THE TEMPORAL SEPARATION OF GENDER IN FLOWERING PLANTS: AN EVOLUTIONARY ANALYSIS

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Abstract

The temporal separation of gender in flowering plants:
An evolutionary analysis

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Dichogamy, the temporal separation of gender within a flower, occurs in two forms: protandry, male function before female function, and protogyny, the converse. Dichogamy is found in over 250 families and cannot be explained solely by its role in avoiding inbreeding. The primary goal of my thesis is to investigate the interference-avoidance hypothesis for the evolution of dichogamy. I accomplish this goal through a comprehensive, evolutionary evaluation of dichogamy, including functional, genetic, comparative, and theoretical analyses of this important floral character.

To evaluate the function of protandry, I manipulated protandry and inflorescence size in the plant *Chamerion angustifolium* (Onagraceae) and monitored male and female reproductive success and pollen dispersal. Protandrous plants had a twofold siring advantage relative to adichogamous plants. However, this advantage did not increase linearly with inflorescence size. Furthermore, two negative consequences of simultaneous hermaphroditism were that anthers impeded pollinator's access to stigmas and pollinators spent more time foraging on hermaphroditic flowers, relative to female flowers. These functional analyses demonstrate that, in addition to reducing inbreeding, protandry can increase pollen export, thereby enhancing male fitness.

I conducted genetic analyses of protandry in *C. angustifolium* using a paternal half-sib design and an artificial selection experiment. The duration of male phase was heritable ($h^2 = 0.27$) and I detected no genetic correlation between male-phase duration and floral size. However, male-phase duration was positively correlated with floral display size. Moreover, male- and female-phase durations were negatively correlated. A conceptual model showed this gender trade-off produced fitness landscapes favourable to protandry's evolution.

To gain a broader perspective on the evolution of dichogamy, I conducted a phylogenetic, comparative analysis of dichogamy and self-incompatibility. I found that protandry was positively correlated with self-incompatibility and protogyny with self-compatibility. Furthermore, dichogamy changes repeatedly throughout the phylogeny. These results support the interference-avoidance hypothesis for the evolution of protandry and inbreeding avoidance for protogyny.

Collectively, this research suggests that protandry enhances male fitness through reductions in both within- and between-flower interference, while protogyny reduces inbreeding. This work has revealed unexpected fitness benefits to protandry

and helps to explain the wide taxonomic and ecological distribution of this trait in flowering plants.

Dedication

This thesis is dedicated to Catherine Shepard for teaching me the value of curiosity and the strength of the human spirit. She is missed.

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Chapter 1

Adaptation, pollination biology, and dichogamy

1.1 Exploring adaptation

> Evolution is henceforth the magic word by which we shall solve all the riddles that surround us. *Ernst Haeckel*

Our understanding of the tremendous depth and breadth of biological diversity was greatly enhanced by Darwin's (1859) insights into the evolutionary process. In particular, his concept of adaptation has provided a mechanistic explanation for the structures and functions of organisms. Although the definition is controversial (West-Eberhard, 1992), generally an adaptation is a characteristic of an organism whose form is the result of selection to perform a particular function that enhances fitness (Futuyma, 1998; Williams, 1966). The concept of adaptation was integrated with population genetics and phylogenetic methods during the Modern Synthesis of the 1930s and 1940s, which led to a renaissance in the biological sciences.

Adaptationist thinking is a powerful method for explaining natural diversity. However, Gould and Lewontin (1979) offered an influential, if controversial, critique of the methods used by evolutionary biologists. In particular, Gould and Lewontin were concerned with the profusion of "adaptive stories" being presented as objective research. In effect, many researchers were constructing elaborate stories based on evolutionary principles without rigorous examination of alternate explanations for organismal structure and function (some similar criticisms were made by Williams (1966)). Many of

these explanations assumed *a priori* that the characters under study were both optimally suited to their environment and a product of natural selection. Another common fallacy was the atomization of organisms into discrete characters that were expected to evolve independently of each other. Furthermore, the historical context of evolution was often ignored, which led to the conflation of current utility with historic selection pressures.

Although the exceptional rhetoric and many of the arguments made by Gould and Lewontin (1979) were subsequently countered (Dennet, 1995; Smith, 1982; Williams, 1985, 1992), the critique of evolutionary methods appropriately strengthened the study of evolutionary biology. A new adaptationism emerged from this period of evaluation requiring a more comprehensive and holistic approach (e.g., Baum and Larson, 1991) that includes analyses of function, genetics, and history. The following sections discuss each of these analyses and their relevance to the study of adaptation. Since this thesis will consider each of these categories of analysis in more detail, I limit the following to brief outlines. Any individual study cannot include all of the categories. However, when considered together across several studies of the same character, this holistic approach provides a potent framework for any investigation of character evolution.

1.1.1 Functional analysis

The typical analysis of adaptation is an analysis of function. Since fitness is the metric of evolution, functional analyses begin with an assessment of the influence on survival and/or fecundity of the character of interest. This assessment confirms that the character affects fitness and is potentially influenced by natural selection. The particular form of adaptation found can then be examined by two typologies of explanation: ultimate and proximate. Ultimate explanations explore why the putative adaptation affects fitness or why it performs a particular function. For example, the character may enhance survival, increase fecundity, or provide a competitive advantage. The proximate explanation is concerned with how the character performs its function or the mechanics and methods used to produce the ultimate function. For example, the adaptation may increase a metabolic pathway's efficiency or change the absorbence-spectrum of a pigment. These typologies of explanation are complimentary, in fact both are required to properly investigate the functional significance of a character. Perhaps the most efficient approach for studying function is through experimental manipula-

tions (e.g., Endler, 1986) in which the putative adaptation is modified and the consequences for function or fitness are monitored. Arguments from engineering analyses (e.g., Niklas, 1992) can also be informative.

Although many evolutionary analyses begin and end with functional tests, such an analysis does not provide a complete picture of the adaptive significance of a character. Genetic and historical analyses also contribute to a full evolutionary analysis.

1.1.2 Genetic analysis

Natural selection acts upon the phenotype, while the genotype is the hereditary material. However, phenotypes can rarely be directly mapped to genotypes creating a disconnect between function and genealogy (Falconer and Mackay, 1996; Lynch and Walsh, 1998). Phenotypic plasticity allows a single genotype to produce multiple phenotypes (Schlichting and Pigliucci, 1998). Conversely, developmental canalization and other constraints can produce identical phenotypes from multiple genotypes. Furthermore, the constituent genes of the genotype are inter-connected in a tangled web of epistasis and linkage that feeds through developmental processes to produce the phenotype (Schlichting and Pigliucci, 1998). These realities of biology can complicate our understanding of the evolutionary consequences of adaptation through several mechanisms.

First, selection for a phenotype can result in evolutionary change only if heritable, genetic variation exists for that phenotype (Falconer and Mackay, 1996; Fisher, 1930). This is succinctly expressed by the well known Breeder's equation:

$$R = h^2 S, \tag{1.1}$$

where R is the response to selection, S is the strength of selection, and h^2 is the heritability of the trait (often estimated from a parent-offspring regression).

