

— Novel Ideas and Pilot Projects —

AGGREGATE ENANTIOSTYLY: FLORAL VISITOR INTERACTIONS WITH A PREVIOUSLY UNREPORTED FORM OF FLORAL DISPLAY

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Abstract—Floral herkogamy, or the spatial separation of reproductive structures, plays an important role in the evolution of plant mating systems. One form of herkogamy is enantiostyly, or the alternate presentation of stamens to the left and right of the floral axis. Theoretically, enantiostyly increases outcrossing rate by promoting reciprocal pollen deposition and export, but empirical evidence of this is lacking. Understanding how pollinators interact with enantiostylous flowers will illuminate the mechanisms by which enantiostyly encourages outcross pollination. We quantified style deployment within and among plants, of *Allionia incarnata* L. (Nyctaginaceae), a species with a new kind of enantiostyly we call “aggregate enantiostyly”. *A. incarnata* is the first species known to have “compound blossoms”, with different flowers each containing a left- or right-handed style. We quantify the autonomous selfing rate and describe floral visitor interactions with this unique floral structure. We found equal frequency of left- and right-handed blossoms within and among plants. Common floral visitors, *Hemiarctus ceraunus* and *Halictus* sp., interacted with either anthers or stigmas, but not both, indicating effective outcrossing at the flower level. Outcrossing produced more fruits than selfing. The order in which floral visitors interacted with reproductive structures indicates that enantiostyly may increase pollen export. However, we did not find evidence that enantiostyly affected pollen deposition. Our study provides an intriguing first look at the reproductive ecology of a species with a new unique form of herkogamy, and provides evidence on the adaptive significance of enantiostyly.

Keywords: *Allionia incarnata*; enantiostyly; herkogamy; pollination; outcrossing

INTRODUCTION

Floral trait evolution to keep apart the sex organs is widespread in nature, and is thought to have evolved to discourage self-pollination. Herkogamy, the spatial separation of anthers and stigmas within or across flowers, is thought to be a mechanism by which plants avoid self-fertilisation (particularly among outcrossing taxa with some degree of self-compatibility), but also to avoid interference such as stigma clogging even in self-incompatible species (Webb & Lloyd 1986). Included within herkogamy is enantiostyly (also called enantiomorphy), which is defined by the alternate presentation of styles to the left and right side of the floral axis, sometimes called left- and right-“handedness” (Dulberger & Ornduff 1980; Jesson & Barrett 2002). Monomorphic enantiostyly (both forms of enantiostylous flowers are found on the same individual) occurs in 25 genera across 10 families (Jesson 2002) and its function is thought to be promotion of outcrossing relative to putative ancestral straight styled flowers. Dimorphic enantiostyly (different forms found on different plants) is reported for just 4 genera in 3 families (Jesson 2002) and is assumed to promote intermorph outcrossing.

There has been much discussion about the adaptive significance of enantiostyly. Presumably, monomorphic enantiostyly promotes outcrossing by increasing pollinator precision (Webb & Lloyd 1986). As pollinators forage among flowers, they should encounter left- and right-handed flowers. In doing so, pollen from any given flower sticks to one side of the pollinator’s body and is less likely to be deposited on other flowers with the same orientation on the same or other plants. Meanwhile, pollen from a previous flower, which is present on the other side of the pollinator’s body, is deposited onto the stigma, avoiding within-flower selfing, possibly reducing geitonogamy and increasing the precision of pollen export and receipt (Dulberger 1981). Enantiostyly may also promote specialised pollination, as the separation of anthers from stigmas encourages efficient pollen-feeding (Neal et al. 1998). Furthermore, the separation of anthers from stigmas should increase pollination effectiveness, because pollinators are able to feed from anthers in a way that reduces stigma damage and avoids stigma clogging with self-pollen (Neal et al. 1998). Overall, however, the pollination ecology of enantiostylous species is very poorly understood empirically.

