

ABSTRACT

Title of Dissertation: EVALUATING THE EFFECTS OF
POLLINATOR MEDIATED SELECTION ON
PATTERNS OF FLORAL VARIATION

Carolina Diller, Doctor of Philosophy, 2017

Dissertation directed by: Professor Dr. Charles B. Fenster, Biology

Angiosperm flowers astonish for their high morphological diversity. More importantly, flower shape variation has a significant reproductive and evolutionary role. Here, I studied the relationship between flower shape and pollination precision, i.e. the precise transfer of pollen across flowers by pollinators. In the first two chapters, I studied the role of one floral trait, corolla chirality, in three species of *Hypericum* (Hypericaceae). Unfixed corolla chirality is the presence of pinwheel arrangement of petals, both right and left rotated, within an individual. Specifically, I evaluated whether corolla chirality promotes disassortative mating between flower morphs through directed movement of pollen and pollinators between flowers. This precise pollination mechanism could increase outcrossing rates by reducing geitonogamous pollinations. Nevertheless, pollinators were indifferent to corolla chirality and thus pollination for unfixed corolla chirality is similar to radially

symmetric flowers with a generalized (non-precise) pollination system. In chapter 3, I performed a macroevolutionary analysis on multiple key flower traits. I hypothesized that flower traits with precise pollinations due to precise fit with their pollinators or due to increased pollination specialization will be under uniform directional selective pressures and thus be less variable than flowers with less precise pollination system. I found that flowers with lateral orientation or bilateral symmetry were significantly less variable than their alternative states (vertical and radial, respectively). Thus, I demonstrate that traits that restrict pollinator landing and movement play an important role in pollination precision. In chapter 4, I quantified patterns of genetic variation available for pollination precision to evolve in a male reproductive trait (i.e. stamen height) using fast-cycling *Brassica rapa*. The match of anthers and stigmas to the contact area on the pollinator body conveys precise pollen transfer. My results suggest that individual mean stamen height can evolve to match the population mean pistil height (presence of additive genetic variation), but that some level of imprecision will remain due to lack of additive genetic variation for within-individual stamen height variation. In summary, floral traits vary in their role in pollination precision and the evolution of pollination precision may be constrained by the types and amounts of genetic variation.

EVALUATING THE EFFECTS OF POLLINATOR MEDIATED SELECTION
ON PATTERNS OF FLORAL VARIATION

by

Carolina Diller

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2017

Advisory Committee:

Professor Charles B. Fenster, Chair

Professor Michele R. Dudash

Associate Professor Alexa Bely

Associate Professor Jeffrey W. Shultz

Associate Professor Nathan Swenson

Distinguished Scientist and Curator of Botany National Museum of
Natural History Dr. John W. Kress

© Copyright by
Carolina Diller
2017

Dedication

To my parents.

Acknowledgements

Above all, I'd like to thank my advisor Charlie Fenster. Thank you for encouraging me to apply to grad school at Maryland, for teaching me how science is done and how to be a scientist, for sharing your experience with me, for treating me as a colleague all along the process, for keeping the door always open, for believing in me and my project, and mostly, for not just being an advisor, but also a true mentor. Thank you. I feel very fortunate to have been your student.

Thanks to my committee: Alexa Bely, Michele Dudash, John Kress, Jeff Shultz and Nate Swenson for signing up, for your time and commitment and for your insightful comments. You've pushed me further, taught me how to communicate with a broader scientific community and forced me to think outside my narrow field of research. Special thanks to Michele and John. To Michele for following up closely my dissertation progress and providing support and insight whenever the opportunity arose. To John for encouraging me to apply for the CIC/SI fellowship and for hosting me at his lab at the National Museum of Natural History. I felt incredibly welcomed and am very grateful for having had the opportunity to work amongst such great collections.

Thanks to all my lab members: Kevin Barry, Jason Berg, Sara Konkel, Abby Kula, Clark Rushing, Andy Simpson, Callie Stanley, Alyssa Stewart, Frank Stearns, Mao-Lun Weng and Juannan Zhou. I've earned a lot from your comments, discussions and advice. Special thanks to Andy Simpson, Mao-Lun Weng for teaching me phylogenetic and bioinformatics skills. Also thanks to Paul Montalvo, Nicola Seitz, Daniel Brizuela and Delmis Umancor for their help collecting data and

Robbin C. Moran, Brad Boyle, W. Scott Armbruster, Christophe Pélabon and Thomas F. Hansen for friendly and very helpful reviews to chapters of my dissertation. I'm incredibly grateful for the many people outside my lab that selflessly dedicated their time and knowledge with me: Paula Casanova, Silvia Alvarez, Eliezer Gurarie, Mónica Carlsen, Caroline Puente-Lelièvre, Marina Strelin and Benjamin Potter.

I am especially fortunate for my parents, my brother and grandmother's unconditional love and support. It is easy to venture outside ones comfort zone with such a strong safety net, which I'm so blessed to have. Por eso y por mucho más, de todo mi corazón, gracias.

Last but not least, to my dear friends who have shared this journey with me: Luigi Alvarado, Paula Casanova, Eduardo Zattara, Silvia Alvarez, Claire Fortunel, Karina Herrera, Maria Teresa Perez, Alyssa Stewart, Ellie Spadafora, Nicola Seitz and Juannan Zhou. Each one of you has reached out and many of you have gone all out to support and care for me. For that, I am always grateful. And finally, my solid and best friend from overseas who never gives up on me: Agustina Aliaga.

My dissertation research was funded by the University of Maryland (Ann G. Wylie Dissertation Fellowship and Eugenie Clark Summer Fellowship), the Committee on Institutional Cooperation/ Smithsonian Institution Predoctoral Fellowship (CIC/SI) and the Margaret Walton Scholarship for Mountain Lake Biological Station (MLBS).

Table of Contents

Dedication	ii
Acknowledgements	iii
Table of Contents	v
List of Tables	vii
List of Figures	viii
Introduction	1
Chapter 1: Corolla chirality in <i>Hypericum irazuense</i> and <i>H. costaricense</i>	
<i>(Hypericaceae)</i> : parallels with monomorphic enantiostyly	5
Abstract.....	5
Introduction.....	6
Materials and Methods.....	9
Results.....	12
Discussion.....	13
Chapter 2: Corolla chirality does not contribute to directed pollen movement in	
<i>Hypericum perforatum (Hypericaceae)</i> : mirror image pinwheel flowers function as	
radially symmetric flowers in pollination	20
Abstract.....	20
Introduction	21
Materials and Methods	24
Results	33
Discussion	35
Chapter 3: Evaluating the role of floral traits in pollination precision – flower	
orientation matters.	48
Abstract.....	48
Introduction.....	49
Materials and Methods.....	52

Predictions.....	60
Results.....	64
Discussion.....	75
Chapter 4: Selection response for stamen length adaptive accuracy in <i>Brassica rapa</i>	102
Introduction.....	103
Materials and Methods.....	107
Results.....	121
Discussion.....	125
Conclusion	143
Appendices	146
Bibliography	191

List of Tables

TABLE 1.1 Number of sinistrorse/left and dextrose/right flowers for each ultimate branch level.....	16
TABLE 2.1 Pollinator species captured and identified while visiting <i>Hypericum perforatum</i> flowers at Mountain Lake Biological Station (MLBS), VA.....	39
TABLE 2.2 Model selection for pollinator behavior (movement within flowers) while visiting <i>Hypericum perforatum</i> flowers at Mountain Lake Biological Station, VA.....	40
TABLE 3.1 List of floral trait code and their categories.	83
TABLE 3.2 Summary of single floral trait analyses - one way ANOVA test statistics	86
TABLE 3.3 Summary of combined floral trait analyses	88
TABLE 4.1 Mean scaled inaccuracy index and accuracy components for stamen length (individual level accuracy) in <i>Brassica rapa</i>	131
TABLE 4.2 Mean scaled inaccuracy index and accuracy components for stamen length at different levels of analysis in <i>Brassica rapa</i> for the base populations (generation 0).....	135

List of Figures

FIGURE 1.1. <i>Hypericum irazuense</i>	17
FIGURE 1.2. <i>Hypericum irazuense</i> . The white arrows are pointing to what was considered as the ultimate/terminal branch levels.	18
FIGURE 2.1. <i>Hypericum perforatum</i> flower	41
FIGURE 2.2. Explanation of the terminology of right and left chirality in <i>Hypericum perforatum</i> at the Mountain Lake Biological Station.	42
FIGURE 2.3. Pollinator behavior observed at the Mountain Lake Biological Station on <i>Hypericum perforatum</i>	44
FIGURE 2.4. Pollinator visitation rate on right and left flowers of <i>Hypericum perforatum</i> as measured at the Mountain Lake Biological Station	45
FIGURE 2.5. Pollinator behavior observed on right and left flowers of <i>Hypericum perforatum</i> at the Mountain Lake Biological Station.	46
FIGURE 3.1. Phylogenetic distribution of all floral traits.....	94
FIGURE 3.2. Flower Orientation.....	96
FIGURE 3.3. Stamen and Pistil Exsertion.....	97
FIGURE 3.4. Summary of combined floral trait analyses.....	98
FIGURE 4.1. Simplified diagrams of the selected traits in <i>Brassica rapa</i> : stamen bias and stamen length variation	137
FIGURE 4.2. Simplified diagram of a <i>Brassica rapa</i> flower showing floral measurements.....	138
FIGURE 4.3. Selection response for stamen bias in <i>Brassica rapa</i>	139
FIGURE 4.4. Stamen length variation (variance) for <i>Brassica rapa</i> between inbred and outbred lines	140

Introduction

The main motive behind this dissertation is to increase our understanding of the origins and maintenance of patterns of floral diversity. Angiosperm flowers astonish for their high morphological diversity, however, equally surprising is how certain floral traits are conserved at higher taxonomic hierarchies. To understand the processes resulting in this balance between diversity and canalization of floral traits, here I study the sources and patterns of floral variation at different evolutionary and ecological scales. Ultimately, I hope this body of work sheds light on the role of selection exerted by ecological agents, as well as the underlying genetic and developmental context on patterns of floral variation.

Within this framework, I focus on the relationship between flower shape and pollination precision. Precise pollination is the efficient transfer of pollen between conspecifics by pollinators and thus has a major role in plant reproductive fitness. Flower shape may influence the diversity of pollinators, as well as the area of the pollinators' body that comes into contact with male and female reproductive parts (anthers and stigma, respectively). Thus, flower shape has a major impact on pollination precision. Increased pollination precision may have evolutionary outcomes such as increased plant reproductive isolation, and also potentially increased plant reproductive success by improving pollen transfer efficiency. A thorough understanding of the role of different floral traits in pollination precision will increase our understanding of their contribution to angiosperm diversification.

An example of a conserved, but also highly understudied, floral trait across angiosperms, is unfixed corolla chirality. Unfixed corolla chirality is the presence of

pinwheel arrangement of petals, both right and left rotated, within an individual. This character state is present in 19 families across the angiosperms, however we do not know what evolutionary mechanism is responsible for the maintenance of this trait. In light of the flower shape-pollination precision framework of this thesis, in chapter 1 and 2, I explore the role of corolla chirality in the pollination biology of three sister species of *Hypericum* (Hypericaceae). Specifically, I evaluated whether mirror image corolla chirality promotes disassortative mating between flower morphs through directed movement of pollen and pollinators between flowers. I framed these questions concerning floral chirality because seemingly analogous floral traits such as enantiostyly (mirror image flowers on the same plant) contribute to precise pollination and increased outcrossing rates. Consequently, floral chirality could lead to increased outcrossing rates by reducing geitonogamous pollinations and thus increase plant reproductive success. Nevertheless, pollinators were indifferent to corolla chirality and thus my results suggest that pollination for unfixed corolla chirality is similar to radially symmetric flowers with an open and generalized (non-precise) pollination system.

In chapter 3, I expand my focus and evaluate the effect of eight different floral traits on pollination precision for 327 angiosperm species. I utilize phenotypic variation for flower size (across individuals) as an indicator of the degree of pollination precision. I hypothesize that flower traits that impose more precise pollination due to precise fit with their pollinators or due to increased pollination specialization will be under uniform directional selective pressures and thus be less variable than flowers that result in less precise pollination system. The results of a

phylogenetically controlled analysis demonstrate that flower orientation and flower symmetry are the main traits of the traits studied that are responsible for pollination precision as measured by amounts of phenotypic variation. Flowers with lateral orientation or bilateral symmetry had significantly less variation than their alternative states (vertical and radial, respectively). Thus I find evidence that traits that are responsible for restricting pollinator landing and movement play a bigger role in pollination precision than traits that affect pollinator specialization. To my knowledge this is the first time that floral orientation has been comprehensively demonstrated to contribute to pollination precision.

In chapter 3, I explore the effects of pollinator mediated selection on phenotypic variation, specifically variation across individuals. However, there are multiple additional hierarchies of variation in plants such as within individuals, across flowers, within flowers, etc. Potentially all of these levels could affect pollination precision and thus be under pollinator mediated selection. But, can all of these levels of variation respond to selection? How precise can pollination evolve to be? If precise pollination conveys increased reproductive success through increased pollen transfer, will all levels of flower variation respond to selection through reduced variation?

In chapter 4, I address these questions by exploring genetic variation of two sources of variation for stamen height that may influence the precision of pollen transfer of a plant: the mean departure to its optimum height (referred to as stamen bias) and the variation within individuals. I assigned the optimum height as the populations average pistil height. Thus, departure of an individual's mean stamen height from the population pistil height implies that on average, the pollen deposit

and pick up area differ on the pollinator body. A mismatch in the contact area on the pollinator body in turn signifies less accurate pollen transfer or pollination precision for that individual. Along those lines, greater variation in stamen height across flowers for an individual translates into greater number of flowers departing from the stigma contact area on a pollinator, reducing the plants pollination precision even more. In this chapter, I performed two artificial selection experiments and an inbreeding vs outbreeding experiment using *Brassica rapa* as my study system. Overall, I found evidence for additive genetic variation for stamen bias, but not for within-individual stamen variation. Instead, I found some evidence suggesting that within-individual stamen variation is under non-additive genetic variation. These results suggest that not all levels of variation may respond to a selective agent such as pollinators, and thus pollination will remain imprecise to a certain degree.

With these four chapters, I explore the role of flower traits in pollination precision from different perspectives. In the first two chapters, I study the role of one floral trait (i.e. corolla chirality) at the species level, in chapter 3, I perform a macroevolutionary analysis on multiple key flower traits and finally in chapter 4, I study the genetic variation available for pollination precision to evolve in a male reproductive trait (i.e. stamen height) using fast-cycling *Brassica rapa*. In summary, I am unable to demonstrate that floral chirality is a mechanism promoting precise pollination but that bilateral symmetry and horizontal floral orientation do promote precise pollination. Finally, I explore how evolutionary response to selection may be constrained by types and amounts of genetic variation in the evolution of floral shape means and variances.

Chapter 1: Corolla chirality in *Hypericum irazuense* and *H. costaricense* (Hypericaceae): parallels with monomorphic enantiostyly

Abstract

Corolla chirality in *Hypericum irazuense* and *H. costaricense* (Hypericaceae): parallels with monomorphic enantiostyly. J. Torrey Bot. Soc. XXX: 000 000. 20XX.— Symmetry is an important floral character and is often associated with the degree of pollination specialization. Typically floral symmetry is fixed within a species. Rotational symmetry occurs in two different corolla variations according to the direction in which the petals overlap. This study describes the pattern of polymorphism in corolla chirality of *Hypericum irazuense* and *H. costaricense*. The individuals of these species exhibit flowers with corollas that rotate either counter clockwise (left/sinistrorse) or clockwise (right/dextrorse). Studying this pattern of floral expression is the first step in gaining a better understanding of whether this trait polymorphism is maintained due to an adaptive advantage or as a byproduct of a developmental genetic constraint. We quantified the proportion of dextrorse and sinistrorse flowers for *H. irazuense* and *H. costaricense*. We also examined whether the chirality of a flower is independent of the corolla chirality of its neighbor flower at the ultimate branch level, i.e., a group of terminal branches that are connected by the same node. We demonstrate that the proportion of sinistrorse and dextrorse flowers is equal among individuals within a population of *H. irazuense* and *H. costaricense*.

In addition we show that in these populations, the forms are randomly distributed at the ultimate branch level indicating that the identity of a flower type is not determined by the chirality type of its neighbor flower. The 1:1 proportion of chirality types among individuals suggests that the maintenance of both flower forms may convey a reproductive benefit. But, the random distribution of flower type at the ultimate branch level challenges this interpretation. Further studies are needed to address the adaptive significance, if any of this pattern of dextrorse and sinistrorse flowers.

Key Words: contort aestivation; enantiomorphy; floral symmetry; polymorphism; rotational symmetry.

Introduction

Phylogenetic and experimental studies have demonstrated that flower symmetry is correlated with diversification rates (Sargent 2004) and reproductive success (Gómez et al. 2006). Floral symmetry is also associated with the degree of pollination specialization and it is therefore fundamental to our understanding of angiosperm diversification, e.g., bilateral symmetry (zygomorphy) is correlated with increased specialized pollination (Faegri and van der Pijl 1979; Fenster et al. 2004; Gómez et al. 2006; but see Herrera, 1996). The shift to a reduced set of pollinators increases the opportunity for prezygotic reproductive isolation and thus greater speciation rates for species with zygomorphic flowers relative to those with actinomorphic flowers (Sargent, 2004; van der Niet and Johnson, 2012).

Within some species, floral symmetry polymorphisms are observed (Endress, 1999). One example is enantiostyly, a type of asymmetry in which the style is deflected either to the right or to the left (Neal et al., 1998). Some species have both style morphs present within an individual (monomorphic-enantiostyly) while others manifest the style morphs on separate plants (dimorphic-enantiostyly) (Jesson and Barrett, 2003). The disposition of right and left-styled flowers within monomorphic-enantiostylous individuals ranges from both morphs present with alternating right and left flowers within each inflorescence (e.g., *Solanum rostratum* and *Chamaecrista fasciculata*; Todd 1882; Fenster 1995, respectively), only one morph present within each inflorescence (e.g., *Monochoria australasica*; Jesson and Barrett 2003), or both morphs randomly distributed (e.g., *Heteranthera mexicana*; Jesson et al. 2003). The proportion of left and right-styled flowers on each individual is equal for most monomorphic-enantiostylous species (Todd, 1882; Gao et al., 2006). The fact that in some species the disposition of right and left styled flower morphs is determined by positional cues, whereas in others it is random, challenges our understanding of the developmental basis and adaptive significance of symmetrical polymorphism (Jesson et al., 2003).

Another example of symmetry polymorphism within species is rotational symmetry. It is expressed in flowers with contort aestivation and is often correlated with flowers that have asymmetric petals (Schoute, 1935; Endress, 1999). This particular aestivation may be expressed as two morph types: sinistrorse/left or counter clockwise and dextrorse/right or clockwise. In sinistrorse flowers every corolla lobe overlaps its clockwise neighbor (Scotland et al., 1994) or similarly, the left side of

each petal overlaps its neighbor (Endress, 1999). The opposite is true for dextrorse flowers. Species with rotational symmetry may either be fixed dextrorse or sinistrorse or unfixed. When species are unfixed, every individual has both dextrorse and sinistrorse flowers present. To our knowledge there is no case similar to dimorphic enantiostyly for rotational symmetry in which the two morphs are present within a species but separated on different plants.

To the best of our knowledge, only two studies have examined rotational symmetry for 31 unfixed species in the Malvaceae (Davis, 1964; Ghosh and Davis, 1978), and both studies demonstrated an equal proportion of sinistrorse and dextrorse morphs which was stable across seasons. Some species had a slight excess of sinistrorse morphs, as might be expected from chance alone, given that a large number of species were sampled and no corrections were made for multiple contrasts. Endress (2001) suggested that unfixed rotational symmetry is under epigenetic control since the directionality of the rotation seems to be a result of chance or is influenced by the symmetry of the inflorescence. Endress (2001) reports that in monochasial impartial inflorescences both morphs regularly alternate while in racemose inflorescences the sequence of the two morphs is not predictable. Yet for groups other than the Malvaceae, there is no documentation of the frequency of dextrorse and sinistrorse flowers at either the individual or population level.

Hypericum (*Hypericaceae*) in the Rosid I clade provides an example of rotational symmetry in a lineage distantly related to Malvaceae in the Rosid II clade (APG III 2009). Of the nearly 500 species of *Hypericum* (Nürk and Blattner 2010), several exhibit unfixed rotational symmetry with both morphs present in the same

individual. Here we evaluate the pattern of expression of dextrorse and sinistrorse flowers of *H. irazuense* and *H. costaricense* to assess (1) if the chirality morphs occur in a 1:1 ratio among individuals in a population and (2) whether dextrorse and sinistrorse flowers are randomly distributed across the branches or conditioned by the chirality type of its neighbor flower. Whether corolla chirality is randomly distributed within an individual or dependent on the form of its neighbor flower, as has been documented in *Oxalis* (Endress, 1999, 2001), may have implications for understanding the adaptive significance or developmental basis of this phenomenon.

Materials and Methods

Study Site

We studied sympatric populations of *H. irazuense* and *H. costaricense* in Cerro Vueltas páramo in the Cordilleras Talamanca of Costa Rica (N9°34'041'' W83°45'283'' at 3393.64m.a.s.l). Páramo is high altitude neotropical vegetation occurring above treeline and characterized by the presence of low shrubs and herbs. The páramos of Central America are dominated by the families Asteraceae and Poaceae and include a high proportion of endemic species, such as *Chusquea subtesellata*, *Hypericum irazuense*, *H. strictum*, *Comarostaphylis arbutoides*, *Escallonia myrtilloides*, *Valeriana prionophylla*, *Pernettya prostrata*, *Senecio oestedianus* and *Vaccinium consanguineum* (Kappelle and Horn, 2005).

Species Studied

Hypericum irazuense and *H. costaricense* are shrubs up to 4 m and 1 m tall, respectively. Both species have bright yellow flowers exhibiting dextrorse and sinistrorse chirality, 2-2.5 cm and 1-1.5 cm across respectively. The petals are asymmetrical, and the chirality can be easily identified by a hooked apex on each petal (Fig. 1.1). We do not know the common pollinators of these species, although one bumblebee *Bombus* spp. was observed to visit *H. irazuense* during a four-day period in the field. This observation is consistent with the common pollinators documented for *H. punctatum* (Robertson, 1928), which shares the same floral traits as *H. irazuense* and *H. costaricense*.

Field Sampling

For each species all individuals surveyed constituted one population, and both populations (*H. irazuense* and *H. costaricense*) were sympatric. We used two sampling approaches to examine the pattern of expression of dextrorse and sinistrorse flowers for *H. irazuense* and *H. costaricense*. First, we quantified the ratio of dextrorse and sinistrorse flowers at the population level. For this we surveyed the chirality forms in 81 and 49 individuals for *H. irazuense* and in *H. costaricense*, respectively. Second, in order to determine whether the observed distribution of dextrorse and sinistrorse flowers was conditioned by the chirality type of its closest neighbor flower, we first described the branching pattern of 50 individuals based on Strahler's method for describing branching networks (Strahler, 1957; Uylings et al., 1975). Essentially this method provides a guideline to classify branches in a

hierarchical fashion in which the main branch is classified as order 1 and the connecting branches are labeled in subsequent order until the terminal branches. This allowed us to designate equivalent branch groups that we could compare across the population. A branch group was defined as a set of terminal branches that are connected to the same node (Fig. 1.2). By using this method we focused the chirality of closest neighboring flowers that are flowering simultaneously. We are interested in the chirality of closest simultaneous flowers because this is the level which would most likely influence pollinator behavior and thus provide an adaptive explanation. We only analyzed branch groups with two open flowers because most branches only had two flowers open at a time. We sampled 238 branches on 50 individuals in *H. irazuense* and 23 branches on 20 individuals in *H. costaricense*. The average number of branch groups with 2 open flowers for each individual was 4.8 for *H. irazuense* and 1.8 for *H. costaricense*.

Statistical Analysis

To evaluate the total proportion of dextrorse and sinistrorse flowers at the population level for each species we used a Student's t-test. The total proportion of sinistrorse flowers was quantified for each individual. Observed proportions were arcsine square root transformed and compared to the arcsine of the square root of 0.5 which represents equal proportion of dextrorse and sinistrorse flowers at the population level. Each individual was treated as a replicate for this test (see Supplementary Rcode 'Ch1_a' and Supplementary Raw Data 2 and 3).

To determine whether the proportion of dextrorse and sinistrorse flowers is random at the ultimate branch level, we performed a Pearson Chi-square test. According to a random expectation of the distribution of chirality types, each branch that produces two flowers may produce either two dextrorse (probability = $0.5 \times 0.5 = 0.25$), two sinistrorse (probability = 0.25), or one dextrorse and sinistrorse flower (probability = $(0.5 \times 0.5) + (0.5 \times 0.5) = 0.5$). The observed proportion of dextrorse and sinistrorse flowers on each branch was therefore compared to the random expectations. The proportion of dextrorse and sinistrorse flowers at each ultimate branch group was the replicate for this analysis. (see Supplementary Rcode 'Ch1_b').

All analyses were conducted with R: A Language and Environment for Statistical Computing (R Development Core Team 2008).

Results

To examine the proportion of dextrorse and sinistrorse at the population level we quantified a total of 454 flowers as dextrorse and 493 flowers as sinistrorse across 81 individuals of *H. irazuense* (mean = 11.7 flowers per individual, STDERR = 1.0). For *H. costaricense* a total of 66 dextrorse and 58 sinistrorse flowers were assessed across 49 individuals (mean = 2.53 flowers per individual, STDERR = 0.3). At the population level, both *H. irazuense* and *H. costaricense* exhibited no significant departure from an equal proportion of sinistrorse and dextrorse flowers (mean = 0.52, $t = 1.09$, $p = 0.278$, $df = 80$ and mean = 0.46, $t = 0.69$, $p = 0.489$, $df = 48$, respectively).

At the ultimate branch level, both *H. irazuense* and *H. costaricense* exhibited no significant departure from random distribution of dextrorse and sinistrorse flowers (Table 1.1, $\chi^2=1.17$, $p = 0.56$, $df = 2$ and $\chi^2 = 0.61$, $p = 0.74$ $df = 2$, respectively). That is to say, the presence of one chirality type does not influence the chirality type of its neighbor flower within a set of terminal branches.

Discussion

The presence of both chirality morphs within an individual raises the question whether this polymorphism is maintained due to a reproductive advantage or whether it is adaptively neutral. For both *Hypericum* species we found that the two chirality morphs had an equal proportion among individuals at the population level and these were expressed randomly at the ultimate branch level.

Unlike monomorphic enantiostyly where the adaptive significance has been investigated (Fenster, 1995; Jesson and Barrett, 2002a), the maintenance of chirality polymorphism within a species remains unknown. Enantiostyly is a type of reciprocal herkogamy (Webb and Lloyd, 1986b) that promotes outcrossing with outcrossing rates highest for dimorphic enantiostyly, lower for mono enantiostyly but still higher than for straight-styled flowers (Jesson and Barrett, 2002a). While this result explains the advantage of enantiostyly over non-enantiomorphic flowers, it doesn't explain why most enantiomorphic flowers are monoenantiomorphic when dimorphic enantiostyly has the highest outcrossing rate. Jesson et al. (2003) suggested that the evolution of dienenantiostyly may be developmentally constrained by the lack of positional cues in the bud that signal the formation of both left and right styled flowers within an individual.

The chirality in *Hypericum* is similar to monomorphic enantiostyly in that both dextrorse and sinistrorse morphs are present within the individual. The random distribution of dextrorse and sinistrorse flowers suggests that the maintenance of this polymorphism does not have an adaptive significance since floral developmental stability has been demonstrated to be maintained by natural selection (reviewed in Fenster and Galloway, 1997). However, we cannot exclude the possibility that the random distribution of chirality within branches is adaptive given that monomorphic enantiostyly has an adaptive advantage of an increased outcrossing rate compared to nonenantiomorphic flowers (Jesson and Barrett, 2002a). This advantage is present for monomorphic enantiostylous species independent of whether they have an alternate (*Solanum rostratum* and *Chamaecrista fasciculata*) or a random distribution (not associated with positional cues, e.g., *Heteranthera mexicana*) of right and left styled flowers.

If unfixed chirality has an adaptive significance, one could speculate that each flower morph is associated with different pollinator preference and behavior or pollen incompatibility, directed pollen movement, etc. However, corolla chirality differs from enantiostyly in that, other than the rotation of the corolla itself, the flowers are radially symmetrical. Thus, it is difficult to envision how differences in corolla rotation could direct the movements or behavior of pollinators, even when there is a highly specialized pollination system as has been recorded for some species of *Hypericum* (Robson 1972). For example, heterostyly has been described for several species of *Hypericum* but there is no difference in the number of legitimate and illegitimate pollen grains on either pin or thrum morphs (Ornduff, 1975) concordant

with the idea that radially symmetric and upwardly oriented flowers have relatively imprecise pollination (Fenster et al., 2009), and unlikely to promote pollination either within or between rotational morphs. In addition, while *H. irazuense* and *H. costaricense* may have UV- absorbent pigments concentrated in the anthers and ovary as has been shown for *H. calycinum* and *H. edisonianum* (presumably to attract and orient pollinators), this would not differ between dextrorse and sinistrorse flowers (Gronquist et al. 2001). However, in the absence of a careful analysis of flower anatomy, mating system and pollinator behavior an adaptive link between pollination and the maintenance of this floral polymorphism cannot be rejected.

Unfixed corolla chirality is common within the Rosids; however, it is fixed within the Asterids (Endress, 2001). The differential expression of corolla chirality across the angiosperms opens the question as to whether it is a result of developmental constraint related to phylogeny or an adaptive advantage related to pollination and subsequent reproductive success (Endress, 1999).

Whereas zygomorphy and its relevance to pollination specialization (Sargent, 2004; van der Niet and Johnson, 2012) and mating system evolution (Gómez et al., 2006) is well known, we remain largely ignorant of the adaptive significance of unfixed corolla chirality although it is present in at least 19 angiosperm families (Endress, 1999). Here we have extended previous observations of equal proportions of chirality morphs in Malvaceae (Davis, 1964; Ghosh and Davis, 1978) to Hypericaceae and we have shown that species with unfixed chiral flowers are similar to some cases of monomorphic enantiostyly in that chirality is randomly distributed among flowers within a plant. The next step is to determine if equal proportions of

chirality morphs are found in other families with unfixed corolla chirality and if there is an adaptive significance for either male or female function to explain the maintenance of this floral polymorphism.

Table 1.1. Number of sinistrorse/left and dextrorse/right flowers for each ultimate branch level with two open flowers in *Hypericum irazuense* and *H. costaricense*. Fifty individuals were evaluated for *H. irazuense* and twenty individuals for *H. costaricense* in the same location at the Páramo in Costa Rica.

Species	Both Dextrorse	Dextrorse & Sinistrorse	Both Sinistrorse	Total # of Branches
<i>H. irazuense</i>	70	112	56	238
<i>H. costaricense</i>	4	14	5	23



Figure 1.1. *Hypericum irazuense* (A) sinistrorse/left flower (B) dextrorse/right flower. Note that the hooks on the apex of each petal point in the opposite direction of rotation. Scale bar = 1 cm.



Figure 1.2. *Hypericum irazuense*. The white arrows are pointing to what was considered as the ultimate/terminal branch levels. This picture represents a group of ultimate branches. Flowers within this group were compared to analogous branch groups across the population. Scale bar = 1 cm.

Chapter 2: Corolla chirality does not contribute to directed pollen movement in *Hypericum perforatum* (*Hypericaceae*): mirror image pinwheel flowers function as radially symmetric flowers in pollination

Abstract

- **Premise of the study:** Corolla chirality, the pinwheel arrangement of petals within a flower, is found throughout the Core Eudicots. In 15 families, different chiral type flowers (i.e. right or left rotated corolla) exist on the same plant, and this condition is referred to as unfixed/enantiomorphic corolla chirality. There are no investigations on the significance of unfixed floral chirality on directed pollen movement even though analogous mirror image floral designs, e.g., enantiostyly, has evolved in response to selection to direct pollinator and pollen movement. Here we examine the role of corolla chirality on directing pollen transfer, pollinator behavior and its potential influence on disassortative mating.
- **Methods:** We quantified pollen transfer and pollinator behavior and movement for both right and left rotated flowers in two populations of *Hypericum perforatum*. In addition, we quantified the number of right and left rotated flowers at the individual level.
- **Key results:** Pollinators were indifferent to corolla chirality resulting in no difference in pollen deposition between right and left flowers.

- **Conclusions:** Corolla chirality had no effect on pollinator and pollen movement between and within chiral morphs. Unlike other mirror image floral designs, corolla chirality appears to play no role in promoting disassortative mating in this species.

Key words: disassortative mating; enantiomorphic contort aestivation; floral symmetry; mirror image; pollination; rotational symmetry

Introduction

Floral symmetry design plays a prominent role in plant mating system patterns (Barrett, 2002b), pollination systems (Faegri and van der Pijl, 1979; Johnson and Steiner, 2000; Fenster et al., 2004), pollen transfer efficiency (Gómez et al., 2006), and angiosperm diversification rates (Sargent, 2004; van der Niet and Johnson, 2012). Symmetry patterns are often associated with directed pollinator movement and consequently pollen movement. For example, bilateral symmetry is associated with predictable placement of pollen on a pollinators body while asymmetric mirror image flowers (enantiostyly) found on the same plant not only place pollen on specific parts of a pollinator's body but also direct pollen on to opposite sides of a bees body, greatly reducing the opportunity for selfing through geitonogamy (Sprengel, 1793; Barrett, 2002b; Jesson and Barrett, 2002a, 2005; Fenster et al., 2009).

Another, and highly understudied floral symmetry pattern is present in flowers with contort aestivation and results from the mutual covering of petal flanks in the flower bud (aestivation pattern). In contort aestivation each petal overlaps only one of

its neighbor petals (Schoute, 1935; Scotland et al., 1994). According to how the petals overlap, the corollas rotate clockwise or counter clockwise (also known as left and right respectively) and are chiral to each other (see Fig. 2.2 for further explanation on terminology) (Schoute 1935; Scotland et al. 1994; Endress 2001). Corolla chirality is visibly distinguishable when the aestivation pattern is still present after anthesis and mainly when individual petals are asymmetric conveying the corolla a pinwheel appearance (Endress, 1999, 2001). Endress (2001) summarizes the phylogenetic distribution of contort flowers across the angiosperm clade and distinguishes unfixed species (both right and left flowers are found on the same individual) from fixed species (all individuals of that species exhibit only one floral form). Note that no species have been observed where individuals are fixed for either right or left flowers, e.g., in no species are some individuals left and the remaining individuals right in the same population. Endress (2012) also refers to unfixed contort floral morphology as enantiomorphic but henceforth we refer to this condition as unfixed corolla chirality. Most fixed species are asterids and most unfixed species, such as *Hypericum*, are rosids (Endress, 2001, 1999). Very little is known, of the adaptive biology underlying unfixed corolla chirality, although it is present within eight taxonomic orders and fifteen families within the rosids (Endress, 1999).

Similar to monomorphic enantiostyly (Todd, 1882; Gao et al., 2006), most unfixed chiral species have a 1:1 ratio of flower morphs within individuals (Davis, 1964; Davis and Ramanujacharyulu, 1971; Diller and Fenster, 2014). In addition, Diller and Fenster (2014) found that the corolla chirality of a flower in two neotropical *Hypericum* species, *H. irazuense* and *H. costaricense*, is independent of

the chirality of its closest neighbor flower, indicating a random distribution of corolla types within an individual. Some monomorphic enantiostylous species, such as *Heteranthera mexicana*, also present a random distribution of morph types as in *H. irazuense* and *H. costaricense* (Jesson et al., 2003). Despite the similarities to monomorphic enantiostyly, we do not know if chirality variation has a parallel influence on pollen movement.

Corolla chirality differs from monomorphic enantiostyly by not having a reciprocal stamen to pistil arrangement between flowers. While enantiostyly increases outcrossing by the differential placement of pollen on the pollinator resulting from the alternate deviation of the style and stamen among flowers on the same individuals (Jesson and Barrett, 2002a), species with unfixed corolla chirality do not have reciprocal placement of anthers and stigmas. However, the reduction of geitonogamous self-pollination could still result if pollinators behave differently on right and left flowers resulting in differential pollen placement on the pollinator's body. Honey bees and bumble bees can distinguish flowers by differences such as location (Makino and Sakai, 2007), corolla shape and size (Galen, 1996; Galen and Cuba, 2001) color (Schemske and Bradshaw, 1999), scent (Cnaani et al., 2006) and symmetry (Giurfa et al., 1996; Gómez et al., 2006). Given this variety of sensory differences that honey bees and bumble bees respond to, it is conceivable that bumble bees may also respond to the direction of pinwheel rotation of flowers, which is exceptionally visible in *H. perforatum* due to their asymmetric petals. Thus we investigate the question of whether corolla chirality leads to asymmetric pollen-movement between right and left flowers similar to enantiostylous flowers.