Second, if selection favours a particular genetic variant (allele), changes in this allele's frequency will also change genes correlated with that trait. Consequently, selection for character change can cause correlated changes in other characters. These correlated changes may retard evolutionary change if they have negative fitness consequences. In a more sophisticated version of the Breeder's equation (Lande, 1979; Lande and Arnold, 1983), R is measured as a collection of inter-related characters, heritability is replaced by a

genetic variance-covariance matrix of characters (the **G** matrix), and selection is represented by a vector of selection gradients on each character (β). The change in trait means ($\mathbf{z}$) due to direct selection on traits and indirect selection through correlations among traits becomes a matrix product:

$$\boldsymbol{\Delta}\hat{\mathbf{z}} = \mathbf{G}\beta. \tag{1.2}$$

Artificial selection experiments and the pattern of character variation among known relatives allow investigations of the quantitative-genetic basis for adaptations (Falconer and Mackay, 1996; Lynch and Walsh, 1998). Such analyses reveal the number of genes controlling a character, their modes of action (i.e., dominant, recessive), and connections with other characters. Since **G** often constrains evolution (e.g., Arnold, 1992; Schluter, 1996) understanding this genetic basis is essential.

1.1.3 Historical analysis

The consistent appearance of similar solutions to the same challenge is strong evidence for evolution by natural selection. The comparative method tests for such convergences across groups of species and has a long history in evolutionary biology (Mayr, 1982). However, another of Darwin's (1859) important insights into the evolutionary process was that life is connected through a series of ancestor-descendant relationships. This means that species cannot be treated as independent data points in a comparative analysis (Felsenstein, 1985). Recently, a great deal of effort has gone into constructing phylogenetic comparative-methods (Crawford, 1990; Harvey and Pagel, 1991; Hillis et al., 1996) that restore the utility of species comparisons. In addition, these phylogenetic methods recognize that adaptations are strongly tied to history (Gould and Vrba, 1982). Consequently, the putative adaptation is compared to the ancestral state from which it arose and must enhance fitness relative to this ancestral state to qualify as an adaptation. These modern comparative analyses can also test the generality of targeted experimental tests across taxonomic groups. This permits, in specific circumstances, inferences about adaptation in taxonomic groups that have not benefited from explicit functional and genetic tests.

1.2 Pollination biology

Darwin not only revolutionized biology, he also created an enduring framework for the study of pollination biology. First, Darwin applied evolutionary thinking to orchid flowers (Darwin, 1862) and later heterostyly (a complex floral polymorphism, Darwin, 1877) to demonstrate the explanatory power of natural selection for the evolution of form. Darwin (1876) also provided a selective context for evolution by demonstrating that inbred progeny were often inferior to outbred progeny (now known as inbreeding depression; Charlesworth and Charlesworth, 1987; Husband and Schemske, 1996). Consequently, much of floral biology, he argued, could be interpreted as the result of evolution to minimize inbreeding. This early framework and subsequent research has made pollination biology one of the most dynamic fields of evolutionary biology and has motivated the research programs of many plant evolutionary-biologists to this day. In particular, it combines animal behaviour with plant biology to understand plants' impressive variation in form, life-history, sexuality, sex expression, and mating systems (Proctor et al., 1996; Richards, 1986). Furthermore, plants are conspicuous, sessile, and easily manipulated, making them excellent experimental organisms.

1.3 Dichogamy

One floral trait with extensive variation and wide taxonomic distribution is dichogamy, the temporal separation of male and female function within hermaphroditic flowers (Bertin and Newman, 1993; Lloyd and Webb, 1986; Proctor et al., 1996). Dichogamy occurs in two forms: protandry, when male function precedes female function, and the converse, protogyny. Historically, dichogamy has been interpreted as a mechanism for reducing inbreeding (e.g., Darwin, 1862). However, a survey by Bertin (1993) found that self-incompatible (SI) plants, which are incapable of inbreeding, were as likely to be dichogamous as were self-compatible (SC) plants. This led to the recent reinterpretation of dichogamy as a more general mechanism for reducing the impact of pollen-pistil interference on pollen import and export (reviewed in Barrett, 2002; Bertin and Newman, 1993; Lloyd and Webb, 1986). Unlike the inbreeding-avoidance hypothesis, which focused on female function, this interference-avoidance hypothesis considers both

gender functions.

In many hermaphroditic species, the close physical proximity of anthers and stigmas makes interference unavoidable, either within a flower or between flowers on an inflorescence. Within-flower interference, which occurs when either the pistil interrupts pollen removal or the anthers prevent pollen deposition, can result in autonomous or facilitated self-pollination (Lloyd and Schoen, 1992; Lloyd and Webb, 1986). Between-flower interference results from similar mechanisms, except that the interfering structures occur on different flowers within the same inflorescence and it requires pollinator activity. This results in geitonogamous pollination, the transfer of pollen between flowers of the same individual (de Jong et al., 1993; Lloyd and Schoen, 1992). In contrast to within-flower interference, geitonogamy necessarily involves the same processes as outcrossing: pollinator attraction, reward provisioning, and pollen removal. Therefore, between-flower interference not only carries the cost of self-fertilization (inbreeding depression; Charlesworth and Charlesworth, 1987; Husband and Schemske, 1996), but also reduces the amount of pollen available for export (pollen discounting; Harder and Wilson, 1998). Because pollen discounting diminishes outcross siring success, interference avoidance may be an important evolutionary force in floral biology (Barrett, 2002; Harder and Barrett, 1995, 1996; Harder and Wilson, 1998).

Dichogamy may reduce between-flower interference by minimizing the temporal overlap between stigmas and anthers within an inflorescence. Harder et al. (2000) demonstrated experimentally that dichogamy both reduced rates of self-fertilization and enhanced outcross siring success through reductions in geitonogamy and pollen discounting, respectively. However, to implicate between-flower interference convincingly in the evolution of dichogamy, the advantage conferred by dichogamy should depend on daily inflorescence size. Large inflorescences attract more pollinators, potentially enhancing reproductive success by increasing pollen import and export (Bell, 1985; Geber, 1985; Klinkhamer and de Jong, 1990; Queller, 1983; Schemske, 1980; Schmid-Hempel and Speiser, 1988). However, large inflorescences also increase the opportunities for both geitonogamy and pollen discounting, so that the opportunity for between-flower interference increases with inflorescence size (Harder and Barrett, 1996). Consequently, the evolution of floral display size may represent a compromise between maximizing pollinator visitation and minimizing geitonogamy and pollen discounting (Barrett et al., 1994; Holsinger, 1996; Klinkhamer and de Jong,

1993; Snow et al., 1996).

Protandry may be particularly relevant to this compromise, because it often results in an inflorescence structure with female phase flowers positioned below male phase flowers (Bertin and Newman, 1993). Given the tendency of many insect pollinators to forage upwards through inflorescences (Galen and Plowright, 1988), protandry may enhance pollen export by reducing between-flower interference (Darwin, 1862; Harder et al., 2000). Furthermore, this enhanced pollen export should increase as floral display size increases, because between-flower interference should increase with floral display size. These effects of protandry on between-flower interference may decouple the benefits of large inflorescences from the consequences of geitonogamy and pollen discounting. Such a decoupling would provide a significant reproductive advantage through increased pollinator visitation and siring success. However, there is a conspicuous absence of research into the role of protandry in pollination. This absence is particularly acute in light of the discussion of adaptive analyses presented earlier.

Although protandry and protogyny are often considered together, they may provide different evolutionary advantages and disadvantages. In protogynous species, stigmatic receptivity before anther dehiscence insures a period of female function prior to the release of any pollen from the same flower. In contrast, protandry does not necessarily insure any period of stigma presentation free of self-pollen, though it is likely to reduce the abundance of same-flower self-pollen during stigma receptivity. This difference suggests that, while both forms of dichogamy could potentially reduce pollen-pistil interference, protogyny is likely to be more effective at reducing inbreeding. If this logic is correct, protogyny is more likely to be associated with SC, where inbreeding is not prevented by other means, while protandry will be associated with SI, where avoidance of inbreeding is not an issue. Despite the importance of these hypotheses for understanding components of floral biology, they have received relatively little attention since Lloyd and Webb (1986), Bertin (1993), and Bertin and Newman (1993), with one experimental test of protandry (Harder et al., 2000) and one of protogyny (Griffin et al., 2000), and a review of interference by Barrett (2002).

