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FLORAL SYMMETRY AFFECTS BUMBLEBEE APPROACH CONSISTENCY IN ARTIFICIAL FLOWERS

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Abstract—Bilateral symmetry has evolved from radial symmetry in several floral lineages, and multiple hypotheses have been proposed to account for the success of this floral plan. One of these hypotheses posits that bilateral symmetry (or, more generally, a reduced number of planes of floral symmetry) allows for more precise pollen placement on pollinators. Greater precision would maximize the efficacy of pollen transfer to conspecifics, while minimizing reproductive interference amongst plant species. Despite the intuitiveness of this hypothesis, it has little experimental support. Here, we tested whether a reduction in the number of floral planes of symmetry (as in the transition from radial to bilateral symmetry) increases the potential precision of pollen placement. We analyzed video recordings of bumblebees (*Bombus impatiens*) visiting artificial flowers to determine whether consistency in flower entry angle differed between radial (round) and disymmetric (rectangular) "flowers". We observed more consistent entry angles for disymmetric flowers than for radial flowers, with entry angles to radial flowers 43% more variable on average (standard deviations of 30° vs. 21°). Bees trained on flowers with an intermediate (square) morphology exhibited a slight, non-significant preference for radial symmetry over disymmetry. Our results show that disymmetry—an evolutionarily intermediate form of floral symmetry—has the potential to increase pollen transfer to conspecific stigmas, relative to radial symmetry. Thus, evolutionary reduction in the number of planes of floral symmetry likely provides benefits in terms of pollen delivery, as suggested by the pollen-placement-accuracy hypothesis. These findings offer insight into the evolution of floral symmetry.

Keywords: artificial flowers, Bombus, floral symmetry, pollen placement, preference, zygomorphy

INTRODUCTION

Bilateral floral symmetry (zygomorphy) has evolved from the ancestral state of radial symmetry (actinomorphy) multiple times across the angiosperms (Donoghue et al. 1998; Damerval & Nadot 2007; Hileman 2014; reviewed by Citerne et al. 2010). Various hypotheses have been put forth to explain this evolutionary trend (reviewed by Neal et al. 1998). For example, greater distinctiveness of zygomorphic flowers may promote pollinator fidelity (Ostler & Harper 1978), or pollinators may have an innate preference for bilateral symmetry (Leppik 1953). While greater pollinator fidelity towards bilateral flowers has been observed (Fenster et al. 2004), evidence for floral-symmetry preference is mixed (e.g., West & Laverty 1998), with previous studies finding pollinator preferences for both radial (Free 1970; Wignall et al. 2006) and bilateral (Leppik 1953; Rodríguez et al. 2004; Lázaro et al. 2008) floral symmetry.

Another hypothesis, the pollen-placement-accuracy (or pollen position) hypothesis, states that flowers with fewer planes of symmetry promote more consistent entry by pollinators into the flower, such that the plant's reproductive parts—both male and female—make more consistent contact with certain parts of pollinators' bodies (Leppik 1972; Macior 1974). This consistency would maximize the efficiency of pollen transfer while minimizing reproductive interference amongst pollinator-sharing floral species (Muchhala & Thomson 2010). Precise placement of reproductive parts reduces pollen waste, increases the accuracy of stigma contact with pollinators (Armbruster et al. 2009), and reduces the chances of stigma-clogging by heterospecific pollen, thereby potentially increasing reproductive output of both the pollen parent and the seed parent (Morales & Traveset 2008; Armbruster & Muchhala 2009). This should benefit plant fitness by maximizing the amount of potential reproduction per unit of floral reward produced (Muchhala et al. 2010; Muchhala & Thomson 2012; Armbruster 2014).

Despite the logic underlying the pollen-placement-accuracy hypothesis, there is little experimental evidence to support it. We therefore set out to test this hypothesis experimentally, by training laboratory-reared bumblebees to visit artificial flowers, and measuring the angle at which these bees entered both radially symmetric and disymmetric "flowers". We used disymmetry, in which flowers possess only two planes of symmetry, to represent an evolutionary step from radial towards bilateral symmetry, in which flowers possess a single plane of symmetry. Indeed, disymmetry appears to be an evolutionarily intermediate stage between radial and bilateral floral symmetries in some plant lineages (Endress 1999; Friis et al. 2011). We expected pollinators to enter disymmetric flowers at a more consistent angle than radially symmetric flowers. We also tested for a symmetry preference in our bees, as a preference for radial symmetry

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*Corresponding author: jforrest@uottawa.ca across several pollinator groups could help explain the persistence of radial symmetry in spite of less efficient pollen transfer.