Here we test whether unfixed corolla chirality is adaptive by decreasing geitonogamy through imposed directionality on pollen and pollinator movement, which to our knowledge has not been previously examined. We performed this study on *Hypericum perforatum*, an invasive plant of N. America, and specifically asked: Is pollinator behavior or pollen movement influenced by chirality type? In addition, given that corolla chirality is an understudied trait, we quantified the frequency and distribution of right and left flowers within individuals to link this study with other studies on chirality ratios for floral traits.

Materials and Methods

Site Description

This study was conducted at Mountain Lake Biological Station, Virginia from June 21st to July 10th 2012 using two populations, one along a roadside (N37° 22' 360'', W80° 31' 533'') and the other by an artificial pond (N37° 22' 459'', W80° 31' 350''), which we will refer to as population A and B, respectively.

Species Description

Hypericum perforatum L. (Hypericaceae) is a perennial shrub with flowers in a thyrse inflorescence, having five petals, three carpels, three stigmas and numerous stamens (Fig. 2.1) (Crompton et al., 1988; Stevens, 2007). In addition, every individual has two floral chiral types. These flowers can be easily distinguished by the shape and direction of their petals. Each petal is asymmetric with one straight side

while the other is rounded and serrated. When an open flower is looked at from above, the right morphotype have the rounded side of every petal located on the right (Fig. 2.2F) and flowers of the left morphotype have it located to the left of every petal (Fig. 2.2E). *Hypericum perforatum* plants are typically 0.3-0.9 m tall (Crompton et al., 1988) and the mean number of open flowers per individual in our study site was 5.05 with a range of 1 to 15 flowers in population A (n = 22 individuals) and 8.53 with a range of 1 – 29 flowers per individual in population B (n = 35 individuals).

Hypericum perforatum is native to Europe and has been present in the US since 1793, given incomplete herbarium records or historical documents on the presence of the species (Muhlenberg, 1793; Sampson and Parker, 1930). It is self-compatible (Molins et al., 2014) as well as a pseudogamous facultative apomict (Matzk et al., 2001; Barcaccia et al., 2006; Molins et al., 2014). The latter means that *H. perforatum* can reproduce both sexually or asexually. Asexual reproduction in *H. perforatum* is characterized by the formation of an embryo without meiotic reduction nor fertilization (apomixis) (Barcaccia et al., 2006). Fertilization is however usually still required for the endosperm formation (pseudogamy) (Barcaccia et al., 2006). Crompton et al. (1988) state that fruit derived from selfed or cross-pollinated flowers developed equally well in the greenhouse. Our observations confirmed these results for population B, but seed production was significantly greater for flowers cross pollinated by hand vs that had been excluded from pollinators in population A (Appendix 2.1A). In addition, we found no differences between right or left flowers in seed production for plants that were either: 1) excluded from pollinators with bags,

2) open-pollinated or 3) cross-pollinated by hand (Appendix 2.1C and D) as well as no differences in pollen and ovule number (Appendix 2.2 and 2.3), respectively.

Because our study focuses on potential differences between right or left flowers and whether unfixed chirality mediates non-random pollinator or pollen movement, our investigations should not be unduly affected by the fact that the species is a facultative apomict. Many flowering plant species are facultative selfers yet have floral morphologies associated with promoting precise pollination and outcrossing (e.g., Fenster and Martén-Rodríguez, 2007). Analogously it is reasonable to assume that there are traits that promote outcrossing and precise pollination in a facultative apomict such as *H. perforatum*. Finally, we detected pollen limitation in both populations (Appendix 2.1B). The presence of both pollen limitation and greater seed production for cross pollinated flowers vs flowers excluded by pollinator in at least one population may suggest strong selection for floral traits related to promote outcrossing in *H. perforatum* (Knight et al., 2005 and references therein).

Data Sampling

Distribution of chirality types—

To quantify the ratio of right and left chiral flowers within individuals, we counted the number of left and right flowers for 55 individuals (22 individuals in population A and 35 individuals in population B).

Chirality, pollinator interactions and pollen movement —

Pollinator visitation and preference—To determine whether pollinators discriminate among chirality types, we observed pollinator visitation for 23.5 hours with 19 video observations (9 in population A and 10 in population B). Durations of each video observation consisted of 1 hour or 1.5 hours. The video observations were performed on 15 different plants and on 41 right flowers and 43 left flowers. Since we were not able to identify the pollinators to the species level in the video observations, we classified them into three groups corresponding to large bees, small bees and syrphid flies (for species examples see Table 2.1). These groups were formed with the assumption that the pollinator size and behavior are similar within a group and could potentially differ between groups. For each pollinator visit, we recorded the chirality of the flower visited. We captured nine pollinators throughout the study and identified them to the species level (Table 2.1).

Pollinator sequence: Movement between flowers—We evaluated whether pollinators moved between right or left flowers in a random or non-random pattern. We examined the same video observations as above and registered for each pollinator observed the sequence in which they visited the flowers that were recorded in our videos. Each video period viewed at least one left and one right flower, and most more than one (with an average of right = 2.38 and left = 2.46 flowers). As an example, if two left and one right flower(s) were observed, and if a bee first visited the left flower, next the right, and then a left flower again, then we would report this visitation bout as a left-right-left sequence. Again, we identified the pollinator to pollinator group (Table 2.1) and not to the species level.

Pollinator behavior: Movement within flowers—We also examined how pollinators behaved when visiting a right and a left flower. With the same video observations as above, we registered how each pollinator moved during each floral visit. We identified four movement categories: right rotation, left rotation, both rotations and no rotation (Fig. 2.3).

Pollen transfer—To evaluate the direction of pollen transfer from right and left flowers, we dyed the anthers of 14 flowers of one chirality type with a pink fluorescence powder during the morning. Later in the afternoon, nearby right and left flowers were collected from within a radius of 0.5 m of the dyed flower. All three stigmas were removed and checked under a fluorescent microscope for dye transfer, an analog of pollen movement (e.g., Fenster et al., 1996). Two days later, the experiment was repeated for the opposing chirality type at that same population. This two-day cycle was repeated 5 times. The period between experiments was implemented to avoid the carryover of fluorescence powder from the previous experiment with the opposite chirality type. During this experiment we dyed a total of 140 flowers (70 right and 70 left flowers) and collected 138 left flowers and 139 right flowers on 19 individuals in population A and 18 individuals in population B.

Data Analysis

All analyses were conducted with R: A Language and Environment for Statistical Computing (R Development Core Team, 2008) with nlme (Pinheiro J, Bates D, DebRoy S, 2016), multcomp (Hothorn et al., 2008) and glmmADMB packages. We checked for the underlying assumptions to all statistical tests applied in

this study and when the assumptions were not met we transformed the data accordingly.

Distribution of chirality types—

To determine if the ratio of right and left flowers within an individual deviates from a 1:1 ratio, we calculated the proportion of left flowers for each individual and then compared the mean value (across all individuals) to 0.5 with a Student's t-test. Observed proportions and the 0.5 expectation were arcsine square root transformed and each individual was treated as a replicate for this test. See Supplementary R code 'Ch_2_a' and Supplementary Raw Data Ch2_1.

Chirality, pollinator interactions and pollen movement—

Pollinator visitation and preference— To evaluate whether pollinators prefer to visit each chirality type differentially, we performed a mixed model ANOVA (nlme package, R) and a post hoc Tukey test (multcomp package, R) on the visitation rates between right and left flowers. In this analysis, population and video observation were assigned as random factors (video observation nested within population) and chirality and pollinator group as a fixed factor.

In the analysis, each video observation represented a replicate. We calculated a standardized visitation rate for each video observation by:

$$\frac{\sum \text{Right flowers visited}}{\sum (\text{Right flowers observed} * \text{hours observed})} \quad \text{and} \quad \frac{\sum \text{Left flowers visited}}{\sum (\text{Left flowers observed} * \text{hours observed})}$$

Essentially, for each video observation, we summed the number of right or left flowers visited and divided it by the total number of right or left flowers observed

multiplied by the number of hours observed to generate a visitation rate metric. See Supplementary R code ‘Ch_2_b’ and Supplementary Raw Data Ch2_2 and Ch2_3.

Pollinator sequence: Movement between flowers—To assess whether pollinators visit right and left flowers in a random sequence, we performed a mixed model ANOVA (nlme package, R) on the number of transitions that pollinators performed between right and left flowers. In this analysis, population and video observation were assigned as random factors (video observation nested within population) and chirality transition as a fixed factor. There were four possible chirality transitions: right to right (R-R), right to left (R-L), left to right (L-R) and left to left (L-L). The question addressed here is similar to the studies by Waser (1986) and Hopkins and Rausher (2012) on pollinator constancy or pollinator movement, but the analysis differs given that we did not experimentally standardize for the number of right and left flowers to which pollinators were exposed to in each given video recording. To account for the fact that the videos differed in the number of right and left flowers observed as well as total number of visits, we calculated the deviation of the observed frequencies for each chirality transition from the expected, with the assumption that the pollinators move equally between right and left flowers. We calculated the expected frequencies by first calculating the expected proportion of flowers visited by each pollinator group in each video observation. For this, we multiplied the conditional probability of moving from one flower to the other by the probability of being on either a right or left flower. For further details and a worked through example please see Appendix 2.4 and Supplementary Material 2.1.

We constructed a parameter to measure an absolute deviation from expected random movement among chiral types for each camera. We subtracted the observed frequencies from expected frequencies (based on no bias of transition) and performed a square root transformation on the data. For this analysis, we eliminated videos for which less than 20 flower transitions were observed to assure that the deviations from the expected are not an artifact of low sample size. Thus, the range of flower transitions observed across all videos included in this analysis was 25 to 169 for the 11-15 camera observations, depending on the analyses. We asked whether there was a difference in transition probabilities among the four possible bee/flower transitions, i.e., right to right, right to left, left to left and left to right. That is, each of the transition sequences was treated as an independent replicate in our analyses. See Supplementary R code ‘Ch_2_c’ and Supplementary Raw Data Ch2_4.

Pollinator behavior: Movement within flowers—To test whether pollinators behave differently on right and left flowers we performed a Generalized Linear Mixed Model (glmmADMB package, R) and a post hoc Tukey test (multcomp package, R). We fitted the data to a negative binomial distribution appropriate for our data and modeled pollinator behavior type (see Fig. 2.3) and chirality as fixed factors and population and video as random factors (with video nested within population). We tested the explanatory power of chirality and behavior by constructing a series of nested mixed models and comparing each model to the previous one using likelihood-ratio tests. In addition, we chose to err on the side of the most conservative model by selecting a model with the least number of parameters within 3.22 units from the lowest AIC (Burnham and Anderson, 2010; Fenster et al., 2015). For every video

observation we added the number of visits for each floral chirality and type of behavior combination observed. Then we standardized these frequencies by dividing it by the number of flowers observed * hours of observation for each video. For example, two right and one left flower(s) were filmed for a period of 1.5 h during one video observation and a total of 24 pollinators visited the right flowers while 20 pollinators visited the left flower. In both cases in this example, the pollinators moved without rotation (NR) while visiting the flowers. In order to calculate the standardized visitation rates for no rotation, both on a right and left flower, we divided 24 by two right flowers * 1.5 h and 20 by one left flower * 1.5 h respectively. Each video observation was treated as a replicate for this test (n = 19). We also performed this analysis for large and small bees separately, but only report results for all pollinators combined due to no differences found among the main pollinator groups observed in this study. See Supplementary R code ‘Ch_2_d’ and Supplementary Raw Data Ch2_5.

Pollen transfer—Each stigma’s fluorescence intensity was classified from a scale of one to five; with one being very little and five being very strong. Only one observer made these measurements, reducing measurement error due to potential differences in the qualitative assessment of fluorescent intensity by different observers. The mean of the fluorescence intensity of the three stigmas was calculated for each flower. We used the fluorescence intensity as a proxy for pollen transfer (Kearns and Inouye, 1993; Fenster et al., 1996) and performed a mixed model ANOVA (nlme package, R) to test potential differences in the quantity of pollen transfer and deposition between right and left flowers. In this analysis, Population and

Block were random factors and Donor (the chirality of the flower on which we applied the fluorescent dye on the anthers) and Recipient (the chirality of the flower on which we qualitatively estimated the fluorescent dye on the stigmas) were fixed factors. We blocked our data every two consecutive experiment days (to control for climatic and other unexplained variation) resulting in five blocks. Each day we applied fluorescent dye to only one type of floral chirality, therefore, each block contains one right and left donor treatment. We standardized the mean fluorescence intensity for each flower with the highest fluorescence intensity of that given day to reduce the block interaction effect. Then we averaged the fluorescence intensity for each individual within each block and performed an arc sine transformation in order to improve the normality of the data. Our replicate level was the average fluorescence intensity for each individual within each block, resulting in a total of 60 right donor individuals, 69 left donor individuals, 65 right recipient individuals and 64 left recipient individuals in the analysis.

A significant donor effect indicates that right and left flowers donate pollen differentially, and a significant recipient effect indicates that right and left flowers are receiving pollen differentially. A significant interaction effect could demonstrate greater likelihood of transfer to opposite chirality. See Supplementary R code ‘Ch_2_e’ and Supplementary Raw Data Ch2_6.

Results

Distribution of chirality types—

We observed 174 right flowers and 221 left flowers in both populations, 45 right and 60 left flowers in population A, and 129 right and 161 left flowers in

population B. The proportion of left flowers within an individual differed marginally significantly from 0.5 in population B (mean = 0.64, 95% CI [0.44, 0.67], $t = 2.07$, $df = 33$, $P = 0.05$), but did not when both populations were combined as well as for population A (mean = 0.41, 95% CI [0.25, 0.59], $t = 0.99$, $df = 54$, $P = 0.326$; mean = 0.56, 95% CI [0.44, 0.67], $t = 0.99$, $df = 20$, $P = 0.33$ respectively).

Chirality, pollinator interactions and pollen movement—

*Pollinator visitation and preference—*During the 23.5 hour video observations the visitation rate was significantly different among pollinator groups ($F_{3,76} = 39.3$, $P < 0.001$, $n = 19$ video observations, see Fig. 2.4) with the visitation rate for large bees statistically different from the other pollinator groups (Tukey, $P < 0.001$, see Fig. 2.4). However, there was no significant difference between the visitation rate for right (mean \pm SE for the number of right flowers observed divided by the number of right flowers observed multiplied by hours of observation: 20.48 ± 2.47) and left (mean \pm SE for the number of left flowers observed divided by the number of left flowers observed multiplied by hours of observation: 19.31 ± 2.82) flowers when all pollinator groups were combined ($F_{1,76} = 0.13$, $P = 0.72$, $n = 19$ video observations).

*Pollinator sequence: Movement between flowers—*Pollinators moved between right and left flowers at equal proportions. All deviations from the observed chirality transitions performed by the pollinators (i.e. right to right, right to left, left to right and left to left) to the expected, did not differ significantly from one another ($F_{3,42} = 0.94$, $P = 0.43$, $n = 15$ video observations). The same was found when analyzing the

two pollinator groups with the highest visitation rate separately: large bees ($F_{3, 42} = 0.67$, $P = 0.56$, $n = 15$ video observations) and small bees ($F_{3, 30} = 1.26$, $P = 0.3$, $n = 11$ video observations).

Pollinator behavior: Movement within flowers—We found that pollinators do not adopt the four observed behaviors (rotate to the right “R”, to the left “L”, not rotate “NR” or rotate to the left and right “R+L”; see Fig. 2.3) equally while visiting the flowers. The model with behavior type as a single fixed factor was the only one with a significantly lower AIC (likelihood ratio test; $\chi^2 = 218.7$, $P < 0.001$; see Table 2.2). The post hoc Tukey test indicates a significant difference between no rotation (NR) and the rest of the pollinator behavior types ($P < 0.001$, see Fig. 2.5), because of a significantly higher number of non-rotating (NR) visits to flowers. In addition, the post hoc Tukey’s test shows no significant differences in pollinator behavior between right and left flowers ($P = 0.53 - 1$; see Fig. 2.5).

Pollen transfer—There was no significant difference between the amount of fluorescence dye (analog of pollen) donated ($F_{1, 118} = 1.26$, $P = 0.26$) or received ($F_{1, 118} = 0.5$, $P = 0.5$) between right and left flowers.

Discussion

This is the first study to explore whether corolla chirality imposes directionality on pollen movement. Differential pollen movement could occur if differences in corolla chirality prompts pollinators to interact differently on right and left flowers resulting in differential pollen placement on the pollinator’s body. However, all of our observations indicate no effect of corolla chirality on pollen

movement. Pollinators are indifferent to corolla chirality and consequently there is no difference in pollen deposition between right and left flowers. Overall, we find no evidence that corolla chirality is an adaptation promoting precise pollination. We found a 1:1 ratio of left and right flowers within individuals, concordant with ratios found for other species with unfixed corolla chirality (Davis, 1964; Davis and Ramanujacharyulu, 1971; Diller and Fenster, 2014).

Different patterns of pollinator movement on right and left flowers may translate into differential pollen placement on the pollinator's body and thus an increase in pollen carry over distance and a reduction in geitonogamy. Our hypothesis was that pollinators may move clockwise or counterclockwise, depending on the rotational pattern of the flower, leading to different pollen placement on the body of the pollinator. Bilateral symmetric flowers are considered to increase pollinator directionality by forcing pollinators to approach the flower from only one direction and thus is thought to also increase pollen movement efficiency and directionality (Neal et al., 1998; Gómez et al., 2006). A comparable process could result in *H. perforatum* flowers if, for instance, pollinators have an innate preference to move in a circular mode from the rounded side towards the straight side of the petal. In this example, pollinators would move right (clockwise) in the left flowers and left (counter clockwise) in the right flowers. Differential rotational movement while probing for pollen on the flower could potentially result in differential pollen placement on the right and left area of the pollinator's body. However, we find no evidence that unfixed chirality is associated with directed pollinator movement on the flower. Bees treat the different chiral types indiscriminately, mostly moving straight

across the flower (no rotation), and when turning on the flower, their turns are unrelated to chiral type. In this sense, the interaction of *H. perforatum* with pollinators resembles that of typical vertically oriented, radially symmetric flowers, which are less capable of directing pollinator movement compared to bilateral flowers (Fenster et al., 2009).

Reciprocal herkogamy, such as heterostyly or enantiostyly are reproductive strategies that increase pollen movement in flowers with radially symmetric corollas (Barrett, 2002b). Heterostylous flowers have anthers and stigmas that differ reciprocally in position among flowers of different individuals and is usually associated with incompatibility between morph types. Pollen–stigma incompatibility between right and left flowers could have also contributed to pollen movement directionality in *H. perforatum* despite the lack of reciprocal positioning of anthers and stigmas. We find no pollen – stigma incompatibility between right and left flowers (Appendix 2.1E), but these results need to be interpreted with caution given that we did not emasculate the flowers, and because *H. perforatum* is a facultative apomict. Heterostyly evolved in three species of *Hypericum* that also have corolla chirality, but the incompatibility between stamen-pistil height morphs is not complete in the most studied species, *H. aegypticum* (Ornduff, 1975). However, *H. aegypticum* does not have a strong petal asymmetry associated to corolla chirality and more importantly to our study, the study makes no mention that the two heterostylous morphs correlate with a specific chirality type.

While studies are lacking to fully comprehend to what extent plant mirror image structures (both vegetative and reproductive) differ in their genetic control and

developmental pathways, it seems that there is some variation in the degree to which they are genetically or environmentally determined. Cross experiments show no inheritance in the direction of leaf phyllotaxy (Allard, 1946; Davis, 1962; Hashimoto, 2002), in contrast to Mendelian genetic control for the expression of style orientation in the dimorphic enantiostylous *Heteranthera multiflora* (Jesson and Barrett, 2002a; b) and the direction of twisting of pods for *Medicago turberculata* and *M. litoralis* (Lilienfeld and Kihara, 1956). Additional studies successfully induced spiraled roots, stems and flower organs in *Arabidopsis thaliana* through mutations (see references in Hashimoto, 2002). Relevant to the discussion of unfixed corolla chirality is that the direction of the helical growth can be either fixed or random depending on the genes disrupted (Hashimoto, 2002). To our knowledge, there are no other studies on the genetic control for mirror structures when both morphotypes are expressed within an individual. Nevertheless, even if the chiral identity of a new shoot or a new flower bud is environmentally determined (such as by the symmetry of the entire inflorescence (Endress, 1999, 2001)), this does not exclude the possibility for unfixed mirror image structures to be adaptive as in monomorphic enantiostyly.

Most of the examples of chirality in vegetative structures and other reproductive structures also challenge an adaptive explanation and instead suggest a neutral hypothesis (e.g., leaf phyllotaxy, stem twisting, cone spirality, fruit arrangement in sunflower heads or circumnutation, i.e. the helical movement of plant organs) (Allard, 1946; Davis and Ramanujacharyulu, 1971; Davis and Davis, 1987; Minorsky, 1998; Klar, 2002; Edwards et al., 2007; Stolarz, 2009). An exception to this, apart from enantiostyly, is anisophylly with dorsiventral shoot symmetry, i.e. the

pair of dorsal leaves are smaller than the pair of ventral leaves. This type of vegetative mirror image is thought to reduce leaf shade of dorsal leaves to the ventral leaves and thus increase photosynthetic surface area (Dengler, 1999; Muelbert et al., 2010).

This study provides evidence that unfixed corolla chirality, unlike mirror image enantiostyly, does not represent an adaptation associated with promoting disassortative mating between floral morphs, or directed movement of pollen between flowers. Instead, our findings demonstrate that unfixed corolla chirality may be similar to other radial symmetrical flowers with open and generalized pollination systems and consequently does not direct pollen movement between flowers. It remains to be determined whether these findings are generalizable to other major clades where corolla chirality is also found.

Table 2.1. Pollinator species captured and identified while visiting *Hypericum perforatum* flowers at Mountain Lake Biological Station (MLBS), VA. For the analyses, we classified the pollinators into three groups corresponding to large bees, small bees and syrphid flies because of the expectation that pollinator size and behavior are similar within a group and would likely differ between groups. This is not an exhaustive list of possible species diversity visiting *H. perforatum* at MLBS, but an example of the most common visitors.

Pollinator Groups	Species
Apidae (large bee)	<i>Bombus griseocollis</i>
	<i>Bombus impatiens</i>
	<i>Bombus perplexus</i>
	<i>Bombus sp.</i>
Colletidae + Halictidae (small bee)	<i>Augochlora pura</i>
	<i>Lassioglossum cressonii</i>
	<i>Lassioglossum viridatum</i>
Syrphidae (syrphid flies)	<i>Hylaeus affinis</i>
	Unidentified to species

Table 2.2. Model selection for pollinator behavior (movement within flowers) while visiting *Hypericum perforatum* flowers at Mountain Lake Biological Station, VA. Models in the table are arranged by increasing complexity starting with the null model. The null model includes the random factors, as intercept only models are not possible when fitting a generalized linear mixed model (glmmADMB package, R). Video is nested within population. “Test” indicates which models are tested in the likelihood ratio test. χ^2 and *P* values are outputs from the likelihood ratio test. The AIC value in bold, indicates the model with the least number of parameters and within 3.22 units from the lowest AIC. Models were selected based on likelihood ratio tests and corroborated with AIC values.

<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>P value</i>
A. Null = Population + Video	-713.90	1437.8	-	-	-
B. Chirality + Population + Video	-713.71	1439.4	A vs. B	0.392	0.5312
C. Behavior + Population + Video	-604.37	1224.7	A vs. C	218.68	< 0.001
D. Behavior + Chirality + Population + Video	-603.92	1225.8	D vs. C	0.880	0.3482
E. Behavior + Chirality + Behavior * Chirality + Population + Video	-603.90	1231.8	E vs. D	0.050	0.9971



Fig. 2.1. *Hypericum perforatum* flower with a syrphid fly (for scale) at the Mountain Lake Biological Station. This flower is an example of a right flower (see Fig. 2.2 for an explanation of terminology).

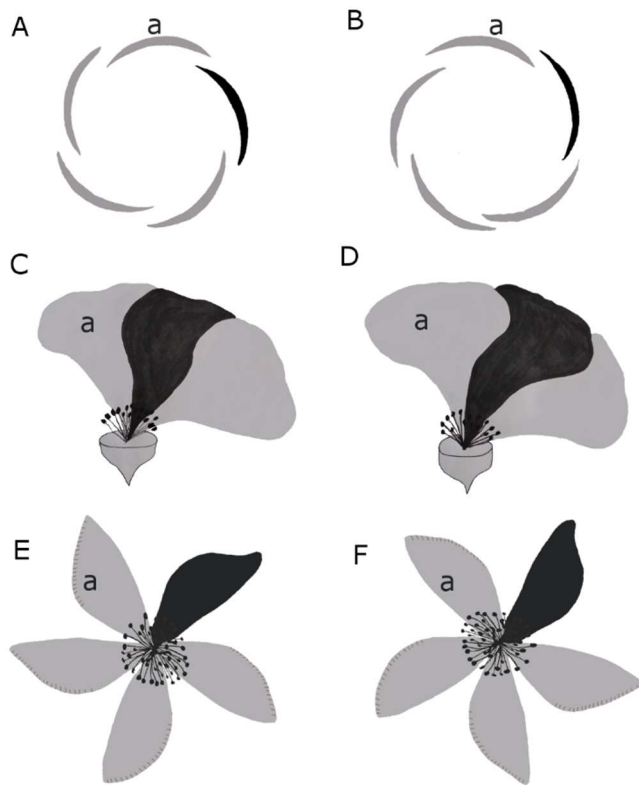


Fig. 2.2. Explanation of the terminology of right and left chirality in *Hypericum perforatum* at the Mountain Lake Biological Station. Flower diagram at flower bud stage. **(A)** Left flower. Each petal overlaps its clockwise neighbor petal (e.g. petal “a” overlaps the shaded petal). Alternatively, if viewed from the side, the left of each petal overlaps its neighbor petal. **(B)** Right flower. Each petal overlaps its counter-clockwise neighbor petal (e.g. the shaded petal overlaps petal “a”). Alternatively, if viewed from the side, the right of each petal overlaps its neighbor petal. Flower diagram at anthesis (open flower) **(C)** left flower **(D)** right flower. Simplified flower diagram (not to scale) at anthesis for *H. perforatum* (E and F). Overlap of petals is less evident in open flowers of *H. perforatum*. However, corolla chirality is still visually distinguishable due to petal asymmetry associated to chirality. Petals have

one rounded and serrated side and one straight side. The rounded section defines the direction of the pinwheel rotation. **(E)** Left flower: pinwheel rotation is counter-clockwise; **(F)** right flower: pinwheel rotation is clockwise. Right or left flowers are defined by the direction of overlap of petals, and not by the direction of the pinwheel rotation. Notice that the circular direction of the pinwheel and the overlap of petals in *H. perforatum* are opposite, i.e. flowers with petals that overlap in a clockwise direction have a counter-clockwise rotating pinwheel. However, both are “left” flowers, thus to avoid confusion in terminology referencing to petal overlap or corolla (pinwheel) rotation, here we define chiral morphs by right and left flower instead of clockwise and counter-clockwise.

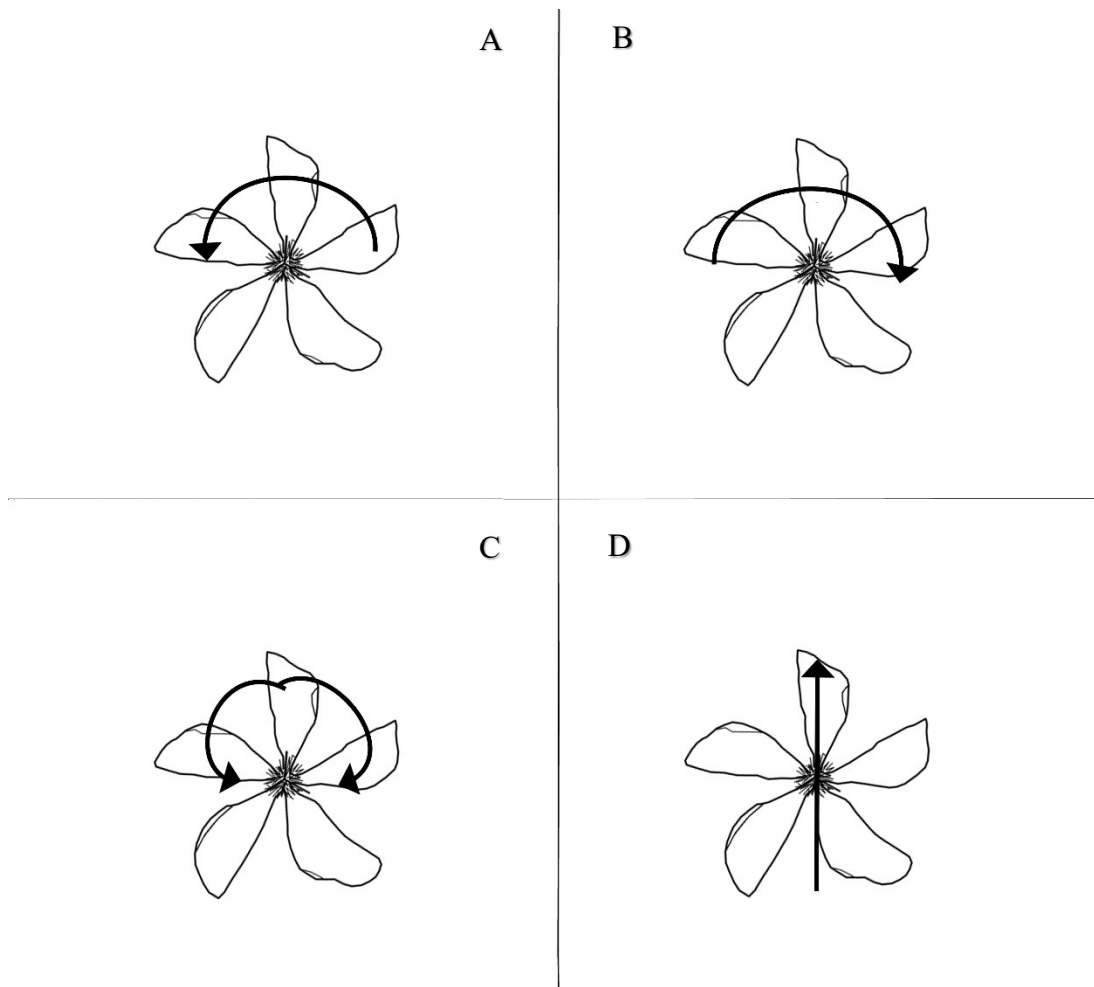


Fig. 2.3. Pollinator behavior observed at the Mountain Lake Biological Station on *Hypericum perforatum*. Arrows indicate pollinator movement. **(A)** left rotation **(B)** right rotation **(C)** right and left rotation (pollinator rotates right, then turns and rotates left or vice versa) **(D)** no rotation (pollinator starts at one side and travels in a straight line before leaving the flower).

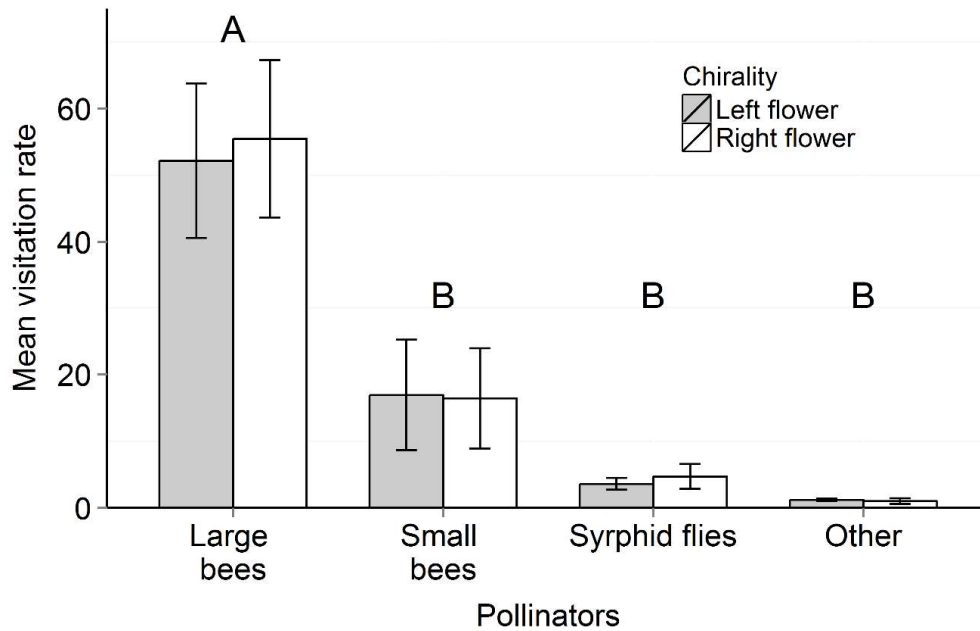


Fig. 2.4. Pollinator visitation rate on right and left flowers of *Hypericum perforatum* as measured at the Mountain Lake Biological Station. Mean visitation rate (number of visits observed for right or left flowers/ number of right or left flowers observed * hours of observation) for each pollinator group and chirality combination. The visitation rate was significantly different among pollinator groups (mixed ANOVA on all pollinator groups combined, $P < 0.001$, $n = 19$ video observations) with the visitation rate for large bees statistically different to the other pollinator groups (Tukey, $P < 0.001$, see “A” and “B” on graph). Pollinators did not discriminate between right and left flowers (mixed ANOVA on all pollinator groups combined, $P = 0.72$). Error bars represent SE.

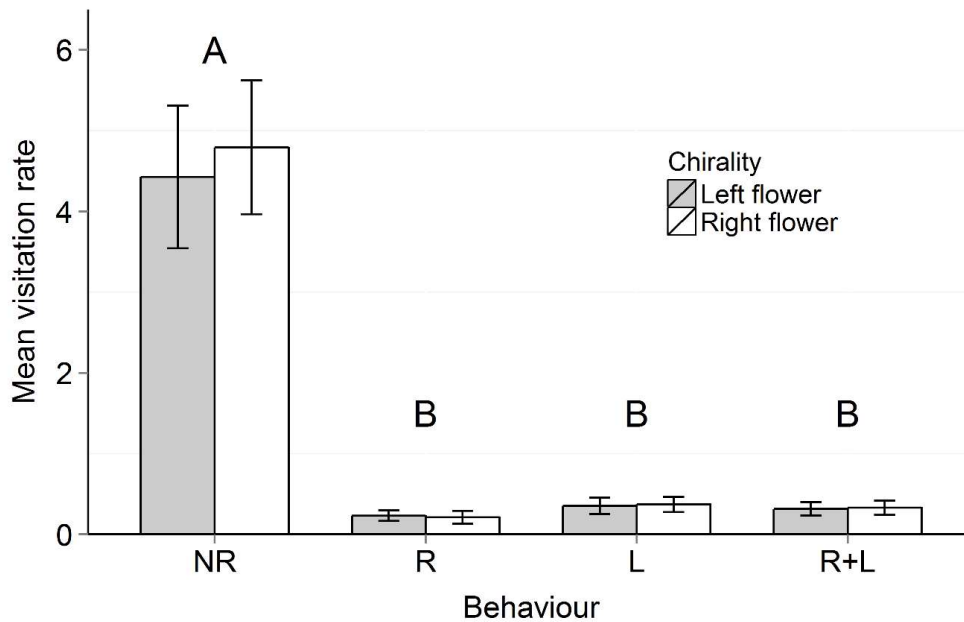


Fig. 2.5. Pollinator behavior observed on right and left flowers of *Hypericum perforatum* at the Mountain Lake Biological Station. Mean visitation rates of all pollinators combined on right and left flowers with each of the following behavior types: NR = no rotation, L = left rotation, R = right rotation, R+L = right and left rotation (see Fig. 2.3). “A” and “B” are significantly different from each other according to Tukey’s test ($P < 0.001$), indicating that pollinators preferred not to rotate (NR) while visiting a flower. There are no significant differences in pollinator behavior between right and left flowers (models that included chirality as an explanatory variable did not have a significantly lower AIC value). Error bars represent SE.

Chapter 3: Evaluating the role of floral traits in pollination

precision – flower orientation matters.

Abstract

Background and Aims: Pollination precision, the process of ensuring that pollen is transferred to conspecifics, impacts plant reproductive isolation and individual plant reproductive success. Precise pollination may result from restricted landing and movement of pollinators on flowers or through increased pollination specialization. More precise pollination is hypothesized to result in more uniform selective pressures relative to less precise pollination, resulting in reduced phenotypic flower size variation. Several studies have explored the relationship between flower shape and pollination precision and have shown that bilateral symmetry, contributes to increased pollination precision as quantified by reduced flower size variation relative to radially symmetric flowers. Here we perform a phylogenetically informed meta-analysis on data collected from twelve papers (published from 1960- 2015) that address the relationship between patterns of floral variation and pollination system, in addition to our own measurements on herbarium samples. This study extends on previous studies, by incorporating more floral traits as predictors of pollination precision as well as the inclusion of more species.