1.4 This thesis

The goal of my thesis is to conduct an evolutionary analysis of the adaptive significance of protandry. In particular, I test the interference-avoidance hypothesis for the evolution of protandry in a variety of contexts. Following the outline for the study of adaptation presented above, the thesis is divided into six additional chapters:

- Chapter 2 explores the ultimate functional significance of protandry with an experimental manipulation of protandry at different inflorescence sizes.
- Chapter 3 follows up with an analysis of the proximate mechanisms by which protandry provides the fitness benefits described in Chapter 2.
- Chapter 4 uses a variety of techniques from quantitative genetics to uncover the genetic basis of protandry and estimate genetic correlations among several floral traits.
- Chapter 5 takes a comparative approach to the study of dichogamy (both protandry and protogyny) and its relationship with self-incompatibility.
- Chapter 6 uses conceptual modelling to summarize the previous four chapters and integrate their results.
- Chapter 7 is a general summary of the thesis.

Overall, this thesis is a comprehensive, evolutionary analysis of a widespread floral trait. Accordingly, this research makes important contributions to our understanding of the evolutionary ecology of plant reproduction.

Chapter 2

The functional significance of protandry

As a first step in establishing the adaptive significance of protandry, I experimentally evaluate the interference-avoidance hypothesis for the evolution of protandry in *Chamerion angustifolium* (Onagraceae). Protandry is expected to enhance male reproductive success by reducing between-flower interference (Darwin, 1862; Harder et al., 2000). Since between-flower interference accumulates with inflorescence size, protandry should benefit large floral displays by reducing the frequencies of both geitonogamy, the transfer of pollen within an inflorescence (de Jong et al., 1993; Lloyd and Schoen, 1992), and pollen discounting, reductions in pollen export (Harder and Wilson, 1998).

I constructed artificial populations containing manipulated protandrous and adichogamous (simultaneous hermaphrodite) plants for each of three different inflorescence-size classes. Subsequent tracking of allozyme markers, associated with the dichogamy treatments, allowed me to infer the rate of self-fertilization and siring success of the two dichogamy treatments for each inflorescence size. Specifically, I tested three predictions, each an important component of interference avoidance: 1) Geitonogamy (estimated from female outcrossing-rates) increases with inflorescence size, 2) Protandry enhances outcross siring-success, and 3) The enhanced siring success provided by protandry increases with inflorescence size.

2.1 Materials and methods

2.1.1 Study species

Chamerion (=Epilobium) *angustifolium* (L.) Holub (fireweed) is an herbaceous, perennial member of the Onagraceae (Evening Primrose family), distributed widely in the northern temperate region. Individual plants are approximately 2 m tall, with indeterminate inflorescences bearing from 10 to 15 open flowers at a time, each with four, 10–15 mm long, pink petals (Husband and Schemske, 2000). A flower has eight anthers which shed blue-green or yellow pollen held together with viscine threads (Myerscough, 1980). Initially the style is strongly deflexed away from the anthers and the four stigma lobes are closed. This male phase lasts for two to three days. The style then straightens moving the stigma upward to the centre of the flower, the stigma lobes spread apart, and female phase begins (Galen and Plowright, 1988). This protandry and the acropetalous (maturing from base to tip) development of the inflorescence, produces an inflorescence with female-phase flowers at the base and male-phase flowers toward the apex (Galen and Plowright, 1988). However, in the absence of pollinators, pollen remains viable within the anthers, resulting in hermaphroditic flowers.

Chamerion angustifolium is self-compatible (Mosquin, 1967), but pollinators (primarily *Bombus* spp.) are required for full seed set (Benham, 1969). Allozyme electrophoresis revealed that in natural populations approximately 45% of the available ovules were self-fertilized (primary female outcrossing-rate; measured female outcrossing-rate=0.06), presumably through geitonogamy (Husband and Schemske, 1995). Furthermore, inbreeding depression was estimated at 0.95, so that selfed progeny experience a 95% reduction in fitness. This combination of protandry, potential for geitonogamy, and high inbreeding depression makes *C. angustifolium* an ideal species for studying the functional significance of dichogamy.

2.1.2 Experimental design

My experiments involved arrays of 16 potted plants, arranged in a 4x4 grid with 0.5 m between each pot. Each array was assigned one of two dichogamy treatments (adichogamous plants only, or 8 protandrous and 8 adichogamous plants) and one of three inflorescence-size treatments (2, 6, or 10 open flowers) as described below. The uniform arrays (adichogamous

plants only) were used to verify that the transmission of marker genotypes was not biased in any way in the absence of the floral manipulation and to investigate the influence of inflorescence size on the response variables in the absence of protandry differences. Each treatment combination was replicated four times resulting in a total of (3 inflorescence-size treatments x 2 dichogamy treatments x 4 replicates=) 24 arrays.

During the summer of 1999 I germinated seeds collected from two populations in northern Wyoming (populations D2 and D6; Husband and Schemske, 1997). I chose these populations because resident plants are predominantly diploid and genetically variable with respect to phosphoglucose isomerase (PGI EC 5.3.1.9) and malate dehydrogenase (MDH EC 1.1.1.37), which I used as markers to quantify male siring success (PGI) and female outcrossing-rates (MDH). Young leaves from these plants were used to assay PGI genotypic frequencies with horizontal, starch-gel electrophoresis (following Husband and Schemske, 1995). I grew the plants to flowering and performed crosses to generate 150 families homozygous for alternate alleles of PGI. All experimental treatments were applied independently of a plant's MDH genotype for the purpose of estimating female outcrossing-rates. I germinated the seeds from these crosses, re-assayed them to confirm their genotypes, and transferred the seedlings to 4 L pots.

When constructing an experimental array, I randomly chose 8 plants from each of the two pools of plants homozygous for alternate PGI genotypes. To limit variation in nectar properties, pollen production, and size among flowers within an inflorescence, I removed all secondary shoots and non-hermaphrodite flowers from each plant. I placed the plants within a large nylon-mesh tent (4 m x 4 m x 2.1 m, Basic Screen House, Canadian Tire Corporation) in an alternating checkerboard pattern according to their PGI genotype. I then applied experimental treatments to the plants. For the inflorescence-size treatments, I removed flowers randomly to produce a uniform array of either 2, 6, or 10 flowered inflorescences. In mixed arrays, I randomly assigned one genotypic class within an array to be protandrous and the other to be adichogamous. For the protandrous class, I removed the styles from flowers in the upper half of the inflorescence with forceps. The adichogamous class was left unmanipulated. Therefore, each array contained plants of the same inflorescence size class, with 8 protandrous plants (all homozygous for the same PGI allele) alternating with 8 adichogamous plants (homozygous for the other PGI allele). Dichogamy classes were randomly assigned to the PGI genotypes. For uniform arrays, I chose plants as

in the mixed arrays, but left all stigmas intact.

My choice of experimental manipulations of inflorescence size was bounded by ten flowers, the largest I could reliably produce, and two flowers, the minimum number required to produce any between-flower interference. My dichogamy treatment has the benefit of maintaining both equal flower number and pollen production between protandrous and adichogamous plants. However, it does double the ratio of pollen to stigmas and halves the number of fruits available on protandrous plants. This could have important consequences for female reproductive success if I considered total seed production as a measure of female fitness. Alternatively, completely dichogamous plants could have been produced with only staminate and pistillate flowers and twice the floral display size (Harder et al., 2000). However, given my focus on outcross siring-success, it was more important to maintain display size and pollen production. Considered together, my experimental manipulations do mimic the natural protandry in *C. angustifolium* and represent a range of inflorescence sizes that an individual plant would experience throughout a season.