Here we describe a new form of enantiostyly which we call *aggregate enantiostyly*, defined as enantiostyly constructed by differential deployment of left and right handed flowers within a “compound blossom” consisting of more than one flower. In order to understand the role of enantiostyly in general—and this unique manifestation of it—with regard to

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outcrossing success and pollinator specialization, we observed the foraging of floral visitors to *Allionia incarnata* in response to the relative placement and display of floral sexual organs. We scored visits in which a floral visitor touched only anthers or only stigmas. Such visits would not contribute to within-flower selfing. Additionally, we tested for autonomous self-compatibility in this system, and measured the distribution within and among plants of the proportion of left- and right-handed styles, to shed light on the role of this unique deployment of enantiostyly in geitonogamous pollination.

MATERIALS AND METHODS

Study system

Allionia incarnata L. (Nyctaginaceae), hereafter *Allionia*, is a perennial herb ranging from the Southwestern United States and Northwestern Mexico to Central and South America. Plants are prostrate, and range in size from small with a single 3-flowered, capitulate umbel, which we term a “blossom,” to large, 1m or more diameter plants, with many blossoms (Turner 1994). We use “blossom”, as it is an ecological term referring to the pollination unit (Faegri and van der Pijl 1979). Blossoms are approximately 2.5 cm wide and are actinomorphic, but actually comprise three smaller perfect, zygomorphic flowers (Fig. 1; Phillips 1976). Each flower contains 4-7 stamens, 1 style, and 1 ovule (Turner 1994). Stamens are located in a cluster in the center of the blossom and are not deflected left or right. Styles are enantiomorphic and are generally arranged in a “Y” shape, with two parallel “upper” styles pointing toward each other and away from the stamens, and one “lower” style pointing away from stamens and the upper styles (Fig. 1A, B; 2A, B). The lower style in some cases points left (like a lower case “y”) and sometimes right, resulting in “left-handed” and “right handed” (3-flowered) blossoms. Each of the three flowers contains a single nectary, and common floral visitors consume both pollen and nectar (S. K. Richman, *pers. obs.*). In Southern Arizona, plants flower sporadically from March–November in response to rain (Phillips 1976). Blossoms are open for a single day, beginning in the early morning and closing by early afternoon (Phillips 1976). Conclusive evidence of self-compatibility in *Allionia* is lacking; however, Phillips (1976) gives anecdotal evidence of some selfing due to hand pollination. Upon fertilisation, each blossom produces three separate 1-seeded fruits enveloped by the perianth (anthocarps). Very little is known about the ecology of *Allionia*; indeed, only species descriptions exist in the literature.

Floral visitor ecology

Fieldwork was conducted in November 2012, at two sites in Tucson Mountain Park (TMP), Tucson, Arizona, USA (32.253980, -111.196960; 32.229446, -111.073830), and in April 2017 at a single site in Ironwood Forest National Monument (IFNM), Marana, Arizona, USA (32.359780, -111.467309). All sites contained 30-50 plants.

Floral visitor observations: We performed once-weekly, thirty-minute observations of single floral visits at both TMP sites for three weeks, recording floral visitor frequency. We identified each floral visitor to lowest feasible taxonomic unit

A



B



FIGURE 1. Photographs of *Allionia incarnata* L. (A) and (B) represent left- and right-handed blossoms, respectively. Each *Allionia* blossom consists of three zygomorphic flowers, each with an enantiomorphic style. Arrows point to styles, which are deflected either to the left or to the right. However, all three styles are generally not deflected in the same direction, resulting in two parallel styles and the third style pointing away from the other two. Styles are white tipped, making pollen visible with the naked eye as seen on the top-left style in (A). Photos by Bob Torrez

on the wing. The most common visitors were identified at least to genus. We observed floral landing positions for the two most frequent visitor species (*Hemiargus ceraunus* (Lepidoptera, Lycaenidae), *Halictus* sp. (Hymenoptera, Halictidae)), noting whether the visitor landed on the top, bottom, left, right, or middle of the blossom (Fig. 2C). The observer stood < 1 m from the blossoms, to ensure careful observation of visitor behavior. To determine whether the two most common visitors were likely to contribute to intra-floral self-pollination or not, we recorded the order in which visitors made contact with *Allionia* anthers or stigmas. Floral visits in which visitors contacted (touched with any part of their body) only anthers, only stigmas, or stigmas before anthers were classified as “flower-level outcrossing”; visits in which visitors contacted anthers and then stigmas were classified as “flower-

level selfing". Below we use "s/o" as shorthand for this distinction in visit outcome.