Methods: We performed a phylogenetic generalized least square analysis on 327 angiosperm species. We evaluated the effects of flower shape on flower size variation

across individuals. We used flower size variation as a proxy for pollination precision, with reduced flower size variation as an indicator of increased pollination precision and consistent pollinator mediated selection. Floral traits included in our analysis were: flower symmetry, perianth fusion, stamen merosity, flower tube, flower orientation and stamen and pistil exertion.

Key Results: Flower orientation and symmetry have significant effects on flower size variation. Specifically, flowers with lateral orientation and bilateral symmetry are less variable in flower size, than their alternate states. In addition, the effects of flower orientation and flower symmetry are independent of the state of the other floral traits analyzed in this study.

Conclusions: We find evidence that pollination precision is mostly determined by flower orientation and flower symmetry, traits that alter the landing and movement of pollinators on the flower. Flower orientation, a previously overlooked trait, is as important a floral trait in determining pollination precision as floral symmetry.

Key words: flower size variation; flower symmetry; flower tube; perianth fusion; phenotypic variation; phylogenetic generalized least squares; pollinator mediated selection; stamen and pistil exertion; stamen merosity

Introduction

The vast diversity of floral shapes are associated with different reproductive, mating and pollination strategies (Faegri and van der Pijl, 1979; Fenster et al., 2004; Willmer, 2011). Pollination precision has direct effects on the reproductive success

and isolation of plant species (Sargent, 2004; van der Niet and Johnson, 2012). Precise pollination results in increased reproductive isolation from sympatric species through the reduction of the set of pollinators visiting the species and or by the precise location in which the pollen is deposited and removed from the pollinator's body, reducing the loss of pollen to interspecific plant individuals (Darwin, 1877; Grant, 1949; Stebbins, 1951; Berg, 1960; Zhang et al., 2012). We expect certain floral shapes such as bilateral symmetry, fused petals, lower merosity, etc., to promote precise pollination as they restrict the pollinator's position when visiting a flower, increase pollination specialization or restrict the position of the reproductive organs relative to the pollinator (Bessey, 1915; Stebbins, 1951; Berg, 1958, 1959, 1960; Fenster, 1991; Armbruster et al., 1999; Wolfe and Krstolic, 1999; Fenster et al., 2004; Ushimaru et al., 2006; van Kleunen et al., 2008; Herrera et al., 2008; Gong and Huang, 2009; Nikkeshi et al., 2015). While the link between floral shape and pollination precision has been addressed since discussion of the relationship between flower structure and function Sprengel (1793) and especially so within an adaptive context, e.g., Darwin (1862) and Stebbins (1951), there is limited evidence of the contribution of specific floral traits to pollination precision.

Pollination precision may influence the type of selection exerted on the flowers, with more uniform selection associated with precise pollination systems and variable and contrasting selective pressures associated with less precise pollination systems. Thus we expect those trait states associated with more precise pollination to have lower phenotypic variation across individuals than alternative trait states. That is to say, precise pollination only works if all flowers within that species are very

similar in size and shape across individuals. The effects of pollination precision on flower size variability have been explored for over 50 years with many studies that support this hypothesis, a.k.a. the pollinator mediated stabilizing selection, PMSS hypothesis (Berg, 1958, 1959, 1960; Fenster, 1991; Armbruster et al., 1999; Wolfe and Krstolic, 1999; Ushimaru et al., 2006; van Kleunen et al., 2008; Herrera et al., 2008; Gong and Huang, 2009; Rosas-Guerrero et al., 2011; Lázaro and Totland, 2014; Nikkeshi et al., 2015, but see Herrera, 1996). The two main floral traits that usually are studied in relation to floral size variation are floral symmetry and floral fusion. However, many more floral traits, such as floral orientation and stamen merosity, have been invoked to play a role in pollination precision (Stebbins, 1951; Berg, 1960; Fenster et al., 2009; O'Meara et al., 2016). In addition, few studies have examined the effect of different floral trait combinations on floral size variation (but see Berg, 1960; Armbruster et al., 1999; Herrera et al., 2008; Nikkeshi et al., 2015), although there is evidence of selection acting on trait combinations at below (e.g., Fenster et al., 2015) and above the species level (O'Meara et al., 2016). Therefore evidence attributed to one trait may in fact be a signal of a combination of traits.

Here we perform a phylogenetically informed meta-analysis of twelve studies (published 1958 - 2015) that have addressed the relationship between patterns of floral variation and pollination system (292 angiosperm species). Furthermore, we included an additional 51 species with our own measurements. With this data set, we evaluate the effects of eight morphological and functional floral traits on flower size variation, as a proxy for pollination precision. Specifically, we perform a phylogenetic least squares analysis to determine which floral traits (i.e. symmetry,

perianth fusion, stamen merosity, flower orientation etc.) and their interactions or combined effects explain the difference in floral size variation across individuals observed across angiosperm species. Thus, we provide a more powerful macroevolutionary analysis than previous studies both with the inclusion of more floral traits as predictors of trait variation as well as the inclusion of more species.

Materials and Methods

Data Set:

We collected data for 303 angiosperm species from twelve studies that test the relationship between hypothesized pollination precision and flower size variation since 1958 to 2015 (see Supplementary Material Table 3.1 for a summary of these studies). In addition, we measured a total of 122 species on herbarium specimens from the National Herbarium, NMNH, Smithsonian Institution (see Supplementary Material Table 3.2). Only three species overlapped with the data set collected from previous published studies (*Deutzia crenata*, *Lonicera japonica* and *Sisyrinchium angustifolium*). In the case of unisexual or imperfect flowers, only male flowers were measured because male and female flowers may be subject to different types of sexual selection and presumably pollinator mediated selection for pollination precision may be stronger on male flowers than on female flowers (Charnov, 1979; but see Wilson et al., 1994). All species names were verified for spelling and synonyms with ‘The Plant List’ (Anon, 2013).

To assess the reliability of floral measurements on dry and pressed flowers versus fresh flowers, we measured and collected fresh flowers for 9 species, pressed

and dried them and measured them again. We measured all flower whorls and estimated the coefficient of variation for each species both for the fresh and dry measurements. Correlations coefficients between fresh and dry measurements for the mean of the CV per species ranged from 0.80 to 0.99 across all whorls. All measurements were performed with a 0.01 mm dial caliper.

Flower Trait Matrix

We searched images and illustrations on the web for all species (see Supplementary Material Table 3.3 for image sources). From these images, we recorded the following traits for every species: perianth symmetry, functional symmetry, tube presence, perianth fusion, functional tube, stamen merosity, stamen & pistil exertion and flower orientation (see Table 3.1 for further description on character states and definitions). Figure 3.1 illustrates the phylogenetic distribution of these floral traits across the phylogeny used in this study.

Notes on the construction of the trait matrix:

1. Rosas-Guerrero et al. (2011) reports CV values for different floral traits. We used the CV values for corolla tube length given that this is both a trait involved in pollination precision and is comparable with other papers that we used in this analysis (instead of, for instance, long stamen).
2. Berg (1958) also reports values of CV for different floral whorls. Berg only reports values of CV on flower diameter for *Fragaria viridis*, *Geranium pretense* and *Epilobium angustifolium*, thus we had no choice but to use these

values. We used values of petal length CV for *Anemone nemorosa*, *Papaver sp.* and *Cosmos bipinnata*; and values of tube length for the remaining species.

3. van Kleunen et al., (2008) report values of both flower length and flower width. We use their values of flower length (among individual variation) in our analysis.
4. Composite flowers & *Daucus carota*: most papers that included composite flowers (see Supplementary Material Table 3.8) recorded them as radial flowers. Because we distinguish between morphological and functional symmetry and because we do not know whether they measured the radial or the bilateral florets within the composite flowers, we decided to record the composite flowers as ‘NA’ (no answer) for morphological/perianth symmetry and as radial for functional symmetry. Exceptions were *Saussurea alpine* and *Scorzoneroides autumnalis* where we were certain that florets were only radial or bilateral respectively. However, for both cases we noted them as functionally radial flowers.
5. In addition, inflorescences such as composite flowers and euphorbia flowers were excluded from the ‘stamen merosity’ analysis and recorded as ‘NA’ given that we did not distinguish between morphology and function for this trait. That is to say an oligoandrous floret may function as a polyandrous flower if considered at the inflorescence level.
6. ‘NA’ was also recorded for any character for which we were uncertain of the character state.

7. We excluded species that are wind pollinated as the focus of our study is the effect of pollinator mediated selection on flower trait variation (Berg papers: *Triticum aestivum*, *Hordeum* sp., and *Leymus arenarius*; Armbruster paper: *Cyperus* sp. and Poaceae).
8. There were several species in the Gong and Huang (2009) study that did not explicitly indicate the genus name of the species. We excluded these species from the analysis given that we were not certain of the species identity.
9. We disagree with Berg's (1959, 1960) morphological designation for *Nicotiana alata* as dorsiventral (aka bilateral) and instead designated it as radially symmetrical for our analysis.
10. *Iris* flowers: Herrera (1996) and Herrera et al., (2008) include one *Iris* species each and designate them as radially symmetric. Nikkeshi et al., (2015) also includes an *Iris* species, but designates it as bilaterally symmetric. Here we designated all *Iris* flowers (3 species) as radially symmetric for 'perianth symmetry', but as bilateral for 'functional symmetry'.

Coefficient of Variation (CV) values for flower size variation

The coefficient of variation for flower size variation across individuals was our proxy for the amount of uniform selection imposed by varying levels of pollination precision across species. We collected values of coefficient of variation directly from the literature or asked the authors for these values whenever values were not reported in the published manuscript. Values were averaged if a species was repeated across studies.

We calculated the values of CV for the species we measured ourselves at the National Herbarium. Whenever possible, we measured multiple whorls for each species and then calculated the CV values for all whorls. For each species, we then selected only one whorl to include in the analyses. Whorls were selected on the basis of their equivalence to the flower whorls measured by the previous papers as well as for their relevance in pollination (see Supplementary Material Table 3.2). Because of uneven availability in herbarium collections, sample sizes vary across the 122 species and range from 3 to 32 individuals per species. We considered each herbarium sheet as a separate individual. Whenever multiple flowers were measured, we calculated the mean value per individual. We measured every whorl twice to account for measurement error. We evaluated the increase of variance with the increase in sample size for a random subsample of 36 species of our total of 122 species (Fenster, 1991). We observed that the variance of most species plateaued with more than 19 individuals, leaving us with 51 of the 122 species initially measured on herbarium specimens. Most studies included in our analysis from the literature (nine of twelve) sampled above 19 individuals per species or showed evidence of variance stabilization (see Supplementary Material Table 3.1).

Phylogenetic Tree

We downloaded DNA sequences for *rbcl* and *matK* genes for all species from NCBI GenBank database (see Supplementary Material Table 3.4 for a sample of accession numbers). These regions were selected as they were densely sampled across the relevant taxa, alignable across a broad range of seed plant taxa, and known to

have high levels of phylogenetic signal and species discrimination (Chase et al., 1993; Hilu et al., 2003; Hollingsworth et al., 2009). The resulting matrix included 364 matK sequences, and 365 rbcL sequences, across the 418 species (12.8% missing data). We aligned sequences with the Multiple Sequence Alignment Software MAFFT version 7.187 (Kato and Standley, 2013) available in CIPRES - Cyberinfrastructure for Phylogenetic Research (Miller et al., 2010). We merged DNA sequences if multiple sequences were available for a species with Mesquite version 3.04 (Maddison and Maddison, 2015) and manually discarded any sequence that seemed unreliable either due to quality of sequencing or questionable identity (corroborated by blasting them in GenBank). The alignments were then inspected by eye using Geneious 7.1 (Kearse et al., 2012), trimmed, and any uninformative indels were removed. Appropriate substitution models for each alignment were assessed using SMS (Lefort et al., 2016), using the Akaike Information Criterion. This selected the GTR + Γ + I as the highest scoring model for both alignments. We concatenated the 2 genes in Mesquite and performed an ultrametric tree with BEAST v.1.8.3 (Drummond and Rambaut, 2007; Suchard and Rambaut, 2009) in CIPRES - Cyberinfrastructure for Phylogenetic Research (Miller et al., 2010).

We ran two Monte Carlo Markov chains (MCMC) with 300 million generations each and sampled every 30,000 generations to obtain 10,000 trees from each run. We partitioned our DNA sequences by our two genes and specified the Yule process as our tree prior, an uncorrelated relaxed clock and GTR + Γ + I nucleotide substitution model (estimated base frequencies) with 4 sites categories for each gene. We constrained our taxa for the major clades (magnoliids, monocots,

eudicots, core eudicots, rosids and asterids) since the purpose of our phylogeny was not to re-infer the phylogenetic relationships among species, but to estimate the relative branch lengths necessary for the phylogenetic generalized least squares analysis (Stevens, 2001; APG, 2009). We assessed the quality of the posterior samples for both runs for convergence, mixing and adequate burn in with TRACER v1.6 (Rambaut A, Suchard MA, 2014). Each individual 300M run had a few parameters with ESS (Effective Sample Size) below 200 (the standard cut off value to determine an adequate number of draws from the posterior in the sample). However, the combination of both runs did meet the standard quality values for all species included in our comparative analysis. We combined both 300M MCMC runs with LogCombiner v1.8.3 and summarized the tree file with TreeAnnotator v1.8.3.

We did not find DNA sequences in the Genbank database for all our species, so we searched for substitute sister species within the same genus to increase our total sampling size (see Supplementary Material Table 3.5 for the list of substituted species). Nevertheless, in some cases we also failed to find sequences for alternative sister species which forced us to exclude these species from our analysis even though we had morphological and quantitative data (values of CV) available for them. This reduced the total number of species from 343 (292 species from literature data and 51 of our own measurements) to 327 species for the final analysis (see Supplementary Material Figure 3.6 for tree plot and Supplementary Material 3.7 for tree newick file). The final phylogeny used for the analysis described below covers 34 taxonomic Orders and 71 taxonomic Families.

Analysis

To determine the effect of each floral trait (see Table 3.1) on flower size variation, we performed a phylogenetic generalized least squares (pgls) analysis (caper, R) to account for non-independence of the residuals due to phylogenetic relatedness (Orme et al., 2013). We estimated Pagel's λ for every model, a parameter used to model phylogenetic signal in the residuals to avoid overcorrecting for phylogeny (Revell, 2010; Mundry, 2014; Symonds and Blomberg, 2014). A Pagel's λ value of zero indicates no phylogenetic signal, while a value of one indicates a strong phylogenetic signal in the residuals. In the single floral trait analyses (described below), we also explored performing our analyses both assuming traits evolve with a Brownian motion model of evolution (i.e. with a strong phylogenetic signal) as well as a non-phylogenetically corrected analysis and report this in Appendix Table 3.1 and 2. All analyses were conducted with the statistical software R version 3.2.4 (R Development Core Team, 2008).

We performed ANOVA tests on the 'pgls' models with a single explanatory factor (floral trait) each. In addition, we explored combined trait analyses based on common flower combination found throughout the angiosperms. See Supplementary R code 'Ch_3_a' and Supplementary Raw Data Ch3_1.

PREDICTIONS

Multiple non-exclusive mechanisms may influence pollination precision: 1. reduction of pollinator diversity (pollination specialization), 2. restriction in the angle of approach/landing, 3. restriction of pollinator's movement on or in the flower, 4. restriction of the contact area of reproductive parts (stamen and pistil) to the pollinator's body. Below we discuss how each floral character state may influence pollination precision and thus our predictions on flower size variability (see Table 3.1 for further description on character states and definitions).

The focus of this study is on the functional effects of specific floral features on the flower-pollinator relationship quantified by flower size variability. The floral traits analyzed in this study were selected based on their potential role in pollination precision. Therefore differences in floral size variability for these traits are used as a signal of pollinator mediated selection on floral size variability, due to different levels of pollination precision. Nevertheless, flower size variability can be also be affected by genetic and developmental constraints as well as by environmental factors (Fenster and Galloway, 1997; Armbruster, Hansen, et al., 2009). This may be particularly important to keep in mind when interpreting the effects of stamen merosity and perianth fusion on flower size variability. Flowers with reduced stamen merosity and fused petals could be less variable due to greater genetic and developmental constraints associated with greater morphological integration, rather than due to a more precise pollination system. Below we outline our prediction for each character state in greater detail.

Single floral traits:

Perianth symmetry: Pollinators are bilateral and thus align themselves with the bilateral plane of bilateral flowers. Therefore, we predict bilateral symmetric flowers to be less variable than radially symmetric flowers as bilaterally symmetric flowers restrict the pollinators approach, also resulting in restricted pollen placement (Neal et al., 1998).

Functional symmetry: Similar to perianth symmetry, we also expect less variability in bilateral flowers when defined by function. Species with radial perianth may function as bilateral flowers during pollination, for example due to bilateral placement of stamens (see Table 3.1). Therefore some species that have radial perianth are classified as bilateral species under this category (see Supplementary Material Table 3.8). As a consequence, the effect observed for ‘functional symmetry’ may even be stronger than for ‘perianth symmetry’.

Tube presence: Flowers with a tube will be less variable than flowers without a tube. The presence of a tube may restrict the movement of a pollinator while visiting a flower or and increase pollinator specialization. Some pollinators may be excluded from visiting flowers with tubes if their body size and shape does not permit it, such as pollinators with a short proboscis.

Flower orientation: Flowers with lateral and pendant orientation will be less variable than flowers with vertical orientation or with flowers that do not have a defined orientation (positioned variably in a vertical, lateral or pendant position). As confirmed by Fenster et al. (2009), flowers with lateral (horizontal) and pendant orientation are approached in a single angle while vertical (facing the sky) flowers are approached by all angles, suggesting more diffuse pollinator mediated selection for vertical flowers. With a similar logic, flowers that are oriented in all directions should be more variable than or as variable as vertical flowers. Flowers that are positioned either vertically or laterally should be either as variable as vertical flowers or be in between flowers that are exclusively oriented either vertically or laterally. Flowers that are positioned both laterally and pendants should be either 1. equal to lateral and pendant flowers (if they have the same value of variability) 2. as variable as lateral flowers (if lateral flowers are more variable than pendant flowers) 3. or a value in between lateral and pendant flowers (if lateral flowers are more variable than pendant flowers).

Stamen merosity: Oligoandrous flowers (number of perianth parts \geq number of stamen) will be less variable than polyandrous flowers (number of perianth parts $<$ number of stamen). This could be due to either: 1) because of developmental stability associated with the oligomerization and fixation of flower parts, 2) because the reduction of stamen number may imply a restricted and more precise placement of pollen on the pollinator body (thus less pollen wastage as well).

Stamen and pistil exertion: Exsertion is correlated with exposure. While the term ‘exsertion’ suggests protrusion out of a tube like structure, species labeled as exserted (category type 4) includes species with exposed reproductive parts, independent of whether they have floral tubes. That is to say, category type 4 (exserted) includes species both with open or tubular flowers with exposed stamens and pistils. Thus, we predict that categories with greater exertion (categories 2, 3 and 4; see Table 3.1) will be more variable than categories with less exertion (categories 0, 1, 5 and 6; see Table 3.1). Exposed stamens and pistils are free to be visited by a greater range of pollinators. In addition, greater exposure may result in pollen being placed (and lost) on a broader and more variable area on pollinators leading to less precise pollination than flowers with less exposed reproductive parts.

Perianth fusion: Flowers with fused perianth will be less variable than flowers with an unfused perianth because of greater developmental stability associated with fusion of parts (Specht and Howarth, 2015). In many cases, perianth fusion may also signify the presence of a tube. Therefore, flowers with fused perianth may also be less variable due to the correlated effects of ‘tube presence’ that not only restricts the type of pollinators but also the movement of pollinators when visiting a flower (thus also restricting contact area on pollinator body).

Functional tube: Flower size variability will increase in the following order: flowers with a full tube, flowers with a partial tube, flowers without a tube. The rationale is that flowers with a full tube (i.e., the tube that restricts access to possible nectaries at

the base of the ovary as well as to the contact of anthers and stigma) will restrict the number of pollinator species or functional pollinator groups more than species with a partial tube (i.e., the tube restricts pollinators access to nectaries at the base of the ovary, but not to the contact of anthers and stigma) and even more so than species without a tube.

Combination of floral traits: As emphasized by Stebbins (1951), the angiosperm flower represents a subset of the possible floral character states. For example, many floral character combinations rarely occur throughout the angiosperms, such as perianth fusion or bilateral symmetry with polyandry (Stebbins 1951). Therefore, analyzing stamen merosity in relation to perianth fusion and flower symmetry would not be possible due to lack of representative species. In addition, we did not analyze the combination of ‘stamen and pistil exertion’ and ‘functional tube’, as exerted categories of ‘stamen and pistil exertion’ will correspond to ‘unfused’ and ‘partial tube’ categories of ‘functional tube’ and thus performing this analysis is redundant. Conversely, some character combinations such as perianth fusion or tube presence with flower symmetry are found in high prevalence suggesting adaptive functionality. The lack and prevalence of certain floral combinations suggests that examining character state combinations will more likely reveal patterns of selection.

Results

Single floral trait analyses

We summarize the results for one way ANOVA tests for each floral trait in Table 3.2 and below we discuss each trait in detail:

Perianth Symmetry on Floral Size Variability

There was a total of 186 radial and 126 bilateral species. Species with bilateral flowers (defined exclusively by the perianth) were significantly less variable than radial species (mean \pm SE = 13.2 ± 0.68 ; 14.5 ± 0.53 respectively; $p = 0.035$; $F_{1, 310} = 4.5$, Pagel's $\lambda = 0.31$).

Functional Symmetry on Floral Size Variability

There was a total of 186 radial and 140 bilateral species. Species with bilateral flowers (defined by one or more than one whorl) were significantly less variable than radial species (mean \pm SE: 12.8 ± 0.63 ; 15.7 ± 0.61 respectively; $p < 0.001$, $F_{1, 324} = 11.63$, Pagel's $\lambda = 0.33$).

Perianth Fusion on Floral Size Variability

There were a total of 146 unfused and 180 fused species. Species with unfused and fused perianth whorls did not differ in flower size variability (mean \pm SE: 14.3 ± 0.56 ; 14.5 ± 0.67 respectively; $p = 0.75$, $F_{1, 324} = 0.1$, Pagel's $\lambda = 0.36$).

Tube Presence on Floral Size Variability

There were a total of 67 species without a tube and 259 with a tube. Species with a tube like structure were less variable (mean \pm SE: 14.1 ± 0.51) than species without a tube (mean \pm SE: 15.6 ± 0.89). However, this difference was not statistically significant ($p = 0.127$; $F_{1, 324} = 2.33$, Pagel's $\lambda = 0.33$).

Functional Tube on Floral Size Variability

There were a total of 66 species with no functional tube, 124 species with partial tube and 128 species with a full tube. Species with no tube had the highest mean flower size variability (mean \pm SE: 15.61 ± 0.9), species with the 'full tube' the

lowest (mean \pm SE: 13.46 ± 0.7) and species with a ‘partial tube’ the intermediate values (mean \pm SE: 14.2 ± 0.65), however these differences were not statistically significant ($p = 0.255$, $F_{2, 315} = 1.37$, Pagel’s $\lambda = 0.352$).

Stamen Merosity on Floral Size Variability

There were a total of 278 oligoandrous and 30 polyandrous species in this analysis. Oligoandrous and polyandrous species did not differ in flower size variability (mean \pm SE: 14.1 ± 0.46 and 13.69 ± 1.02 respectively; $p = 0.415$, $F_{1, 306} = 0.67$, Pagel’s $\lambda = 0.36$).

Flower Orientation on Floral Size Variability

Species with different flower orientations differed in flower size variation ($p < 0.001$; $F_{5, 318} = 5.88$, Pagel’s $\lambda = 0.282$). The post hoc Tukey test indicated that flowers with lateral orientation had significantly less variation than vertical flowers (Tukey, $p < 0.01$) and with flowers that had no defined orientation (all) (Tukey, $p < 0.001$). In addition, flowers with ‘all’ orientations were significantly more variable than flowers with both lateral and pendant orientation (Tukey, $p = 0.042$) and marginally significant to flowers with pendant orientation only (Tukey, $p = 0.06$; see Fig. 3.2).

Stamen and Pistil Exsertion on Floral Size Variability

Species with different degrees of ‘stamen and pistil exsertion’ marginally differ in flower size variation ($p = 0.055$, $F_{6, 312} = 2.08$, Pagel’s $\lambda = 0.42$, see Fig. 3.3). After further examination of our data and state characters, we identified that category ‘6’ (i.e. stamen and pistil exserted but covered- salvia type flower) to be misleading given that all 23 species within this category also happen to have (without exception)

lateral orientation, bilateral symmetry, fused perianth and the presence of a tube. If we exclude this category from the analysis, the results are non-significant ($p = 0.85$, $F_{5, 290} = 0.4$, Pagel's $\lambda = 0.41$).

Comparison of phylogenetic (Brownian evolution and Pagel) and non-phylogenetic analyses for single floral traits

The results reported above were performed by estimating the phylogenetic signal of the residuals (Pagel's λ) to account for the non-independence of species due to phylogenetic relationships. The estimated values for Pagel's λ ranged from 0.282 to 0.42 across all single trait analyses (see Appendix Table 3.2). Zero indicates no phylogenetic signal (equivalent to a non-phylogenetic analysis) and values equal to one reflect a strong phylogenetic signal in the residuals (equivalent to a Brownian evolutionary model). Thus, analyses performed with values of Pagel's λ on the low range will be similar to results obtained with the non-phylogenetic analyses and analysis with values for Pagel's λ on the higher range observed (but still below 0.5) will be intermediate between the non-phylogenetic analyses and analyses performed assuming strong phylogenetic signal (i.e. Brownian evolutionary model).

All three analyses types (i.e. non-phylogenetic, estimated Pagel's λ and Brownian evolution), show flower orientation as an important explanatory variable for differences in flower size variation (see Appendix Table 3.1 and 3.2). In addition, all three analyses agree with the overall trend that flowers with lateral orientation are less variable than flowers oriented in all directions (i.e. category type '0') or with vertical orientation. Bilateral flowers are significantly less variable than radial flowers

both when the analysis is performed by estimating the phylogenetic signal of the residuals (Pagel's λ) and with a non-phylogenetic analysis, but not if a strong phylogenetic signal is assumed (Brownian). 'Stamen and pistil exertion' (with all 7 categories included) is significant under the Brownian evolutionary model, marginally for the analyses run with Pagel's λ and not significant under the non-phylogenetic analysis. The post hoc Tukey test shows that flowers of category '6' are significantly less variable than flowers of categories '0', '1', '3', '4', and '5'. Category '6' are flowers with stamen and pistil exerted but covered with a petal (like a salvia type flower). Species that fall under this category also have lateral orientation, bilateral symmetry, fused perianth and the presence of a tube. Thus the reduced variability of category '6' may reflect the combination of this suite of traits rather than by the type of exertion alone. Finally, 'tube presence' and 'functional tube' are marginally significant under the non-phylogenetic analyses. In both cases, flowers with a tube like structure are less variable than flowers without a tube.

Combined floral trait analyses

Figure 3.4 and Table 3.3 summarizes the results for all the analyses discussed below and Appendix Table 3.3 presents the sample sizes, mean values and standard errors. Due to limited sample size for all trait combinations, in many cases we present results for only some categories of 'flower orientation' and 'stamen and pistil exertion'. We specify whenever we did this below for each case.

Stamen and Pistil Exsertion & Stamen Merosity on Floral Size Variability

We found no combined effects of ‘stamen merosity’ and flowers with or without exserted reproductive parts (category types 0 and 4). Both main and interaction effects were not significant (‘stamen merosity’: $F_{1,200} = 0.09$, $p = 0.76$; ‘stamen and pistil exsertion’: $F_{1,200} = 0.04$, $p = 0.85$, interaction: $F_{1,200} = 0.2$, $p = 0.66$, Pagel’s $\lambda = 0.5$). See Appendix Table 3.3.a for descriptive statistics.

Stamen and Pistil Exsertion & Tube Presence on Floral Size Variability

Due to lack of sample size for all trait combinations, we were only able to analyze the effects of ‘tube presence’ on flowers with exserted stamens and pistils (category type 4). Flowers with exserted reproductive parts and no tube were more variable (mean \pm SE: 15.16 ± 0.9179 , $n = 62$) than flower with exserted reproductive parts and with a tube (mean \pm SE: 14.03 ± 0.74 , $n = 77$), however this difference was not statistically significant ($F_{1,137} = 2.8$, $p = 0.096$, Pagel’s $\lambda = 0.69$). See Appendix Table 3.3.b for descriptive statistics.

Stamen and Pistil Exsertion & Perianth Symmetry on Floral Size Variability

These analyses were possible only after removing category ‘6’ (i.e. stamen and pistil exserted but covered- salvia type flower) of ‘stamen and pistil exsertion’. Both main effects, ‘perianth symmetry’ and ‘stamen and pistil exsertion’, were not significant ($F_{1,277} = 1.69$, $p = 0.19$, Pagel’s $\lambda = 0.36$ and $F_{5,277} = 0.36$, $p = 0.87$, Pagel’s $\lambda = 0.36$, respectively) as was the interaction ($F_{5,277} = 0.45$, $p = 0.81$, Pagel’s $\lambda = 0.36$). See Appendix Table 3.3.c for descriptive statistics.

Stamen and Pistil Exsertion & Functional Symmetry on Floral Size Variability

These analyses were possible only after category '6' (i.e. stamen and pistil exserted but covered- salvia type flower) was removed for 'stamen and pistil exsertion'. The main effect of 'functional symmetry' was significant ($F_{1, 284} = 7.76$, $p < 0.01$, Pagel's $\lambda = 0.44$), but the main effect of 'stamen and pistil exsertion' as well as the interaction were not and ($F_{5, 284} = 0.4$, $p = 0.85$, Pagel's $\lambda = 0.44$ and $F_{5, 284} = 0.62$, $p = 0.68$, Pagel's $\lambda = 0.44$, respectively). See Appendix Table 3.3.d for descriptive statistics.

Stamen and Pistil Exsertion & Perianth Fusion on Floral Size Variability

This analysis was possible with all categories of 'stamen and pistil exsertion' except, category '6' (i.e. stamen and pistil exserted but covered- salvia type flower). Both main effects, 'perianth fusion' and 'stamen and pistil exsertion', were not significant ($F_{1, 284} = 0.18126$, $p = 0.6706$, Pagel's $\lambda = 0.41$ and $F_{5, 284} = 0.4$, $p = 0.85$, Pagel's $\lambda = 0.41$ respectively) as was the interaction ($F_{5, 284} = 1.1$, $p = 0.38$, Pagel's $\lambda = 0.41$). See Appendix Table 3.3.e for descriptive statistics.

Stamen and Pistil Exsertion & Flower Orientation on Floral Size Variability

The analysis of these trait combinations was only possible with categories 'lateral' and 'vertical' for 'flower orientation' and categories '0' (not exserted) and '4' (exserted) 'stamen and pistil exsertion'. Both the interaction term and the main effect of 'stamen and pistil exsertion' were not significant ($F_{1, 132} = 0.004$, $p = 0.94$ and $F_{1, 132} = 0.02$, $p = 0.88$, Pagel's $\lambda = 0.33$, respectively). Concordant with the single factor analysis, however, the main effect of 'flower orientation' was significant

($F_{1,132} = 7.24$, $p = 0.008$, Pagel's $\lambda = 0.33$). See Appendix Table 3.3.f for descriptive statistics.

Perianth Fusion & Tube Presence on Floral Size Variability

Both main effects, 'perianth fusion' and 'tube presence', were not significant ($F_{1,322} = 0.096$, $p = 0.76$, Pagel's $\lambda = 0.35$ and $F_{1,322} = 2.31$, $p = 0.13$, Pagel's $\lambda = 0.35$, respectively) as was the interaction ($F_{1,322} = 0.703$, $p = 0.40$, Pagel's $\lambda = 0.35$). See Appendix Table 3.3.g for descriptive statistics.

Flower Orientation & Stamen Merosity on Floral Size Variability

There was no combined effect of 'stamen merosity' and 'flower orientation' (with flowers oriented either only vertically or vertical and laterally) as both main effects and interaction were not significant ('stamen merosity': $F_{1,112} = 0.67$, $p = 0.42$, Pagel's $\lambda = 0.35$); 'flower orientation': $F_{1,112} = 0.4$, $p = 0.53$, Pagel's $\lambda = 0.35$, interaction effect: $F_{1,112} = 0.19$, $p = 0.67$, Pagel's $\lambda = 0.78$). See Appendix Table 3.3.h for descriptive statistics.

Flower Orientation & Perianth Symmetry on Floral Size Variability

We analyzed the effects of 'perianth symmetry' on flowers oriented both lateral and vertically (category type 2) as well as flower that are exclusively oriented laterally (category type 3). The main effect of 'flower orientation' was significant ($F_{1,196} = 11.78$, $p < 0.001$, Pagel's $\lambda = 0.25$), but the main effect of 'perianth symmetry' and the interaction effects were not significant ($F_{1,196} = 1.22$, $p = 0.28$ and $F_{1,196} = 1.85$, $p = 0.17$, Pagel's $\lambda = 0.25$ respectively). See Appendix Table 3.3.i for descriptive statistics.

Flower Orientation & Functional Symmetry on Floral Size Variability

We analyzed the effects of ‘functional symmetry’ on flowers oriented both lateral and vertically (category type 2) as well as flower that are exclusively oriented laterally (category type 3). The main effect of ‘flower orientation’ was significant ($F_{1, 199} = 11.85$, $p < 0.001$, Pagel’s $\lambda = 0.26$), but the main effect of ‘functional symmetry’ and the interaction effects were not significant ($F_{1, 199} = 0.0003$, $p = 0.99$ and $F_{1, 199} = 1.01$, $p = 0.32$, Pagel’s $\lambda = 0.26$ respectively). See Appendix Table 3.3.j for descriptive statistics.

Flower Orientation & Tube presence on Floral Size Variability

We analyzed the effect of ‘tube presence’ on flowers oriented in all categories except for flowers of type 4 and 5 (pendant and lateral or pendant only). Again, the main effect of ‘flower orientation’ was significant ($F_{3, 286} = 9.2$, $p < 0.0001$, Pagel’s $\lambda = 0.28$), but the main effects of ‘tube presence’ and the interaction were not ($F_{1, 286} = 0.00002$, $p = 0.99$ and $F_{3, 286} = 0.34$, $p = 0.8$, Pagel’s $\lambda = 0.28$ respectively). See Appendix Table 3.3.k for descriptive statistics.

Flower Orientation & Functional Tube on Floral Size Variability

We analyzed the effect of ‘functional tube’ on flowers oriented in category types 1, 2 and 3, i.e. vertical, vertical and lateral and only lateral. The main effect of ‘flower orientation’ was significant ($F_{2, 254} = 9.04$, $p < 0.001$, Pagel’s $\lambda = 0.3$), but the main effects of ‘functional tube’ and the interaction were not ($F_{2, 254} = 0.15$, $p = 0.86$ and $F_{4, 254} = 0.39$, $p = 0.81$, Pagel’s $\lambda = 0.3$ respectively). See Appendix Table 3.3.l for descriptive statistics.

Flower Orientation & Perianth Fusion on Floral Size Variability

We analyzed the effect of ‘perianth fusion’ on flowers oriented in all categories except for flowers of type 5 (pendant). Again, the main effect of ‘flower orientation’ was significant ($F_{4, 304} = 6.98$, $p < 0.0001$, Pagel’s $\lambda = 0.28$), but the main effects of ‘perianth fusion’ and the interaction were not ($F_{1, 304} = 0.02$, $p = 0.88$ and $F_{4, 304} = 1.55$, $p = 0.19$, Pagel’s $\lambda = 0.28$ respectively). See Appendix Table 3.3.m for descriptive statistics.

Perianth Fusion & Perianth Symmetry on Floral Size Variability

We found no combined effect between ‘perianth fusion’ and ‘perianth symmetry’ as only the main effect of ‘perianth symmetry’ was significant ($F_{1, 307} = 4.47$, $p = 0.035$, Pagel’s $\lambda = 0.37$) but the main effect of ‘perianth fusion’ and the interaction term were not ($F_{1, 307} = 0.0065$, $p = 0.94$ and $F_{1, 307} = 0.35$, $p = 0.55$, Pagel’s $\lambda = 0.37$, respectively). See Appendix Table 3.3.n for descriptive statistics.