After constructing the arrays, I introduced a hive of bumble bees (*Bombus impatiens*, Natupol-Plus Class A Hive, supplied by Koppert Biological Systems) experienced with *C. angustifolium* into the tent and allowed one bee to forage freely within the array at one time. I monitored individual bees while they foraged, noting the number of flowers visited per inflorescence, the vertical direction of movement within an inflorescence, the time spent foraging per inflorescence, and the number of plants visited during a foraging bout. For this study, I defined a foraging bout as a minimum of three plant visits with less than 1 min of non-foraging between flower visits. After a foraging bout, I recaptured the bee and released a new bee into the array. Each experimental run was concluded after 25 separate foraging bouts had occurred within the array (approximately 6 h elapsed time). To ensure that pollen was not transferred into the hive and between arrays, I isolated all bees after each experimental run.

At the end of an experimental run I removed all but two adichogamous flowers randomly from each plant. This removal of flowers minimized differences in resource allocation across the treatments, because all treatment plants required the same level of investment. Otherwise, ten-flowered inflorescences might have set fewer seeds than two-flowered inflorescences because of greater resource demands rather than because of pollination differences resulting from interference between sexual organs. The only

exception involved two-flowered protandrous plants, as only one flower per inflorescence could produce a fruit because the style had been removed from the other flower.

2.1.3 Seed set

Once fruits matured, I harvested them and counted the seeds within each fruit. The contents of each fruit were scanned digitally with a flatbed scanner and the image was analyzed with Northern Eclipse software (Empix Imaging, Inc., 1996). Based on area and perimeter, I categorized each object in the scanned image as one of three objects: full seed (ϕ, area>0.019 cm^2, 0.6 cm<perimeter<1.05 cm), aborted seed (β, 0.005 cm^2<area<0.019 cm^2), and debris that was excluded from the analysis (area>0.044 cm^2, perimeter>1.06 cm). To ensure that these categories were characterized accurately, I compared the digitally analyzed counts to visual counts under a dissecting microscope for the first 83 fruits. The correlation between the two was close to one with a significant paired t-test (y=1.09x, R^2=0.98, t=57.3, P<0.0001; t=3.34, df=82, P<0.01), consequently I used digital analysis for all subsequent seed counts.

Relative seed set was calculated for each fruit as:

$$\frac{\sum \phi}{\sum \phi + \sum \beta}. \tag{2.1}$$

These values were averaged for each treatment and fruit position within an array, and arrays were used as the unit of replication in the statistical tests. Although most aborted seeds are larger than unfertilized ovules, slight overlap may occur. Therefore, I may have overestimated relative seed set slightly. However, this upward bias should apply equally to all treatments.

2.1.4 Female outcrossing-rates

I estimated the proportion of a treatment group's seeds that were sired by outcrossed pollen (t) within the mixed arrays from the segregation of MDH allozymes among seeds from each fruit collected. I assayed the MDH genotype of six seeds from each fruit with cellulose-acetate-gel electrophoresis (Hebert and Beaton, 1989) during the winter of 2001. I homogenized seeds in 12 μL of *Decodon* extraction buffer (Eckert and Barrett, 1993),

centrifuged the homogenate for 5 min at 15,000 rpm, and applied the supernatant to 76 x 76 mm gel plates (Titan III, Helena Laboratories, Texas). A tris-citrate, pH 8 buffer system was used with running conditions of 250 V, 25 mA for 35 min.

To estimate female outcrossing-rates, I used Ritland's (1986) maximum likelihood program, MLTR (version 0.9), with Newton-Raphson iteration using the most likely parent as the maternal genotype. I ran two analyses, one for the dichogamy treatment and one for the inflorescence-size treatment. In these analyses, I used the grouping function of the computer program to estimate female outcrossing-rates for the different classes within a given treatment (i.e., protandrous and adichogamous or two-, six-, and ten-flowered inflorescence estimates) with the arrays pooled to increase the number of progeny arrays in the estimate. I calculated standard errors of the female outcrossing-estimates from the standard deviations of 1,000 bootstrap estimates of the mean, using the progeny array (seeds from an individual fruit) as the unit of resampling.

I inferred levels of geitonogamy from the female outcrossing-rate. However, Husband and Schemske (1995) found that for *C. angustifolium*, inbreeding depression was largest during seed maturation (inbreeding depression at this stage estimated as 0.87). Consequently, inbreeding depression probably eliminated some fraction of the progeny produced through selfing from the progeny arrays used to estimate female outcrossing. In particular, homozygous progeny may occur less frequently than in the absence of inbreeding depression. Considering this caveat, my female outcrossing-estimates should be interpreted with caution as they may be overestimates. To supplement this measure of geitonogamy, I also analyzed whether the frequency of aborted seeds increased across inflorescence sizes.

2.1.5 Outcross siring-success

I estimated siring success for the protandrous and adichogamous plants of all inflorescence-size treatments using the PGI locus. Because the two dichogamy classes were fixed for different PGI alleles in each array, I estimated siring of seeds on the competing class directly as the frequency of heterozygotes (ϵ), relative to the number of within-class outcrosses. By including within-class transfers in the calculation I am also controlling for individual variation in seed production. Homozygous offspring (γ) result from within-class pollen transfer; however, within-plant (selfing) cannot be distinguished

from between-plant mating using the PGI locus. To estimate the frequency of within-class outcrosses, I corrected the number of homozygotes with the female outcrossing-rate estimate of each dichogamy treatment. For example, the relative siring success of protandrous plants would be calculated as the number of progeny on adichogamous plants sired by protandrous plants (ϵ) relative to the number of adichogamous-sired progeny (γ x t) on adichogamous plants,

$$\frac{\epsilon_{adichogamous}}{\gamma_{adichogamous} x t_{adichogamous}}. \tag{2.2}$$

I then averaged these siring-success estimates within arrays to obtain two estimates for each array, one each for the dichogamous and adichogamous plants. These array estimates were used as the unit of replication in the statistical analyses, giving four replicate estimates for each dichogamous class at each inflorescence-size treatment.

It is important to point out that the outcross siring-success of a particular dichogamy class is calculated from the progeny genotypes of the alternate class. In the absence of differences in pollen export, homozygotes and heterozygotes should be equally frequent, giving an outcross siring-success estimate of 1. However, the female outcrossing-rate correction removes self-fertilized seeds from the siring-success estimate. This biases the siring-success estimate towards between-class pollen transfers. An unbiased estimator of the null-model of no pollen export differences then becomes 1.14 heterozygotes/homozygotes (8 between-class plants/7 within-class plants). Because I predict that protandrous plants will export more pollen, there should be an excess of heterozygous progeny among the offspring of adichogamous plants and a deficiency among the offspring of protandrous plants (i.e., a siring success estimate greater than 1.14 for protandrous plants and less than 1.14 for adichogamous plants).

2.1.6 Statistical analysis

I performed all statistical tests with SuperANOVA (version 1.11 Abacus Concepts, 1991) with the array as the experimental unit. Because protandrous and adichogamous plants were not statistically independent within mixed arrays, I used split-plot ANOVAs (Chapter 29 of Neter et al., 1996) to compare their pollinator-visit frequencies, seed-set estimates, and siring-success estimates. I designated arrays as plots, with inflorescence size as

the whole-plot factor and dichogamy class (protandrous or adichogamous) as the split-plot factor. To test the inflorescence effect, I used array nested within inflorescence size as the error term. I used two-way ANOVAs to compare uniform and mixed arrays for pollinator visit duration and frequency, and seed set. Siring-success estimates were square-root-transformed and both aborted and relative seed-set measures log-transformed to meet the assumption of normality. I used Tukey-Kramer post-hoc comparisons to test for significant differences between means. The direction of pollinator movement within inflorescences (i.e., up or down) could not be transformed adequately, so I used the non-parametric Wilcoxon rank test.