Plant-level style directionality and pollen deposition to stigmas: We surveyed 112 blossoms across 30 plants at the IFMN site, quantifying style direction for each stigma on each blossom. To determine whether style direction and position affected pollen receipt, we scored the presence or absence of pollen on stigmas with different orientations. Pollen is visible with the naked eye (Fig. 1A), as stigma tips without pollen are white while stigmas with pollen are yellow.

Testing for self-compatibility: On April 1, 2017, we haphazardly selected 5 plants at the IFNM site, and marked 6 blossoms/plant, checking to make sure stigmas were free of pollen. We divided blossoms into *autonomous selfing* and *open pollination* treatments ($N = 3$ blossoms/treatment/plant). Blossoms in the *autonomous selfing* treatment were enclosed in bridal veil, while blossoms in the *open pollination* treatment were left unmanipulated. On April 15, 2017, we collected all available anthocarps, checked for the presence of seeds, and weighed all anthocarps containing seeds (mg) using a microbalance (SmartWeigh GEM20 high-precision milligram scale).

Statistical analyses

Analyses of self and outcross pollination visits were performed with a three-way contingency table to using the frequencies of 2 flower-level self and outcross (s/o) states \times 2 visitor species \times 5 landing positions. Because the contingency table was large (20 cells), sample size per cell was smaller than recommended for standard procedures, so we relied on parametric bootstrapping to generate null distributions and P -values. We first generated expected probabilities using the observed proportion of visits that were s/o, at each position, and involving each species. We then used these probabilities (that assume no statistical associations between variables) to generate 2,000 random data sets. We then analysed the random data sets to generate a frequency distribution of χ^2 values under the assumption of no association among variables and used it to test our observed field data. To analyse the observed data, we used a glm (poisson family, log link) with s/o, species (two most common visitor species only), landing location, and all possible pairwise interactions as fixed effects. The three-way interaction of all explanatory variables was not significant, and was therefore excluded from the final model.

We determined whether the proportion of blossoms with right-handed bottom styles on all plants with 2+ flowers ($N = 16$) differed from an expected value of 50:50 using a Pearson's Chi-squared test; we determined the p-value using a permutation test of 2,000 replicates. Effect of style position and direction on pollen receipt was analyzed at the stigma level using glm (binomial family, logit link) with the presence of pollen as the response variable and style position (top, bottom) and blossom handedness (left, right) as crossed fixed effects, and plant added as a fixed covariate. Significance of individual model terms were determined using a Likelihood Ratio Test. To test for differences in seed set between autonomous selfing and open pollination, we used glm (binomial family, logit link) with the proportion fruit set (number of fruits present/number of ovules (3)) as the response variable and self vs open as the fixed effect, with plant added as a fixed covariate. Significance of individual model terms was determined using a Likelihood Ratio Test. We compared average anthocarp weight (mg) between treatments using ANOVA, treating plant as a fixed covariate. All analyses were performed in R version 3.3.1 (R Core Team 2016).

RESULTS

Floral visitor observations: We observed 93 single floral visits by individuals from 4 taxonomic groups. The most frequent floral visitors were lepidopterans ($N = 64$, 42 of which were *Hemiargus ceraunus* butterflies), and *Halictus* sp. bees ($N = 18$); rare species included dipterans and other hymenopterans. Proportions of contacts with anthers versus stigmas by each species are shown in Tab. I. Of the two most common visitor species, *Hemiargus* made 100% flower-level outcrossing visits while 69% of the *Halictus* visits were flower-level outcrossing, a significant difference between the two taxa, as indicated by the difference in frequency of visits by each species, of overall s/o landings, and their interaction (χ^2_1 species = 10.470, $P = 0.001$, χ^2_1 s/o = 39.006, $P < 0.001$, χ^2_1 species*s/o = 4.888, $P = 0.03$). The two most common species also differed in their use of landing positions, with potential implications for outcrossing. *Hemiargus* landed on all possible floral sections but least frequently in the middle of the blossom where the stamens are. In contrast, *Halictus* sp. was never observed to visit the left or top edges of the blossom and seldom landed on the right edge, landing mostly in the middle or bottom of the blossom (χ^2_4 species*landing position = 18.032 $P = 0.001$; Fig. 2C, Fig. 3). There was no significant interaction of s/o and landing position.