Perianth Fusion & Functional Symmetry on Floral Size Variability

We found no combined effect between ‘perianth fusion’ and ‘functional symmetry’ as only the main effect of ‘functional symmetry’ was significant ($F_{1, 320} = 10.99$, $p = 0.001$, Pagel’s $\lambda = 0.35$) but the main effect of ‘perianth fusion’ and the interaction term were not ($F_{1, 320} = 0.0035$, $p = 0.952$ and $F_{1, 320} = 0.0242$, $p = 0.876$, Pagel’s $\lambda = 0.35$, respectively). See Appendix Table 3.3.o for descriptive statistics.

Tube Presence & Perianth Symmetry on Floral Size Variability

We found no combined effect between ‘tube presence’ and ‘perianth symmetry’ as only the main effect of ‘perianth symmetry’ was significant ($F_{1, 307} = 4.52$, $p = 0.034$, Pagel’s $\lambda = 0.35$) but the main effect of ‘tube presence’ and the

interaction term were not ($F_{1, 307} = 1.4$, $p = 0.236$ and $F_{1, 307} = 1.48$, $p = 0.225$, Pagel's $\lambda = 0.35$, respectively). See Appendix Table 3.3.p for descriptive statistics.

Tube Presence & Functional Symmetry on Floral Size Variability

We found no combined effect between 'tube presence' and 'functional symmetry' as only the main effect of 'functional symmetry' was significant ($F_{1, 320} = 11.071$, $p = 0.001$, Pagel's $\lambda = 0.34$) but the main effect of 'tube presence' and the interaction term were not ($F_{1, 320} = 0.416$, $p = 0.5193$ and $F_{1, 320} = 1.224$, $p = 0.269$, Pagel's $\lambda = 0.34$, respectively). See Appendix Table 3.3.q for descriptive statistics.

Tube Presence & Stamen Merosity on Floral Size Variability

There was no combined effect between 'tube presence' and 'stamen merosity'. The main effect of 'tube presence' was marginally significant ($F_{1, 303} = 3.77$, $p = 0.053$). In agreement to our prediction, flowers with a tube like structure were less variable than flowers without a tube. The main effect of 'stamen merosity' and the interaction term were not significant ($F_{1, 303} = 0.68$, $p = 0.41$ and $F_{1, 303} = 0.025$, $p = 0.874$, Pagel's $\lambda = 0.37$, respectively). See Appendix Table 3.3.r for descriptive statistics.

Functional Tube & Perianth Symmetry on Floral Size Variability

We found no combined effect between 'functional tube' and 'perianth symmetry' as we found no significant effects ('perianth symmetry': $F_{1, 299} = 3.144$, $p = 0.077$; 'functional tube': $F_{2, 299} = 1.225$, $p = 0.295$; interaction term: and $F_{2, 299} = 1.286$, $p = 0.277$, Pagel's $\lambda = 0.31$). See Appendix Table 3.3.s for descriptive statistics.

Functional Tube & Functional Symmetry on Floral Size Variability

We found no combined effect between ‘functional tube’ and ‘functional symmetry’ as only the main effect of ‘functional symmetry’ was significant ($F_{1,311} = 8.862$, $p = 0.0031$, Pagel’s $\lambda = 0.35$), but the main effect of ‘functional tube’ and the interaction term were not ($F_{2,311} = 1.4033$, $p = 0.247$ and $F_{2,311} = 1.0892$, $p = 0.3378$, Pagel’s $\lambda = 0.35$, respectively). See Appendix Table 3.3.t for descriptive statistics.

Discussion

The phylogenetically controlled analyses across 327 angiosperm species reveals that flower orientation and symmetry significantly explain patterns of flower size variation, congruent with the expected selection pressures associated with different degrees of pollination precision. In contrast, species that differ in stamen merosity, perianth fusion, tube presence (but see below) and the degree of stamen and the pistil exertion, do not significantly differ in the amount of flower size variation across individuals. The assumption of our hypothesis is that the effect of floral traits on flower size variation is the consequence of the type of pollinator mediated selection (i.e. uniform or diffuse) due to differing levels of pollination precision. Therefore, our results show that flower orientation and flower symmetry contribute to the degree of pollination precision.

Our results demonstrate floral orientation as the main trait responsible for differences in flower size variation across species. This is especially evident in the ‘combined floral trait’ analyses where repeatedly the main effect of ‘flower

orientation' was the only significant effect, independent of the other flower traits with which it was analyzed (see Fig. 3.4). In other words, floral orientation has a major influence on pollination precision independently of the all other floral traits, even floral symmetry. This result is congruent with other studies that have previously noted the importance of floral orientation in pollination precision (e.g. Berg, 1960; Fenster et al., 2009; Ushimaru et al., 2009; Nikkeshi et al., 2015), although neither of the studies included in our analysis (Supplementary Material Table 3.1) in fact explicitly test for flower orientation. Furthermore, our *post hoc* tests show that values of flower size variation for the different orientation states (i.e. vertical, lateral, pendant, etc.) are also in the direction as expected based on the study by Fenster et al. (2009). Flowers with vertical orientation are more variable than lateral and pendant flowers. In addition, flowers with no defined orientation (i.e. can be oriented vertical, lateral and pendant) are more variable than vertical flowers, although this is not statistically significant.

While not as strong an effect as floral orientation, floral symmetry also contributes to differences in flower size variability across species. In addition, the values follow the direction expected by the pollination precision framework, with bilateral species being less variable than radial species. Our results therefore agree with many other studies that found this same trend (Wolfe & Krstolic 1999, Ushimaru et al. 2006, Herrera et al. 2008, van Kleunen et al. 2008, Gong & Huang 2009, Nikkeshi et al. 2015). In this study we made the distinction between perianth symmetry and functional symmetry, and found a stronger effect on flower size variability for functional symmetry, reinforcing the importance of function (versus

morphology) in pollinator mediated selection. In fact, most of the previous studies that examine the effect of symmetry on floral trait variability actually evaluate what we defined as ‘functional symmetry’ (see Supplementary Material Table 3.8), however, to our knowledge, this distinction has not been previously explicitly made.

Our single trait analyses show that ‘tube presence’, ‘functional tube’ and ‘perianth fusion’ do not have significant effects on flower size variability. However, we did find evidence for the effect of ‘tube presence’ on flower size variability in our combined analysis with ‘stamen merosity’. The two analyses, the single trait analysis for ‘tube presence’ and the combined analysis of ‘tube presence’ with ‘stamen merosity’, differ in that the later data set does not include composite flowers because we did not assign values for stamen merosity to them (see methods: floral trait matrix). If we conduct the single trait analysis for ‘tube presence’ without composite flowers (Asteraceae), we detect a marginal effect for ‘tube presence’ ($F_{1, 308} = 3.03$, $p = 0.08$), but not for ‘functional tube’ or ‘perianth fusion’. Thus, it is solely the presence of a tube that affects pollination precision, independently of whether the reproductive parts are inside or outside of the tube (demonstrated by the lack of effect of ‘functional tube’) or whether the tube is fused or not (revealed by the lack of significance for ‘perianth fusion’). The latter supports our assumption that flower size variation in this study reflects the effect of pollinator precision and not that of developmental instability. *In contra*, if perianth fusion had played a role in explaining flower size variation instead of the presence or lack of tube, caution should have been taken in the interpretation of our results as evidence for the evolutionary effects of different levels of pollination precision.

As Herrera et al., (2008) point out, petal fusion by itself does not guarantee more precise pollination and emphasizes the importance of distinguishing the different floral tubes types such as short or long tubes. The reasoning being that short tubes do not necessarily restrict pollinator approach and pollen wastage as long tubes do (Fenster, 1991). In this study we attempted to address this by including the ‘functional tube’ trait. However, because we distinguished ‘partial tube’ versus ‘full tube’ by whether the lack or presence of exertion of the reproductive parts and not by the relative lengths of the floral tubes, it is likely that we failed to quantify the effects of tube length on pollinator specialization. Moreover, we did not distinguish between open and closed tubes, which could also play an important role in the degree of pollination precision. Nevertheless, we do find weak evidence supporting the importance of tube-like structures in flowers in increasing pollination precision.

Contrary to our expectations, ‘stamen merosity’ and ‘stamen and pistil exertion’ were not important predictors of pollination precision in our study. Moreover, the reduced floral variability observed for polyandrous versus oligoandrous species also disagrees with the existing pollination precision framework (Stebbins, 1951; O’Meara et al., 2016). Perhaps, it is not stamen number (merosity), but stamen symmetry or stamen fusion that matters. However, our results could also reflect an inaccurate value due to the low sampling of polyandrous species. Furthermore, we quantified the effects of ‘stamen merosity’ and ‘stamen and pistil exertion’ on flower size variation measured (mostly) on perianth traits. However, we suggest that pollination precision also implies increased floral integration (Armbruster, Pélabon, et al., 2014). Moreover, many studies have shown that corolla

size and stamen height (size) to be highly correlated and heritable traits (i.e., subject to selection response) (Conner and Via, 1993; Carr and Fenster, 1994; Campbell, 1996). Therefore, effects of pollinator mediated selection due to pollination precision should be evident across all floral whorls involved in pollination.

The fact that species share the same level of pollination precision independently of stamen number or whether the stamens and pistils are exposed or not, may indicate that pollination precision, is mostly determined by floral traits that alter the landing and movement of pollinators on the flower versus traits that facilitate pollination specialization or reduce pollen wastage. While it is difficult to conceive that oligoandry does not convey increased pollination precision through the reduced contact area with the pollinators body, it is also apparent that if the pollinator's movement isn't constrained by floral traits such as orientation and symmetry, the effects of oligoandry on pollination precision are likely lost when pollen is transferred across the pollinators body as it freely moves around the flower. Thus our signal, floral size variation, may only detect effects of pollinator mediated selection due to pollination precision at a gross scale. Floral traits that affect finer scale precision, such as area and placement of pollen on pollinators, may not be detected in our study. Another caveat is our definition of oligoandry as \leq to perianth number. Perhaps a more restrictive definition, that is, with fewer stamens, corresponding to orchids or some Zingiberales, may have demonstrated the hypothesized relationship of fewer stamens with lower floral size variation.

The combined analyses of 'stamen and pistil exertion' and 'tube presence' hints at another reason why 'stamen and pistil exertion did not explain differences of

flower size variability across species. The analysis is performed only on species with exertion type 4, i.e. with both stamen and pistils fully exerted. Therefore this ‘combined analysis’ is in fact a one way ANOVA test of the effect of ‘tube presence’ on flower size variation on a controlled subsample of species with exerted reproductive parts. Species with exerted reproductive parts (category ‘4’), however engulf species with very different flower forms such as open, radial and vertical flowers, but also tubular, bilateral and lateral flowers. Our sample size does not provide the power to analyze a combined analysis of all four traits. Nevertheless, while not significant ($p = 0.096$), flowers with exerted reproductive parts and a tube were less variable than flower with exerted reproductive parts and no tube. Flowers with exerted reproductive parts and a tube may manifest more precise pollination as the reproductive parts can only be contacted by a relatively smaller set of pollinators, i.e., only bigger pollinators. These results highlight the fact that ‘stamen and pistil exertion’ categories include flower shapes with contrasting pollination precision and thus may explain the lack of explanatory power found for ‘stamen and pistil exertion’ on flower size variation across species.

Perhaps the most striking result from this study in the context of the current literature is that it was not a combination of traits that best explained the differences in flower trait variation (our proxy for the degree of pollination precision), but rather orientation by itself. Flower orientation is mentioned occasionally as a contributor to pollination precision, however it is usually left out in the major discussions of pollination syndromes or seen as a secondary trait when compared to floral symmetry. It could be that our combined trait analysis did not detect a symmetry

effect because these models have less degrees of freedom in comparison when traits were tested separately. Nevertheless, our results indicate at the very least that flower orientation should be considered with the same weight as floral symmetry, in terms of pollination precision and how that translates to pollinator mediated selection.

The lack of trait combination effects challenges the increasing evidence that flowers respond to pollinator mediated selection via trait combinations (e.g., Stebbins, 1951; Fenster et al., 2015; O'Meara et al., 2016). Genetic studies demonstrate the importance of pleiotropy and epistasis underlying flower traits and trait correlations (Conner, 2002; Kelly, 2005; Smith, 2015). In addition, developmental studies demonstrate that certain genes and regulatory systems affect more than one floral whorl (Kalisz et al., 2006; Litt and Kramer, 2010; Smith, 2015). However, it may be that our results make evident a known caveat of comparative approaches. Because natural selection favors certain combination of traits (Fenster et al., 2015) and or due to their effect on diversification (O'Meara et al., 2016), the alternate character traits are either underrepresented or absent, limiting the capacity to compare character alternate states.

The discussion on the relationship between floral traits and pollination precision relates to studies on key innovations responsible for angiosperm radiations. Many of the traits analyzed in this study such as bilateral symmetry, fusion and oligomerization are either considered as key innovations or at the very least as representative traits of large and derived angiosperm clades (Bessey, 1915; Stebbins, 1951; Sargent, 2004; Soltis et al., 2005; Endress, 2011; O'Meara et al., 2016). Our results support the idea of bilateral symmetry enhancing precise pollination and thus

reproductive isolation, which could translate into the evolutionary radiations that label bilateral symmetry a key innovation. However, we did not find evidence supporting this type of evolutionary mechanism for reduced stamen number or sympetaly (i.e. petal fusion). Again, it would be interesting to consider flower orientation in studies that study key innovations.

The advantage of a larger data set achieved here by merging data from previous published studies with our own measurements, is the inclusion of more floral traits and trait combinations as well as increased statistical power. Nevertheless, the merging of different datasets also conveys the disadvantage of increased error due to the fact that some studies differed in their choice of the unit of measurement. For instance, Lázaro and Totland, (2014) measure flower width and Gong and Huang, (2009) use area measurements of floral visual units while most studies measure petal or corolla length (see Supplementary Material Table 3.1). Finally, we also observed some major differences in CV values for the same species not only across studies of different authors, but also by the same authors (e.g. Herrera, 1996 and Herrera et al., 2008). If anything however, this implies that the trends observed in this study are conservative.

In addition to the eight traits studied in this analysis it would be interesting to evaluate the effect of factors such as pollinator diversity and mating/breeding system similar to other studies (Armbruster et al., 1999; Ushimaru et al., 2006; Gong and Huang, 2009; Rosas-Guerrero et al., 2011; Lázaro and Totland, 2014; Nikkeshi et al., 2015). Including pollinator diversity would help pinpoint whether pollination precision is accomplished specifically by the restriction of pollinator diversity instead

of the restriction of pollinator movement (or any of the other mechanisms outlined in the methods). However, obtaining data on pollinator diversity in a macroevolutionary analysis may be hampered by the fact that some studies demonstrate specificity to pollinator visitors is community dependent (Lazaro et al., 2008). Including the mating and breeding system in the analysis would elucidate the relative importance of pollination precision in the overall reproductive fitness of the species and thus the strength of pollinator mediated selection on flower size variation (Mazer and Delesalle, 1998). While we excluded species that are exclusively wind pollinated, other factors such as mixed mating systems and different types of compatibility systems for which we did not account for may also play an important role determining the relative strength of pollinator mediated selection on flower size variation. Future studies controlling for pollinator diversity and mating/breeding system may reveal the importance of additional floral traits in pollination precision, other than flower orientation and symmetry.

In summary, we show strong evidence for the role of flower orientation and symmetry and weak evidence for the role of tube presence, in pollination precision through their effects on pollinator mediated selection on flower size variability. Specifically, bilateral and lateral oriented flowers restrict pollinator movement and landing on flowers and therefore, we conjecture, induce uniform and consistent selective pressures that translate into reduced flower size variability. Additionally, bilateral symmetry and the presence of a tube, may increase pollination specialization which will further focus the selective pressure on flowers. We reiterate, the importance of flower orientation in pollination precision as this trait has eluded major

studies discussing adaptive traits in response to pollination (Stebbins, 1951; Chartier et al., 2014; O'Meara et al., 2016), perhaps due to its low taxonomic importance.

Table 3.1. List of floral trait code and their categories. All species in this study were coded for the following floral traits: stamen and pistil exertion, flower orientation, stamen merosity, perianth symmetry, perianth fusion, tube presence, functional symmetry and functional tube.

Trait	Code	Definition
Stamen and Pistil Exsertion	0	Not exposed
	1	Stigma or anthers slightly exposed
	2	Only stigma or anthers exposed, the other not
	3	Only stigma or anthers exposed, the other slightly
	4	Both stigma and anthers exposed
	5	Both stigma and anthers slightly exposed
	6	Exserted from tube, but covered (e.g. by a petal)
Flower Orientation	0	All orientations (vertical, lateral and pendant)
	1	Vertical
	2	Vertical-Lateral

	3	Lateral
	4	Lateral-Pendant
	5	Pendant
Stamen Merosity	0	Oligoandry: number of perianth parts \geq number of stamen
	1	Polyandry: number of perianth parts $<$ number of stamen
Perianth Symmetry	0	Radial
	1	Bilateral
Perianth Fusion	0	Perianth (the whorl (sepals or petals) that was measured) Unfused
	1	Perianth (the whorl (sepals or petals) that was measured) Fused
Tube Presence	0	No tube
	1	Tube present
Functional Symmetry	0	Radial: perianth and androecium are radial or the inflorescence functions as a radial flower (e.g. composite flowers)

	1	Bilateral: perianth (sepal/calyx and/or petals/corolla) and or androecium are bilateral or florets function as bilateral flowers (e.g. Iris flowers)
Functional Tube	0	Unfused/Open: even if perianth is fused, there is no morphological structure (e.g. tube) restricting the access to possible nectaries at the base of the ovary and to anthers and stigma
	1	Partial Tube: presence of a tube (fused or not; any whorl) that it may restrict pollinators access to nectaries at the base of the ovary, but not to the contact of anthers and stigma (anther and stigmas are both exerted). *Species that are visibly buzz pollinated were categorized as Unfused.
	2	Full tube: tube presence (fused or not; any whorl) that restricts access to possible nectaries at the base of the ovary as well as to the contact of anthers and stigma (anther and stigma are within the tube)

Table 3.2. Summary of single floral trait analyses - one way ANOVA test statistics for the phylogenetic generalized least squares analysis on the effects of floral traits on flower size variation across ~ 327 angiosperm species. See Table 3.1 for further description on flower trait category description. Bilateral flowers are significantly less variable than radially symmetric flowers, both when symmetry is defined exclusively by the perianth or by various floral whorls (functional symmetry). Flowers oriented laterally were significantly less variable than flowers with vertical orientation or with a non-defined orientation (i.e. vertical, lateral and pendant orientation). See fig. 3.2 for further information on the effects of flower orientation on flower size variation across species. Pagel's λ was estimated to account for the correct non-independence of the residuals due to phylogenetic relatedness. A Pagel's λ value of zero indicates no phylogenetic signal, while a value of one indicates a strong phylogenetic signal in the residuals. Values in bold highlight significant effects ($p < 0.05$).

Floral Trait	Denom df	Num df	F	p value	Pagel's λ
Perianth Symmetry	310	1	4.5	0.035	0.31
Functional Symmetry	324	1	11.63	< 0.001	0.33
Perianth Fusion	324	1	0.1	0.75	0.36
Tube Presence	324	1	2.33	0.127	0.33
Functional Tube	315	2	1.37	0.255	0.352
Stamen Merosity	306	1	0.67	0.415	0.36
Flower Orientation	318	5	5.88	< 0.001	0.282
Stamen and Pistil Exsertion	312	6	2.08	0.055	0.42
Stamen and Pistil Exsertion, without category '6'	290	5	0.4	0.85	0.41

Table 3.3. Summary of combined floral trait analyses. Phylogenetic generalized least squares analysis of the effect of floral traits (see Table 3.1) on flower size variation (mean among- individual coefficient of variation) across 327 angiosperm species. Due to lack of sample size for all trait combinations, sample size varies for each analysis, ranging from 116 to 325 species. Appendix table 3.3 reports the detailed sample sizes. All analyses are two way ANOVA tests, except for the combined effect of ‘tube presence’ and ‘stamen and pistil exertion’, marked with an asterisk (*) below. This analysis tests the effect of ‘tube presence’ exclusively on category type ‘4’ from ‘stamen and pistil exertion’, i.e. flowers with exerted stamens and pistils. Pagel’s λ was estimated to account for the correct non-independence of the residuals due to phylogenetic relatedness. A Pagel’s λ value of zero indicates no phylogenetic signal, while a value of one indicates a strong phylogenetic signal in the residuals. Values in bold highlight significant effects ($p < 0.05$).

Explanatory Factors		Source of Variance	Denom df	Num df	F	p value	Pagel's λ
Flower Orientation	Perianth Symmetry	Flower Orientation	196	1	11.78	<0.001	0.25
		Perianth Symmetry	196	1	1.219	0.270	0.25
		Interaction	196	1	1.853	0.1749	0.25
Flower Orientation	Functional Symmetry	Flower Orientation	199	1	11.85	0.0007	0.26
		Functional Symmetry	199	1	0.0003	0.9857	0.26
		Interaction	199	1	1.0137	0.3152	0.26
Flower Orientation	Perianth Fusion	Flower Orientation	304	4	6.9813	<0.0001	0.28
		Perianth Fusion	304	1	0.0223	0.881	0.28
		Interaction	304	4	1.549	0.1879	0.28
Flower Orientation	Tube Presence	Flower Orientation	286	3	9.267	<0.0001	0.28
		Tube Presence	286	1	0.00002	0.996	0.28
		Interaction	286	3	0.34123	0.7955	0.28

Flower Orientation	Functional Tube	Flower Orientation	254	2	9.05	0.0002	0.3
		Functional Tube	254	2	0.1527	0.858	0.3
		Interaction	254	4	0.3927	0.8138	0.3
Flower Orientation	Stamen Merosity	Flower Orientation	112	1	0.39	0.53	0.78
		Stamen Merosity	112	1	0.66	0.42	0.78
		Interaction	112	1	0.19	0.67	0.78
Flower Orientation	Stamen and Pistil	Flower Orientation	166	1	7.94	0.0054	0.34
	Exsertion						
		Stamen and Pistil	166	5	1.33	0.26	0.34
		Exsertion					
		Interaction	166	5	1.38	0.23	0.34
Stamen and Pistil	Perianth Symmetry	Stamen and Pistil	277	5	0.36	0.87	0.36
	Exsertion	Exsertion					
		Perianth Symmetry	277	1	1.69	0.19	0.36

		Interaction	277	5	0.45	0.81	0.36
Stamen and Pistil	Functional Symmetry	Stamen and Pistil	284	5	0.4	0.85	0.44
Exsertion		Exsertion					
		Functional Symmetry	284	1	7.76	<0.01	0.44
		Interaction	284	1	0.62	0.68	0.44
Stamen and Pistil	Perianth Fusion	Stamen and Pistil	284	5	0.4	0.85	0.41
Exsertion		Exsertion					
		Perianth Fusion	284	1	0.181	0.67	0.41
		Interaction	284	5	1.1	0.38	0.41
Stamen and Pistil	Stamen Merosity	Stamen and Pistil	200	1	0.0379	0.845	0.5
Exsertion		Exsertion					
		Stamen Merosity	200	1	0.089	0.764	0.5
		Interaction	200	1	0.1956	0.658	0.5

Stamen and Pistil	Tube Presence	Tube Presence *	137	1	2.8043	0.0963	0.69
Exsertion							
Perianth Fusion	Tube Presence	Perianth Fusion	322	1	0.096	0.76	0.35
		Tube Presence	322	1	2.31	0.13	0.35
		Interaction	322	1	0.703	0.40	0.35
Stamen Merosity	Tube Presence	Stamen Merosity	303	1	0.68	0.41	0.37
		Tube Presence	303	1	3.77	0.053	0.37
		Interaction	303	1	0.025	0.874	0.37
Perianth Fusion	Perianth Symmetry	Perianth Fusion	307	1	0.0065	0.94	0.37
		Perianth Symmetry	307	1	4.47	0.035	0.37
		Interaction	307	1	0.35	0.55	0.37
Perianth Fusion	Functional Symmetry	Perianth Fusion	320	1	0.0035	0.952	0.35
		Functional Symmetry	320	1	10.99	0.001	0.35
		Interaction	320	1	0.0242	0.876	0.35

Tube Presence	Perianth Symmetry	Tube Presence	307	1	1.4	0.236	0.35
		Perianth Symmetry	307	1	4.52	0.034	0.35
		Interaction	307	1	1.48	0.225	0.35
Tube Presence	Functional Symmetry	Tube Presence	320	1	0.416	0.5193	0.34
		Functional Symmetry	320	1	11.071	0.001	0.34
		Interaction	320	1	1.224	0.269	0.34
Functional Tube	Perianth Symmetry	Functional Tube	299	2	1.225	0.295	0.31
		Perianth Symmetry	299	1	3.144	0.077	0.31
		Interaction	299	2	1.286	0.277	0.31
Functional Tube	Functional Symmetry	Functional Tube	311	2	1.4033	0.247	0.35
		Functional Symmetry	311	1	8.862	0.0031	0.35
		Interaction	311	2	1.0892	0.3378	0.35

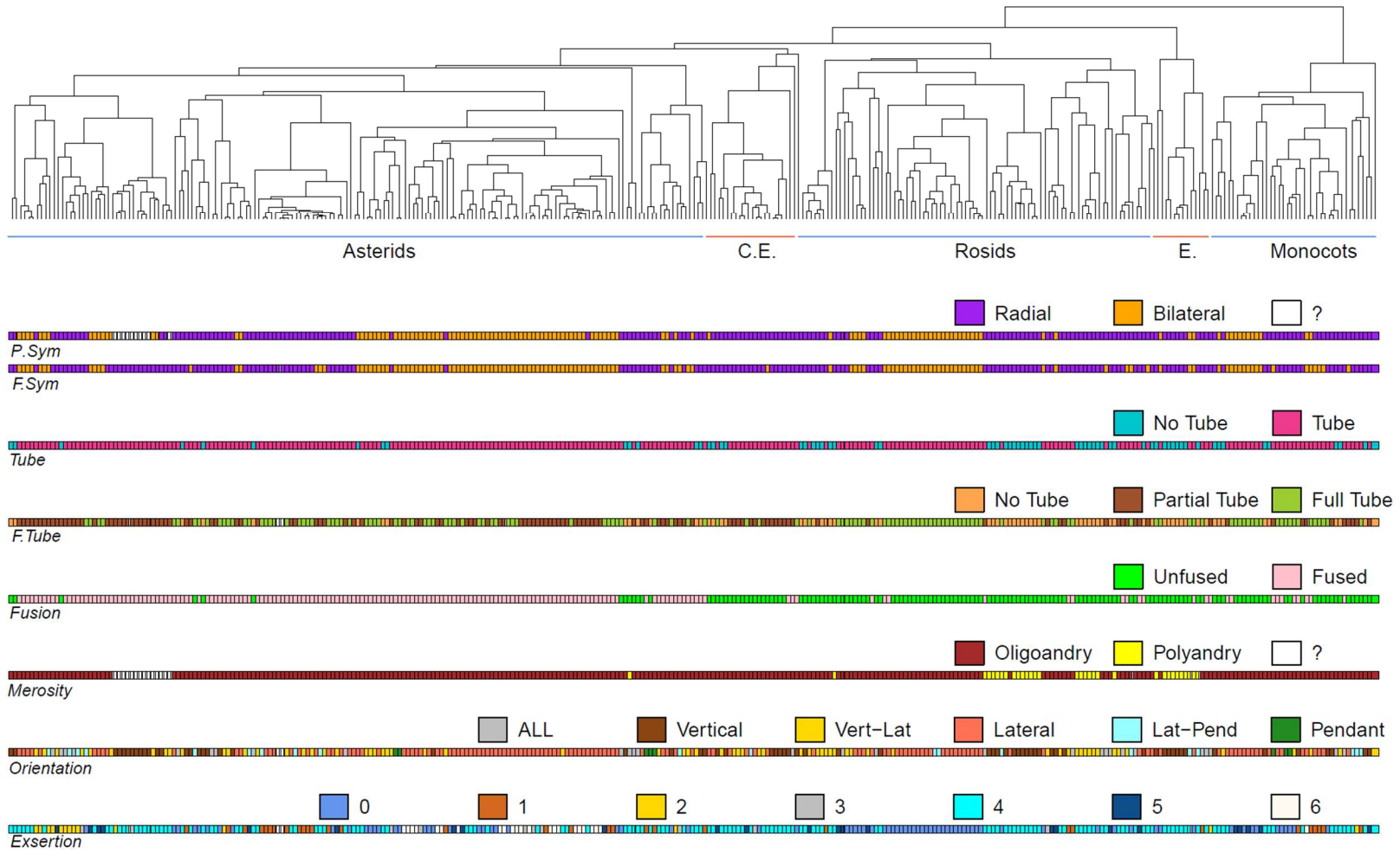


Fig. 3.1.

Fig 3.1. Phylogenetic distribution of all floral traits analyzed in this study across 327 angiosperm species (34 Orders and 71 Families). See Table 3.1 for further description of trait and trait categories. The non-calibrated ultrametric tree is performed with *rbcl* and *matk* genes in BEAST. Outgroup species have been pruned for this figure. See Supplementary Material 3.6 for tree plot with readable tip labels). Traits were coded for all species through images and illustrations. Major taxa clades (asterids, rosids, C.E: core eudicots, E: eudicots and monocots) are defined as by APG website (Stevens, 2001). Orientation = flower orientation; F. Sym. = functional symmetry; P. Sym. = perianth symmetry; Tube = tube presence; Fusion = perianth fusion; F. Tube = functional tube; Merosity = stamen merosity; Exsertion = stamen and pistil exsertion. Vert-Lat = vertical and lateral; Lat – Pend = lateral and pendant, ? = unknown trait category, ‘O’ = not exposed, ‘1’ = stigma or anthers slightly exposed, ‘2’ = only stigma or anthers exposed, the other not, ‘3’ = only stigma or anthers exposed, the other slightly, ‘4’ = both stigma and anthers exposed, ‘5’ = both stigma and anthers slightly exposed, ‘6’ = exserted from tube, but covered (e.g. by a petal).

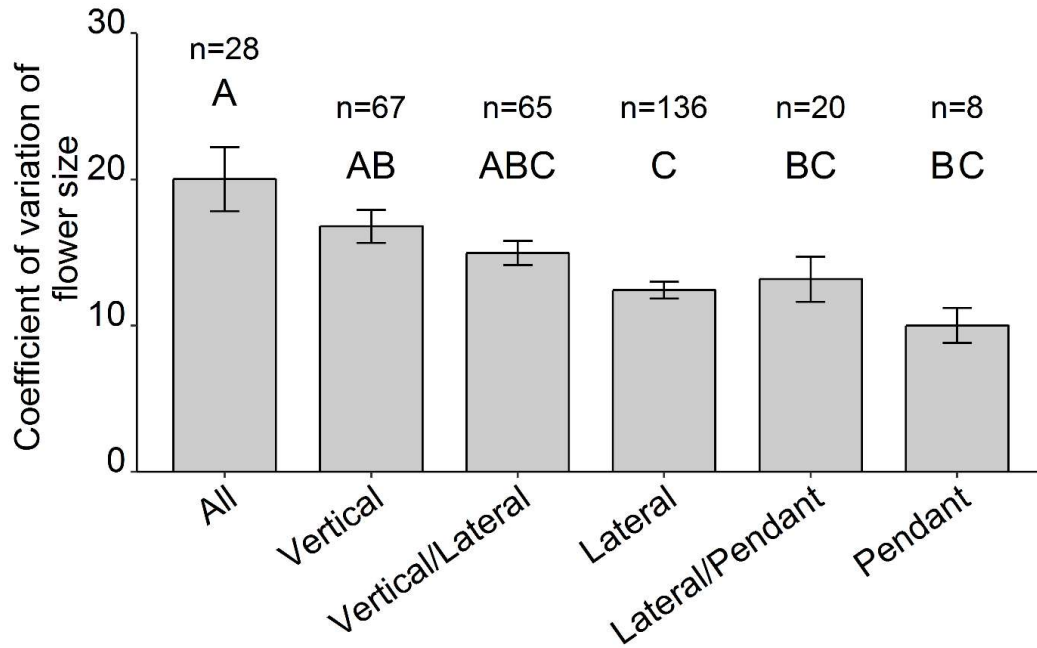


Fig. 3.2. Flower Orientation. Flowers with lateral orientation are significantly less variable than vertical flowers (Tukey, $p < 0.01$) and with flowers that had no defined orientation (all) (Tukey, $p < 0.001$). Flowers with ‘all’ orientations are significantly more variable than flowers with both lateral and pendant orientation (Tukey, $p = 0.042$) and marginally significant to flowers with pendant orientation only (Tukey, $p = 0.06$). Error bars are SE, n = number of species. Letters represent Tukey test.

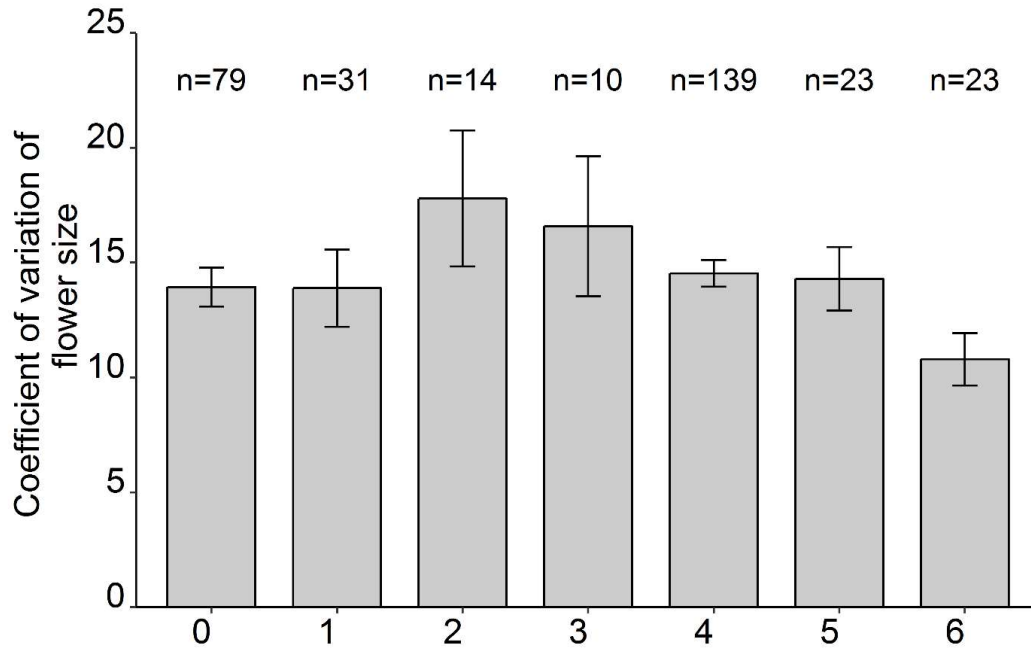


Fig. 3.3 Stamen and Pistil Exsertion. ‘0’ = not exposed, ‘1’ = stigma or anthers slightly exposed, ‘2’ = only stigma or anthers exposed, the other not, ‘3’ = only stigma or anthers exposed, the other slightly, ‘4’ = both stigma and anthers exposed, ‘5’ = both stigma and anthers slightly exposed, ‘6’ = exserted from tube, but covered (e.g. by a petal). Error bars are SE, n = number of species.