I tested differences between female outcrossing-estimates by inspecting the distributions of bootstrap estimates and calculating 95% confidence intervals of the t estimate as the interval from the 2.5-percentile to the 97.5-percentile of the bootstrap distribution. For a two-tailed test with α=0.05, I considered the estimates significantly different if the confidence intervals did not overlap.

2.2 Results

2.2.1 Pollinator observations

The duration of bee visits and the number of flowers visited by a bee differed significantly with inflorescence size (Table 2.1; Figure 2.1). The duration of visits to individual plants increased linearly with the number of open flowers and all treatment means differed significantly from each other. Based on the between-array analysis, bees visited significantly fewer flowers on two-flowered inflorescences than on either the six- or ten-flowered inflorescences, which did not differ (Table 2.1c, Figure 2.1). Furthermore, bees visited proportionally fewer flowers on either the six- or ten-flowered inflorescences relative to the two-flowered (i.e., ten-flowered inflorescences did not receive five times as many visits as two-flowered).

Bees moved up during 72% (95% CI =3%) of all vertical movements within inflorescences. Neither dichogamy treatment (non-parametric Wilcoxon rank test, X^2=0.15, df=1, P>0.50) nor inflorescence size (X^2=1.06, df=2, P>0.50) affected this preference.

Bee behaviour did not differ between dichogamy treatments. The array-type-by-inflorescence-size interactions were not significant for either the du-

Table 2.1: Analysis of variation in *Bombus impatiens* visits in experimental arrays of *Chamerion angustifolium*. a) Two-factor ANOVA between mixed and uniform arrays of foraging duration per inflorescence visit; b) Split-plot analysis within mixed arrays of flower visits/inflorescence visit, inflorescence size is tested against Array [Inflorescence size] error; c) Two-factor ANOVA between mixed and uniform arrays of flower visits/inflorescence visit. Treatment means are presented in Figure 2.1.

Source of variation	d.f.	SS	F	P
a) Duration of inflorescence visits				
Array type	1	0.03	1.63	>0.20
Inflorescence size	2	0.69	17.90	<0.01
Array type X inflorescence size	2	0.01	0.04	>0.95
Residual	18	0.35		
b) Flowers visited per inflorescence - mixed arrays				
Inflorescence size	2	10.31	17.74	<0.01
Array [Inflorescence size]	9	2.61	0.29	<0.01
Dichogamy	1	0.01	0.04	>0.85
Dichogamy X inflorescence size	2	0.01	0.34	>0.70
Residual	9	0.14		
c) Flowers visited per inflorescence - both array types				
Array type	1	0.20	1.34	>0.25
Inflorescence size	2	11.42	38.86	<0.01
Array type X inflorescence size	2	0.11	0.39	>0.65
Residual	18	2.64		

ration of inflorescence visits or the number of flowers visited per inflorescence in the between-array comparison. Also, neither the dichogamy effect nor dichogamy-by-inflorescence-size interaction were significant in the within-array comparison.

2.2.2 Seed set

Relative seed set (Table 2.2, Figure 2.2) estimated from 713 fruits (71 seeds per fruit on average) decreased significantly with increasing inflorescence size, based on the two-factor ANOVA of both mixed and uniform arrays. However, the split-plot ANOVA showed no decrease in seed set for the mixed

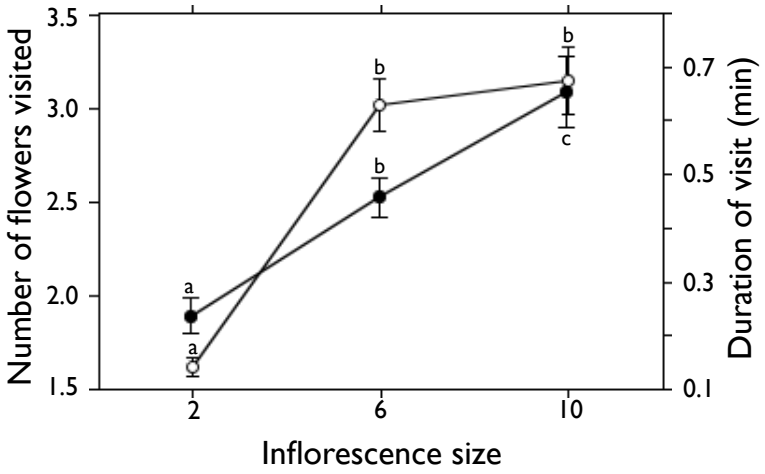


Figure 2.1: Mean (±SE) frequency (open circles) and duration (filled circles) of visits by *Bombus impatiens* to inflorescences of three sizes. Values are calculated from the averages of the eight replicate size-class arrays, irrespective of dichogamy treatments. Means sharing letters within a response variable do not differ significantly (α=0.05). Statistical analyses are presented in Table 2.1 and the text.

arrays alone. Dichogamy treatment did not affect seed set either between or within arrays (Table 2.2). Similar results were obtained with the absolute number of full seeds as the response variable (between arrays: $F_{1,18}$=0.11, P>0.70; within arrays: $F_{1,9}$=0.89, P>0.3. The vertical position of the fruit within the inflorescence did not affect seed set ($F_{46,1}$=0.10, P>0.70).

The number of aborted seeds per fruit differed significantly among inflorescence sizes ($F_{2,18}$=5.90, P<0.01). Tukey-Kramer post-hoc tests showed that six-flowered inflorescences aborted significantly more seeds (mean±SE; 178±8) than two-flowered (116±11), but not ten-flowered (142±9) inflorescences. Two- and ten-flowered treatments did not differ significantly.

2.2.3 Female outcrossing-rate

Estimated female outcrossing-rates (Table 2.3), assayed from 767 progeny, for the dichogamy and inflorescence-size treatments were generally high (mean t=0.94±0.11). Protandrous plants outcrossed marginally more than

Table 2.2: Analysis of variation in relative seed set between experimental manipulations of dichogamy and inflorescence size: a) Split-plot analysis of mixed arrays with inflorescence size tested against Array [Inflorescence size] error; b) Two-factor ANOVA between mixed and uniform arrays. Treatment means are presented in Figure 2.2.

Source of variation	d.f.	SS	F	P
a) Mixed arrays only				
Inflorescence size	2	0.51	2.04	>0.15
Array [Inflorescence size]	9	0.13	4.89	<0.05
Dichogamy	1	0.01	0.18	>0.85
Dichogamy X inflorescence size	2	0.01	0.23	>0.75
Residual	9	0.02		
b) Both array types				
Array type	1	0.01	0.33	>0.55
Inflorescence size	2	0.11	8.14	<0.01
Array type X inflorescence size	2	0.01	0.84	>0.40
Residual	18	0.12		

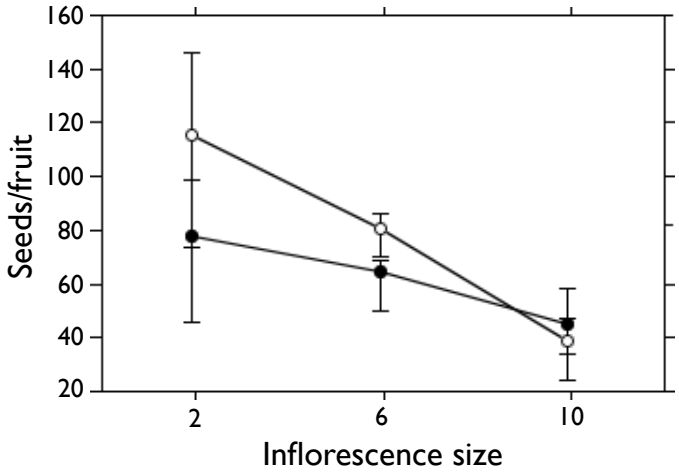


Figure 2.2: Mean (±SE) proportion of ovules setting seed for three inflorescence-size classes and two array types (open circles are uniform arrays, closed circles are mixed arrays). Statistical analyses are presented in Table 2.2 and the text.

