_{\text{ethological}} = 1 - (\text{observed heterospecific transitions} / \text{expected heterospecific transitions}) / (\text{observed conspecific transitions} / \text{expected conspecific transitions}).$$

Again, the expected number of heterospecific transitions is two-thirds of the total, while the expected number of conspecific transitions is one-third of the total.

Pollen transfer

Previous work using fluorescent dye as a pollen analogue has revealed that the primary pollinators of both *I. fulva* and *I. nelsonii* are ruby-throated hummingbirds. However, due to the morphological differences between the two species, the degree to which pollen could be transferred during interspecific pollination bouts is unknown. On 12 April 2013, arrays were set up in which two flowers were placed ≈ 0.5 m apart, and fluorescent dye was dusted on all three anthers of a focal flower. After a hummingbird legitimately visited the focal flower and then legitimately visited the other flower in the two-flower array, the second flower was collected and stored on ice. The flower was then examined with an ultraviolet light in a dark room for the presence of dye on the stigmatic surface. Both flowers were replaced after each pollination bout, and three different colours of dye were alternatively used after each pollination bout to reduce the chance that dye carryover could affect the results.

RESULTS

Floral preference

Iris nelsonii has larger and showier flowers than *I. fulva*, and hummingbirds did show a general trend towards preferentially visiting this species. Because we initially hypothesized that hummingbirds would prefer *I. nelsonii* flowers over those of *I. fulva*, we report the results of both one- and two-tailed tests below. Hummingbirds did not appear to discriminate among the two *Iris* species when first entering the experimental array. Of the 41 visitation bouts recorded, 23 first-flower visits were to *I. nelsonii* and 18 to *I. fulva*, which is not significantly different (sign test: $P = 0.533$, 0.266; two- and one-tailed test respectively). Hummingbirds did tend to visit slightly more *I. nelsonii* flowers (1.24 ± 0.14 s.e.) than *I. fulva* flowers (0.79 ± 0.12) during their individual foraging bouts, but again this is not significantly different (paired t -test: $t = 1.451$, $P = 0.154$, 0.077; two- and one-tailed test respectively). When examining the total number of pollination units visited per foraging bout, hummingbirds visited the pollination units of the showier *I. nelsonii* flowers significantly more often than those of *I. fulva* (2.27 ± 0.30 , 1.66 ± 0.24 units respectively; $t = 1.747$, $P = 0.044$), yet this is only a marginally significant difference when a more stringent two-tailed test is applied ($P = 0.088$). Of the 41 bouts recorded in this study, hummingbirds visited *I. nelsonii* flowers exclusively in 12 of the bouts, and *I. fulva* flowers exclusively in eight of the bouts.

Flower constancy

This study revealed strong evidence of flower constancy, such that the species identity of the first flower visited was a strong indicator of the second flower to be visited. However, reproductive isolation due to flower constancy was quite asymmetric. Of all bouts where at least two independent flowers were visited, hummingbirds next visited the conspecific flower 57.1% of the time, significantly more often than the 33.3% expected (binomial test: $N = 28$, $P = 0.008$), and $RI_{\text{ethological}}$ was calculated to be 0.625. This was largely due to the

fact that when hummingbirds visited *I. nelsonii* first, they also next visited the other *I. nelsonii* flower in the array 68.8% of the time, again significantly more than the 33.3% expected (binomial test: $N = 16$, $P = 0.004$), and $RI_{\text{ethological}}$ for *I. nelsonii* first-visits was calculated as 0.773. Hummingbirds that visited *I. fulva* first did not subsequently visit the other *I. fulva* in the array more than expected. In fact, of the 12 bouts in which *I. fulva* was visited first, 36.8% ($N = 5$) of the subsequent flowers that were visited were *I. fulva*, which is not significantly different from 33.3% (binomial test: $N = 12$, $P = 0.368$). $RI_{\text{ethological}}$ for *I. fulva* first-visits was therefore not calculated.