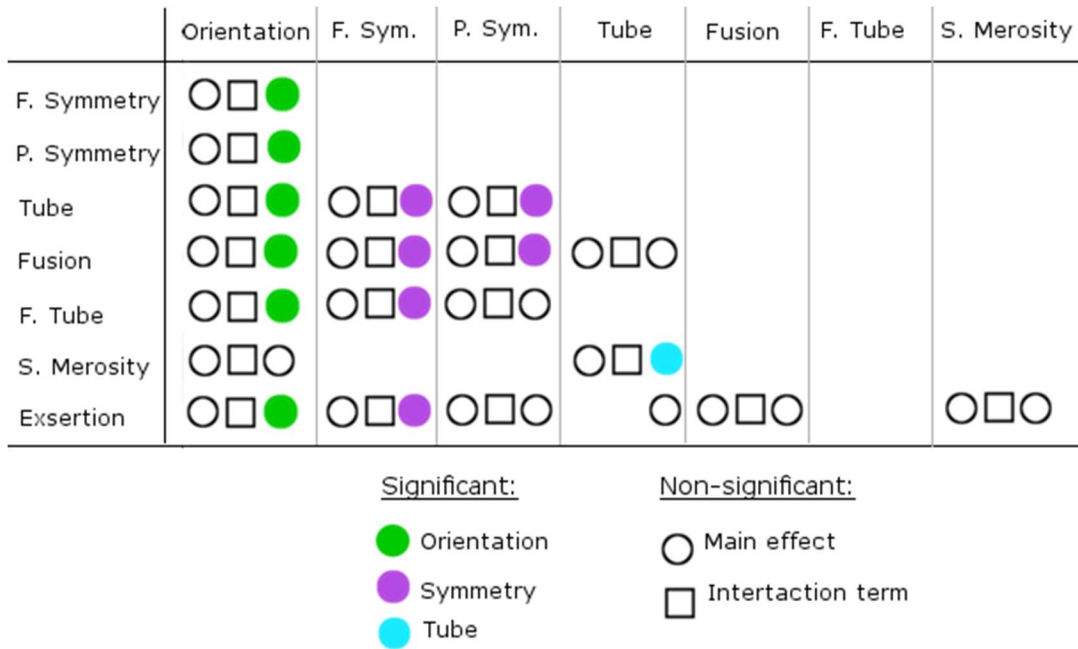


Fig. 3.4 Summary of combined floral trait analyses. Phylogenetic generalized least squares analysis of the effect of floral traits (see Table 3.1) on flower size variation (mean among- individual coefficient of variation) across 327 angiosperm species. Due to lack of sample size for all trait combinations, sample size varies for each analysis, ranging from 116 to 325 species. Table 3.2 reports two-way ANOVA test statistics and Appendix table 3.3 reports the detailed sample sizes. Circles are main effects and squares represent the interaction term. Empty shapes are non-significant and filled shapes significant effects. For each cell, the first circle represents the main effect for the floral trait named in the row and the last circle represents the main effect of floral trait labeled in the column. The square represents the row by column interaction. Due to lack of sample size for all trait combinations, many analyses could not be performed (blank cells). All analyses are two-way ANOVA tests, except for the combined effect of ‘tube presence’ and ‘stamen and pistil exsertion’, marked with

a single circle for that cell. This analysis tests the effect of ‘tube presence’ exclusively on category type ‘4’ from ‘stamen and pistil exertion’, i.e. flowers with exerted stamens and pistils. Only main effects for ‘flower orientation’, ‘flower symmetry’ and ‘tube presence’ were significant across all combined analyses. Bilateral flowers are less variable than radial flowers, lateral flowers less variable than vertical oriented flowers or flowers oriented in all directions; and flowers with a tube are less variable than flowers without a tube. Orientation = flower orientation; F. Sym. = functional symmetry; P. Sym. = perianth symmetry; Tube = tube presence; Fusion = perianth fusion; F. Tube = functional tube; S. Merosity = stamen merosity; Exsertion = stamen and pistil exertion.

Chapter 4: Selection response for stamen length adaptive accuracy in *Brassica rapa*

Abstract

- **Background and Aims:** Many studies demonstrate pollinators as important selective agents of floral design. However, lack of sufficient genetic variation may limit floral traits from evolving to their adaptive optimum. The serial expression of flowers, reflecting the modular construction of plants suggests that flowers on the same plant may experience different environments. Hence, we quantify the genetic contribution to two sources of variation that may lead to maladaptation of stamen height using fast-cycling *Brassica rapa* (Brassicaceae). We consider maladaptation at two levels: 1) the target deviance (bias) that results from conflicting selective pressures, fluctuating selection or genetic/developmental constraints and is quantified as the difference between the trait mean and the optimum mean value, and 2) the individual level imprecision (variation) that may result from developmental instability and is quantified as the within-individual variance of the trait under study.
- **Methods:** To quantify the contribution of additive genetic variation to phenotypic variation of stamen bias and stamen length variation, and hence the potential of both to respond to selection to reduce maladaptation, we selected for increased and decreased values for these two floral traits, using within-family and mass selection experimental designs. To determine if non-additive genetic variation

contributes to phenotypic variation for stamen bias and variation we examined the role of inbreeding (full and half sib crosses vs. random mating) on trait expression.

- **Key Results:** Based on the response to both artificial selection experiments performed in this study, we show additive genetic variance for stamen bias, but not for stamen height within-individual variation in flowers of *B. rapa*. Our results from the inbred-outcrossed comparison suggest that non-additive genetic variation may be contributing to phenotypic variation for within-individual stamen height variation as well as other floral parts.
- **Conclusions:** This study suggests that stamen accuracy in *B. rapa* could potentially evolve towards increased accuracy by eliminating any target deviance (bias). Nevertheless, some inaccuracy would remain due to the developmental instability (variation) within-individuals that did not respond to our imposed selection.

Key words: artificial selection; developmental instability; conflicting selective pressures; genetic variation; maladaptation; pollination efficiency; pollinator mediated selection

Introduction

Many studies provide evidence for the importance of pollinator mediated selection on flower shape and diversity in the angiosperms (reviewed in Fenster et al., 2004; Sargent, 2004; Smith et al., 2008; Martén-Rodríguez et al., 2010; Rosas-

Guerrero et al., 2011; Schiestl and Johnson, 2013; Lázaro and Totland, 2014). Moreover, the diversity of floral designs associated with biotic pollination has been historically interpreted as a consequence of pollinator mediated selection to increase the successful transfer of pollen across conspecifics (Stebbins, 1950, 1951; Faegri and van der Pijl, 1979; Fenster et al., 2004). For instance, the relative position of the anthers to the stigma both within and among flowers can determine the efficiency of pollen removal and deposition and thus is a good example of a trait under strong pollinator mediated selection (Webb and Lloyd, 1986b; Harder and Barrett, 1993; Armbruster, Corbet, et al., 2014). In theory, the matching placement of anthers and stigmas will increase pollen transfer between flowers and individuals as they contact the same area on the pollinator's body. However, anthers and stigmas do not always contact the same place on the pollinator due to conflicting selective pressures such as selection against self-pollination in self-compatible species or pollen competition and sexual interference in outcrossing species (Webb and Lloyd, 1986b; Barrett, 1990, 2002a). In fact, there is considerable phenotypic variation within flowering plant species in the positioning of anthers and stigmas (Barrett, 2002b; Endress, 2010, 2011) emphasizing the complexity of the genetic basis and selective pressures responsible for the mismatch of floral organs from an adaptive optimum.

For evolutionary response to pollinator mediated selection to take place, flower traits require underlying additive genetic variation, the component of genetic variation most responsible for the resemblance between parent and offspring (Falconer and Mackay, 1996). Several studies have corroborated additive genetic variation for both anther and stamen position (Conner and Via, 1993; Campbell et al.,

1994; Carr and Fenster, 1994; Campbell, 1996; Ashman, 1999; Motten and Stone, 2000; Conner et al., 2003, 2009, 2011; Hansen et al., 2003a; Caruso, 2004).

Furthermore, many of these studies show that the relative position of anthers and stigmas to each other or to a floral trait that controls the pollinator's position (e.g. corolla tube or nectary) are highly heritable traits and with considerable additive genetic coefficient of variation (Houle, 1992; Hansen et al., 2011) and thus can respond to selection. Strong directional selection could reduce a traits' additive genetic variation (Falconer and Mackay 1996; but see Carter et al. 2005). However, the presence of conflicting selective pressure (Campbell, 1989; Galen and Cuba, 2001; Delph et al., 2004; Sahli and Conner, 2011; Sletvold et al., 2015) and fluctuating selective pressures (Fenster and Dudash, 2001; Price et al., 2005; Campbell and Powers, 2015) suggests the absence of additive genetic variation in flower traits to be unlikely. Moreover, even if flowers were subject to uniform selective pressures, environment by gene interactions (Murren and Dudash, 2012; Spigler and Kalisz, 2013), non-additive genetic effects (Carr and Fenster, 1994; Ashman, 1999), as well as developmental instability (Paxman, 1956; Barrett and Harder, 1992; Moller, 1996) may reduce the effectiveness of selection. For instance, increased developmental instability or lack of developmental canalization (i.e. increased phenotypic plasticity) not only reduces the accuracy of a trait and thus the fitness, but also its heritability (Falconer and Mackay, 1996; Tonsor et al., 2013).

Genetic constraints due to correlated traits under opposing selection (Hansen et al., 2003b; Caruso, 2004; Delph et al., 2004; Pélabon et al., 2004; Ashman and Majetic, 2006; Hansen and Houle, 2008; Walsh and Blows, 2009; Sahli and Conner,

2011) may also limit selection response. Correlation across flower whorls is very common and ensures flowers to function as a single reproductive unit (reviewed in Waitt and Levin 1998; and Ashman and Majetic 2006). However, correlations may also increase the conflicting selective pressures for each given flower part due to their different reproductive functions (i.e. pollen deposit vs pollen transfer) (e.g. Sahli and Conner 2011). In summary, it is important to understand the genetic basis of the floral traits as well as the correlation across floral whorls when evaluating the potential for response to selection mediated by pollinators.

Armbruster and colleagues (Armbruster et al., 2004; Hansen et al., 2006; Pélabon and Hansen, 2008; Armbruster, Hansen, et al., 2009; Armbruster, Pélabon, et al., 2009) recognize that selection not only acts on the mean of a trait, but also on its variance. They introduce the concept of “adaptive inaccuracy” to measure the degree of mismatch, or maladaptation, for a given trait due to conflicting selective pressures, fluctuating selection or genetic/developmental constraints. Maladaptation may originate from two sources (deviation from the target and imprecision) and selection acts directly on the sum of these two components and indirectly on each (Hansen et al., 2006; Armbruster, Hansen, et al., 2009; Armbruster, Pélabon, et al., 2009).

There needs to be genetic variation for both components to contribute to the evolution of adaptive accuracy. To our knowledge, there are no studies that simultaneously investigate the genetic basis of both components. Therefore, the question remains, can a trait evolve to be more or less accurate? That is to say, do both components of adaptive accuracy have genetic variation for selection to either eliminate or increase the degree of maladaptation? To answer this last question, we

performed a selection experiment on two components of male function involved in pollen transfer. We used response to selection as a method to quantify genetic variation for the degree to which individual stamen length deviates from its optimum (bias) and stamen length variation within an individual (variation across flowers within an individual). Specifically, we performed an artificial selection experiment on a model rapid generation species (*Brassica rapa*) to answer: 1) Does stamen bias and within-individual variation respond to selection and 2) Does non-additive genetic variation contribute to stamen bias and within-individual variation? Because an important contribution to genetic constraints to selection response is pleiotropy, in this case a correlated response to selection we also asked, 3) Does selecting on stamen variation within an individual have a correlated response on the variation of other floral parts?

Materials and Methods

Data Sampling and Experimental Design

STUDY SYSTEM AND GROWING CONDITIONS

We used rapid-cycling *Brassica rapa* (Brassicaceae) to conduct our selection experiments. We purchased seeds of the standard strain from The Wisconsin Fast Plants® Program (Crucifer Genetics Cooperative, University of Wisconsin, Madison; Williams and Hill 1986). *Brassica rapa* has the typical hermaphroditic flower of Brassicaceae, with four unfused sepals and petals, and tetradynamous stamen (two short stamens and four long stamens). During the flowering period, 2-3 flowers open per day and individuals produce approximately 10 to 30 flowers. The rapid- cycling

B. rapa are self-incompatible with a life cycle of 30-45 days, making it an ideal study system for selection experiments.

We performed the experiments at the University of Maryland greenhouse facilities. Plants were grown in 10*10 cm pots with Sunshine LC1 soil, 18-25.5° Celsius, watered daily, no fertilizers added, and sprayed as needed (M-Peede EPA # 62719-515 at 2%) against white flies usually at least once during each generation with 14-16 hrs of light. The experiments were performed during the months of August-May from 2011-2015. Experiments were not performed during the summer months of June and July due to the difficulty of maintaining temperatures beneath ~25° Celsius at the greenhouse. We observed that temperatures > 25° C resulted in plants producing sterile pollen.

TRAITS SELECTED AND MEASUREMENTS

We selected for increased and decreased values for two floral traits: stamen bias and stamen length variation. Stamen bias is the difference between the mean stamen height of an individual to its optimum, which we defined here to be the mean pistil height of the population. Stamen length variation is the variation of stamen height within an individual. We selected in both directions for both traits in addition to maintaining a control line (see Fig. 4.1). Both stamen bias and stamen height variation were measured based on the long stamens of the flower. While this paper is framed by the male function adaptive accuracy of a flower, we excluded the short stamen of this analysis to simplify the selection experiments and also because the role

of the short stamens in pollination in *Brassica* flowers is still unclear (Kudo, 2003; Conner et al., 2009).

We measured one of the four long stamens for each flower measured in addition to its pistil height. Every measurement was taken twice in order to quantify and to control for measurement error. We performed two selection experiments (further details of each design below) for which we measured 2 flowers per individual in the within-family selection experiment and 5 flowers per individual in the mass selection experiment. To control for developmental variation due to flower age we measured flowers on their first day of anthesis. We did this by removing all the flowers on all plants with forceps and returning the next day to measure the freshly opened flowers. We did not entirely control for spatial placement within the inflorescence nor day within the flowering period, although most flowers were measured or collected within the first week of flowering. At the very least, not completely controlling for these factors will make the results of this study more conservative, that is, less likely to quantify genetic variation.

Measurements were performed with a 0.01 mm dial caliper. With these measurements we calculated stamen bias and stamen length variation for each individual as follows:

$$\textit{Stamen bias} = \mu \textit{ pistil height of the population} - \mu \textit{ stamen height of individual}$$

with μ = mean. Values for stamen bias may be positive or negative depending on whether the stamen length is longer or shorter than the pistil height of the population. Since our goal for the high-bias treatment was to increase the distance

between the anthers and the stigma, we ensured that we always selected for positive values (stamen height < pistil height) to avoid a positive and a negative bias to cancel out and give offspring with low-bias. The choice of positive values was arbitrary, and we could have used the same rationale with negative values. For example, if we had two individuals, individual A with a value of -0.10 and individual B with a value of 0.05, even though the absolute value of individual A is larger than individual B, we selected individual B as the parental generation for the next generation. This was not an issue for low-bias (we selected the lowest value independently of whether it was a positive or negative value).

$$\text{Within individual stamen length variation} = \frac{sd}{\mu} = \frac{\sqrt{\frac{\sum_{i=1}^n (x_i - \mu)^2}{n - 1}}}{\mu}$$

with sd = standard deviation among flowers within an individual; μ = mean stamen height across flowers measured within an individual; x_i = height of one long stamen within a flower; n = number of flower measured per individual. The above formula is the coefficient of variation of stamen length and is provided to clarify how within-individual stamen length variation was calculated. We calculated these values with R (stats and raster package, R). Note that while we calculated the coefficient of variation to select for stamen length variation, this measure of variation was completely concordant with measurements using the variance of stamen length for each individual used in our analysis (see below under “Stamen variation calculation and measurement error” in the Data Analysis section).

FIRST SELECTION EXPERIMENT: WITHIN-FAMILY SELECTION

To quantify how stamen bias and within-individual variation would respond to increased and decreased selection, we selected for four generations with a within-family selection design of 49 families. We cross pollinated the base population and then sowed six seeds per family. Their maternal line defined family lines because we did not keep record of the seedling's sire identity (i.e. seedlings may have been either full or half sibs). From these six seedlings, we randomly assigned one of them as the control line. Then we measured two flowers per individual for all the remaining seedlings and assigned them their corresponding treatments (high and low stamen bias and variation, in total four plants) as explained above. The additional seedling of the six initial seeds planted per family, was grown as a backup in case not all germinated. For each following generation, we sowed two individuals per selection line for each family and measured two flowers on each individual. As an example, if these two individuals belonged to the high-bias selection line, we calculated their bias and chose the individual with the highest bias (biggest difference between stigma and anther). Hand cross pollinations were performed randomly among family lines within treatment lines. For example, we crossed a high-bias individual from one family with another high-bias individual from another family, and so forth. A within-family selection experiment has the advantage of maintaining the effective population size (Falconer and Mackay, 1996) and therefore reducing the loss of self-incompatible alleles in the population. Despite these precautions, we did lose maternal lines for all treatments in every generation throughout the experiment.

To determine if selecting for increased and decreased stamen length variation had a correlational response on the variation of the other floral parts we collected two flowers per individual and stored these flowers in 70% ethanol (in 1.5 Eppendorf tubes) for both the base population and the last generation of selection of the within-family selection experiment. We measured seven additional floral traits: sepal length, corolla tube length, petal length, petal width, short stamen length, long stamen length, and pistil length with a 0.01 cm precision caliper (see Fig. 4.2). We measured one representative organ for every flower for those flower parts composed of more than one meristem (sepal, petal and stamen whorls). We only examined correlational response for high and low stamen variation treatments given that stamen bias is the difference between pistil and stamen height and thus we do not expect nor could we predict how selecting in either direction for stamen bias could impact bias for other flower organs. On the contrary, selecting for high or low stamen variation may also increase or reduce the variation of the other floral organs, if all floral organs are highly integrated, i.e. with high levels of phenotypic correlation among functionally or developmentally related traits (Olson and Miller, 1958).

SECOND SELECTION EXPERIMENT: MASS SELECTION

To verify if the selection response obtained with the within-family selection experiment is repeatable and possibly enhanced under a different selection design we performed a mass selection design for the second selection experiment. Because mass selection designs have the disadvantage of reducing the effective population size drastically, and increasing the loss of self-incompatible alleles, we planned this

experiment for at most two generations. The main difference with the first selection experiment other than the selection design itself, was that we measured 5 flowers per individual instead of two flowers. We hoped that this would increase the precision of treatment assignment for each individual.

We selected for both stamen bias and within-individual stamen length variation on our base population, but then performed the following generations of selection for stamen bias and variation separately. We first selected two generations of stamen length variation. Once we completed the stamen variation selection experiment we performed one generation of selection for stamen bias. This experiment started with a base population of 100 individuals from 50 maternal lines. For each generation, we selected the top/bottom half of the population (i.e. 50 individuals with the largest or smallest values for the first generation and 25 individuals with the largest or smallest values for the second generation of selection). For both sections of this experiment (stamen bias and stamen length variation), all generations were divided into blocks and randomized, except for the base generation, which was blocked but not randomized. The base population was divided into 7 blocks, the first generation in both experiments (stamen bias and stamen length variation) had 5 blocks and the second generation for stamen length variation had 3 blocks. The drop of block number across generations was in response to the decrease of individuals through this selection experiment.

INBREEDING VS OUTBREEDING EXPERIMENT

To determine if non-additive genetic variation contributes to phenotypic variation for stamen bias and within-individual stamen height variation we performed inbred and outbred crosses. Specifically, we wished to document whether the two traits, stamen bias and stamen height variation, were affected by inbreeding depression, which reflects both dominance and epistatic sources of genetic variation, in addition to a small component of additive genetic variation (Falconer and Mackay, 1996). We planted six individuals each for 30 maternal plants in the greenhouse. Seedlings were either full or half sibs, since seeds were taken from either one or two fruits, for which we did not take note of sire identity. Once these plants flowered, we performed four crosses on every plant: two outbred and two inbred. Outbred crosses were conducted by pollinating a flower with pollen from an individual belonging to a different family and inbred crosses were conducted by pollinating a flower with pollen from one of six individuals that were the sibs (either full or half sib crosses). We did not perform self-crosses (with flower on the same individual) because *B. rapa* is self-incompatible. In the following generation, we selected two individuals per family and planted one outcrossed and one inbred offspring for each individual. We had 2 blocks, and assigned to each block one individual of each family with both of its treatments. Appendix Fig. 4.1 summarizes the breeding procedure for this experiment.

For this experiment, we have two data sets. The first data set consists of fresh flowers measured in the greenhouse (five flowers per individual for stamen length and two for pistil length). The second consists of flowers collected and stored in 70%

ethanol (two flowers per 1.5 Eppendorf tube) until measured. We collected four flowers per individual and measured all floral traits: sepal length, corolla tube length, petal length, petal width, short stamen length, long stamen length, and pistil length (see Fig. 4.2). In many cases flowers measured freshly at the greenhouse were also collected and stored in ethanol. Both data sets are measurements after one generation of inbred and outbred crosses.

Data Analysis

STAMEN VARIATION CALCULATION AND MEASUREMENT ERROR

During the selection experiments we assigned high and low stamen length variation treatments by calculating the coefficient of variation of stamen length for each individual. The coefficient of variation, however, is a ratio and thus one cannot disentangle whether the results are due to a change in the standard deviation or to the mean. Therefore we performed all the analyses for stamen length variation with both the coefficient of variation and with the within individual variance, but report only the variance given that these results do not differ.

$$\textit{Within individual stamen length variance} = \frac{\sum_{i=1}^n (x_i - \mu)^2}{n - 1}$$

μ = mean stamen height across of flowers measured within an individual; x_i = height of one long stamen within a flower; n = number of flower measured per individual. The variance was calculated in R with the ‘aggregate’ function (R Development Core Team, 2008).

Because we took all measurements twice, we took the average of these two measurements to reduce the effects of measurement error on our estimates. To verify the quality of our measurements and variable estimates we also estimated the percent of measurement error (Bailey and Byrnes, 1990). We performed a mixed model (both with nlme and lme4 packages, R) with the fixed effect equal to one (fixed intercept) and each flower measured as a random variable (Bates et al., 2015; Cox, 2016; Pinheiro et al., 2016). We found that the percent of measurement error estimates ranged from 0.23% to 6.5% and a median of 1.01% across all datasets (see Appendix Table 4.1). Thus our results presented below are true biological variation.

Procedure for all analyses below

We tested the significance of each explanatory variable by constructing a series of nested mixed models (lme4 package, R) and comparing each model to the previous one using likelihood-ratio tests (Bates et al., 2015). We verified the model selection results based on likelihood ratio tests with AIC values. We selected models with the least number of parameters and within 3.22 units from the lowest AIC (Burnham and Anderson, 2010; Fenster et al., 2015). If a model that best fit the data had the explanatory variable of interest, then we performed a post hoc Tukey test (lsmeans package, R) to examine all possible contrasts or sequential Bonferroni correction (Holm, 1979; Lenth, 2016) across the subset of contrasts of interest. Detailed statistics on the model selection for all analyses are included in the Appendix Tables 4.2 – 4.9.

Finally whenever two data sets were available (measurements taken in the greenhouse as well as on flowers collected and preserved in 70% alcohol), we analyzed both data sets. We only report the results for the two data sets when results differed among them (inbreeding vs outbreeding experiment). Appendix Table 4.1 shows that measurement error for all data sets was very similar, thus differences between data sets may reflect weak evidence in the effects analyzed.

SELECTION RESPONSE

Stamen bias and stamen length variation

To determine if stamen bias and stamen length variation responded to selection we tested whether high and low selection lines for both stamen traits differed significantly. Stamen bias and stamen length variation mean values for each individual in each generation of selection were calculated based on the values prior to selection.

The examination for selection response constitutes of a total of four model selection analyses. The first two analyses are the model selection analyses for stamen bias and stamen length variation for the within-family selection experiment. Here we constructed mixed models with ‘selection line’ (i.e. high and low selection lines) and ‘generation’ (four generations) as fixed factors and ‘family’ as a random factor. Even though we did block all generations in this experiment, we lacked that information for all the generations to incorporate into the analysis. Missing the block term in our models for this analysis makes this result more conservative.

The remaining two analyses are the model selection analyses for stamen bias and stamen length variation for the mass selection experiment. Here, we also constructed mixed models with ‘selection line’ (i.e. high and low selection lines) and ‘generation’ (four generations) as fixed factors, except for the analysis of stamen bias in the mass selection experiment that only had one generation of selection, and thus only had selection line as fixed factor. For the mass selection experiment we assigned both ‘family’ and ‘block’ as random factors. Family was nested within blocks in this analysis as each block did not necessarily have a representative of every family due to the experimental design of a mass selection experiment.

In all four model selection analyses, if the best selected model included ‘selection line’ as one of its explanatory variables, this would indicate a positive response to selection provided that the mean values of each selection line agree with the direction (i.e. high or low) of selection.

Stamen length variation correlational response for other flower parts (within-family selection experiment only)

To determine whether selecting for increased and decreased stamen variation had a correlational response on the variation of the other floral parts (two flowers per individual), we constructed a mixed model with ‘selection line’ (i.e. high and low stamen length variation) and each ‘floral part’ (see Fig. 4.2) as the fixed factors and ‘block’ and ‘family’ as random factor for generation 0 and family in generation 4. If the best model selected for the fourth generation of selection is the interaction model with ‘selection line’ and ‘floral part’ as explanatory variables, this would indicate

correlational response, especially if the post hoc contrasts indicate that all or some floral parts differ between high and low variation selection lines.

INBREEDING VS OUTBREEDING EXPERIMENT

To compare whether inbred and outbred individuals differed in stamen bias and stamen length variation, for each stamen trait we constructed mixed models with ‘cross type’ (i.e. inbred or outbred) as the fixed factor and ‘block’ and ‘family’ as random factor. A significant inbreeding effect would result in the model with ‘cross type’ as the best model (versus the null model without ‘cross type’ as an explanatory variable). We performed this analysis for both our fresh measurements at the greenhouse and our alcohol preserved flowers data set.

With our alcohol data set, we also evaluated whether inbred and outbred individuals differed in variation for all floral traits, i.e. a correlated inbreeding effect across all floral whorls. We constructed mixed models with ‘cross type’ (i.e. inbred or outbred) and ‘floral part’ (i.e. sepal length, corolla tube length, petal length, petal width, short stamen length, long stamen length, and pistil length) as fixed factors and ‘block’ and ‘family’ as random factors. Evidence for a correlated inbreeding effect across all whorls would result if the main effect model with both ‘cross type’ and ‘floral part’ as explanatory factors is selected as the best model. However, a best model that includes a ‘cross type’ by ‘floral part’ interaction would indicate an inconsistent effect of inbreeding across whorls.

To test for variation across families in stamen length variation, both for inbred and outbred crosses, we compared models with and without ‘family’ as a random

factor. We analyzed the inbred and outbred individuals separately and excluded families from the dataset for which we had measurements for only one individual (instead of two), due to mortality. Thus the inbred dataset included 24 families and the outbred dataset 27 families. Family variation in stamen length variation for the outbred dataset could be due to non-genetic maternal effects, as well as additive and non-additive genetic effects, while variation found in the inbred dataset is evidence for maternal and non-additive genetic variation. We performed this analysis for both our fresh measurements at the greenhouse and our alcohol preserved flowers data set.

INACCURACY INDEX

To compare our results with previous reports on adaptive accuracy, we calculated the mean scaled inaccuracy index following the formula described in Armbruster et al., (2009):

$$= \frac{[(Bias)^2 + Stamen\ length\ variance + Pistil\ length\ variance]}{Stamen\ length\ mean^2}$$

We calculated the individual level male accuracy index for the inbreeding vs. outbreeding experiment as well as for the first and last generation of both of the selection experiments (within family and mass selection experiment). In addition, we calculated the population level male accuracy index for the base generations for each selection experiment to compare how the accuracy components vary depending on the level of analysis.

The individual and population level accuracy differ in how bias and stamen length variation are calculated. For the within individual accuracy, bias is calculated for each individual (i.e. mean population pistil height – mean individual stamen height), these values are squared and then averaged together. Stamen length variation is within individuals (variation across flowers within individuals). For the population level accuracy, bias is calculated by subtracting the mean population level pistil height from the mean population level stamen height, while the stamen variation is the variance of stamen mean height across individuals. In both cases (individual and population accuracy) pistil length variance represents the variation of the optimum value as defined in this study at the population level (variation of pistil length across individuals). Because we had measurements of pistil length for two flowers in the mass selection experiment, we could also calculate the individual level male accuracy index with a different optimum based on the within-individual pistil height variation (instead of the variation of pistil height across individuals).

Results

SELECTION RESPONSE

Stamen bias

For the within-family selection experiment the response is consistent for 4 generations of selection, with greater values for stamen bias in the high-bias than in the low-bias selection lines for each of the 4 generations (Fig. 4.3). The model that included the interaction term between ‘selection line’ and ‘generation’ had the lowest AIC value (likelihood ratio test; $\chi^2 = 15.9$, $p = 0.014$; see Appendix Table 4.2. a),

meaning that the level of selection response varied across the generations of selection. We performed a post hoc test on the 12 contrasts that involved treatment differences within each generation. For generations 2 and 3, the difference of mean bias between high-bias and low-bias is significantly different, after correcting for multiple comparisons (sequential Holm-Bonferroni; $p < 0.01$). In generation 4, the difference between high and low-bias was significantly different before adjusting for multiple contrasts ($p = 0.006$) but barely exceeds the significance cut off after applying the sequential Bonferroni procedure ($p = 0.06$) (Fig. 4.3). This last result is conservative because it reflects a two-tailed test. Since we expected an increase for the up selection lines and a decrease for the down selection lines, a one-tailed approach may be more appropriate (but less conservative), and would indicate a difference at the $p < 0.05$ level. See Supplementary R code ‘Ch_4_a’ and Supplementary Raw Data Ch4_1.

In the mass selection experiment, the model with ‘selection line’ as a factor had the lowest AIC for stamen bias (likelihood ratio test; $\chi^2 = 10.5$, $p < 0.01$; see Appendix Table 4.2. b.). The mean for high-bias was significantly greater than the low-bias line after one round of mass selection (Tukey; $p < 0.01$) (Fig. 4.3). See Supplementary R code ‘Ch_4_b’ and Supplementary Raw Data Ch4_2.

Stamen length variation

No selection response for within-individual stamen length variation was detected in the within-family selection experiment or the mass selection experiment as stamen length variation was not significantly different between high and low-variation lines within or among each generation of selection in either selection

experiment. In both selection experiments, the best model based on the rule of the model with the least number of parameters and within 3.22 units from the lowest AIC, had ‘generation’ as sole fixed factor as the sole explanatory model (see Appendix Table 4.3. a. for model selection results; and Appendix Table 4.4. a. for variance estimates for within-family selection experiment and Appendix Table 4.3. b.; and Appendix Table 4.4. b. for variance estimates for mass selection experiment). See Supplementary R code ‘Ch_4_c’ and ‘Ch_4_d’ and Supplementary Raw Data Ch4_3 and Ch4_4.

We observed no correlational response for stamen length variation on any of the floral traits. Flower variation did not differ between high and low selection lines of stamen length variation after four generations of within-family selection (see Appendix Table 4.5). See Supplementary R code ‘Ch_4_e’ and ‘Ch_4_f’ and Supplementary Raw Data Ch4_5 and Ch4_6.

INBREEDING VS OUTBREEDING EXPERIMENT

We found no significant difference for stamen bias between inbred and outbred lines in both our greenhouse (likelihood ratio test, $\chi^2 = 1.1831$, $p = 0.2767$; see Appendix Table 4.6. a.) and alcohol data set (likelihood ratio test, $\chi^2 = 1.24$, $p = 0.2656$; see Appendix Table 4.6. a.). We summarize the effect sizes for inbreeding effect on stamen bias in Appendix Table 4.7. We did find evidence for inbreeding effect on stamen length variation in our alcohol data set (likelihood ratio test, $\chi^2 = 4.206$, $p = 0.04$; see Appendix Table 4.6.b), but less so for our greenhouse data set (likelihood ratio test, $\chi^2 = 2.986$, $p = 0.084$; see Appendix Table 4.6.b). The post hoc

Tukey test on ‘cross type’ for the alcohol data set confirms that stamen length variation significantly differs between inbred and outbred lines ($t = -2.063$, $df = 81.97$, $p = 0.042$) with higher variation for inbred individuals than for outbred individuals. Results for inbreeding effect on stamen length variation for inbred and outbred individuals are summarized in Fig. 4.4.

We also found evidence for a correlated inbreeding effect across all flower whorls. The likelihood ratio test indicated that the best model was the one with the interaction term between ‘cross type’ and ‘floral part’ (likelihood ratio test, $\chi^2 = 13.242$, $p = 0.04$; see Appendix Table 4.6.c.). A post hoc test on the interaction term shows that all significant contrasts involve pistil length (see Appendix Table 4.8 and Appendix Fig 4.2). Nevertheless, following the criteria of the model with the least number of parameters and within 3.22 units from the lowest AIC (Burnham and Anderson, 2010; Fenster et al., 2015), the model with the main effect of ‘cross type’ was the best (likelihood ratio test, $\chi^2 = 11.909$, $p < 0.001$; see Appendix Table 4.6. c.). The post hoc Tukey test on ‘cross type’, for the main effect model, shows that inbred lines (0.001717, [0.001438, 0.001997]) have significantly greater variation than outbred lines (0.001202, [0.001038, 0.001366]) when values are averaged across all flower parts (Tukey, $t = -3.45$, $df = 746.23$, $p < 0.001$).

Furthermore, our alcohol dataset shows that stamen length variation differed across families for inbred individuals (likelihood ratio test; $\chi^2 = 19.78$, $p < 0.001$; see Appendix Table 4.9. a.), but not for outbred individuals (likelihood ratio test; $\chi^2 = 0$, $p = 1$; see Appendix Table 4.9. b.). This pattern is less clear for the greenhouse data set (see Appendix Table 4.9. a. and b.). See Supplementary R code ‘Ch_4_g’, ‘Ch_4_h’

and ‘Ch_4_i’ and Supplementary Raw Data Ch4_7, Ch4_8, Ch4_9, Ch4_10, Ch4_11, Ch4_12, Ch4_13, Ch4_14 and Ch4_15 for results presented in this section.

INACCURACY INDEX

Table 4.1 summarizes the raw and mean scaled inaccuracy indices for our study as well as its individual components. The mean scaled within-individual male accuracy values range from 0.019 to 0.56. Table 4.2 summarizes the values for inaccuracy across different levels of analysis (within-individual and population level) for the base generation of both selection experiments. The mean scaled inaccuracy indices vary little across different levels of analysis, but the individual components of accuracy (bias, stamen variation and optimum variation) do.

Discussion

The goal of this study was to quantify whether there is additive genetic variance for both components of adaptive accuracy (bias and variation) for stamen height in an individual and consequently whether both components can respond to selection. We show additive genetic variance for stamen bias, but not for stamen height variation in flowers of *B. rapa*. Given the lack of response to selection for stamen length variation we would not expect to see a correlated genetic response for variation of other flower parts, and we did not. However, we did find a general effect of within-individual variation for all floral whorls following inbreeding. Furthermore, non-additive genetic variation may be contributing to among individual variation for phenotypic variation for within-individual stamen height variation as we observed increased variation for stamen length variation in inbred individuals compared to

outbred individuals. This idea is further corroborated by the fact that we also found variation across families for stamen length variation in inbred, but not for outbred individuals. Thus we demonstrate that while a flower functions as a single reproductive unit, different sources of variation of adaptive accuracy are under different genetic control.

The presence of additive genetic variance for stamen bias is not surprising given that many studies have detected genetic variation for mean values of stamen and pistil height in other systems (Carr and Fenster, 1994; Campbell, 1996; Motten and Stone, 2000; Conner et al., 2003; Caruso, 2004; Herlihy and Eckert, 2007). However, this study differs from most studies in that selection was imposed by selecting individuals in relation to the population mean of a different floral trait (pistil height) than the one selected upon (stamen height). Commonly, selection studies select traits in relation to the values of the same trait of other individuals in the population and not in relation to the population mean of a different trait like performed in this study. Our selection approach may better simulate pollinator-mediated selection for increased pollen transfer in outcrossing species. Our assumption is that an individual's mean stamen height should equal the mean pistil height of the population improving the chances of an individual's stamen contacting the same area on the pollinators body that later will contact a conspecific stigma. Therefore, male fitness based on its mean stamen height may depend on the overall population pistil height. Consequently, an individual assigned as high-bias in our selection experiment, because its mean stamen height was lower than the population mean pistil height, may in fact have had flowers with reduced herkogamy i.e. stamen

and pistil height being equal within a flower, simply because the population pistil height was larger than the pistil height of that particular individual.

This lack of concordance between stamen and pistil height within flowers and stamen height to the overall population pistil height reinforces the idea that adaptive accuracy in pollen transfer is also affected by the variance of the optimum, in this case the population pistil height (Armbruster et al., 2004; Hansen et al., 2006; Pélabon and Hansen, 2008; Armbruster, Hansen, et al., 2009; Armbruster, Pélabon, et al., 2009). If a population has a high pistil height variance (both across individuals and flowers within-individuals) the probability of an individual's anther's contacting the same area as another individual's stigmas are lower due to an imprecise area of contact on the pollinators body, even if an individual's mean stamen height matches the population mean pistil height (no bias). Closer scrutiny of the components constituting the inaccuracy index reveals that the variance of the optimum (at the population level) is significantly high for our study population ranging from approximately 30 to 60% of the total inaccuracy value (see Table 4.1). Our results show, however, that stamen bias responds to selection in *B. rapa*, regardless of the other sources of variation influencing adaptive accuracy.

The lack of additive genetic variance for stamen height variation within-individuals for *B. rapa* was unexpected, particularly because Paxman (1956) and Barrett and Harder (1992) found a genetic basis for stamen height variation within-individuals for *Nicotiana rustica* and *Eichhornia paniculata* respectively. However, general conclusions on the genetic basis of developmental stability in plant traits cannot be made given that the literature is still scarce and conflicting. In addition,

most studies on the genetic basis of plant developmental stability measure the fluctuating asymmetry of organs, which could arguably be under different genetic control than among repeated modules within-individuals (e.g., flowers) (Evans and Marshall, 1996; Moller, 1996; Pélabon et al., 2004).