Table 2.3: Estimated female outcrossing-rates (t; ±SE) for dichogamy, inflorescence size, and position categories.

Treatment	t
Dichogamy	
protandrous	1.14±0.40
adichogamous	0.92±0.13
Inflorescence size	
two-flowered	0.84±0.39
six-flowered	1.99±0.07
10-flowered	0.90±0.25
Position	
bottom	0.95±0.10
top	0.84±0.13

adichogamous plants (two-tailed P<0.10). Based on the 95% CI of the female outcrossing-estimates, neither inflorescence size nor flower position had a significant effect on outcrossed seed production (except for the extremely high value for six-flowered inflorescences).

Mean (±SE) parental inbreeding (F) for the inflorescence treatment was estimated as 0.08±0.26 and for the dichogamy treatment as 0.22±0.16. Neither F estimate differed significantly from 0 (P>0.50 and 0.10, respectively).

2.2.4 Outcross siring-success

Protandrous plants had an average (±SE) siring success of 1.38±0.15 on adichogamous plants, whereas adichogamous plants had an average siring success of 0.66±0.13 on protandrous plants. Although the female outcrossing-rate did not differ significantly between dichogamy treatments, these estimates were used to correct the siring success estimates. Because the outcrossing-rate correction has the effect of increasing the siring-success of hermaphroditic plants, it makes my test of siring-success differences between dichogamy treatments a conservative one. In the absence of this correction, the results obtained are statistically identical to the corrected values. In a split-plot ANOVA, I found a significant effect of dichogamy treatment and a marginally significant (P<0.10) dichogamy-by-inflorescence-size interaction on siring success within mixed arrays (Table 2.4, Figure 2.3).

Table 2.4: Split-plot analysis of outcross siring-success in mixed arrays with inflorescence size tested against Array [Inflorescence size] error. Siring-success estimates were square-root transformed to meet the assumption of normality. Treatment means are presented in Figure 2.3.

Source of variation	d.f.	SS	F	P
Siring success				
Inflorescence size	2	0.01	0.01	>0.95
Array [Inflorescence size]	9	0.49	1.15	>0.90
Dichogamy	1	0.87	18.38	<0.05
Dichogamy X inflorescence size	2	0.34	3.61	>0.05
Residual	9	0.43		

Inflorescence-size had no significant effect on siring success. In pure arrays, the mean siring success of the two PGI genotypes did not differ significantly ($F_{22,1}$=0.05, P>0.80). Consequently, no biases were detected in the movement of PGI alleles.

2.3 Discussion

Despite a long-standing interest in dichogamy, very few studies have experimentally evaluated the functional significance of the temporal separation of gender in flowering plants (Griffin et al., 2000; Harder et al., 2000). This study represents the first experimental test of the influence of inflorescence size on the significance of protandry for outcross siring-success. I found that protandry conferred, on average, a twofold siring advantage relative to adichogamous plants. However, in contrast to my prediction, this benefit did not increase with inflorescence size. Furthermore, although the selfing rate did not change, seed set did decrease with increasing inflorescence size, which, as I discuss below, suggests that geitonogamy increased in large inflorescences. These results have important consequences for our understanding of the functional significance of protandry and for the role of interference avoidance in the evolution of floral form.

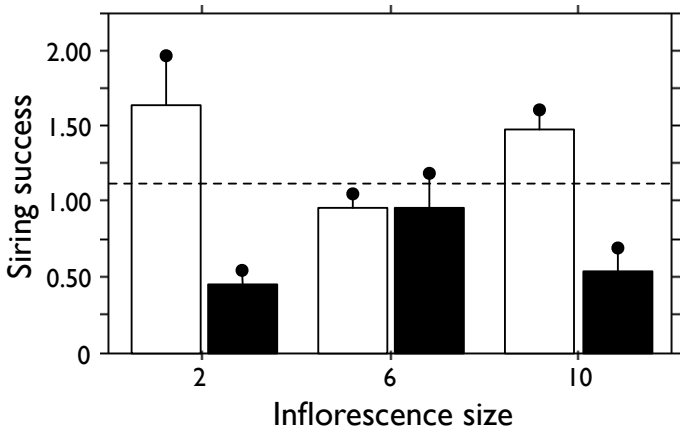


Figure 2.3: Outcross siring-success (proportion of seeds sired, +SE) for protandrous and adichogamous plants at three different inflorescence sizes. Open bars represent the success of protandrous plants at siring seeds of adichogamous plants (i.e., protandrous siring success). Closed bars represent the success of adichogamous plants at siring seeds of protandrous plants. The dashed line represents the null-model of equivalent siring success after the outcrossing-rate correction. Statistical details are presented in Table 2.4. Values are back-transformed for ease of interpretation.

2.3.1 Pollen discounting and the functional significance of protandry

Lloyd and Webb (1986) suggested that dichogamy may have evolved to reduce interference between male and female function within a flower. Harder and Barrett (1995; 1996) further proposed that dichogamy may play a more important role in reducing between-flower interference and pollen discounting than within-flower interference. A few correlative studies have detected an influence of dichogamy on self-fertilization (a specific type of interference; Brunet and Eckert, 1998; Holtsford and Ellstrand, 1992; Schoen, 1982). However, only one other study (Harder et al., 2000) has experimentally tested the more general hypothesis that dichogamy reduces between-flower interference and pollen discounting. In one year of their study, Harder et al. (2000) found that protandrous *Eichhornia paniculata* (Pontederi-

aceae) plants sired 56% of the outcrossed seeds relative to adichogamous plants. This study of *Chamerion angustifolium*, a species with more intense inbreeding depression, also detected a significant siring-success advantage for protandrous plants.

A critical prediction of the between-flower interference hypothesis is that the advantage conferred by dichogamy should increase with inflorescence size. Although this study revealed a strong outcross siring advantage of protandry, the siring advantage was surprisingly high in the two-flowered treatment and inflorescence size had no significant effect on this advantage. This could be due to a lack of statistical power in detecting an inflorescence-size-by-dichogamy interaction, but the trend is not in the predicted direction. Alternatively, the siring advantage in the two-flowered treatment could result if the magnitude of pollen carryover differed between the dichogamy treatments due to the extra stigma in adichogamous plants. If this was the case, pollen exported from an adichogamous plant to a neighbouring protandrous plant may have remained on the pollinator to be exported to another adichogamous plant (the next nearest neighbour). This would have the effect of increasing within-class pollen transfer for adichogamous plants and thus reducing the apparent siring success of protandrous plants. Conversely, this effect would increase pollen export from protandrous to adichogamous plants, enhancing the apparent siring success of protandrous plants. Although these two effects may not cancel each other out entirely, I do not consider the difference large enough to explain the very large siring-success difference between two-flowered protandrous and adichogamous plants. This complication is a consequence of my experimental-design decision to hold floral display size constant between dichogamy treatments. However, avoiding this complication by maintaining stigma to anther ratios would have caused more significant differences in pollinator attraction and, consequently, pollen export.