Pollen transfer

In total, 27 foraging bouts were made to the two-flower experimental arrays set up to ascertain mechanical pollinator isolation. Previous datasets show that hummingbirds are efficient at pollen transfer between *I. nelsonii* flowers [pollen transfer in 80.0% of bouts (Taylor *et al.*, 2012)] and between *I. fulva* flowers [pollen transfer in 70.3% of bouts (J.P. Shaw *et al.*, unpublished)], so efforts were focused on interspecific transfer instead of collecting more data on intraspecific transfer. Two intraspecific bouts for each species were documented in the current study. All four bouts revealed pollen transfer from the dyed flower to the recipient flower. Twenty-three interspecific bouts were also recorded. Of the 11 bouts in which the *I. fulva* flower was dyed, the recipient *I. nelsonii* flower received the dye on its stigmatic surface in all 11 bouts. For the 12 bouts in which the *I. nelsonii* flower was dyed, the *I. fulva* flower received dye on its stigmatic surface in only 75% ($n = 9$) of the bouts, indicating a potential asymmetry with respect to interspecific pollen transfer, although this asymmetry is not significant (χ^2 2×2 contingency test: $\chi^2 = 3.16$, $P = 0.075$). No effort was made to calculate mechanical pollinator isolation because the sample size of intraspecific bouts in the current study was low. However, there clearly appears to be no reproductive isolation when examining *I. fulva*–*I. nelsonii* pollen transfer, as all interspecific bouts were successful. Furthermore, the 75% *I. nelsonii*–*I. fulva* transfer rate does not dramatically differ from previously published intraspecific *I. nelsonii* transfer rates [80.0% (Taylor *et al.*, 2012)] or intraspecific *I. fulva* transfer rates [70.3% (J.P. Shaw *et al.*, unpublished)].

DISCUSSION

Hybrid species represent rare instances where divergence most likely occurred in initial sympatry and are thus great tools for examining the early stages of speciation. The chance for establishment of a new hybrid lineage is often limited by simple demographic factors in sympatry because all else being equal, the newly formed hybrids are most likely to mate with individuals of either parental species because those individuals, at least initially, will be the most abundant. However, if the newly forming hybrid lineage is ecologically divergent from the parental species, the establishment of a new hybrid species is more likely (Buerkle *et al.*, 2000). For example, floral isolation could potentially reduce the minority disadvantage of new hybrid lineages by increasing the probability that hybrid flowers will mate with one another instead of with either of the parental species.

Like many other identified homoploid hybrid species, *Iris nelsonii* is primarily isolated from its parental taxa by abiotic habitat characteristics (Randolph, 1966; Taylor *et al.*, 2011). However, despite this strong habitat isolation, *I. nelsonii* does occasionally occur in local sympatry with all three of its parental species (L. Dietz and N. Martin, unpublished data), so other

barriers are important at preventing gene flow. Floral isolation in the form of pollinator preferences is likely strong between the hummingbird-pollinated *I. nelsonii* and its two blue-flowered bee-pollinated parents, *I. hexagona* and *I. brevicaulis*, but floral isolation between the two hummingbird-pollinated species (*I. fulva* and *I. nelsonii*) had not yet been documented. Floral preference, flower constancy, and pollen transfer success were assayed for hummingbird visitors to experimental arrays of *I. nelsonii* and *I. fulva* on 12 and 13 April 2013. While this experiment was conducted over a relatively short duration, the hummingbird behaviours exhibited during this period are likely representative of hummingbird behaviours throughout the flowering season. At the Lake Martin hybrid zone, flowering began on 22 February 2013 (N. Martin, personal observation), and hummingbirds had ample time to learn how to manipulate Louisiana Iris flowers. Indeed, in the current study, hummingbirds approached and manipulated both *I. nelsonii* and *I. fulva* flowers by inserting their beaks directly under and parallel with the stylar arms (Fig. 1), and is the most common form of approach/manipulation by these pollinators throughout the range of both *I. fulva* and *I. nelsonii* (Martin *et al.*, 2008; Taylor *et al.*, 2012).

Floral preference

Louisiana Iris hybrids receive substantially increased pollinator activity (Wesselingh and Arnold, 2000a; Martin *et al.*, 2008) and are often preferred relative to their parental species by both hummingbird and bee visitors, owing to the fact that the nectar reward is much greater (Wesselingh and Arnold, 2000b). We hypothesized that this increased pollinator visitation of hybrid plants was potentially important during the early establishment of *I. nelsonii* and maintenance of current species boundaries.