Genetic or environmental stress can increase developmental instability (Waddington 1960; Waddington and Robertson 1966; Huether 1968; Barrett and Harder 1992; reviewed in Fenster and Galloway 1997). While we did not impose environmental stress, we did find that inbreeding, a type of genetic stress, increased the levels of within-individual stamen length variation, demonstrating the presence of non-additive genetic control for stamen height variation within-individuals for *B. rapa*. We also found an inbreeding effect on within-individual variation across all flower parts, emphasizing the role of flowers as a single reproductive unit. Nevertheless, Appendix Fig. 4.2, calls for some caution as some whorls may respond to inbreeding more than others. In general, our evidence for inbreeding effects were not strong, perhaps because self-crosses weren't possible due to the incompatibility system of *B. rapa*. Instead, our inbred crosses were either full or half sibs which translates into an inbreeding coefficient of $\frac{1}{4}$ and $\frac{1}{8}$ respectively, instead of $\frac{1}{2}$ expected for self-crosses (Falconer and Mackay, 1996). Lack of additive genetic variance suggests the ability of stamen height in *B. rapa* to evolve towards complete accuracy under non-stressful conditions is limited. More studies are needed on the genetic basis of developmental stability of repeated modules within-individuals to understand the role of developmental stability in increased or reduced levels of adaptive accuracy.

Our inaccuracy values are much smaller than the ones reported for other flowering species by Armbruster and colleagues. Rather than indicating that *Brassica rapa* has more accurate flowers, we believe this reflects the fact that we measured our flowers in a greenhouse and controlled for ontogeny. More importantly, some variation may have been depleted from our study population as the seeds for our study originated from an artificially grown population selected for rapid growth (Williams and Hill, 1986). Therefore we caution any adaptive interpretations of our inaccuracy indexes.

Table 4.2 depicts the differences in each component of the accuracy index depending on whether accuracy is calculated at the individual or at the population level (Armbruster, Hansen, et al., 2009). The values of accuracy do not change depending on the level of analysis, but the percentage of variation explained by bias and stamen length variation do. Bias is a greater source of variation at the within individual level, but less so at the population level and stamen variation plays a larger role at the population level and less at the within individual level. The estimate of the optimum (i.e. pistil variance) at the within individual level also is smaller. Our findings of greater variation at the within individual level versus among individuals agrees with results by Williams and Conner (2001) on *Raphanus raphanistrum*, a sister species to *B. rapa*. Overall, these results highlight the hierarchical attribute of the sources of variation both within one trait (stamen height in the case of this study), but also in relation to multiple levels of sources of variation of other traits (within or among level optimum).

The motivation for this study was to explore the genetic basis of adaptive accuracy of a floral trait that could realistically be under selective pressures for pollination efficiency. Therefore, we worked with a simplified model in which the only factor determining the efficiency of pollen transfer is the spot on which anthers and stigmas contact the pollinator body. It could very well be, however, that a certain level of separation between anthers and stigmas is in fact the optimum if sexual interference and clogging of stigmas is more disadvantageous than the advantage of precise pollen transfer without anther–stigma separation (Webb and Lloyd, 1986a; Barrett, 1990, 2002a; Armbruster, Corbet, et al., 2014). While we realize that stamen accuracy for *B. rapa* may be under very different selective pressures than the ones explored in this study, the results provided here do increase our understanding of the selection response of the sources of variation that add to the total maladaptation of a trait.

The evolutionary role of developmental instability is two sided: it can be considered maladaptive by reducing a trait's fitness and heritability or adaptive as a bet-hedging strategy under unpredictable environmental fluctuations and thus an evolutionary mechanism to shift between adaptive peaks (Fenster and Galloway, 1997; Simons and Johnston, 1997; Tonsor et al., 2013). In this study we explored the genetic basis for both increased and decreased developmental instability for within-individual stamen length in *B. rapa*. Less variable flowers are not necessarily adaptive for all species and may depend on the specific breeding and pollination system of the species (Berg, 1960; Fenster, 1991; Wolfe and Krstolic, 1999; Ushimaru et al., 2006; Nikkeshi et al., 2015).

Brassica rapa is self-incompatible, so this eliminates the problem of within individual selfing as a potential evolutionary factor influencing stamen height. But, *B. rapa* has a generalized pollination system, pollinated by a variety of Hymenoptera, Diptera and Lepidopterans (Rader et al., 2009) and therefore high flower variation may be advantageous to contact pollinators of different body sizes and behaviors. In addition, *B. rapa* has many flowers open at the same time and high-variation in stamen length across flowers of the same individual may be advantageous if this increases the degree of pollen carryover and thus reduces pollen loss to flowers of the same individual (Price and Waser, 1982). The same effect could be accomplished with high within-individual pistil variation. Conversely, Conner and colleagues found evidence for a different evolutionary hypothesis with *Raphanus raphanistrum* (wild radish), a closely related species. They found evidence of stabilizing selection on stamen height and argue that the optimum height, an intermediate level of exertion, increases the contact of the anthers both to small and large pollinators (Conner et al., 2009). However, their study differs to ours because they focused on the individuals mean stamen height (or anther exertion) instead of the within-individual stamen height variation. As Orzack and Sober (1994) and Hansen et al. (2006) point out, the fact that the mean of a trait matches its optimum, does not imply that all individuals within that population are at its optimum state. Likewise, when transferring this principle to the within individual level, not all the flowers within an individual are at its optimum state. This emphasizes the need to include the variance of a trait in question when optimality is tested. Our study shows that even if an individual's mean stamen height were under strong selection, a certain level of maladaptation

would still exist due to lack of additive genetic variance for within-individual stamen height developmental stability in *B. rapa*.

Armbruster and colleagues applied the framework of adaptive accuracy to identify the potential source of maladaptation in the positioning of stamen and pistils in relation to pollen transfer across different species (Armbruster et al., 2004; Armbruster, Hansen, et al., 2009; Armbruster, Pélabon, et al., 2009). Here we explored whether two components of adaptive accuracy may in fact respond to selection and thus whether species potentially could evolve to be accurate given the correct selective pressures. Our findings suggest that stamen accuracy in *B. rapa* could potentially evolve towards increased accuracy by eliminating any target deviance (bias), i.e. the difference between the trait mean and the optimum mean value, but that some inaccuracy would still remain due to the developmental instability (variation) within-individuals which did not respond to our imposed selection. Our study, as with any quantitative genetic study, quantified parameters that may only be specific to the study population; however, this study underlines the evolutionary limitations under which selection can operate due to complex genetic backgrounds.

Table 4.1. Mean scaled inaccuracy index and accuracy components for stamen length (individual level accuracy) in *Brassica rapa* for **a.** within family selection line experiment, **b.** mass selection experiment and **c.** inbreeding vs outbreeding experiment. Bias²: values are calculated for each individual, squared and then averaged across all individual; Stamen length variation: average stamen length variance per individual (across flowers); Optimum variation: pistil length variance at the population level (across individuals); ‘Gen.’ refers to the generation of selection in the selection experiments; ‘n’ refers to number of individuals. Values in parenthesis represent the percentage of the raw inaccuracy index. ‘GH’ refers to measurements taken on fresh flowers on the individual plants in the greenhouse. ‘EtOH’ refers to measurements taken on flowers collected and preserved in 70% alcohol.

a.

<i>Gen.</i>	<i>Treatment / Selection Line</i>	<i>n</i>	<i>Bias², (%)</i>	<i>Stamen length variation, (%)</i>	<i>Optimum variation, (%)</i>	<i>Raw inaccuracy</i>	<i>Mean scaled inaccuracy index</i>
0	-	250	0.005676, (31.3)	0.001108, (6.1)	0.01137, (62.6)	0.018154	0.033789
4	High Bias	50	0.012086, (56.6)	0.001318, (6.2)	0.007956, (37.3)	0.021360	0.052475
	Low Bias	51	0.007457, (44.2)	0.001441, (8.5)	0.007956, (47.2)	0.016854	0.037234
	High Stamen Variation	39	0.006522, (42.8)	0.000761, (4.9)	0.007956, (52.2)	0.015239	0.033966
	Low Stamen Variation	55	0.008554, (47.9)	0.001365, (7.6)	0.007956, (44.5)	0.017875	0.040361
	Control	19	0.007476, (45.2)	0.001122, (6.8)	0.007956, (48.1)	0.016554	0.037359

b.

<i>Gen.</i>	<i>Treatment / Selection Line</i>	<i>n</i>	<i>Bias², (%)</i>	<i>Stamen length variation, (%)</i>	<i>Optimum variation, (%)</i>	<i>Raw inaccuracy</i>	<i>Mean scaled inaccuracy index</i>
0	-	99	0.003042, (31.4)	0.001040, (10.7)	0.005619, (57.9)	0.009701	0.022532
1	High Bias	43	0.013696, (61.1)	0.001246, (5.6)	0.007476, (33.4)	0.022419	0.056239
	Low Bias	48	0.008220, (48.1)	0.001390, (8.1)	0.007477, (43.7)	0.017087	0.038298
	Control	40	0.009658, (52.6)	0.001223, (6.7)	0.007477, (40.7)	0.018358	0.042861
2	High Stamen Variation	19	0.006617, (37.6)	0.003023, (17.2)	0.007977, (45.3)	0.017616	0.046227
	Low Stamen Variation	20	0.005767, (35.2)	0.002633, (16.1)	0.007977, (48.7)	0.016378	0.043886
	Control	18	0.003688, (26.3)	0.002344, (16.7)	0.007977, (56.9)	0.014009	0.035396

c.

<i>Data set</i>	<i>Cross type</i>	<i>n</i>	<i>Bias², (%)</i>	<i>Stamen length variation, (%)</i>	<i>Optimum variation, (%)</i>	<i>Raw Inaccuracy</i>	<i>Mean scaled inaccuracy index</i>
GH	Outbred	56	0.005271, (37.7)	0.000867, (6.2)	0.007829, (28.3)	0.013968	0.027669
	Inbred	56	0.007331, (44.9)	0.001160, (7.1)	0.007829, (47.9)	0.016320	0.032937
EtOH	Outbred	58	0.002478, (28.1)	0.000751, (8.5)	0.005590, (63.4)	0.008819	0.019648
	Inbred	57	0.003224, (32.3)	0.001160, (11.6)	0.005590, (56.1)	0.009973	0.020127

Table 4.2. Mean scaled inaccuracy index and accuracy components for stamen length at different levels of analysis in *Brassica rapa* for the base populations (generation 0) for **a.** within family selection line experiment, **b.** mass selection experiment. ‘Gen.’ refers to the generation of selection during the selection experiments; ‘n’ refers to number of individuals. Values in parenthesis represent the percentage of the raw inaccuracy index. Values in bold highlight the values that are different across levels of analysis. Within individual: Bias²: values are calculated for each individual, squared and then averaged across all individuals; Stamen length variation: average stamen length variance per individual (across flowers); Optimum variation: pistil length variance at the population level (across individuals); Population level: Bias²: values were calculated by subtracting population mean stamen length from the population mean pistil length; Stamen length variation: average stamen length variance across individual; Within individual*: Bias and stamen length variation are calculated as in within individual, but here, the optimum variance is calculated within individual, i.e. mean pistil length variation across flowers. This was only possible for the mass selection experiment as we had measurements for pistil length on two flower per individual, while only one flower per individual for the within family selection experiment.

a.

<i>Gen.</i>	<i>Level of analysis</i>	<i>n</i>	<i>Bias², (%)</i>	<i>Stamen length variation, (%)</i>	<i>Optimum variation, (%)</i>	<i>Raw inaccuracy</i>	<i>Mean scaled inaccuracy index</i>
0	Within individual	250	0.005676, (31.3)	0.001108, (6.1)	0.01137, (62.6)	0.018154	0.033789
	Population	250	0.001798, (10.5)	0.003898, (22.8)	0.01137, (66.6)	0.017065	0.031762

b.

<i>Gen.</i>	<i>Level of analysis</i>	<i>n</i>	<i>Bias², (%)</i>	<i>Stamen length variation, (%)</i>	<i>Optimum variation, (%)</i>	<i>Raw inaccuracy</i>	<i>Mean scaled inaccuracy index</i>
0	Within individual	99	0.003042, (31.4)	0.001040, (10.7)	0.005619, (57.9)	0.009701	0.022532
	Population	99	0.000862 , (9.9)	0.002202 , (25.4)	0.005619, (64.7)	0.008683	0.020168
	Within individual *	99	0.003042, (39.3)	0.001039, (13.5)	0.003651 , (47.2)	0.007733	0.017961

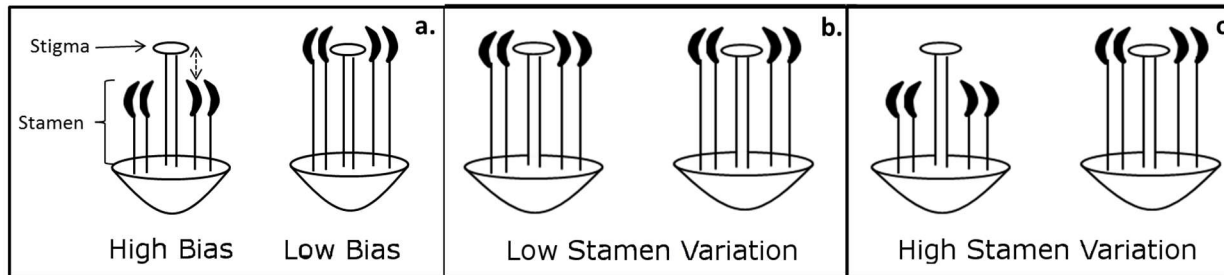


Fig. 4.1. Simplified diagrams of the selected traits in *Brassica rapa*: stamen bias and stamen length variation. All flowers only show the four long stamen. The two short stamen, petals and sepals are not included for simplification. **a.** High and low stamen bias. Bias is the distance (double arrowed line) between the anthers and the stigma. Here, each flower is a representative flower of a different individual. High-bias refers to a big distance between anthers and stigma, while low-bias refers to no or very small vertical distance between anthers and stigma. **b.** Low stamen length variation. These two flowers are collected from the same individual and since both have the same or very similar stamen height, this individual has low stamen variation. **c.** High stamen length variation. The two flowers are collected from the same individual. Because the stamen heights are different in the two flowers, this individual exemplifies high stamen variation.

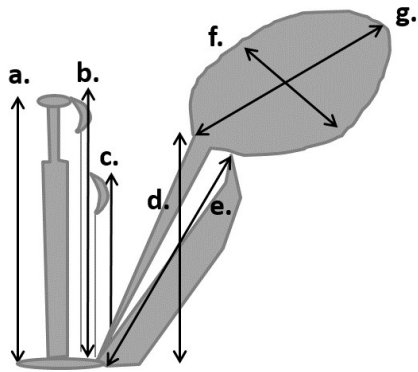


Fig. 4.2. Simplified diagram of a *Brassica rapa* flower showing floral measurements.

a. Pistil length. **b.** Long stamen length. **c.** Short stamen length. **d.** Corolla tube length.

The corolla tube is formed by the claws of the four unfused petals. Petal limbs (the wide section of the petal) are at a right angle to the claws (the narrow part of the

petal). **e.** Sepal length. **f.** Petal width. **g.** Petal length. All measurements were done with a 0.01mm dial caliper.

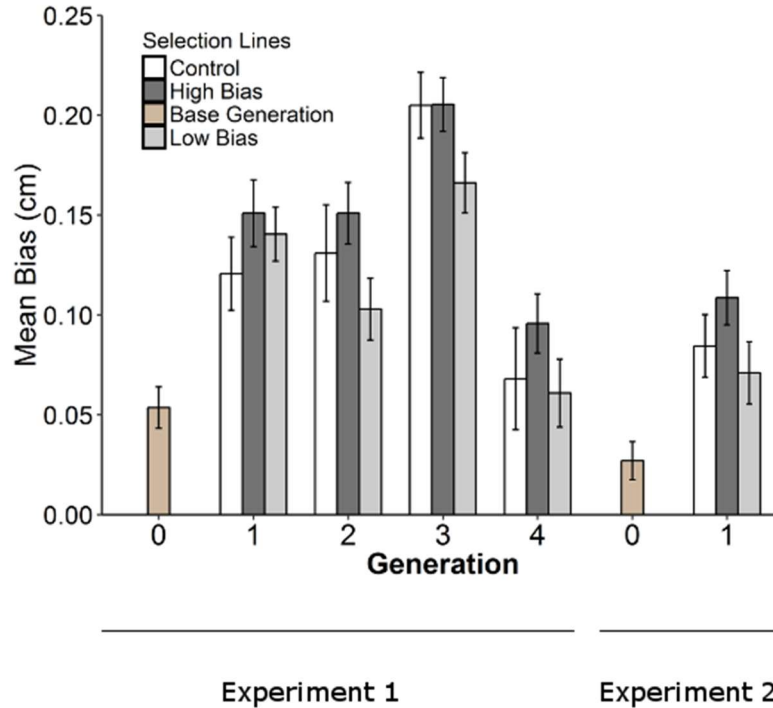


Fig. 4.3. Selection response for stamen bias in *Brassica rapa*. Values of stamen bias for all selection lines for each generation, before selection. The values of stamen bias from each individual were averaged for each generation and selection line. Generation 0 was not included in the analysis, but is kept in graph for visualization purposes. Experiment 1: within-family selection with four generations of selection. The difference of mean bias between high-bias and low-bias is significantly different for generations 2 and 3 (sequential Holm-Bonferroni; $p < 0.01$). In generation 4, the difference between high and low-bias was significant before adjusting for multiple contrasts ($p = 0.006$) but barely exceeds the significance cut off after applying the sequential Bonferroni procedure ($p = 0.06$). Experiment 2: mass selection with one generation of selection. The mean for high-bias was significantly greater than the low-bias line after one round of mass selection (Tukey; $p < 0.01$). Error bars represent 95 % CI.

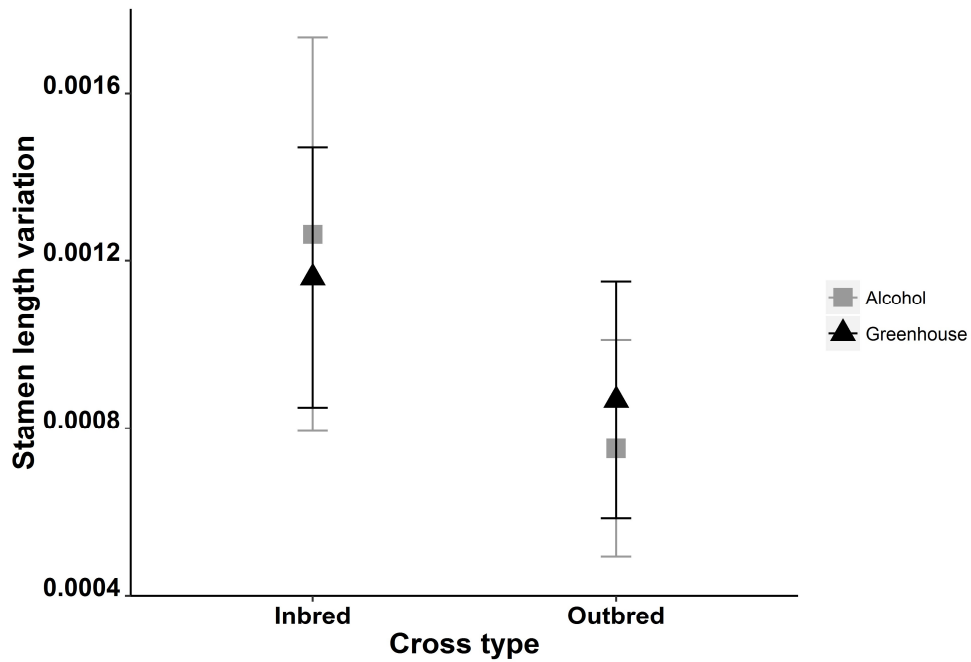


Fig. 4.4. Stamen length variation (variance) for *Brassica rapa* between inbred and outbred lines for both the alcohol and greenhouse data set. Within-individual stamen length variation is higher in inbred than in outbred individuals. This difference is significant for the alcohol dataset (Tukey; $p = 0.04$), but not for the greenhouse data set (likelihood ratio test, $p = 0.084$). Error bars are 95 % CI.

Conclusion

This dissertation moves the field of pollination biology and plant evolution forward by providing a comprehensive view of the role of pollination precision both at macro and micro evolutionary processes. Because studies at macroevolutionary scales are not site, community or species specific, observed patterns are highly informative of grand evolutionary driving forces that shape biodiversity. In chapter 3, I demonstrate that the proposed microevolutionary process of consistent and uniform pollinator mediated selection for species with flower traits that leads to precise pollination systems due to restriction in pollinator type of movement can also explain differences across species and taxonomic success. Specifically, species with laterally oriented and bilaterally symmetric flowers may benefit of increased reproductive isolation and reproductive success versus species with the alternative character states.

Most of the floral traits analyzed in chapter 3 have been previously suggested to have a role in pollination either in restricting pollinator type or pollinator movement. However, we knew nothing of the role of corolla chirality in pollination biology. To determine whether to include corolla chirality into our macroevolutionary analysis (chapter 3), we analyzed whether corolla chirality can direct pollinator behavior and movement within and across flowers and thus increase pollination precision. Because our results with *H. perforatum* show that flowers with corolla chirality function as a typical radially symmetric, vertically oriented and open flower we refrained from including this character in our macroevolutionary analysis.

Microevolutionary studies illuminate the specific evolutionary mechanisms that can later explain the observed macroevolutionary patterns. In chapter 3, I focus

on the effects of pollinator mediated selection on flower size variation due to pollination precision. Specifically, flower size variation across individuals. This is justified as selection is a population level process and differences across individuals are selected upon. However, plants are modular organisms and usually have several flowers per individual. While it is easy to imagine how species with lots of within-individual variation may have less precise pollination systems and thus also reduced reproductive success, I found that within-individual variation does not respond to selection in *B. rapa*, indicating perhaps the limitations of modular organisms to achieve optimum states. Because chapter 4 is a microevolutionary study, I was able to pinpoint that the lack of within-individual response to increased or decreased precision is due to lack of additive genetic variation (and presence of non-additive genetic variation).

The weakness of microevolutionary studies such as chapter 4, is that results are species and population specific. *Brassica rapa* has both radially symmetric and vertical oriented flowers and thus falls under the ‘less precise’ pollination category according to the results provided in chapter 3. Therefore the lack of response for increased or decreased within-individual variation found in chapter 4, may be specific to the pollination system of the species. It would be interesting to test this hypothesis by performing a comparative analysis such as in chapter 3 on within-individual variation, instead of across individual variation. Perhaps species with floral traits that lead to less precise pollination systems, such as radial symmetry and vertical orientation, also have increased within-individual variation because of the lack of additive genetic variation for within-individual variation. Conversely, species with

floral traits that lead to increased pollination systems, such as bilateral symmetry and lateral orientation, may also have reduced within-individual variation plausibly because these species retain additive genetic variation for within-individual variation and thus have the capacity to respond to selection for increased pollination precision. This dissertation highlights the advantages of addressing questions both with a macro and microevolutionary approach. In addition, with this dissertation I not only answer many questions but also provide the basis for concrete new questions that will further advance the field of pollination biology and plant evolution.

Appendices

Appendix 2.1.

Data sets for sections 2.1. A- E*—

*We did not emasculate flowers in the three data sets described below and this should be taken into account when interpreting the results presented in sections appendix 2.1. A-E.

Data set 1— Flower buds were bagged for 13 individuals (6 from population A and 7 from population B). Once opened, a right and left flower was selected per individual whenever possible, and left bagged until the flowers senesced. We ended up with 5 left and 6 right flowers in population A and 7 left and 6 right flowers in population B, resulting in 12 left and right flowers in total.

Data set 2— We cross-pollinated two right and two left flowers on each individual. Each flower morph was once hand-pollinated with pollen from a right flower and once with pollen from a left flower to test for all donor–recipient chirality combinations. After pollinating the flowers we bagged them to assure that no additional pollen was deposited. This experimental design was replicated on 17 individuals (7 from population A and 10 from population B), resulting in 17 left flowers pollinated with another left flower (LXL), 17 left flowers pollinated with a

right flower (LXR), 15 right flowers pollinated with a left flower (RXL) and 16 right flowers pollinated with another right flower (RXR).

Data set 3— For each individual, we added pollen on two right and two left flowers, whenever possible. Each floral chirality received pollen from both a right and left flower of a different individual. In addition, we had two control flowers (a right and left) without pollen addition. All flowers had been previously bagged. This was replicated for thirteen individuals (6 from population A and 7 from population B), resulting in 12 left flowers with pollen supplementation from another left flower (LXL), 13 left flowers supplemented with pollen from a right flower (LXR), 13 right flowers supplemented with pollen from another right flower (RXR), 11 right flowers supplemented with pollen from a left flower (RXL), as well as 13 left and 13 right flowers as control treatments.

2.1. A. Seed production in *H. perforatum*- flowers excluded from pollinators vs flowers cross pollinated— To determine whether cross pollination produces more seeds than seed produced either by selfing or facultative apomixes (flowers excluded from pollinators) we compared data set 1 and 2 independently of whether flowers were right or left. We averaged the number of seeds per individual. Because we found a significant interaction between population and treatment (i.e. excluded vs cross pollinated) (two way ANOVA (stats package, R) with population and treatment as fixed factors; $F_{1,26} = 4.56$, $P = 0.04$) we performed a separate one way ANOVA (stats package, R) for each population. The average seed production per individual for

flowers excluded vs cross pollinated treatments did not differ significantly for population B (excluded: mean \pm SE = 53.75 ± 7.4 , n = 7; cross pollinated: mean \pm SE = 45.2 ± 6.3 , n = 10; $F_{1, 15} = 0.74$, $P = 0.4$) but was marginally significant for population A (excluded: mean \pm SE = 31.8 ± 7.6 , n = 6; cross pollinated: mean \pm SE = 54.1 ± 7 , n = 7; $F_{1, 11} = 0.471$, $P = 0.053$).

2.1. B. Pollen limitation in *H. perforatum*— To determine whether *H. perforatum* was pollen limited, we compared the seed production of flowers with pollen addition vs. control flowers (data set 3), independently of whether flowers were right or left. We averaged the number of seeds per individual. We found no significant interaction effect between population and treatment (i.e. pollen addition and control) ($F_{1, 22} = 2.64$, $P = 0.12$) and thus performed mixed model ANOVA (nlme package, R) with individual and population as random factors (individual nested within population) and treatment (pollen added vs control) as fixed factor on these averages. We found a marginally significant difference ($F_{1, 12} = 4.66$, $P = 0.052$) in the seed production between flowers with added pollen (48.5 ± 3.8 , n = 13) and control flowers (38.6 ± 3.31 , n = 13), indicating that flowers are pollen limited.

2.1. C. Chirality and pollinator exclusion— We used data set 1 to determine whether seed production differed between right and left flowers when excluded from pollinators. Because our data set is unbalanced, we checked for an interaction effect between treatment (i.e. chirality) and population with a two way ANOVA (stats package, R) with population and treatment as fixed factors. No interaction was found

($F_{1,20} = 0.0008$, $P = 0.98$) so we performed a mixed model ANOVA (nlme package, R) with individual and population as random factors (individual nested within population) and chirality as fixed factor. We found no significant difference in seed production between right and left flowers when they were excluded from pollinators (right flowers: mean \pm SE = 38.8 ± 7.72 , left flowers: 46.1 ± 5.73 , $F_{1,10} = 1.797$, $P = 0.21$).

2.1. D. Chirality and pollen limitation (open pollination vs cross pollination by hand)—We used data set 3 to check whether right and left flower had differential pollen limitation. Here again, we checked for an interaction effect between treatment and population with a two way ANOVA (stats package, R) with population and treatment as fixed factors. When no interaction was found ($F_{5,63} = 1.2$, $P = 0.32$) we performed two separate mixed model ANOVA (nlme package, R) with individual and population as random factors (individual nested within population) and treatment as fixed factor. Treatment had six levels: control/open pollination right flower, control/open pollination left flower, pollen added from left flower to right flower, pollen added from left flower to left flower, pollen added from right flower to left flower and pollen added from right flower to right flower. Right and left flowers showed no pollen limitation ($F_{5,57} = 1.62$, $P = 0.17$). Flowers produced the same amount of seed independently of whether the pollen supplied was taken from a right or left flower.

2.1. E. Pollen incompatibility associated with chirality pollen donor - recipient combinations—We used data set 2 to test for pollen-stigma incompatibility associated with chirality. We compared seed production between right and left flowers after cross pollinating them with various combinations of pollen from the two chirality types (i.e. left flowers pollinated with pollen from another left flower [LXL], left flower pollinated with a pollen coming from a right flower [LXR], right flowers pollinated with pollen coming from a left flower [RXL] and right flowers pollinated with pollen from another right flower [RXR]). When no interaction was found between population and treatment ($F_{3, 57} = 0.376$, $P = 0.77$) we performed a mixed model ANOVA (nlme package, R) with individual and population as random factors (individual nested within population) and cross-pollination treatment combinations as a fixed factor. We found no significant difference whether the pollen donor or recipient was a left or right flower in the outcrossing treatments ($F_{3, 45} = 0.71$, $P = 0.55$). Flowers produced the same amount of seed independently of whether the pollen supplied was taken from a right or left flower (mean \pm SE: right donor and recipient: 51.38 ± 6.6 , right donor and left recipient: 48.8 ± 6.378 , left donor and right recipient: 46.7 ± 5.72 , left donor and recipient: 51.4 ± 5.01).

Appendix 2.2. Chirality and pollen number—To determine whether right and left flowers differ in pollen number, we collected right and left flowers from 21 individuals (11 from population A and 10 from population B). These flowers were previously bagged flower buds and belonged to the same inflorescence. For each flower we selected three dehisced and untouched stamens and placed the stamens in a 1.5 ml Eppendorf tube in 0.1 ml of aniline blue dye. After mixing the solution with a pipette we took two samples of 10 microliters from the 0.1 ml pollen solution with aniline blue dye and quantified pollen production with a hemocytometer. We counted the pollen on the four 1 mm² hemocytometer squares and calculated the mean of the two solution samples for each flower. We performed a mixed model ANOVA (nlme package, R) with population and individual as random factors (individual nested within population) and chirality as the fixed factor to compare the number of pollen per flower between right and left flowers. No significant differences were detected for pollen number between right (mean \pm SE for pollen grains per anther: 160 \pm 14.24) and left (mean \pm SE for pollen grains per anther: 157 \pm 15.93) pairs of flowers ($F_{1, 20} = 0.025$, $P = 0.87$).

Appendix 2.3. Chirality and ovule number—To determine whether right and left flowers differ in ovule number we collected one right and left flower for each of 20 individuals (10 individuals from each population), dissected the three carpels and counted ovule number under a dissecting scope. For 8/20 individuals we collected adjacent right and left flowers, controlling for developmental timing. No differences were found between the two sampling methods (adjacent right and left flowers within an individual vs non-adjacent flowers but still within the same individual), and so we report the combined data. We performed a mixed model ANOVA (nlme package, R) with population and individual as random factors (individual nested within population) and chirality as the fixed factor to compare the number of ovules per flower between right and left flowers. No significant differences were detected in ovule number between right (mean \pm SE = 245 ± 7.80) and left (255.1 ± 6.67) flowers ($F_{1,19} = 1.43$, $P = 0.25$).

Appendix 2.4. Pollinator sequence: Movement between flowers—

Further explanation on how we calculated expected proportions for the analysis on pollinator sequence: If the video camera was set to observe four right flowers and two left flowers, the expected proportion given that the visit sequence is random would be:

	<i>Probability of being on either a Right (R) or Left flower (L)</i>	<i>Conditional Probability of moving to the next flower of given chirality type</i>	<i>Final expected proportions</i>
R-R	4/6	3/5	6/15
R-L	4/6	2/5	4/15
L-R	2/6	4/5	4/15
L-L	2/6	1/5	1/15

In the above example, if a bee is on a right chiral flower then there are only five flowers to which it can move and three of them are right, the pollinator has 3/5 probability to move to another right flower and 2/5 probability to visit a left flower. In addition, the probability of visiting a right flower in this given scenario of four right flowers and two left flowers is 2/3 (4 right flowers/6 total flowers). Therefore the final expected proportion of visitation sequences by a pollinator from a right chiral flower to another right chiral flower is $2/3 * 3/5 = 6/15$. With these expected proportions we calculated expected frequencies, by multiplying the expected

proportion with the total number of flower transitions made by a pollinator during that video observation. We were only interested in pollinators moving away from the flower and therefore did not include pollinators immediately returning to the same flower as a new visit.

Appendix Table 3.1. Non-phylogenetically corrected analyses statistics performed with single floral trait as explanatory factors. See Table 3.1 for further description of floral trait categories. Values in bold highlight significant effects ($p < 0.05$).

Floral Trait	F	p value
Perianth Symmetry	4.88	0.0278
Functional Symmetry	9.9	0.0017
Perianth Fusion	0.21	0.64
Functional Tube	2.33	0.096
Tube Presence	3.03	0.0825
Stamen Merosity	0.39	0.53
Flower Orientation	6.3	<0.001
Stamen and Pistil Exsertion	1.6	0.13

Appendix Table 3.2. Phylogenetic generalized least squares analysis statistics performed with single floral trait as explanatory factors both with Brownian evolutionary model and with the estimated Pagel's λ to account for the correct non-independence of the residuals due to phylogenetic relatedness. A Pagel's λ value of zero indicates no phylogenetic signal, while a value of one indicates a strong phylogenetic signal in the residuals. See Table 3.1 for further description of floral trait categories. Values in bold highlight significant effects ($p < 0.05$).