MATERIALS AND METHODS

Experimental design

Colonies of captive-reared bumblebees (*B. impatiens* Cresson) were connected to a 60 × 60 × 180 cm flight cage by a gated tunnel that allowed for controlled entry and exit of individual bees. During initial training, bees were allowed to forage freely on artificial flowers until consistent foraging began and reliable foragers could be individually marked using dots of coloured paint applied to the dorsum of the thorax. Flowers consisted of 1.5 mL Eppendorf tubes with the lids removed, ringed by blue plastic 11.4 cm^2 "corollas" (details below). Training flowers were filled with 30% (*w/w*) sucrose solution ("nectar") and were presented in the horizontal plane (i.e., corollas parallel, and Eppendorf tubes perpendicular, to the floor of the cage) to allow us to fill Eppendorf tubes to the brim during initial training. Training flowers were replaced every second day to avoid build-up of sucrose residue or bee scent marks on corollas.

Two colonies were used in this study. The first colony was used to compare entry-angle variability between radial and disymmetric flowers. The second colony was used both to measure entry-angle variability and to assess floral symmetry preference. For the first colony, training flowers were identical to our radially symmetric flowers (described below). For the second colony, we used 3.4 × 3.4 cm square corollas for training in an effort to minimize bee bias towards a particular symmetry before completion of the preference test. Pollen was provided to the colonies daily.

For testing entry-angle consistency, 5.5 × 2.1 cm blue plastic rectangles were used to represent corollas of disymmetric flowers, and circles with radii of 1.9 cm represented corollas of radially symmetric flowers (Fig. 1). The surface area of all corollas (both experimental and training) was the same (11.4 cm^2). Flowers (corollas and Eppendorf tubes) were washed with warm water and detergent, rinsed, and dried prior to each foraging bout. The first colony, of which 6 bees were individually observed (15 Dec. 2014–30 Jan. 2015), foraged on a setup consisting of 9 flowers that were either entirely radial or entirely disymmetric. The centre of each flower was 8–10 cm from the centre of the closest neighbouring flower. The second colony, of which 10 bees were individually observed (13 Mar.–2 Apr. 2015), foraged on a setup consisting of a combination of radial and disymmetric flowers (10 of each symmetry) in a 5 × 4 matrix. Flowers were set up in a pattern that allowed for an equal ratio of neighbouring radial and disymmetric flowers. For both colonies, flowers were presented in a single vertical plane (i.e., corollas perpendicular, and Eppendorf tubes parallel, to the floor of the cage) (Fig. 1). Each flower contained 3.5 µL of nectar at the start of a foraging bout and was refilled as soon as it was emptied by a bee. For the first colony, each bee performed 2–3 foraging bouts per flower symmetry (approximately 60 visits per symmetry per bee; Appendix 1), with alternation of symmetries between foraging bouts and alternation of starting symmetries among individual bees. For the second colony, three foraging bouts per bee were performed overall (approximately 44 visits per symmetry per bee; Appendix 1). Each individual bee's foraging bouts were recorded by video camera and still photographs were extracted as the bee entered a flower. Entry was identified as insertion of the entire thorax of the bee into the Eppendorf tube. Any entry attempts or movements that did not meet this criterion were ignored and not measured. ImageJ (National Institutes of Health, Version 1.49k, 2014) was used to measure the angle at which bees entered flowers by drawing two lines (one for the vertical midline of the flower and one for the midline of the bee) and measuring the angle between these lines (Fig. 2). The midline from the centre of the flower to the bottom of the flower was considered to be 0°, and arbitrary designations of positive for left of 0° and negative for right of 0° were used to measure directionality. This meant that entries left of the midline theoretically ranged from 0°–180°, and entries right of the midline could range from −180°–0°. For testing flower symmetry preference, we counted the number of flower entries per symmetry by the 10 bees (from the second colony) that foraged on the mixed (10 radial: 10 disymmetric) array.