TABLE I. Proportion of landings by each visitor species that resulted in contact of one or more floral reproductive structures. "Anther only" and "Stigma only" indicate visits in which visitors contacted anther(s) but not stigma(s), and vice-versa. "Anther first" indicates visits in which visitors contacted anther(s) but not stigma(s), and "Stigma first" indicated visits in which visitors contacted stigma(s) but not anther(s).

Reproductive contact	Visitor species	
	<i>Halictus</i> sp. (Hymenoptera, Halictidae) $N = 18$	<i>Hemiargus. Ceraunus</i> (Lepidoptera, Lycaenidae) $N = 42$
Anther only	0.38	0.71
Stigma only	0	0.29
Anther first	0.39	0
Stigma first	0.23	0

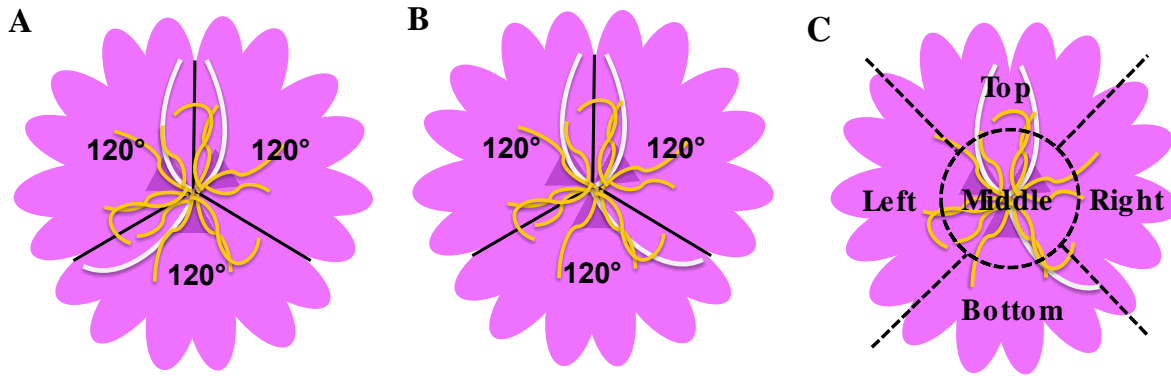


FIGURE 2. Floral illustration of *Allionia incarnata* L. (A) and (B): Typical display of flowers, anthers (yellow), and stigmas (white). Each flower provides 120° of the 360° blossom. This third style will be either (A) left- or (B) right "handed". (C): Division of the blossom into 5 "positions" in which a pollinator can land: top, bottom, left, right, and middle.