Floral Trait	Brownian model		Pagel		
	F	p value	F	p value	λ
Perianth Symmetry	0.348	0.55	4.49	0.035	0.31
Functional Symmetry	2.78	0.096	11.63	<0.001	0.33
Perianth Fusion	0.457	0.499	0.1	0.753	0.364
Functional Tube	0.695	0.499	1.37	0.255	0.352
Tube Presence	0.096	0.757	2.33	0.127	0.33
Stamen Merosity	0.08	0.78	0.67	0.414	0.36
Flower Orientation	2.46	0.0326	5.88	<0.001	0.282
Stamen and Pistil Exsertion	6.47	<0.001	2.08	0.055	0.42
Stamen and Pistil Exsertion without category '6'	1.67	0.14	0.4	0.85	0.41

Appendix Table 3.3. Summary of sample size, mean CV values and standard errors for combined trait analyses. **a)** stamen and pistil exsertion & stamen merosity, **b)** stamen and pistil exsertion & tube presence, **c)** stamen and pistil exsertion & perianth symmetry, **d)** stamen and pistil exsertion & functional symmetry, **e)** stamen and pistil exsertion & perianth fusion, **f)** stamen and pistil exsertion & flower orientation, **g)** perianth fusion & tube presence, **h)** flower orientation & stamen merosity, **i)** flower orientation & perianth symmetry, **j)** flower orientation & functional symmetry, **k)** flower orientation & tube presence, **l)** flower orientation & functional tube, **m)** flower orientation & perianth fusion, **n)** perianth fusion & perianth symmetry, **o)** perianth fusion & functional symmetry, **p)** tube presence & perianth symmetry, **q)** tube presence & functional symmetry, **r)** tube presence & stamen merosity, **s)** functional tube & perianth symmetry, **t)** functional tube & functional symmetry. N = species number, CV = mean coefficient of variation for flower size, SE = standard error.

a)

Stamen and Pistil Exsertion	Stamen Merosity	N	CV	SE
0 (No exsertion)	Oligoandry	72	13.79500	0.9229851
0 (No exsertion)	Polyandry	5	14.96800	2.20377
4 (Exserted)	Oligoandry	102	14.5293	0.71165
4 (Exserted)	Polyandry	25	13.4284	1.154797

b)

Stamen and Pistil Exsertion	Tube Presence	N	CV	SE
4 (Exserted)	No Tube	62	15.1619	0.91793
4 (Exserted)	Tube	77	14.028	0.74070

c)

Stamen and Pistil Exsertion	Perianth Symmetry	N	CV	SE
0	Radial	27	15.4907	1.14076
1	Radial	26	13.8815	1.98030
2	Radial	10	18.8960	3.95942
3	Radial	5	10.808	2.0587
4	Radial	101	14.180	0.61919
5	Radial	12	15.1233	1.77325
0	Bilateral	51	13.1202	1.16733
1	Bilateral	5	13.9260	1.94742
2	Bilateral	3	14.666	4.95255
3	Bilateral	4	18.327	2.758790
4	Bilateral	28	14.5021	1.61472
5	Bilateral	11	13.3990	2.19828

d)

Stamen and Pistil Exsertion	Functional Symmetry	N	CV	SE
0	Radial	25	15.7300	1.15596
1	Radial	23	14.628	2.19199
2	Radial	11	18.6481	3.59000
3	Radial	5	17.8880	5.20370
4	Radial	103	14.8266	0.63256
5	Radial	12	15.1233	1.77325
0	Bilateral	53	13.09679	1.13782
1	Bilateral	8	11.7612	1.60828
2	Bilateral	3	14.6666	4.95255
3	Bilateral	5	15.2820	3.720433
4	Bilateral	36	13.696	1.32245
5	Bilateral	11	13.3990	2.19828

e)

Stamen and Pistil Exsertion	Perianth Fusion	N	CV	SE
0	Unfused	42	13.5097	1.2578
1	Unfused	6	10.3183	1.5355
2	Unfused	2	20.4850	1.0550
3	Unfused	2	12.22000	0.7200
4	Unfused	78	15.0029	0.6980
5	Unfused	13	14.6761	1.61127
0	Fused	36	14.4436	1.1772
1	Fused	25	14.7456	2.02456
2	Fused	12	17.346	3.46313
3	Fused	8	17.6762	3.74812
4	Fused	61	13.9342	0.97450
5	Fused	10	13.8080	2.48199

f)

Stamen and Pistil Exsertion	Flower Orientation	N	CV	SE
0	Vertical (1)	9	17.4366	1.62769
4	Vertical (1)	44	15.6625	1.09421
0	Lateral (3)	46	12.62413	1.1824
4	Lateral (3)	37	12.6421	0.99904

g)

Perianth Fusion	Tube Presence	N	CV	SE
Unfused	No Tube	60	15.8875	0.87947
Unfused	Tube	85	13.18106	0.7047903
Fused	No Tube	7	13.340	4.1383
Fused	Tube	173	14.548	0.6787

h)

Flower Orientation	Stamen Merosity	N	CV	SE
Vertical (1)	Oligoandry	36	15.597	1.1976
Vertical (1)	Polyandry	18	14.763	1.4158
Lateral/Vertical (2)	Oligoandry	54	15.244	0.9379
Lateral/Vertical (2)	Polyandry	8	12.6050	1.98086

i)

Flower Orientation	Perianth Symmetry	N	CV	SE
Lateral/Vertical (2)	Radial	49	14.04653	0.774613
Lateral/Vertical (2)	Bilateral	14	17.78714	2.59462
Lateral (3)	Radial	33	11.28697	0.66923
Lateral (3)	Bilateral	104	12.78058	0.72304

j)

Flower Orientation	Functional Symmetry	N	CV	SE
Lateral/Vertical (2)	Radial	50	14.42680	0.74259
Lateral/Vertical (2)	Bilateral	16	16.1931	2.4774341
Lateral (3)	Radial	27	11.6940	0.78759
Lateral (3)	Bilateral	110	12.59918	0.688024

k)

Flower Orientation	Tube Presence	N	CV	SE
All (0)	No Tube	8	21.1537	2.4883
All (0)	Tube	18	19.344	3.20388
Vertical (1)	No Tube	30	16.508	1.3949
Vertical (1)	Tube	35	17.457	1.7853
Lateral/Vertical (2)	No Tube	20	13.5835	1.40151
Lateral/Vertical (2)	Tube	46	15.4078	0.9997
Lateral (3)	No Tube	8	10.9200	1.5085
Lateral (3)	Tube	129	12.5138	0.60192

d)

Flower Orientation	Functional Tube	N	CV	SE
Vertical (1)	No Tube	29	16.4879	1.443770
Vertical (1)	Partial Tube	19	15.1668	1.80204
Vertical (1)	Full Tube	15	15.9093	1.26292
Lateral/Vertical (2)	No Tube	19	13.6873	1.4732613
Lateral/Vertical (2)	Partial Tube	24	15.2945	1.422072
Lateral/Vertical (2)	Full Tube	22	15.4277	1.44493
Lateral (3)	No Tube	8	10.920	1.50851
Lateral (3)	Partial Tube	55	13.0045	0.82037
Lateral (3)	Full Tube	72	12.0311	0.8515664

m)

Flower Orientation	Perianth Fusion	N	CV	SE
All (0)	Unfused	12	19.705	1.739497
All (0)	Fused	14	20.0692	4.13086
Vertical (1)	Unfused	40	14.764	1.02628
Vertical (1)	Fused	25	20.62720	2.355345
Lateral/Vertical (2)	Unfused	30	15.2513	1.219757
Lateral/Vertical (2)	Fused	36	14.5247	1.1122894
Lateral (3)	Unfused	49	11.8473	0.91604
Lateral (3)	Fused	88	12.7401	0.7344936
Lateral/Pendant (4)	Unfused	11	15.404	2.18979
Lateral/Pendant (4)	Fused	9	10.4611	1.90870

n)

Perianth Symmetry	Perianth Fusion	N	CV	SE
Radial	Unfused	104	14.932	0.58127
Radial	Fused	81	13.9959	0.95685
Bilateral	Unfused	40	12.2435	1.249800
Bilateral	Fused	86	13.600	0.81841

o)

Functional Symmetry	Perianth Fusion	N	CV	SE
Radial	Unfused	98	15.299	0.62231
Radial	Fused	86	15.9652	1.099385
Bilateral	Unfused	47	12.218	1.08802
Bilateral	Fused	93	13.168	0.783392

p)

Perianth Symmetry	Tube Presence	N	CV	SE
Radial	No tube	60	15.9423	0.91758
Radial	Tube	125	13.8410	0.64421
Bilateral	No tube	6	9.865	1.62417
Bilateral	Tube	120	13.3348	0.71181

q)

Functional Symmetry	Tube Presence	N	CV	SE
Radial	No Tube	62	16.1845	0.91993
Radial	Tube	122	15.3191	0.7939
Bilateral	No Tube	5	8.638	1.30332
Bilateral	Tube	135	13.0056	0.652946

r)

Stamen Merosity	Tube Presence	N	CV	SE
Oligoandry	No Tube	39	16.5253	1.29378
Oligoandry	Tube	238	13.6533	0.48855
Polyandry	No Tube	27	14.0377	1.10786
Polyandry	Tube	3	10.5100	1.4100473

s)

Perianth Symmetry	Functional Tube	N	CV	SE
Radial	No Tube	59	15.9379	0.9270
Radial	Partial Tube	66	13.4266	0.8194
Radial	Full Tube	56	14.4962	1.072345
Bilateral	No Tube	6	9.86500	1.62417
Bilateral	Partial Tube	47	14.25106	1.10804
Bilateral	Full Tube	71	12.6419	0.92714

t)

Functional Symmetry	Functional Tube	N	CV	SE
Radial	No Tube	61	16.184	0.92934
Radial	Partial Tube	67	14.712	0.88501
Radial	Full Tube	51	14.947	1.1382
Bilateral	No Tube	5	8.63800	1.1382
Bilateral	Partial Tube	57	13.6126	0.96951
Bilateral	Full Tube	76	12.4610	0.88150

Appendix Table 4.1. Percent measurement error estimates between the two measurements taken on all *Brassica rapa* flowers in this study. ‘GH’ refers to measurements taken on fresh flowers on the individual plants in the greenhouse. ‘EtOH’ refers to measurements taken on flowers collected and preserved in 70% alcohol. ‘Gen’ refers to the number of generations selection was imposed for each selection experiment. ‘n’ refers to the number of flowers measured per individual. ‘% M.E’ refers to the percentage of measurement error. See Fig. 4.2 for description on flower parts. Within and among flower variance estimates were calculated by running mixed models with the fixed effect equal to one (fixed intercept) and each flower measured as a random variable (Cox, 2016). Because only one flower per individual was measured for pistil length in the within-family selection experiment (greenhouse data set), the among-flower variance is actually the among-individual variance.

Experiment	Data set				n	Among flower variance	Within flower variance	% M. E.
	GH	EtOH	Gen	Flower part				
Within-family selection experiment	√		0	Long stamen	2	4.356 e-03	8.354 e-05	1.86
	√		0	Pistil	1	1.133 e-02	8.645 e-05	0.757
	√		1	Long stamen	2	4.958 e-03	8.526 e-05	1.69

	√		1	Pistil	1	9.740 e-03	6.556 e-05	0.67
	√		2	Long stamen	2	4.690 e-03	1.504 e-04	3.1
	√		2	Pistil	1	1.155 e-02	7.119 e-05	0.61
	√		3	Long stamen	2	4.668 e-03	4.631 e-05	0.98
	√		3	Pistil	1	9.543 e-03	4.823 e-05	0.5
	√		4	Long stamen	2	3.879 e-03	4.510 e-05	1.15
	√		4	Pistil	1	8.096 e-03	3.402 e-05	0.42
		√	0	Long stamen	2	4.457 e-03	2.300 e-04	4.9
		√	0	Pistil	2	6.547 e-03	5.918 e-05	0.9
		√	0	Sepal	2	2.498 e-03	7.786 e-05	3.02
		√	0	Corolla tube	2	2.805 e-03	8.939 e-05	3.08
		√	0	Petal length	2	4.132 e-03	7.576 e-05	1.8
		√	0	Petal width	2	4.369 e-03	5.283 e-05	1.19
		√	0	Short stamen	2	5.936 e-03	5.767 e-05	0.96

		√	4	Long stamen	2	4.043 e-03	3.259 e-05	0.799
		√	4	Pistil	2	9.283 e-03	2.658 e-05	0.285
		√	4	Sepal	2	2.421 e-03	3.17 e-05	1.29
		√	4	Corolla tube	2	2.255 e-03	5.247 e-05	2.27
		√	4	Petal length	2	4.087 e-03	2.701 e-05	0.656
		√	4	Petal width	2	3.524 e-03	2.388 e-05	0.67
		√	4	Short stamen	2	4.340 e-03	2.217 e-05	0.51
Mass selection experiment	Stamen bias & variation	√	0	Long stamen	5	2.986 e-03	6.569 e-05	2.15
	Stamen bias & variation	√	0	Pistil	2	7.584 e-03	4.95 e-05	0.65
	Stamen bias	√	1	Long stamen	5	3.55 e-03	3.04 e-05	0.85
	Stamen bias	√	1	Pistil	2	9.160 e-03	2.074 e-05	0.23

	Stamen variation	√		1	Long stamen	5	2.519 e-03	3.062 e-05	1.2
	Stamen variation	√		2	Long stamen	5	4.109 e-03	2.940 e-05	0.71
Inbreeding experiment		√		-	Long stamen	5	3.931 e-03	6.009 e-05	1.5
		√		-	Pistil	2	1.003 e-02	8.429 e-05	0.83
			√	-	Long stamen	4	4.441 e-03	6.548 e-05	1.45
			√	-	Pistil	4	8.478 e-03	7.982 e-05	0.93
			√	-	Sepal	4	2.284 e-03	8.084 e-05	3.42
			√	-	Corolla tube	4	1.442 e-03	1.004 e-04	6.5
			√	-	Petal length	4	3.875 e-03	7.12 e-05	1.8
			√	-	Petal width	4	4.682 e-03	5.234 e-05	1.11
			√	-	Short stamen	4	5.794e-03	5.915e-05	1.01

Appendix Table 4.2. Model selection for stamen bias for *Brassica rapa* for the two selection experiments: **a.** within-family selection experiment. **b.** mass selection experiment (family is nested within block). ‘Selection Line’ refers to the selection treatments imposed: high stamen bias, low stamen bias and control line. ‘Generation’ refers to the number of generations selection was imposed (4 generations for within-family selection experiment and one generation for the mass selection experiment). Models in the table are arranged by increased number of parameters starting with the null model. “Test” indicates which models are tested in the likelihood ratio test. χ^2 and p values are outputs from the likelihood ratio test. The AIC value in bold indicated the model with the least number of parameters and within 3.22 units from the lowest AIC. The null model includes the random factors, as intercept only models are not possible when fitting a generalized linear mixed model (lme4 package, R). Models were selected based on likelihood ratio tests and corroborated with AIC values.

a.

<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
A. Family	766.52	-1527.0	-	-	-	-
B. Selection Line + Family	780.13	-1550.3	-	-	-	-
C. Generation + Family	864.1	-1716.2	A vs. C	195.2	3	<0.001
D. Selection Line + Generation + Family	881.6	-1747.1	D vs. C	34.9	2	<0.001
E. Selection Line + Generation + Selection Line*Generation + Family	889.5	-1751.1	D vs. E	15.93	6	0.014

b.

<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
A. Block + Family	14.0	-420.0	-	-	-	-
B. Selection Line + Block + Family	219.3	-426.5	A vs. B	10.49	2	<0.01

Appendix Table 4.3. Model selection for within-individual stamen length variation for *Brassica rapa* for the two selection experiments: **a.** within-family selection experiment. **b.** mass selection experiment (family is nested within block). ‘Selection Line’ refers to the selection treatments imposed: high stamen length variation, low stamen length variation and control line. ‘Generation’ refers to the number of generations selection was imposed (4 generations for within-family selection experiment and two generations for the mass selection experiment). Models in the table are arranged by increased number of parameters starting with the null model. “Test” indicates which models are tested in the likelihood ratio test. χ^2 and p values are outputs from the likelihood ratio test. The AIC value in bold indicated the model with the least number of parameters and within 3.22 units from the lowest AIC. The null model includes the random factors, as intercept only models are not possible when fitting a generalized linear mixed model (lme4 package, R). Models were selected based on likelihood ratio tests and corroborated with AIC values.

a.

<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
A. Family	2707.6	-5409.2	-	-	-	-
B. Selection Line + Family	2709.9	-5409.8	A vs. B	4.584	2	0.1011
C. Generation + Family	2711.0	-5410.0	A vs. C	6.767	3	0.0797
D. Selection Line + Generation + Family	2713.5	-5411.0	C vs. D	4.9918	2	0.08242
E. Selection Line + Generation + Selection Line*Generation + Family	2720.6	-5413.1	D vs. E	14.115	6	0.02838

b.

<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
A. Block + Family	905.01	-1802.0	-	-	-	-
B. Selection Line + Block + Family	905.13	-1798.3	A vs. B	0.2475	2	0.8836
C. Generation + Block + Family	920.89	-1831.8	A vs. C	31.776	1	< 0.001
D. Selection Line + Generation + Block + Family	921.02	-1828.0	D vs. C	0.2461	2	0.8842
E. Selection Line + Generation + Selection Line*Generation + Block + Family	921.66	-1825.3	-	-	-	-

Appendix Table 4.4. Estimates for within-individual stamen length variation in *Brassica rapa* for the two selection experiments: **a.** within-family selection experiment and **b.** mass selection experiment (family is nested within block). Stamen length variation was calculated by the variance of stamen length among flowers of an individual. Two flowers were measured per individual for the within-family selection experiment and five flower per individual for the mass selection experiment. ‘n’ stands for number of individuals and ‘Generation’ refers to the number of generations selection was imposed for each selection experiment. Trt. = treatment.

a.

<i>Generation</i>	<i>Trt./ Selection Line</i>	<i>Variance: Mean, 95% CI [,]</i>	<i>n</i>
Base	-	0.001108 [0.000903,0.001313]	250
1	High stamen variation	0.002129, [0.001087, 0.003170]	68
	Control	0.000887, [0.000513, 0.001260]	38
	Low stamen variation	0.001619, [0.001139, 0.002099]	73
2	High stamen variation	0.001253, [0.000739, 0.001768]	60
	Control	0.001132, [0.000662, 0.001601]	28
	Low stamen variation	0.001109, [0.000762, 0.001456]	72
3	High stamen variation	0.001072, [0.000626, 0.001519]	51
	Control	0.000889, [0.000374, 0.001405]	22
	Low stamen variation	0.002387, [0.001095, 0.004172]	64
4	High stamen variation	0.000761, [0.000474, 0.001047]	39
	Control	0.001122, [0.000513, 0.001731]	19
	Low stamen variation	0.001365, [0.001487, 0.003286]	55

b.

<i>Generation</i>	<i>Treatment/ Selection Line</i>	<i>Variance: Mean, 95% CI [,]</i>	<i>n</i>
Base	-	0.001040 [0.00082, 0.001263]	99
1	High stamen variation	0.000985, [0.000684, 0.001286]	43
	Control	0.001099, [0.000847, 0.001352]	41
	Low stamen variation	0.000949, [0.000708, 0.001190]	47
2	High stamen variation	0.003023, [0.001695, 0.004350]	19
	Control	0.002344, [0.000853, 0.003835]	17
	Low stamen variation	0.002634, [0.001095, 0.004172]	20

Appendix Table 4.5. Model selection for stamen length variation correlational response on other floral part variation in *Brassica rapa* – for within-family selection experiment: **a.** Generation 0 (base generation). **b.** Generation 4. ‘Selection Line’ refers to the selection treatments imposed: high stamen length variation, low stamen length variation and control line. See Fig. 4.2 for description on flower parts. Models in the table are arranged by increased number of parameters starting with the null model. “Test” indicates which models are tested in the likelihood ratio test. χ^2 and p values are outputs from the likelihood ratio test. The AIC value in bold indicated the model with the least number of parameters and within 3.22 units from the lowest AIC. The null model includes the random factors, as intercept only models are not possible when fitting a generalized linear mixed model (lme4 package, R). Models were selected based on likelihood ratio tests and corroborated with AIC values.

a.

<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
A. Family + Block	3058.9	-6109.9	-	-	-	-
B. Selection Line + Family + Block	3059.1	-6108.2	A vs. B	0.3656	1	0.5454
C. Flower Part + Family + Block	3077.4	-6134.9	A vs. C	37.035	6	< 0.001
D. Selection Line + Flower Part + Family + Block	3077.6	-6133.3	D vs. C	0.3661	1	0.5452
E. Selection Line + Flower Part + Selection Line*Flower Part + Family + Block	3079.9	-6125.8	-	-	-	-

b.

<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
A. Family	1342.5	-2679.0	-	-	-	-
B. Selection Line + Family	1342.6	-2677.3	A vs. B	0.2855	1	0.5931
C. Flower Part + Family	1355.7	-2693.4	A vs. C	26.404	6	< 0.001
D. Selection Line + Flower Part + Family	1355.8	-2691.6	D vs. C	0.2253	1	0.635
E. Selection Line + Flower Part + Selection Line*Flower Part + Family	1359.9	-2687.8	-	-	-	-

Appendix Table 4.6. Model selection for inbreeding effects on **a.** stamen bias and **b.** within-individual stamen length variation **c.** whole flower within-individual variation for *Brassica rapa* in the inbreeding vs outbreeding experiment. ‘Cross type’ refers to inbred vs outbred individuals. ‘GH’ refers to measurements taken on fresh flowers on the individual plants in the greenhouse. ‘EtOH’ refers to measurements taken on flowers collected and preserved in 70% alcohol. Models in the table are arranged by increased number of parameters starting with the null model. “Test” indicates which models are tested in the likelihood ratio test. χ^2 and p values are outputs from the likelihood ratio test. The AIC value in bold indicated the model with the least number of parameters and within 3.22 units from the lowest AIC. The null model includes the random factors, as intercept only models are not possible when fitting a generalized linear mixed model (lme4 package, R). Models were selected based on likelihood ratio tests and corroborated with AIC values.

a.

<i>Data set</i>	<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
GH	A. Family + Block	192.87	-377.74	-	-	-	-
	B. Cross type + Family + Block	193.46	-376.92	A vs. B	1.183	1	0.2767
EtOH	A. Family + Block	227.4	-444.7	-	-	-	-
	B. Cross type + Family + Block	226.7	-445.5	A vs. B	1.239	1	0.2656

b.

<i>Data set</i>	<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
GH	A. Family + Block	623.03	-1238.1	-	-	-	-
	B. Cross type + Family + Block	624.52	-1239.0	A vs. B	2.986	1	0.0840
EtOH	A. Family + Block	568.65	-1129.3	-	-	-	-
	B. Cross type + Family + Block	570.75	-1131.5	A vs. B	4.206	1	0.04

c.

<i>Data set</i>	<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
EtOH	A. Family + Block	3702.9	-7397.9	-	-	-	-
	B. Cross type + Family + Block	3707.0	-7403.9	A vs. B	8.04	1	0.0045
	C. Flower Part + Family + Block	3812.4	-7604.8	A vs. C	218.88	6	< 0.001
	D. Cross type + Flower Part + Family + Block	3818.3	-7614.7	D vs. C	11.909	1	< 0.001
	E. Cross type + Flower Part + Cross type *Flower Part + Family + Block	3825.0	-7615.9	E vs. D	13.242	6	0.0393

Appendix Table 4.7. Estimates for stamen bias in inbred and outbred individuals during the inbreeding vs outbreeding experiment for *Brassica rapa*. ‘GH’ refers to measurements taken on fresh flowers on the individual plants in the greenhouse. ‘EtOH’ refers to measurements taken on flowers collected and preserved in 70% alcohol.

<i>Data set</i>	<i>Cross type</i>	<i>Variance: Mean, 95% CI [,]</i>	<i>n</i>
GH	Inbred	0.071029 [0.058098, 0.083960]	56
	Outbred	0.062164 [0.052017, 0.072312]	56
EtOH	Inbred	0.044329 [0.035729, 0.052928]	57
	Outbred	0.037803 [0.029673, 0.045933]	53

Appendix Table 4.8. Post hoc test contrasts with sequential Holm-Bonferonni adjustment for multiple comparisons for the model that includes the ‘flower part’ and ‘cross type’ interaction (Model E in Appendix Table 4.6.c.) for *Brassica rapa*.

Contrast	estimate	SE	df	t.ratio	p.value	alpha	n	alpha/n	signif?	alpha	n	alpha/n	signif?
Outbred,sepal - Inbred,pistil	-3.47E-03	0.0003	748.23	-11.18	<.0001	0.05	49	0.001	yes	0.01	49	0.0002	no
Inbred,sepal - Outbred,pistil	-1.90E-03	0.0003	747.16	-6.221	<.0001	0.05	48	0.001	yes	0.01	48	0.0002	no
Outbred,corolla.tube - Inbred,pistil	-3.72E-03	0.0003	748.23		<.0001	0.05	47	0.001	yes	0.01	47	0.0002	no
Inbred,corolla.tube - Outbred,pistil	-2.45E-03	0.0003	747.16	-8.014	<.0001	0.05	46	0.0011	yes	0.01	46	0.0002	no
Outbred,petal length - Inbred,pistil	-3.42E-03	0.0003	748.73	-10.98	<.0001	0.05	45	0.0011	yes	0.01	45	0.0002	no
Inbred,petal length - Outbred,pistil	-1.72E-03	0.0003	747.16	-5.635	<.0001	0.05	44	0.0011	yes	0.01	44	0.0002	no
Outbred,Petal width - Inbred,pistil	-3.36E-03	0.0003	748.23	-10.83	<.0001	0.05	43	0.0011	yes	0.01	43	0.0002	no
Inbred,Petal width - Outbred,pistil	-1.74E-03	0.0003	747.16	-5.688	<.0001	0.05	42	0.0012	yes	0.01	42	0.0002	no
Outbred,Long stamen - Inbred,pistil	-3.65E-03	0.0003	748.23	-11.75	<.0001	0.05	41	0.0012	yes	0.01	41	0.0002	no
Inbred,Long stamen - Outbred,pistil	-1.78E-03	0.0003	747.16	-5.843	<.0001	0.05	40	0.0012	yes	0.01	40	0.0002	no
Outbred,pistil - Inbred,pistil	-1.37E-03	0.0003	748.17	-4.38	<.0001	0.05	39	0.0013	yes	0.01	39	0.0003	no
Outbred,pistil - Inbred,short stamen	1.71E-03	0.0003	747.3	5.576	<.0001	0.05	38	0.0013	yes	0.01	38	0.0003	no
Inbred,pistil - Outbred,short stamen	3.35E-03	0.0003	748.23	10.78	<.0001	0.05	37	0.0013	yes	0.01	37	0.0003	no
Outbred,corolla.tube - Inbred,short stamen	-6.47E-04	0.000305	747.03	-2.119	0.0344	0.05	36	0.0014	no	0.01	36	0.0003	no
Outbred,corolla.tube - Inbred,petal length	-6.38E-04	0.000304	746.89	-2.099	0.0362	0.05	35	0.0014	no	0.01	35	0.0003	no
Outbred,corolla.tube - Inbred,Petal width	-6.22E-04	0.000304	746.89	-2.046	0.0411	0.05	34	0.0014	no	0.01	34	0.0003	no
Outbred,corolla.tube - Inbred,Long stamen	-5.74E-04	0.000304	746.89	-1.89	0.0591	0.05	33	0.0015	no	0.01	33	0.0003	no
Outbred,Long stamen - Inbred,short stamen	-5.71E-04	0.000305	747.03	-1.871	0.0617	0.05	32	0.0015	no	0.01	32	0.0003	no
Inbred,petal length - Outbred,Long stamen	5.62E-04	0.000304	746.89	1.849	0.0648	0.05	31	0.0016	no	0.01	31	0.0003	no
Inbred,Petal width - Outbred,Long stamen	5.46E-04	0.000304	746.89	1.796	0.0729	0.05	30	0.0016	no	0.01	30	0.0003	no
Outbred,Long stamen - Inbred,Long stamen	-4.98E-04	0.000304	746.89	-1.64	0.1013	0.05	29	0.0017	no	0.01	29	0.0003	no
Inbred,corolla.tube - Outbred,short stamen	-4.65E-04	0.000304	746.89	-1.529	0.1268	0.05	28	0.0017	no	0.01	28	0.0003	no
Inbred,sepal - Outbred,corolla.tube	4.59E-04	0.000304	746.89	1.51	0.1314	0.05	27	0.0018	no	0.01	27	0.0004	no
Inbred,corolla.tube - Outbred,Petal width	-4.49E-04	0.000304	746.89	-1.479	0.1396	0.05	26	0.0019	no	0.01	26	0.0004	no
Outbred,sepal - Inbred,short stamen	-3.96E-04	0.000305	747.03	-1.298	0.1948	0.05	25	0.0019	no	0.01	25	0.0004	no
Outbred,sepal - Inbred,petal length	-3.87E-04	0.000304	746.89	-1.273	0.2034	0.05	24	0.002	no	0.01	24	0.0004	no
Inbred,corolla.tube - Outbred,petal length	-3.87E-04	0.000305	747.35	-1.267	0.2056	0.05	23	0.0021	no	0.01	23	0.0004	no
Inbred,sepal - Outbred,Long stamen	3.83E-04	0.000304	746.89	1.261	0.2079	0.05	22	0.0022	no	0.01	22	0.0004	no
Outbred,sepal - Inbred,Petal width	-3.71E-04	0.000304	746.89	-1.22	0.2229	0.05	21	0.0023	no	0.01	21	0.0005	no
Outbred,petal length - Inbred,short stamen	-3.49E-04	0.000307	747.49	-1.137	0.2559	0.05	20	0.0024	no	0.01	20	0.0005	no
Outbred,sepal - Inbred,corolla.tube	3.39E-04	0.000304	746.89	1.116	0.2647	0.05	19	0.0025	no	0.01	19	0.0005	no
Outbred,petal length - Inbred,petal length	-3.39E-04	0.000305	747.35	-1.112	0.2667	0.05	18	0.0026	no	0.01	18	0.0005	no
Outbred,sepal - Inbred,Long stamen	-3.23E-04	0.000304	746.89	-1.064	0.2875	0.05	17	0.0028	no	0.01	17	0.0006	no
Outbred,petal length - Inbred,Petal width	-3.23E-04	0.000305	747.35	-1.059	0.29	0.05	16	0.0029	no	0.01	16	0.0006	no
Outbred,Petal width - Inbred,short stamen	-2.86E-04	0.000305	747.03	-0.937	0.3491	0.05	15	0.0031	no	0.01	15	0.0006	no
Inbred,petal length - Outbred,Petal width	2.77E-04	0.000304	746.89	0.91	0.3629	0.05	14	0.0033	no	0.01	14	0.0007	no
Outbred,petal length - Inbred,Long stamen	-2.76E-04	0.000305	747.35	-0.904	0.3664	0.05	13	0.0036	no	0.01	13	0.0007	no
Outbred,short stamen - Inbred,short stamen	-2.71E-04	0.000305	747.03	-0.887	0.3752	0.05	12	0.0038	no	0.01	12	0.0008	no
Inbred,petal length - Outbred,short stamen	2.61E-04	0.000304	746.89	0.861	0.3898	0.05	11	0.0042	no	0.01	11	0.0008	no
Outbred,Petal width - Inbred,Petal width	-2.61E-04	0.000304	746.89	-0.857	0.3915	0.05	10	0.0045	no	0.01	10	0.0009	no
Inbred,Petal width - Outbred,short stamen	2.45E-04	0.000304	746.89	0.807	0.4196	0.05	9	0.005	no	0.01	9	0.001	no
Outbred,Petal width - Inbred,Long stamen	-2.13E-04	0.000304	746.89	-0.702	0.483	0.05	8	0.0056	no	0.01	8	0.0011	no
Outbred,sepal - Inbred,sepal	-2.08E-04	0.000304	746.89	-0.684	0.4939	0.05	7	0.0063	no	0.01	7	0.0013	no
Inbred,Long stamen - Outbred,short stamen	1.98E-04	0.000304	746.89	0.652	0.5147	0.05	6	0.0071	no	0.01	6	0.0014	no
Inbred,corolla.tube - Outbred,Long stamen	-1.64E-04	0.000304	746.89	-0.54	0.5893	0.05	5	0.0083	no	0.01	5	0.0017	no
Inbred,sepal - Outbred,petal length	1.60E-04	0.000305	747.35	0.526	0.5993	0.05	4	0.01	no	0.01	4	0.002	no
Inbred,sepal - Outbred,Petal width	9.78E-05	0.000304	746.89	0.322	0.7477	0.05	3	0.0125	no	0.01	3	0.0025	no
Outbred,corolla.tube - Inbred,corolla.tube	8.83E-05	0.000304	746.89	0.29	0.7715	0.05	2	0.0167	no	0.01	2	0.0033	no
Inbred,sepal - Outbred,short stamen	8.26E-05	0.000304	746.89	0.272	0.7857	0.05	1	0.025	no	0.01	1	0.005	no

Table 4.9. Model selection for among-family variation in stamen length variation in *Brassica rapa* for **a.** inbred individuals, and **b.** outbred individuals. ‘GH’ refers to measurements taken on fresh flowers on the individual plants in the greenhouse. ‘EtOH’ refers to measurements taken on flowers collected and preserved in 70% alcohol. Models in the table are arranged by increased number of parameters starting with the null model. “Test” indicates which models are tested in the likelihood ratio test. χ^2 and p values are outputs from the likelihood ratio test. The AIC value in bold indicated the model with the least number of parameters and within 3.22 units from the lowest AIC. The null model includes block as the random factor, as intercept only models are not possible when fitting a generalized linear mixed model (lme4 package, R). Family was assigned as a random factor. Models were selected based on likelihood ratio tests and corroborated with AIC values.

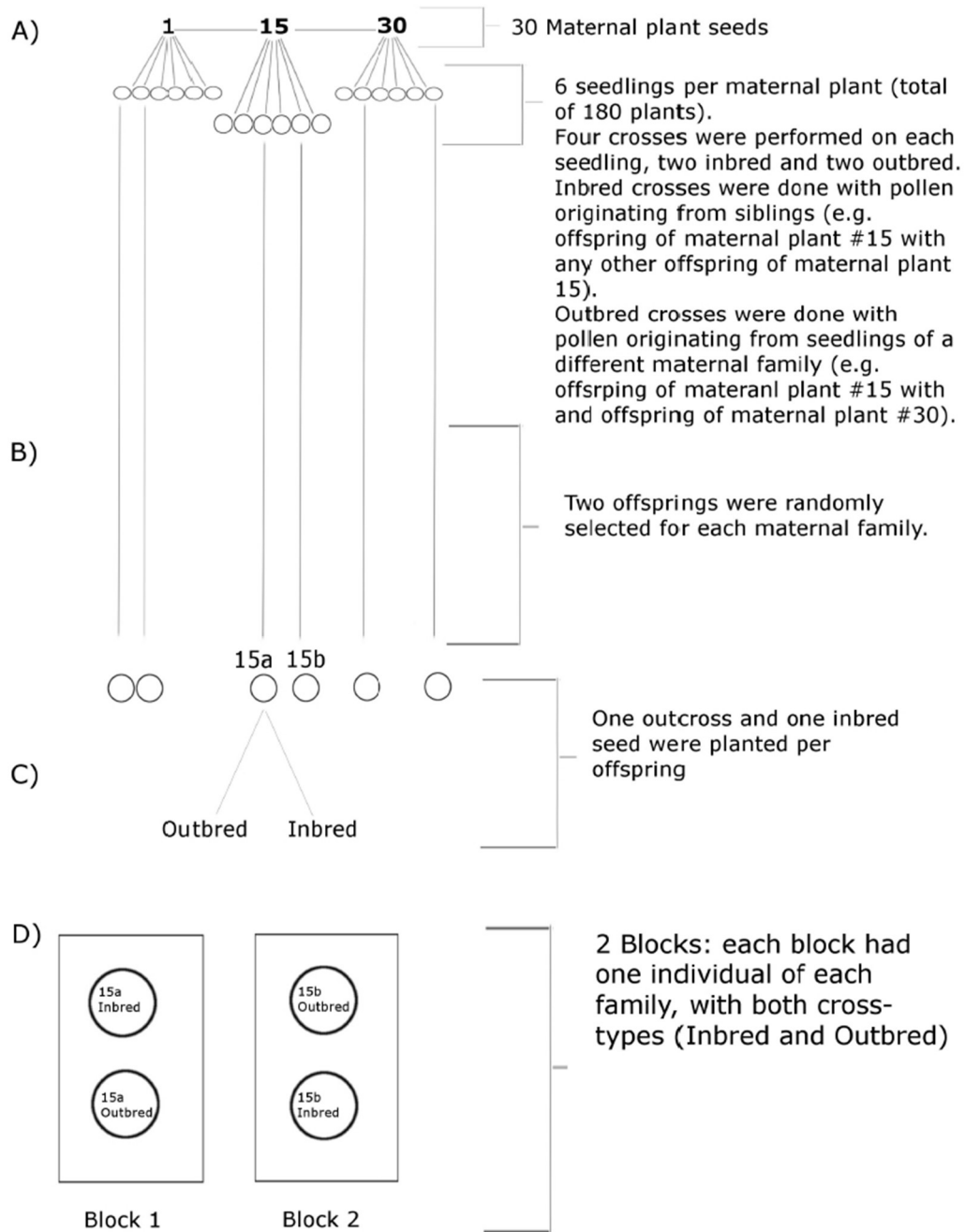
a.

<i>Data set</i>	<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
GH	A. Block	300.56	-595.13	-	-	-	-
	B. Family + Block	301.99	-595.98	A vs. B	2.851		0.0913
EtOH	A. Block	236.75	-467.49	-	-	-	-
	B. Family + Block	246.64	-485.27	A vs. B	19.78	1	< 0.001

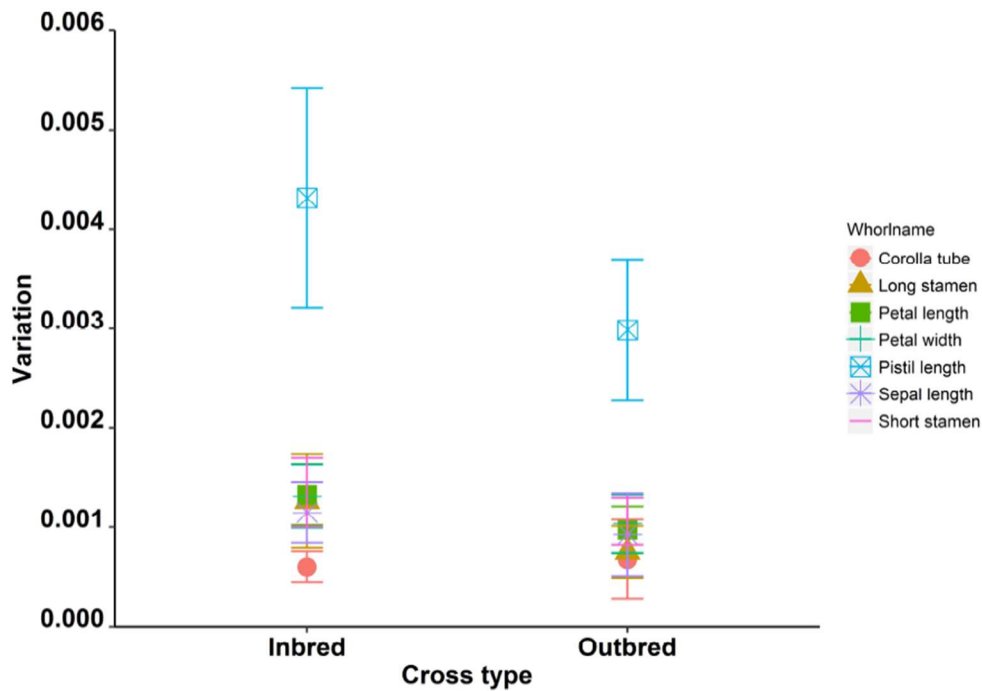
b.