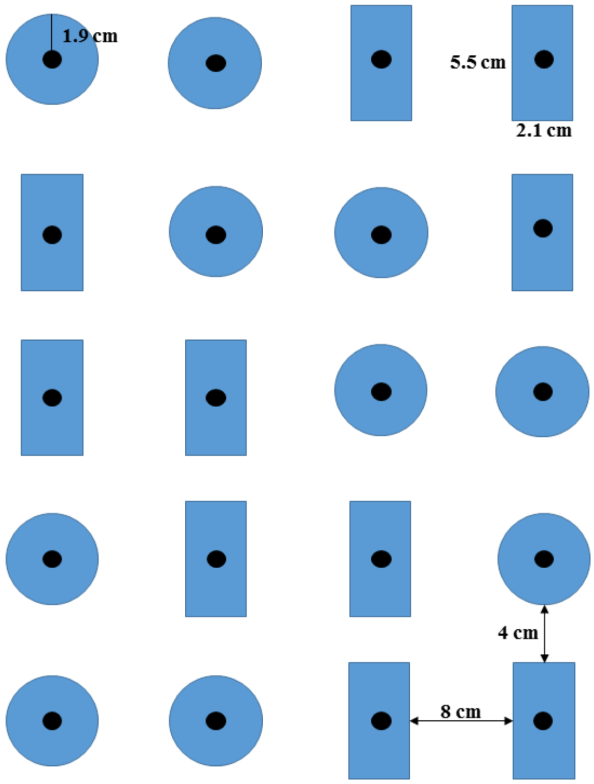

FIGURE 1. Experimental setup of artificial flowers for the second *Bombus impatiens* colony. Note the presence of both radial and disymmetric (rectangular) "flowers". The array was set up in a vertical configuration (i.e., the "flowers" in the top row of the array were closest to the roof of the flight cage; those in the bottom row were closest to the floor; and rectangular flowers were oriented so as to be longest top-to-bottom).

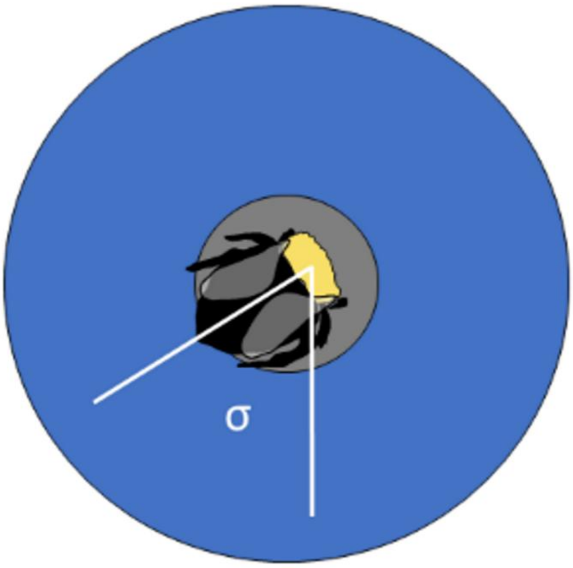

FIGURE 2. Schematic representation of an entry-angle measurement, as performed for each flower visit. Entry angle was measured as the bee's thorax completely entered the flower.

Statistical analyses

For each bee on each symmetry, the standard deviation of entry angle was calculated separately for each foraging bout. We ran linear mixed models using the R (R Core Team, Version 3.1.1, 2014) package lme4 (Bates et al. 2015) to test for an effect of floral symmetry on these standard deviation values, which were normally distributed. We included colony and bee nested within colony as random factors in the models. To test for symmetry preference, we modelled the number of flower visits received (per foraging bout) from bees in the second colony, as a function of floral symmetry and bout number (a categorical factor). Because these visit counts were normally distributed, we again used linear mixed-effects models, with bee as the random factor. For all models, we tested the significance of predictor variables using likelihood-ratio tests of nested models.

RESULTS

Observed flower-entry angles ranged from −140° to 159° for radially symmetric flowers and from −60° to 169° for disymmetric flowers. However, bees typically entered flowers from below: only 12 of 1585 entry angles (< 1%) had absolute values ≥ 90°. Entry angles were more consistent for disymmetric flowers than radial flowers for 15 of the 16 bees tested (Fig. 3). Standard deviations of entry angles were approximately 9° lower (95% C.I. = 6.59–11.32) for bees entering the disymmetric flowers (mean SD = 21°) than for bees entering radial flowers (mean SD = 30°; LMM, $X_1^2 = 24.1$, $N = 16$ bees, $P < 0.0001$). Excluding visits with extreme entry angles (those with absolute values ≥ 90°) and recalculating the standard deviations only strengthened this result (LMM, $X_1^2 = 47.4$, $N = 16$ bees, $P < 0.0001$). There was no significant difference in entry-angle variability between the two colonies, despite their different training regimens ($X_1^2 = 0.10$, $N =$ 16 bees, $P = 0.75$), nor did mean entry angles differ significantly between floral symmetries (1.8° for disymmetric; 3.0° for radial; LMM, $X_1^2 = 0.06$, $N = 16$ bees, $P = 0.80$). Bees showed a non-significant preference for radial flowers (average proportion of visits to radial flowers = 0.53; $X_1^2 = 0.9$, $N = 10$ bees, $P = 0.34$; Fig 4).