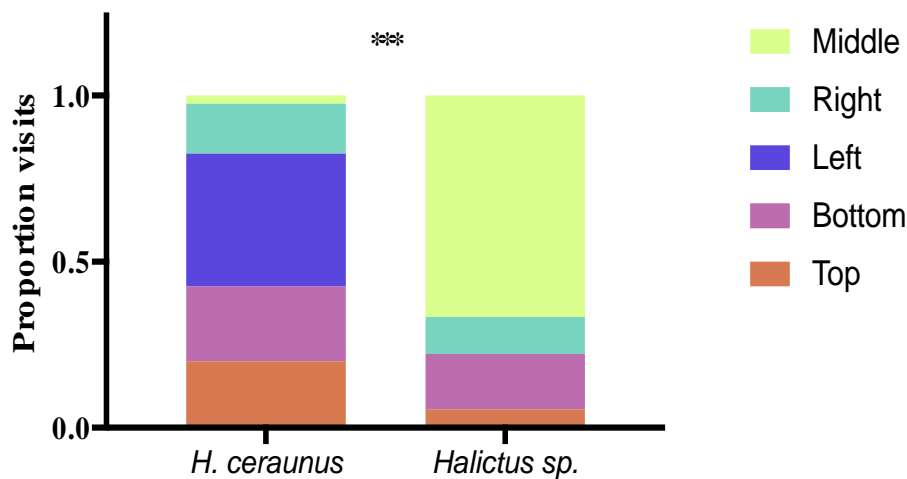


FIGURE 3. Floral landing patterns of the two most common *Allionia* visitor taxa, *Hemiargus ceraunus* and *Halictus*. Bars represent the total visits by each species, colors indicate the different possible landing positions and the proportion of landings to each position. Asterisks indicate a statistically significant difference ($P < 0.001$) between species in overall landing patterns based on Likelihood Ratio Test.

Style directionality and pollen deposition: Among plants, the average proportion of right-handed bottom stigmas was 0.46 ± 0.06 (mean \pm SE; not differing from 0.5 R:L; $\chi^2_1 = 1.265$, $P = 1$). Pollen was present on $46\% \pm 0.07\%$ of both top stigmas, and on $42\% \pm 0.08\%$ of bottom stigmas. There was no effect of style position or direction on pollen deposition, nor was the interaction between style position and direction significant (χ^2_1 position = 2.485, $P = 0.11$, χ^2_1 direction = 2.242 $P = 0.33$; χ^2_1 , position*direction = 0.610, $P = 0.43$). There was, however, a significant difference in overall pollen deposition among plants ($\chi^2_{10} = 38.168$, $P < 0.001$).

Testing for self-compatibility: Blossoms in the *Open pollination* treatment set $95.2\% \pm 3.2\%$ of seeds, while blossoms in the *Autonomous selfing* treatment set $53.3\% \pm 10.1\%$ ($\chi^2_1 = 17.825$, $P < 0.001$). However, in blossoms that developed seeds, anthocarps did not differ in weight between treatments ($F = 2.17$, $P = 0.14$). There was also significant variation among plants in proportion fruit set ($\chi^2_1 = 15.135$, $P = 0.004$).

DISCUSSION

Allionia incarnata has a unique, previously undescribed deployment of enantiostyly across blossoms that we have dubbed "aggregate enantiostyly". Our results support the hypothesis that this new form of enantiostyly reduces self-fertilisation at the flower level, which we have shown to be potentially costly in this system. The landing pattern of *Hemiargus* suggests this role. *Hemiargus* generally landed at the edge of the blossom and walked toward the central nectaries. Because styles display different handedness and are oriented at the top and bottom of the blossom, there is an approximately large 240° stigma-free space on one side of the blossom and a 120° separation on the other (Fig. 1). This causes a greater overall separation of anthers and stigmas than if styles were straight or all with the same handedness, i.e., a roughly 120° uniform separation. Visitors landing on the 240° side would likely promote pollen export, while those landing on the 120° side may also avoid contact with a stigma. In contrast, the probability of stigma contact on a blossom of uniform stigma separation should be equal regardless of

landing position. In contrast to *Hemiargus*, *Halictus*, the second most common visitor, visited blossoms primarily for pollen, focused their landings on the centre of the blossom and then walked outward. This pattern of visitation would promote pollen pickup followed by possible pollen deposition on the same blossom, i.e. autogamous self-fertilisation. The extent to which this behaviour is costly for *Allionia* fitness is not entirely clear. Autonomous selfing blossoms experienced lower fruit set than blossoms that were available for pollination; however, it remains unknown whether this is due to some degree of self-incompatibility in *Allionia*, or due to low autonomous self-pollination.