<i>Data set</i>	<i>Model</i>	<i>loglikelihood</i>	<i>AIC</i>	<i>Test</i>	χ^2	<i>df</i>	<i>p value</i>
GH	A. Block	300.66	-595.32	-	-	-	-
	B. Family + Block	300.66	-593.32	A vs. B	0	1	1
EtOH	A. Block	303.1	-600.21	-	-	-	-
	B. Family + Block	303.1	-598.21	A vs. B	0	1	1

Appendix Fig. 4.1 Breeding procedure for inbreeding vs outbreeding experiment for *Brassica rapa*.



Appendix Fig. 4.2. Within-individual flower variation for *Brassica rapa* between inbred and outbred lines with the alcohol preserved flowers data set. This figure illustrates the contrasts for the post hoc test on the interaction term model (flower part + cross type + flower part*cross type) shown in Appendix Table 4.8. This model was the best model (see Model E in Appendix Table 4.6. c.) according to the likelihood ratio test, but not by the criteria of the model with the least number of parameters and within 3.22 units from the lowest AIC. See Fig. 4.2 for description on flower parts. Error bars are 95 % CI.



Bibliography

- ALLARD, H.A. 1946. Clockwise and counterclockwise spirality in the phyllotaxy of tobacco. *Journal of Agricultural Research* 73: 237–242.
- Anon. 2013. The Plant List. *Version 1*. <http://www.theplantlist.org/>. Available at: <http://www.theplantlist.org/> [Accessed January 1, 2016].
- APG. 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants : APG III. *Botanical Journal of the Linnean Society* 161: 105–121.
- ARMBRUSTER, W.S., S.A. CORBET, A.J.M. VEY, S.-J. LIU, and S.-Q. HUANG. 2014. In the right place at the right time: *Parnassia* resolves the herkogamy dilemma by accurate repositioning of stamens and stigmas. *Annals of Botany* 113: 97–103.
- ARMBRUSTER, W.S., T.F. HANSEN, C. PÉLABON, R. PÉREZ-BARRALES, and J. MAAD. 2009. The adaptive accuracy of flowers: measurement and microevolutionary patterns. *Annals of botany* 103: 1529–45.
- ARMBRUSTER, W.S., C. PÉLABON, G.H. BOLSTAD, and T.F. HANSEN. 2014. Integrated phenotypes: understanding trait covariation in plants and animals. *Philosophical transactions of the Royal Society of London. Series B* 369: 20130245.
- ARMBRUSTER, W.S., C. PÉLABON, T.F. HANSEN, and G.H. BOLSTAD. 2009. Macroevolutionary patterns of pollination accuracy: a comparison of three genera. *New Phytologist* 183: 600–617.
- ARMBRUSTER, W.S., C. PÉLABON, T.F. HANSEN, and C.P.H. MULDER. 2004. Floral integration, modularity and accuracy. Distinguishing complex adaptations from genetic constraints. In K. Pigliucci, M. and Preston [ed.], *Phenotypic integration:*

- studying the ecology and evolution of complex phenotypes, 23–49. Oxford University Press, New York.
- ARMBRUSTER, W.S., V.S. DI STILIO, J.D. TUXILL, T.C. FLORES, and J.L.V. RUNK. 1999. Covariance and decoupling of floral and vegetative traits in nine Neotropical plants: a re-evaluation of Berg's correlation-pleiades concept. *American Journal of Botany* 86: 39.
- ASHMAN, T.L. 1999. Quantitative genetics of floral traits in a gynodioecious wild strawberry *Fragaria virginiana*: Implications for the independent evolution of female and hermaphrodite floral phenotypes. *Heredity* 83: 733–741.
- ASHMAN, T.-L., and C.J. MAJETIC. 2006. Genetic constraints on floral evolution: a review and evaluation of patterns. *Heredity* 96: 343–52.
- BAILEY, R.C., and J. BYRNES. 1990. A new, old method for assessing measurement error in both univariate and multivariate morphometric studies. *Systematic Zoology* 39: 124–130.
- BARCACCIA, G., F. ARZENTON, T.F. SHARBEL, S. VAROTTO, P. PARRINI, and M. LUCCHIN. 2006. Genetic diversity and reproductive biology in ecotypes of the facultative apomict *Hypericum perforatum* L. *Heredity* 96: 322–334.
- BARRETT, S.C.H. 2002a. Sexual interference of the floral kind. *Heredity* 88: 154–159.
- BARRETT, S.C.H. 1990. The evolution and adaptive significance of heterostyly. *Trends in Ecology & Evolution* 5: 144–148.
- BARRETT, S.C.H. 2002b. The evolution of plant sexual diversity. *Nature reviews. Genetics* 3: 274–84.
- BARRETT, S.C.H., and L.D. HARDER. 1992. Floral variation in *Eichhornia paniculata*

- (Spreng.) Solms (Pontederiaceae) II. Effects of development and environment on the on the formation of selfing flowers. *Journal of Evolutionary Biology* 5: 83–107.
- BATES, D., M. MAECHLER, B. BOLKER, and S. WALKER. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67: 1–48.
- BERG, R.L. 1959. A general evolutionary principle underlying the origin of developmental homeostasis. *The American Naturalist* 93: 103–105.
- BERG, R.L. 1958. Further investigations on the role of stabilizing selection in the evolution of the flower. *Botanicheskii Zhurnal* 43: 12–28.
- BERG, R.L. 1960. The ecological significance of correlation pleiades. *Evolution* 14: 171–180.
- BESSEY, C.E. 1915. The phylogenetic taxonomy of flowering plants. *Annals of the Missouri Botanical Garden* 2: 109–164.
- BURNHAM, K.P., and D.R. ANDERSON. 2010. Model selection and multimodal inference: a practical information- theoretic approach. 2nd ed. Springer-Verlag, New York.
- CAMPBELL, D.R. 1996. Evolution of floral traits in a hermaphroditic plant: field measurements of heritabilities and genetic correlations. *Evolution* 50: 1442.
- CAMPBELL, D.R. 1989. Measurements of selection in a hermaphroditic plant : variation in male and female pollination success. *Evolution* 43: 318–334.
- CAMPBELL, D.R., and J.M. POWERS. 2015. Natural selection on floral morphology can be influenced by climate. *Proceedings of the Royal Society* 282: 1–7.
- CAMPBELL, D.R., N.M. WASER, and M. V. PRICE. 1994. Indirect selection of stigma

- position in *Ipomopsis aggregata* via a genetically correlated trait. *Evolution* 48: 55–68.
- CARR, D.E., and C.B. FENSTER. 1994. Levels of genetic variation and covariation for *Mimulus* (Scrophulariaceae) floral traits. *Heredity* 72: 606–618.
- CARTER, A.J.R., J. HERMISSON, and T.F. HANSEN. 2005. The role of epistatic gene interactions in the response to selection and the evolution of evolvability. *Theoretical Population Biology* 68: 179–196.
- CARUSO, C.M. 2004. The quantitative genetics of floral trait variation in *Lobelia*: potential constraints on adaptive evolution. *Evolution* 58: 732–740.
- CHARNOV, E.L. 1979. Simultaneous hermaphroditism and sexual selection. *Proceedings of the National Academy of Sciences of the United States of America* 76: 2480–2484.
- CHARTIER, M., F. JABBOUR, S. GERBER, P. MITTEROECKER, H. SAUQUET, M. VON BALTHAZAR, Y. STAEDLER, ET AL. 2014. The floral morphospace - a modern comparative approach to study angiosperm evolution. *New Phytologist* 204: 841–853.
- CHASE, M.W., D.E. SOLTIS, R.G. OLMSTEAD, D. MORGAN, H. LES, B.D. MISHLER, M.R. DUVALL, ET AL. 1993. Phylogenetics of Seed Plants: an analysis of nucleotide sequences from the plastid gene *rbcL*. *Annals of the Missouri Botanical Garden* 80: 528–580.
- CNAANI, J., J.D. THOMSON, and D.R. PAPA. 2006. Flower choice and learning in foraging bumblebees: effects of variation in nectar volume and concentration. *Ethology* 112: 278–285.

- CONNER, J., and S. VIA. 1993. Patterns of phenotypic and genetic correlations among morphological and life-history traits in wild radish, *Raphanus raphanistrum*. *Evolution* 47: 704–711.
- CONNER, J.K. 2002. Genetic mechanisms of floral trait correlations in a natural population. *Nature* 420: 407–410.
- CONNER, J.K., R. FRANKS, C. STEWART, and S. URL. 2003. Expression of additive genetic variances and covariances for wild radish floral traits: comparison between field and greenhouse environments. *Evolution* 57: 487–495.
- CONNER, J.K., K. KAROLY, C. STEWART, V.A. KOELLING, H.F. SAHLI, and F.H. SHAW. 2011. Rapid independent trait evolution despite a strong pleiotropic genetic correlation. *The American Naturalist* 178: 429–441.
- CONNER, J.K., H.F. SAHLI, and K. KAROLY. 2009. Tests of adaptation: functional studies of pollen removal and estimates of natural selection on anther position in wild radish. *Annals of botany* 103: 1547–56.
- COX, S.B. 2016. 8.6 Model II ANOVA. In *Applied biostatistical analysis using R*, OTexts.
- CROMPTON, C.W., I. V HALL, K.I.N. JENSEN, and P.D. HILDEBRAND. 1988. The biology of Canadian weeds. 83. *Hypericum perforatum* L. *Canadian Journal of Plant Science* 68: 149–162.
- DARWIN, C. 1862. On the various contrivances by which British and foreign orchids are fertilised by insects: and on the good effects of intercrossing. John Murray, London.
- DARWIN, C. 1877. The different forms of flowers on plants of the same species.

- Murrey, London.
- DAVIS, T.A. 1964. Aestivation in Malvaceae. *Nature* 201: 515–516.
- DAVIS, T.A. 1962. The non inheritance of asymmetry in *Cocos nucifera*. *Journal of Genetics* 58: 42–50.
- DAVIS, T.A., and B. DAVIS. 1987. Association of coconut foliar spirality with latitude. *Mathematical Modelling* 8: 730–733.
- DAVIS, T.A., and C. RAMANUJACHARYULU. 1971. Statistical analysis of bilateral symmetry in plant organs. *The Indian Journal of Statistics* 33: 259–290.
- DELPH, L.F., J.L. GEHRING, F.M. FREY, A.M. ARNTZ, M. LEVRI, and S. URL. 2004. Genetic constraints on floral evolution in a sexually dimorphic plant revealed by artificial selection. *Evolution* 58: 1936–1946.
- DENGLER, N.G. 1999. Anisophylly and dorsiventral shoot symmetry. *International Journal of Plant Sciences* 160: S67–S80.
- DILLER, C., and C.B. FENSTER. 2014. Corolla chirality in *Hypericum irazuense* and *H. costaricense* (Hypericaceae): parallels with monomorphic enantiostyly. *Journal of the Torrey Botanical Society* 141: 109–114.
- DRUMMOND, A.J., and A. RAMBAUT. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC evolutionary biology* 7: 214.
- EDWARDS, W., A.T. MOLES, and P. FRANKS. 2007. The global trend in plant twining direction. *Global Ecology and Biogeography* 16: 795–800.
- ENDRESS, P.K. 2001. Evolution of floral symmetry. *Current Opinion in Plant Biology* 4: 86–91.
- ENDRESS, P.K. 2011. Evolutionary diversification of the flowers in angiosperms.

- American Journal of Botany* 98: 370–396.
- ENDRESS, P.K. 2010. Flower structure and trends of evolution in eudicots and their major subclades. *Annals of the Missouri Botanical Garden* 97: 541–583.
- ENDRESS, P.K. 1999. Symmetry in flowers: diversity and evolution. *International Journal of Plant Sciences* 160: S3–S23.
- ENDRESS, P.K. 2012. The immense diversity of floral monosymmetry and asymmetry across angiosperms. *Botanical Review* 78: 345–397.
- EVANS, A., and M. MARSHALL. 1996. Developmental instability in *Brassica campestris* (Cruciferae): fluctuating asymmetry of foliar and floral traits. *Journal of Evolutionary Biology* 9: 717–736.
- FAEGRI, K., and L. VAN DER PIJL. 1979. The principles of pollination ecology. 3rd ed. Pergamon Press, Oxford.
- FALCONER, D., and T.F.C. MACKAY. 1996. Introduction to quantitative genetics. fourth. Longman, Harlow.
- FENSTER, C.B. 1995. Mirror image flowers and their effect on outcrossing rate in *Chaemaecrista fasciculata* (Leguminosae). *American Journal of Botany* 82: 46–50.
- FENSTER, C.B. 1991. Selection on floral morphology by hummingbirds. *Biotropica* 23: 98–101.
- FENSTER, C.B., W.S. ARMBRUSTER, and M.R. DUDASH. 2009. Specialization of flowers: is floral orientation an overlooked step? *The New Phytologist* 183: 497–501.
- FENSTER, C.B., W.S. ARMBRUSTER, P. WILSON, M.R. DUDASH, and J.D. THOMSON.

2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution, and Systematics* 35: 375–403.
- FENSTER, C.B., and M.R. DUDASH. 2001. Spatiotemporal variation in the role of hummingbirds as pollinators of *Silene virginica*. *Ecology* 82: 844.
- FENSTER, C.B., and L.F. GALLOWAY. 1997. Developmental homeostasis and floral form: evolutionary consequences and genetic basis. *International Journal of Plant Sciences* 158: S121–S130.
- FENSTER, C.B., C.L. HASSLER, and M.R. DUDASH. 1996. Fluorescent dye particles are good analogs for hummingbird pollinated *Silene virginica* (Caryophyllaceae). *Canadian Journal of Botany* 74: 189–193.
- FENSTER, C.B., and S. MARTEN-RODRIGUEZ. 2007. Reproductive assurance and the evolution of pollination specialization. *International Journal of Plant Sciences* 168: 215–228.
- FENSTER, C.B., R.J. REYNOLDS, C.W. WILLIAMS, R. MAKOWSKY, and M.R. DUDASH. 2015. Quantifying hummingbird preference for floral trait combinations: the role of selection on trait interactions in the evolution of pollination syndromes. *Evolution* 69: 1113–1127.
- GALEN, C. 1996. Rates of floral evolution: adaptation to bumblebee pollination in an Alpine wildflower, *Polemonium viscosum*. *Evolution* 50: 120–125.
- GALEN, C., and J. CUBA. 2001. Down the tube: pollinators, predators, and the evolution of flower shape in the alpine skypilot, *Polemonium viscosum*. *Evolution* 55: 1963–1971.
- GAO, J.-Y., P.-Y. REN, Z.-H. YANG, and Q.-J. LI. 2006. The pollination ecology of

- Paraboea rufescens* (Gesneriaceae): a buzz-pollinated tropical herb with mirror-image flowers. *American Journal of Botany* 97: 371–376.
- GHOSH, S.S., and T.A. DAVIS. 1978. Foliar spirality and aestivation of flowers in *Hibiscus cannabinus* Linn. *Experientia* 34: 348–350.
- GIURFA, M., B. EICHMANN, and R. MENZEL. 1996. Symmetry perception in an insect. *Nature* 382: 458–461.
- GÓMEZ, J.M., F. PERFECTTI, and J.P.M. CAMACHO. 2006. Natural selection on *Erysimum mediohispanicum* flower shape: insights into the evolution of zygomorphy. *The American Naturalist* 168: 531–45.
- GONG, Y.-B., and S.-Q. HUANG. 2009. Floral symmetry: pollinator-mediated stabilizing selection on flower size in bilateral species. *Proceedings of the Royal Society B: Biological Sciences* 276: 4013–4020.
- GRANT, V. 1949. Pollination systems as isolating mechanisms in angiosperms. *Evolution* 3: 82–97.
- HANSEN, T.F., W. ARMBRUSTER, M. CARLSON, and C. PÉLABON. 2003a. Evolvability and genetic constraints in *Dalechampia* blossoms: genetic correlations and conditional evolvability. *Journal of Evolutionary Biology* 16: 754–766.
- HANSEN, T.F., W. ARMBRUSTER, M. CARLSON, and C. PÉLABON. 2003b. Evolvability and genetic constraints in *Dalechampia* blossoms: genetic correlations and conditional evolvability. *Journal of Experimental Zoology* 296B: 23–39.
- HANSEN, T.F., A.J.R. CARTER, and C. PÉLABON. 2006. On adaptive accuracy and precision in natural populations. *The American Naturalist* 168: 168–181.
- HANSEN, T.F., and D. HOULE. 2008. Measuring and comparing evolvability and

- constraint in multivariate characters. *Journal of Evolutionary Biology* 21: 1201–1219.
- HANSEN, T.F., C. PÉLABON, and D. HOULE. 2011. Heritability is not evolvability. *Evolutionary Biology* 38: 258–277.
- HARDER, L.D., and S.C.H. BARRETT. 1993. Pollen removal from tristylous *Pontederia cordata*: effects of anther position and pollinator specialization. *Ecology* 74: 1059–1072.
- HASHIMOTO, T. 2002. Molecular genetic analysis of left-right handedness in plants. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 357: 799–808.
- HERLIHY, C.R., and C.G. ECKERT. 2007. Evolutionary analysis of a key floral trait in *Aquilegia canadensis* (Ranunculaceae): genetic variation in herkogamy and its effect on the mating system. *Evolution* 61: 1661–74.
- HERRERA, C.M. 1996. Floral traits and plant adaptation to insect pollinators: a devil's advocate approach. In D. G. Lloyd, and S. C. H. Barrett [eds.], *Floral Biology*, 65–87. Chapman & Hall, New York.
- HERRERA, J., M. ARISTA, and P.L. ORTIZ. 2008. Perianth organization and intra-specific floral variability. *Plant biology (Stuttgart, Germany)* 10: 704–10.
- HILU, K.W., T. BORSCH, K. MÜLLER, D.E. SOLTIS, P.S. SOLTIS, V. SAVOLAINEN, M.W. CHASE, ET AL. 2003. Angiosperm phylogeny based on matK sequence information. *American Journal of Botany* 90: 1758–1776.
- HOLLINGSWORTH, P.M., L.L. FORREST, J.L. SPOUGE, M. HAJIBABAEI, S. RATNASINGHAM, M. VAN DER BANK, M.W. CHASE, ET AL. 2009. A DNA barcode

- for land plants. *Proceedings of the National Academy of Sciences of the United States of America* 106: 12794–7.
- HOLM, S. 1979. A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* 6: 65–70.
- HOPKINS, R., and M.D. RAUSHER. 2012. Pollinator-mediated selection on flower color allele drives reinforcement. *Science* 335: 1090–1092.
- HOTHORN, T., F. BRETZ, and P. WESTFALL. 2008. Simultaneous inference in general parametric models. *Biometrical Journal* 50: 346–363.
- HOULE, D. 1992. Comparing evolvability and variability of quantitative traits. *Genetics* 130: 195–204.
- HUETHER, C.A. 1968. Exposure of natural genetic variability underlying the pentamerous corolla constancy in *Linanthus androsaceus* ssp. *androsaceus*. *Genetics* 60: 123–146.
- JESSON, L.K., and S.C.H. BARRETT. 2005. Experimental tests of the function of mirror-image flowers. *Biological Journal of the Linnean Society* 85: 167–179.
- JESSON, L.K., and S.C.H. BARRETT. 2002a. Solving the puzzle of mirror-image flowers. *Nature* 417: 707.
- JESSON, L.K., and S.C.H. BARRETT. 2003. The comparative biology of mirror-image flowers. *International Journal of Plant Sciences* 164: S237–S249.
- JESSON, L.K., and S.C.H. BARRETT. 2002b. The genetics of mirror-image. *Proceedings of the Royal Society of London B: Biological Sciences* 269: 1835–1839.
- JESSON, L.K., J. KANG, S.L. WAGNER, S.C.H. BARRETT, and N.G. DENGLER. 2003.

- The development of enantiostyly. *American Journal of Botany* 90: 183–195.
- JOHNSON, S.D., and K.E. STEINER. 2000. Generalization versus specialization in plant pollination systems. *Trends in Ecology & Evolution* 15: 140–143.
- KALISZ, S., R.H. REE, and R.D. SARGENT. 2006. Linking floral symmetry genes to breeding system evolution. *Trends in Plant Science* 11: 568–573.
- KAPELLE, M., and S.P. HORN. 2005. Páramos de Costa Rica. 1st ed. INBio, Santo Domingo de Heredia.
- KATO, K., and D.M. STANDLEY. 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution* 30: 772–780.
- KEARNS, C.A., and D.W. INOUE. 1993. Techniques for pollination biologists. University Press of Colorado, Niwot.
- KEARSE, M., R. MOIR, A. WILSON, S. STONES-HAVAS, M. CHEUNG, S. STURROCK, S. BUXTON, ET AL. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647–1649.
- KELLY, J.K. 2005. Epistasis in monkeyflowers. *Genetics* 171: 1917–1931.
- KLAR, A.J.S. 2002. Fibonacci's flowers. *Nature* 417: 595.
- VAN KLEUNEN, M., A. MEIER, M. SAXENHOFER, and M. FISCHER. 2008. Support for the predictions of the pollinator-mediated stabilizing selection hypothesis. *Journal of Plant Ecology* 1: 173–178.
- KNIGHT, T.M., J.A. STEETS, J.C. VAMOSI, S.J. MAZER, D.R. CAMPBELL, M.R. DUDASH, M.O. JOHNSTON, ET AL. 2005. Pollen limitation of plant reproduction:

- pattern and process. *Annual Review of Ecology , Evolution and Systematics* 36: 467–497.
- KUDO, G. 2003. Anther arrangement influences pollen deposition and removal in hermaphrodite flowers. *Functional Ecology* 17: 349–355.
- LAZARO, A., S.J. HEGLAND, and Ø. TOTLAND. 2008. The relationships between floral traits and specificity of pollination systems in three Scandinavian plant communities. *Oecologia* 157: 249–257.
- LÁZARO, A., and O. TOTLAND. 2014. The influence of floral symmetry, dependence on pollinators and pollination generalization on flower size variation. *Annals of botany* 114: 157–165.
- LEFORT, V., J.-E. LONGUEVILLE, and O. GASCUEL. 2016. SMS: Smart Model Selector. Available at: <http://www.atgc-montpellier.fr/sms/>.
- LENTH, R. V. 2016. Using lsmeans. *Journal of Statistical Software* 69: 1–33.
- LILIENFELD, F.A., and H. KIHARA. 1956. Dextrality and sinistrality in plants. *Proceedings of the Japan Academy* 32: 626–632.
- LITT, A., and E.M. KRAMER. 2010. The ABC model and the diversification of floral organ identity. *Seminars in cell & developmental biology* 21: 129–37.
- MADDISON, W., and D. MADDISON. 2015. Mesquite: a modular system for evolutionary analysis.
- MAKINO, T.T., and S. SAKAI. 2007. Experience changes pollinator responses to floral display size: from size-based to reward-based foraging. *Functional Ecology* 21: 854–863.
- MARTÉN-RODRÍGUEZ, S., C.B. FENSTER, I. AGNARSSON, L.E. SKOG, and E.A.

- ZIMMER. 2010. Evolutionary breakdown of pollination specialization in a Caribbean plant radiation. *The New Phytologist* 188: 403–417.
- MATZK, F., A. MEISTER, R. BRUTOVSKA, and I. SCHUBERT. 2001. Reconstruction of reproductive diversity in *Hypericum perforatum* L. opens novel strategies to manage apomixis. *The Plant Journal* 26: 275–282.
- MAZER, S.J., and V.A. DELESALLE. 1998. Contrasting variation within and covariation between gender-related traits in autogamous versus outcrossing species: alternative evolutionary predictions. *Evolutionary Ecology* 12: 403–425.
- MILLER, M.A., W. PFEIFFER, and T. SCHWARTZ. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *In* Proceedings of the Gateway Computing Environments Workshop (GCE), 1–8. New Orleans, LA.
- MINORSKY, P. V. 1998. Latitudinal differences in coconut foliar spiral direction: a re-evaluation and hypothesis. *Annals of Botany* 82: 133–140.
- MOLINS, M.P., J.M. CORRAL, O.M. ALIYU, M.A. KOCH, A. BETZIN, J.L. MARON, and T.F. SHARBEL. 2014. Biogeographic variation in genetic variability, apomixis expression and ploidy of St. John's wort (*Hypericum perforatum*) across its native and introduced range. *Annals of Botany* 113: 417–27.
- MOLLER, P.A. 1996. Developmental stability of flowers, embryo abortion, and developmental selection in plants. *Proceedings of the Royal Society* 263: 53–56.
- MOTTEN, A.F., and J.L. STONE. 2000. Heritability of stigma position and the effect of stigma-anther separation on outcrossing in a predominantly self-fertilizing weed, *Datura stramonium* (Solanaceae). *American Journal of Botany* 87: 339–347.
- MUELBERT, A.E., I.G. VARASSIN, M.R. TORRES BOEGER, and R. GOLDENBERG. 2010.

- Incomplete lateral anisophylly in *Miconia* and *Leandra* (Melastomataceae): inter and intraspecific patterns of variation in leaf dimensions. *Journal of the Torrey Botanical Society* 137: 214–219.
- MUHLENBERG, H. 1793. Index florae Lancastriensis. *Transactions of the American Philosophical Society* 3: 157–184.
- MUNDRY, R. 2014. Statistical issues and assumptions of phylogenetic generalized least squares. *In* Modern phylogenetic comparative methods and their application in evolutionary biology, 131–153. Springer Berlin Heidelberg.
- MURREN, C.J., and M.R. DUDASH. 2012. Variation in inbreeding depression and plasticity across native and non-native field environments. *Annals of Botany* 109: 621–632.
- NEAL, P.R., A. DAFNI, and M. GIURFA. 1998. Floral Symmetry and it's role in plant-pollinator systems: terminology, distribution, and hypotheses. *Annual Review of Ecology and Systematics* 29: 345–373.
- VAN DER NIET, T., and S.D. JOHNSON. 2012. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology & Evolution* 27: 353–61.
- NIKKESHI, A., D. KURIMOTO, and A. USHIMARU. 2015. Low flower-size variation in bilaterally symmetrical flowers: support for the pollination precision hypothesis. *American Journal of Botany* 102: 1–9.
- NÜRK, N.M., and F.R. BLATTNER. 2010. Cladistic analysis of morphological characters in *Hypericum* (Hypericaceae). *Taxon* 59: 1495–1507.
- O'MEARA, B.C., S.D. SMITH, W.S. ARMBRUSTER, D. HARDER, C.R. HARDY, L.C.

- HILEMAN, L. HUFFORD, ET AL. 2016. Non-equilibrium dynamics and floral trait interactions shape extant angiosperm diversity. *Proceedings of the Royal Society Biological Sciences* 283: .
- OLSON, E., and R. MILLER. 1958. Morphological integration. University of Chicago Press, Chicago, IL.
- ORME, D., R. FRECKLETON, G. THOMAS, T. PETZOLDT, S. FRITZ, N. ISAAC, and W. PEARSE. 2013. caper: Comparative Analyses of Phylogenetics and Evolution in R.
- ORNDUFF, R. 1975. Heterostyly and pollen flow in *Hypericum aegypticum* (Guttiferae). *Botanical Journal of the Linnean Society* 71: 51–57.
- ORZACK, S.H., and E. SOBER. 1994. Optimality models and the test of adaptationism. *The American Naturalist* 143: 361–380.
- PAXMAN, G.J. 1956. Differentiation and stability in the development of *Nicotiana rustica*. *Annals of Botany* 20: 331–347.
- PÉLABON, C., and T.F. HANSEN. 2008. On the adaptive accuracy of directional asymmetry in insect wing size. *Evolution* 62: 2855–67.
- PÉLABON, C., T.F. HANSEN, M.L. CARLSON, and W.S. ARMBRUSTER. 2004. Variational and genetic properties of developmental stability in *Dalechampia scandens*. *Evolution* 58: 504–514.
- PINHEIRO J, BATES D, DEBROY S, S.D. AND R.C.T. 2016. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-124.
- PRICE, M. V., and N.M. WASER. 1982. Experimental studies of pollen carryover: hummingbirds and *Ipomopsis aggregata*. *Ecology* 54: 353–358.

- PRICE, M. V, N.M. WASER, R.E. IRWIN, D.R. CAMPBELL, and K. ALISON. 2005. Temporal and spatial variation in pollination of a montane herb: a seven-year study. *Ecology* 86: 2106–2116.
- RADER, R., B.G. HOWLETT, S.A. CUNNINGHAM, D.A. WESTCOTT, L.E. NEWSTROM-LLOYD, M.K. WALKER, D.A.J. TEULON, and W. EDWARDS. 2009. Alternative pollinator taxa are equally efficient but not as effective as the honeybee in a mass flowering crop. *Journal of Applied Ecology* 46: 1080–1087.
- R DEVELOPMENT CORE TEAM. 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, AT.
- RAMBAUT A, SUCHARD MA, X.D.& D.A. 2014. TRACER.
- REVELL, L.J. 2010. Phylogenetic signal and linear regression on species data. *Methods in Ecology and Evolution* 1: 319–329.
- ROBERTSON, C. 1928. Flowers and insects. Lists of visitors of four hundred and fifty - three flowers. Carlinville, Il.
- ROSAS-GUERRERO, V., M. QUESADA, W.S. ARMBRUSTER, R. PÉREZ-BARRALES, and S.D. SMITH. 2011. Influence of pollination specialization and breeding system on floral integration and phenotypic variation in *Ipomoea*. *Evolution* 65: 350–364.
- SAHLI, H.F., and J.K. CONNER. 2011. Testing for conflicting and nonadditive selection: floral adaptation to multiple pollinators through male and female fitness. *Evolution* 65: 1457–73.
- SAMPSON, A.W., and K.W. PARKER. 1930. St. Johnswort on range lands of California. *University of California Experimental Station Bulletin* 503: 1–48.
- SARGENT, R.D. 2004. Floral symmetry affects speciation rates in angiosperms.

- Proceedings of the Royal Society of London B: Biological Sciences* 271: 603–608.
- SCHEMSKE, D.W., and H.D. BRADSHAW. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the United States of America* 96: 11910–11915.
- SCHIESTL, F.P., and S.D. JOHNSON. 2013. Pollinator-mediated evolution of floral signals. *Trends in ecology & evolution* 28: 307–15.
- SCHOOTE, J.C. 1935. On corolla aestivation and phyllotaxis of floral phyllomes. *Verh. kon. akad. Wet., Amsterdam. Afd. Natuurk* 34: 1–77.
- SCOTLAND, R.W., P.K. ENDRESS, and T.J. LAWRENCE. 1994. Corolla ontogeny and aestivation in the Acanthaceae. *Biological Journal of the Linnean Society* 114: 49–65.
- SIMONS, A., and M. JOHNSTON. 1997. Developmental instability as a bet-hedging strategy. *Oikos* 80: 401–406.
- SLETVOLD, N., K.K. MORITZ, and J. AGREN. 2015. Additive effects of pollinators and herbivores result in both conflicting and reinforcing selection on floral traits. *Ecology* 96: 214–221.
- SMITH, S.D. 2015. Pleiotropy and the evolution of floral integration. *New Phytologist* 209: 80–85.
- SMITH, S.D., C. ANÉ, and D.A. BAUM. 2008. The role of pollinator shifts in the floral diversification of *Iochroma* (Solanaceae). *Evolution* 62: 793–806.
- SOLTIS, D.E., P.S. SOLTIS, P.K. ENDRESS, and M.W. CHASE. 2005. Phylogeny and evolution of angiosperms. Sinauer Associates Incorporated, Sunderland,

Massachusetts.

- SPECHT, C.D., and D.G. HOWARTH. 2015. Adaptation in flower form: a comparative evodevo approach. *New Phytologist* 206: 74–90.
- SPIGLER, R.B., and S. KALISZ. 2013. Phenotypic plasticity in mating-system traits in the annual *Collinsia verna*. *Botany* 91: 597–604.
- SPRENGEL, C.K. 1793. Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen. Friedrich Vieweg, Berlin.
- STEBBINS, G.L. 1951. Natural selection and the differentiation of angiosperm families. *Evolution* 5: 299–324.
- STEBBINS, G.L. 1950. Variation and evolution in plants. Columbia University Press, New York.
- STEVENS, P.F. 2001. Angiosperm Phylogeny Website. Version 12. Available at: <http://www.mobot.org/MOBOT/research/APweb/> [Accessed July 1, 2012].
- STEVENS, P.F. 2007. Hypericaceae. In K. Kubitzki [ed.], The families and genera of vascular plants Volume IX, 194–201. Springer-Verlag, Berlin Heidelberg.
- STOLARZ, M. 2009. Circumnutation as a visible plant action and reaction: physiological, cellular and molecular basis for circumnutations. *Plant Signaling & Behavior* 4: 380–387.
- STRAHLER, A. 1957. Quantitative analysis of watershed geomorphology. *Transactions- American Geophysical Union* 38: 913–920.
- SUCHARD, M.A., and A. RAMBAUT. 2009. Many-core algorithms for statistical phylogenetics. *Bioinformatics* 25: 1370–1376.
- SYMONDS, M.R., and S.P. BLOMBERG. 2014. A primer on phylogenetic generalised

- least squares.". *In* Modern phylogenetic comparative methods and their application in evolutionary biology., 105–130. Springer Berlin Heidelberg.
- TODD, J.E. 1882. On the flowers of *Solanum rostratum* and *Cassia chamaecrista*. *The American Naturalist* 16: 281–287.
- TONSOR, S.J., T.W. ELNACCASH, and S.M. SCHEINER. 2013. Developmental instability is genetically correlated with phenotypic plasticity, constraining heritability, and fitness. *Evolution* 67: 2923–2935.
- USHIMARU, A., I. DOHZONO, Y. TAKAMI, and F. HYODO. 2009. Flower orientation enhances pollen transfer in bilaterally symmetrical flowers. *Oecologia* 160: 667–674.
- USHIMARU, A., S. KIKUCHI, R. YONEKURA, A. MARUYAMA, N. YANAGISAWA, M. KAGAMI, M. NAKAGAWA, ET AL. 2006. The influence of floral symmetry and pollination systems on flower size variation. *Nordic Journal of Botany* 24: 593–598.
- UYLINGS, H.B.M., G.J. SMIT, and W.A.M. VEITMAN. 1975. Ordering methods in quantitative analysis of branching structures of dendritic trees. *Advances in Neurology* 12: 247–254.
- WADDINGTON, C.H. 1960. Experiments on canalizing selection. *Genetical Research* 1: 140–150.
- WADDINGTON, C.H., and E. ROBERTSON. 1966. Selection for developmental canalisation. *Genetical Research* 7: 303–312.
- WAITT, D.E., and D. A LEVIN. 1998. Genetic and phenotypic correlations in plants: a botanical test of Cheverud's conjecture. *Heredity* 80: 310–319.

- WALSH, B., and M.W. BLOWS. 2009. Abundant genetic variation + strong selection = multivariate genetic constraints: a geometric view of adaptation. *Annual Review of Ecology, Evolution, and Systematics* 40: 41–59.
- WASER, N.M. 1986. Flower constancy: definition, cause and measurement. *The American Naturalist* 127: 593–603.
- WEBB, C.J., and D.G. LLOYD. 1986a. The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. *New Zealand Journal of Botany* 24: 163–178.
- WEBB, C.J., and D.G. LLOYD. 1986b. The avoidance of interference between the presentation of pollen and stigmas in angiosperms II . Herkogamy. *New Zealand Journal of Botany* 24: 163–178.
- WILLIAMS, J.L., and J.K. CONNER. 2001. Sources of phenotypic variation in floral traits in wild radish, *Raphanus raphanistrum* (Brassicaceae). *American Journal of Botany* 88: 1577–1581.
- WILLIAMS, P.H., and C.B. HILL. 1986. Rapid-cycling populations of *Brassica*. *Science* 232: 1385–1389.
- WILLMER, P. 2011. Pollination and floral ecology. Princeton University Press, Princeton, New Jersey.
- WILSON, P., J.D. THOMSON, M.L. STANTON, and L.P. RIGNEY. 1994. Beyond floral Batemanian: gender biases in selection for pollination success. *The American Naturalist* 143: 283–296.
- WOLFE, L., and J. KRSTOLIC. 1999. Floral symmetry and its influence on variance in flower size. *The American Naturalist* 154: 484–488.

ZHANG, F., C. HUI, and A. PAUW. 2012. Adaptive divergence in Darwin's race: how coevolution can generate trait diversity in a pollination system. *Evolution* 67: 548–560.