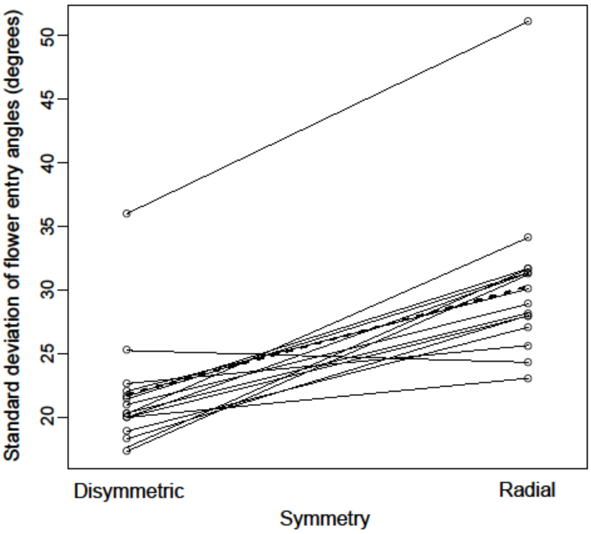

FIGURE 3. Standard deviations of flower entry angles for all bees on disymmetric and radial symmetries. Lines connect radial and disymmetric values for a given individual. The average trend across all bees is depicted by the thick, dashed line. $N = 16$ bees; note overlap between several points and lines.

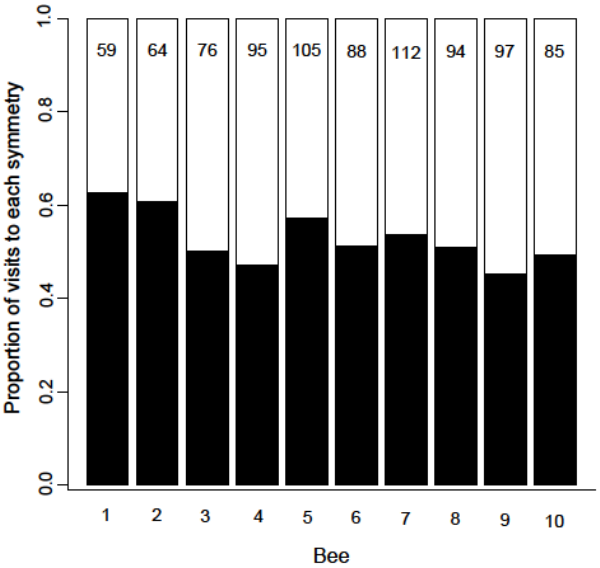

FIGURE 4. Proportion of visits made by bees to flowers of each symmetry. The lower portion of each column (black) represents visits to radial flowers; the upper portion of each column (white) represents visits to disymmetric flowers. Total number of flower visits made by each bee is given at the top of the appropriate column.

DISCUSSION

Floral symmetry affects the consistency with which bees enter flowers. We found more consistent floral entry angles by bumblebees on disymmetric artificial flowers than on radially symmetric flowers. Assuming that more consistent floral entry allows plants to more precisely place pollen onto (and receive pollen from) their pollinators (Armbruster et al. 1994; Wang et al. 2014), disymmetric flowers should have a fitness advantage over radial flowers—perhaps especially in species-rich plant communities where plants share pollinators. If, more generally, flowers with fewer planes of symmetry exhibit more precise pollen placement and therefore more efficient deployment of resources, then the evolution of bilateral symmetry should be favoured. Thus, our results may help explain the evolution, in many plant lineages, of bilateral floral symmetry from ancestrally radial symmetry.