I consider two potential explanations for the unexpected lack of influence of inflorescence size that are related to interference effects. The first is that protandry may provide a siring advantage solely by reducing within-flower interference, rather than between-flower interference. This may occur when the absence of a stigma enhances pollen removal from individual flowers (Harder and Thomson, 1989; Lloyd, 1984), rather than pollen carryover (Robertson, 1992). Stigmas can restrict a pollinator's access to pollen, remove pollen from a pollinator's body as it leaves the flower, or a combination of the two. In this scenario, the enhanced pollen removal provided

by protandry would be independent of inflorescence size and, consequently, I would not detect an inflorescence size influence on siring success.

A second interpretation of these results requires a liberal interpretation of the marginally significant interacting effects of inflorescence size and dichogamy. The apparent bimodal distribution of the siring advantage of protandry (Figure 2.3) may be biologically significant. For this to occur, within-flower interference may decrease with inflorescence size, while between-flower interference increases. The sensitivity of within-flower interference to inflorescence size may be due to changes in pollinator behaviour. If bees visit flowers longer on small inflorescences (e.g., Figure 1of Harder and Thomson, 1989), within-flower interference may increase, perhaps increasing facilitated selfing. Indeed, bees in this study spent more time per flower on small inflorescences (Figure 2.1). In this scenario, protandry enhances pollen removal in two-flowered inflorescences and pollen carryover in ten-flowered inflorescences. Six flowers may be a transition between the determinant of siring success from pollen removal to pollen carryover, consequently, on average, protandry provides no benefit at this inflorescence size. Unfortunately, because I did not compare different inflorescence sizes directly in the same array, this experiment cannot assess these two possibilities. A competition of plants with differing proportions of female- and male-phase flowers, rather than the equal proportion of adichogamous and dichogamous inflorescences used in this study, might also provide insights into these issues. Additionally, an investigation of the actual pollen dynamics (e.g., Rademaker et al., 1997, see Chapter 3) in protandrous relative to adichogamous flowers would be very useful.

2.3.2 Inflorescence size and geitonogamy

An increase in the size of *Chamerion angustifolium* inflorescences increased bee visitation and foraging time within the inflorescence (Figure 2.1). However, ten-flowered plants received fewer than five times the visits to two-flowered plants. This effect may be more exaggerated in natural conditions where the number of foraging bouts is not constrained and inflorescence-size classes compete directly. Similar diminishing gains in per-flower visitation with increasing inflorescence size have been found in numerous other studies (e.g., Barrett et al., 1994; Bell, 1985; Emms and Arnold, 1997; Geber, 1985; Klinkhamer and de Jong, 1990; Queller, 1983; Schemske, 1980; Schmid-

Hempel and Speiser, 1988, see Iwasa et al., 1995 for theoretical considerations).

Despite the increased visitation rates and durations in large inflorescences, I detected no differences between the female outcrossing-rates of the inflorescence-size treatments for *C. angustifolium*. I had expected geitonogamy to increase with inflorescence size and be reflected in a decreasing female outcrossing-rate. However, studies of the relationship between geitonogamy and inflorescence size have been equivocal. As examples, Barrett et al. (1994) found a gradual increase in self-fertilization as inflorescence size increased (3, 6, 9, and 12 flowers) in *Eichhornia paniculata* and Galloway et al. (2002) found an increase in geitonogamous visits with increased display size in the protandrous *Campanula americana* (Campanulaceae). Conversely, no such correlation was found in *Aquilegia caerulea* (Ranunculaceae; Brunet and Eckert, 1998) or *Mertensia ciliata* (Boraginaceae; Geber, 1985).

Inbreeding depression may have obscured the effects of geitonogamy on my estimate of female outcrossing (Husband and Schemske, 1996). In *C. angustifolium*, inbreeding depresses performance by 95%, among the highest documented for any plant or animal (Husband and Schemske, 1995). Such a severe reduction in fitness from selfing makes geitonogamy particularly detrimental in this species. As discussed earlier, this may have influenced my female outcrossing-estimates and my ability to detect changes associated with inflorescence size. In fact, as reflected in the aborted-seed measure, six-flowered inflorescences produced more aborted seeds in their fruits than two-flowered inflorescences. This suggests that geitonogamy is more frequent in these six-flowered inflorescences, but that its measurement is obscured by strong inbreeding depression. Regardless, there does not appear to be a strong influence of inflorescence size on geitonogamy in *C. angustifolium*.

The substantial siring advantage of protandrous plants seems inconsistent with the moderate differences between treatments in geitonogamy, inferred from the female outcrossing-rates. One possible resolution of this apparent inconsistency is that much of the non-exported pollen is not deposited on the stigma (e.g., Rademaker et al., 1997). Rather, interference from the stigma and style may result in pollen being dropped to the ground, deposited on petals or the style, or otherwise wasted, in addition to geitonogamy and self-fertilization. Although the specific mechanism(s) that cause interference are unclear, any of these potential consequences of interference can reduce pollen export. Considered together, as they likely occur in nature, these mechanisms of interference represent potent impediments

to pollen export.

2.3.3 Evolutionary implications

In a survey of the angiosperms, Bertin and Newman (1993, see Chapter 5) found that dichogamy was a common floral trait in a wide variety of families. Although the phylogenetic history of the trait is currently unresolved, this widespread occurrence of dichogamy in disparate families suggests that it serves an important role in the biology of many flowering plants. Given the current evidence for the role of protandry in enhancing siring success, perhaps the avoidance of pollen discounting has played a central role in the evolution of floral form and development.

Chapter 3

Pollen dynamics: A proximate analysis of protandry

Experimental tests of the adaptive significance of dichogamy (Griffin et al., 2000; Harder et al., 2000, Chapter 2) demonstrate the fitness consequences of the temporal separation of gender. However, the mechanical processes that produce these fitness effects (i.e., proximate explanations) are unclear, particularly at the individual-flower level. The primary goal of this chapter is to investigate the unexpected siring advantage of two-flowered, protandrous plants. Since these plants had little opportunity for between-flower interference, the consequences of protandry for within-flower interference may provide the explanation.

The simultaneous presentation of gender in hermaphroditic flowers can affect within-flower pollen dynamics through two major mechanisms: interference with access to gender structures and interference with the action of the gender (Bawa and Opler, 1975; Lloyd and Yates, 1982). For example, the stigma and style (female function) can interfere with access to male function by physically obstructing a pollinator's contact with the anthers. In addition, female function may interfere with the action of male function by removing pollen from a pollinator's body as it leaves a flower. I define these types of interference as male-access interference and male-action interference, respectively. Male function can interfere with female function in analogous ways. Female-access interference occurs when anthers interfere with the contact between a pollinator and the stigma, reducing pollen deposition. Female-action interference can occur if the anthers remove incoming pollen from the pollinator. Of course, these categories of interference may

not be mutually exclusive, co-occurring in a number of ways to reduce the effectiveness of pollination.