Allionia is found from Southwestern US to South America and its flat open blossoms probably provide pollen and nectar resource to many floral visitors, though bees and butterflies likely predominate. The generality of our floral visitation results will depend on the mix of pollinators at different sites and the similarity of their behaviors to those of *Hemiargus* and *Halictus* observed in our study in southern Arizona. Increased outcrossing due to enantiostyly would be adaptive if *Allionia* suffers from inbreeding depression or pollen or ovule discounting associated with selfing. Interestingly, this is the case for self-compatible *Paraboea rufescens* (Gesneriaceae) which is buzz-pollinated by bumble bees that do not discriminate between left- or right-handed flowers and therefore contribute to reciprocal pollen deposition, discouraging self-fertilisation (Gao et al. 2006). Furthermore, it is unclear whether monomorphic enantiostyly promotes or discourages geitonogamous pollination in *Allionia*. Since monomorphic enantiostyly appears to have evolved from straight styled ancestors rather than from dimorphic enantiostyly (Jesson et al. 2003), it is thought that the function of monomorphic enantiostyly is to reduce geitonogamy, even though it does not do so as well as the rarer dimorphic enantiostyly. Because bottom style direction did not differ from 50/50 left- and right-handed stigmas within plants, and the most common pollinator showed no pattern in its landing orientation, it remains unclear how much geitonogamy may be reduced in *Allionia* relative to a putative straight-styled ancestor. Other studies show reduced geitonogamy in monomorphic enantiostylous plants manipulated to display only left- or right-handed flowers (Fenster 1995; Jesson et al. 2003). One caveat with regard to our results is that while we could distinguish allogamy from autogamy at the floral level, we were unable to determine whether allogamous visits resulted in geitonogamy or outcrossing. One way to accomplish this would be to observe pollinators over entire foraging bouts, quantifying within- and between-plant visits and the pattern of pollen pick-up and deposition.

Making the assumption that, within a flower visit, contacting only anthers contributes to male fitness, and contacting only stigmas contributes to female fitness, more floral visits were involved in male fitness via pollen export than female fitness via pollen receipt. Interestingly, theory suggests that flowers with enantiomorphic styles and straight anthers (as is the case in *Allionia*), should have increased allogamous pollen receipt but no difference in pollen export (Jesson et al. 2003). In *Allionia*, the three contiguous enantiostylous flowers in a single blossom may promote pollen export to a

greater degree than would be predicted. In our study, Lepidopteran floral visitors only ever contacted anthers or stigmas within a flower visit, never both. It would seem that the 120° and 240° gaps between stigmas would favor pollen export by Lepidopterans as they moved to the center of the blossom to feed on nectar. The single zygomorphic flowers by themselves would only have one open edge, meaning Lepidopteran pollinators would only approach the flower from one side. In this case, there is a greater chance they would reciprocally contact stigmas upon landing (by visiting left- and right-handed stigmas in succession), making a greater contribution to pollen receipt without necessarily contributing to pollen export (Dulberger 1981; Fenster 1995). Effective pollination studies of the floral visitors would be necessary to fully determine pollen export and receipt.

This system is the first documentation of this pattern of deployment of enantiostyly in compound blossoms. Although our observations tell us how this curious case of enantiostyly is functioning today, we did not compare the functioning of a putative, straight-styled ancestral condition, which would allow stronger inference about adaptation. This could be done with experimental manipulation of style positions or even using the occasional blossoms with all flowers having the same handedness, thus positioning styles 120° apart. It would be interesting to know the putative ancestral condition from comparative phylogenetic studies. For example, when did enantiostyly originate in relation to the origin of multiflowered blossoms? *Allionia* is the only genus of Nyctaginaceae with zygomorphic flower aggregated into an actinomorphic blossom (Douglas & Manos 2007). Species from the putative sister genera to *Allionia* (e.g., *Mirabilis*, *Commiscarpus*) often have small umbels of flowers that are individually actinomorphic except that the single style is usually pressed against a petal, off to the side of the centrally placed anthers. It usually extends well beyond the anthers and petals (approach herkogamy). Since the corolla is otherwise actinomorphic, if flowers open upward this lateral position cannot be called enantiostyly, though there may be many unknown cases of enantiostyly among related species with more horizontally oriented flowers. A deeper understanding of the ecology of this new form of floral display will provide interesting insights into reproductive ecology and floral evolution.

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