Our artificial flowers did not have reproductive structures, and we did not measure actual pollen transfer. Artificial flowers with male and female "reproductive organs" and dye as a pollen analogue (see Fenster et al. 1996; Adler & Irwin 2006; Thomson et al. 2012) could be developed in the future to determine whether transfer rates differ between radial and bilateral flowers. In this study, we have imagined our flowers as having few or bilaterally arranged reproductive structures (e.g., the conjoined stamens or staminal columns of orchids, lobelioids, and many legumes), such that consistent pollinator entry angles would allow consistent dorsal or ventral pollen placement. (Disymmetric corollas combined with numerous, radially arranged reproductive organs would not be expected to increase pollen-placement precision, regardless of their effect on pollinator behaviour.) In fact, Rudall & Bateman (2004) have noted that the evolution of bilateral symmetry is associated with stamen suppression (the development of fewer stamens per flower), and suggested that the latter was a preliminary step towards the development of bilateral symmetry. Stamen suppression frequently results in flowers with a single functional stamen, most commonly positioned at the top of the flower. Alternatively, the evolution towards zygomorphy may have been initiated by a shift in placement of stamens to one side of the flower (e.g., as seems to have occurred in *Passiflora* and in *Schlumbergera* cacti). This evolutionary scenario (reduction or rearrangement of reproductive organs first; selection on corolla symmetry second) was the premise for our current study and would be the logical starting point for future studies measuring actual pollen transfer.

Although several angiosperm lineages have evolved bilateral symmetry, many others have not, and some may have even reverted to radial from bilateral symmetry (Donoghue et al. 1998). This suggests there are benefits to radial symmetry. One possible explanation for the persistence of radial symmetry is that pollinators have a preference for radial flowers. Our bees did show a slight, though non-significant, preference for radial flowers (Fig. 4); but our bees were not completely naïve (having been trained on flowers with square corollas, which they may have perceived as more similar to our disymmetric flowers). In general, although many types of bees have a strong preference for symmetric over asymmetric flowers (Møller 1995; Møller & Eriksson 1995; but see West & Laverty 1998), preferences for a particular symmetry type seem to be idiosyncratic. Previous studies have found no innate preference for radial over bilateral symmetry in *Bombus impatiens* (West & Laverty 1998); a preference for bilateral symmetry in *B. terrestris* (Rodríguez et al. 2004) and other bumblebee species (Leppik 1953; Lázaro et al. 2008); and a preference for radial symmetry in honeybees (*Apis mellifera*) (Free 1970; Wignall et al. 2006).

Even if bumblebees do not consistently prefer radial symmetry, radial flowers may attract more functional groups of pollinators (Fenster et al. 2004). Radially symmetrical flowers may therefore benefit from high and relatively stable visitation rates, at the expense of low pollination efficiency. For this reason, it has been suggested that plants with radially symmetric flowers might be less pollen limited (McLernon et al. 1996; Wolowski et al. 2014); however, syntheses of the available data show no general trend for greater pollen limitation in plants with zygomorphic flowers (Knight et al. 2005; Vamosi et al. 2013). Regardless, radial flowers may have advantages over bilateral flowers that would be apparent only in a more diverse field setting.

In nature, radial flowers are generally oriented in the horizontal plane (parallel to the ground), whereas bilateral flowers are oriented in the vertical plane (Neal et al. 1998). In our study, both symmetries were presented in the vertical plane. It is therefore possible that radial flowers have a pollen-transmission advantage when oriented horizontally, such that pollinators may approach from any direction (Ushimaru & Hyodo 2005; Fenster et al. 2009; Ushimaru et al. 2009; Wang 2010; Wang et al. 2014a; Wang et al. 2014b). It seems likely that bilateral symmetry would have evolved on a background of vertical floral orientation—the background we simulated here. However, floral orientation is an additional factor that could be manipulated in future studies to investigate its functional interaction with floral symmetry. It should also be noted that since we deployed our disymmetric flowers in a vertical orientation, they presented an enhanced "landing platform", or lower lip, compared to the radial flowers. The presence of this landing platform may have contributed to the increased entry-angle consistency that we observed for disymmetric flowers. Bilateral symmetry is often associated with presence of a landing platform in nature—an association we mimicked in this study—but any landing-platform effect could be experimentally decoupled in the future from the effect of floral symmetry by rotating the corollas 90°, such that they are wider than long.

In conclusion, bumblebees (*Bombus impatiens*) have a more consistent angle of entry into disymmetric flowers than radially symmetric artificial flowers. They do not, however, have a significant preference for one symmetry over the other. Our findings provide the first experimental support for the pollen-placement-accuracy hypothesis, and shed light on why bilateral symmetry has evolved from radial symmetry so frequently.

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APPENDICES

Additional supporting information may be found in the online version of this article:

APPENDIX I. Summary of entry angle standard deviation (SD) and preference data for *B. impatiens* on artificial flowers.

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