In the current study, hummingbirds, which are the primary pollinators of both *I. fulva* and *I. nelsonii* (Emms and Arnold, 2000; Wesselingh and Arnold, 2000a; Martin *et al.*, 2008; Taylor *et al.*, 2012), preferred *I. nelsonii* only slightly more than *I. fulva*. They tended to visit more individual *I. nelsonii* flowers than *I. fulva* flowers (though not significantly so), and visited significantly more pollination units of *I. nelsonii* flowers than those of *I. fulva*. However, the lack of strong preference for one species or another suggests that pollinator preference alone would not substantially restrict gene flow between *I. nelsonii* and *I. fulva*.

Flower constancy

Flower constancy is a common foraging strategy (Waser, 1986; e.g. Grant, 1950; Goulson and Cory, 1993; Goulson and Wright, 1998), and this tendency to reduce heterospecific floral visits can result in ethological isolation (Grant, 1994). This assortative visitation combined with increased preferences (above) by pollinators may be particularly important in the early establishment of new homoploid hybrid species where the relative abundance of the new taxon is likely to be low. In the current study, flower constancy by hummingbirds is an asymmetric component of reproductive isolation. When hummingbirds first visited *I. nelsonii* in the experimental arrays (which happened most often owing to the fact that hummingbirds revealed a slight preference for these larger, more showy flowers), the next flower they visited was also *I. nelsonii* 68.8% of the time, a more than twofold increase over what would be expected if hummingbirds were visiting flowers at random. When hummingbirds first visited *I. fulva*, however, they did not subsequently visit the other *I. fulva* in the array more than expected. Therefore, reproductive isolation due to flower constancy was quite

asymmetric (and high), with $RI_{\text{ethological}}$ for *I. nelsonii* first-visits being calculated at 0.773, while there was no observed reproductive isolation for *I. fulva* first-visits. As such, the pollen from a focal *I. nelsonii* flower is likely to be deposited on another *I. nelsonii* flower, but *I. nelsonii* flowers are equally likely to receive pollen from *I. nelsonii* or *I. fulva*. Thus, ethological pollinator isolation in the form of flower constancy may contribute to reproductive isolation between these species when paired with later acting barriers, such as differential pollen tube growth. As *I. fulva* pollen tubes have been shown to be shorter than hybrid and *I. hexagona* pollen tubes on hybrid plants (Carney *et al.*, 1994), and the styles of *I. nelsonii* flowers are likely longer than those of *I. fulva*, the *I. fulva* pollen tubes may not grow long enough to reach *I. nelsonii* ovules after hummingbirds visit *I. nelsonii* during an interspecific foraging bout. Later-acting, post-pollination barriers such as differential pollen germination and pollen tube growth are yet to be investigated between these two species.

Pollen transfer

We further hypothesized that the difference in size and shape of the pollination units of *I. nelsonii* and *I. fulva* would result in mechanical isolation where pollen would not be transferred from the anther of one species to the stigma of the other (Grant, 1994). The results of this study, however, reveal that mechanical isolation does not contribute to reproductive isolation between *I. nelsonii* and *I. fulva*. Using fluorescent dyes as pollen analogues, interspecific dye transfer rates were quite high (100% success from *I. fulva* to *I. nelsonii*, and 75% success from *I. nelsonii* to *I. fulva*), and not different from intraspecific dye-transfer rates observed in other studies on these two species (Taylor *et al.*, 2012; J.P. Shaw *et al.*, unpublished). Even though fluorescent dyes may transfer more readily than pollen (Thomson *et al.*, 1986), the fact that the dye was transferred from the anther of one species to the stigmatic surface of the other (and vice versa) shows that the morphology of the species does not result in differential pollen placement so interspecific pollen transfer is possible, but perhaps not at rates of 100%. Thus, despite the differences in floral morphology between these species, mechanical isolation does not effect reproductive isolation between *I. nelsonii* and *I. fulva*.

CONCLUSION

The establishment and maintenance of homoploid hybrid species is clearly facilitated when the new hybrid lineages are ecologically isolated from their parental species (Gross and Rieseberg, 2005). The present study revealed some asymmetric reproductive isolation due to flower constancy of hummingbirds, but this barrier is clearly not sufficient to completely restrict interspecific gene flow. Other prezygotic barriers resulting in ecogeographic/habitat isolation are primarily responsible for reducing the chance for hybridizing in nature (Randolph, 1966). We are currently performing experiments to determine the strength of those and later-acting barriers between *I. nelsonii* and its parental species.

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