If interference causes an increase in self-pollen deposition, facilitated selfing will occur. This mode of selfing is similar to geitonogamy in that it requires pollinator visitation and the resources required for pollinator attraction (Lloyd and Schoen, 1992). However, as with all of the modes of selfing, the progeny produced through facilitated selfing suffer from inbreeding depression. Furthermore, the pollen involved in facilitated selfing through male-action interference might have otherwise been involved in outcrossing. As discussed in Chapter 1 and Chapter 2, this pollen discounting (Harder and Wilson, 1998) can reduce male reproductive-success significantly. Conversely, if pollen deposition decreases through either female-action or access interference, seed set may become pollen limited. Pollen limitation is common in many species (Larson and Barrett, 2000) and can reduce female reproductive success. Protandry provides periods of male and female function that are interference free. This is particularly true for male function, since pollen is presented in the absence of female function. However, the degree to which protandry can reduce interference on female function is largely dependent on the frequency of pollinator visitation during male phase. If pollinator activity is sufficient to remove the majority of pollen available before the onset of female phase, protandry may effectively reduce interference for both genders. Nonetheless, given the vagaries of pollinator activity, protandry may be most effective at reducing the effects of within-flower interference on male reproductive success.

In this chapter, I evaluate the role of within-flower interference for the evolution of protandry with experimental manipulations of *Chamerion angustifolium*. I begin with a field study comparing rates of pollen deposition in female and hermaphroditic flowers to estimate the degree of within-flower interference produced by male function. A second experiment was conducted indoors with captive bees to more fully explore both male and female forms of interference. This lab experiment involved pairs of flowers with differing combinations of male, female, and hermaphroditic flowers in the pair. The general objective of both experiments is to quantify interference produced by either gender and explore the effects of protandry on within-flower interference.

3.1 Materials and methods

I conducted these experiments with *Chamerion angustifolium*. Refer to Chapter 2 for relevant biological details about this species and growth of research plants.

3.1.1 Field experiment

I conducted this experiment in two populations on the Beartooth Pass, Wyoming (Husband and Schemske, 1997): Crazy Creek and Clay Butte. Crazy Creek is at relatively low elevation (∼2200m) and composed predominately of tetraploid plants. Clay Butte is at high elevation (∼2700m) and is predominately diploid.

To quantify female interference, in August 2000, I presented unvisited, hermaphroditic flowers to foraging bees at both populations, with anthers and filaments either intact or removed with forceps. After a single pollinator visit, I harvested the stigma and style of the flower and stored them in 50% ethanol. I later softened the stigma and style in 1 mL of 1 N NaOH for 36 hours, mounted them on slides, and counted pollen adhering to the stigmatic surface under 10x magnification. In addition, to account for any pollen that detached from the stigmatic surface, I counted the pollen in four, 0.1 mL sub-samples of the NaOH solution. I calculated the total pollen deposition on a stigma as the sum of the stigma count and 10 times the average of the liquid counts.

In the lab experiment, described below, I measured pollen removal in hermaphroditic and male flowers. To compare pollen dynamics in the field and lab settings, I also estimated pollen removal in the field, but for male-phase flowers only. I chose pairs of flowers on a plant and harvested two anthers from one flower of the pair prior to visitation. After a single bee visit, I collected two anthers from the second flower. I stored the anthers in 50% ethanol and counted their pollen with a Beckman-Coulter Multisizer 3 particle counter (Beckman Coulter Canada Inc.) as described below for the lab study.

3.1.2 Lab experiment

This experiment attempts to quantify the amount of pollen transfered between two flowers and the effect of interference on this transfer. Each exper-

imental run included two flowers vertically arranged. Since pollinators tend to forage upwards through an inflorescence (Galen and Plowright, 1988), I positioned the donor flower below the recipient flower. Donor flowers were either hermaphroditic (unmanipulated) or I removed their stigma and style with forceps. This allowed me to assess the effect of the style and stigma on pollen removal. Recipient flowers were either hermaphroditic or I removed their anthers and filaments with forceps. Comparing these two types of recipient flowers provided a measure of the effect of anthers on pollen deposition. I randomly removed one anther from flowers with male function and stored them in 90% ethanol. I then placed the pedicels of both flowers in water-filled micro-centrifuge tubes and suspended them inside a pollinator cage (2m x 2m x 1m) and released a single *Bombus impatiens* worker (from a Natupol-Plus Class A Hive, supplied by Koppert Biological Systems, Mississauga, ON) into the inclosure. As the bee foraged on the pair of flowers, I recorded the amount of time it spent foraging on each flower. I removed the bee once it visited both flowers and harvested stigmas from hermaphroditic flowers and one randomly chosen anther from each flower with male function and stored them in 90% ethanol.

Pollen adhering to stigmas from the lab experiment was quantified as in the field experiment. Pollen that detached from the stigmatic surface and pollen contained in the harvested anthers was counted with a Beckman-Coulter Multisizer 3 particle counter (Beckman Coulter Canada Inc.). I sonicated the samples in 8 mL of ISOTON II solution (Beckman Coulter Canada Inc.) for 15 sec to separate pollen from the viscine threads and disperse individual pollen grains. The particle counter then subsampled 2 mL from the 8 mL sample and counted the number of particles in the solution with diameters from 10 to 120 μM. Pollen of *C. angustifolium* ranges from 65–105 μM (A. Crawford and B. Husband, unpublished data), so I used the number of particles with diameters between 55 and 115 μM to estimate pollen quantities per mL of each sample. For the stigma solutions, I multiplied the per mL estimates by 8 mL per sample to estimate the amount of pollen in the NaOH solutions. For the anther samples, I multiplied the per mL estimates by 8 mL per sample and 8 anthers per flower to estimate the total amount of pollen in each flower sampled. By subtracting the quantity of pollen in the flowers after visitation from the flowers before visitation, I estimated the amount of pollen removed by a single pollinator visit.

3.1.3 Data analysis

Traditional hypothesis testing with the associated alpha levels and null hypotheses are not entirely suitable for the objectives of these experiments (Nester, 1996). Rather than only rejecting null models, I am also concerned with estimating the parameters that describe the relative contributions of male and female structures to floral interference. Considerations of the confidence limits around parameter estimates and the ability of a model to explain the observed biology become the important analytical goals. The primary difficulty with using ANOVAs and variance components for such a task is deciding which model to use for the parameter estimation. Therefore, I adopt an information-theoretic approach to these data (Burnham and Anderson, 1998).

This approach involves constructing biologically informed, *a priori* models to explain the system of interest. Standard regression and ANOVA methods are then used to obtain maximum-likelihood estimates (MLE) of parameters in the model (e.g., the effect of anthers on pollen deposition) and calculate the residual mean-squared error (RMSE) of the total model. Akaike's information criterion (AIC) is then used to rank the candidate models by their ability to explain the data generated from the experiment, weighted by the number of parameters contained within the model (Burnham and Anderson, 1998). With this approach the model with the lowest AIC value is the best explanation for the data. Akaike weights (π_i) for each model (i) are normalized log likelihoods that are used to determine the relative suitability of each model. To assess model-selection uncertainty (Burnham and Anderson, 1998), I generated 10,000 bootstrap datasets (Efron and Tibshirani, 1993) and re-calculated the Akaike weights of the candidate models for each dataset. I then tallied the number of times each model had the lowest AIC value and used these selection probabilities as a measure of the robustness of model selection. If the model with the highest Akaike weight, or equivalently the lowest AIC value, is consistently chosen by the bootstrap analysis it is a robust model for parameter value inference. In other words, the Akaike weight indicates how well a particular model is supported by the data, while the bootstrap values indicate how sensitive this support is to the particular dataset used.

The Akaike weights are also used to generate parameter estimates ($\hat{\theta}_a$) for components of the R models as weighted averages of each model's MLE

parameter estimates (θ_i):

$$\hat{\theta}_a = \sum_{i=l}^{R} \pi_i \theta_i, \tag{3.1}$$

and 95% confidence intervals from the individual parameter confidence intervals ($\theta_{i,L}$ and $\theta_{i,U}$):

$$\hat{\theta}_L = \sum_{i=l}^{R} \pi_i \theta_{i,L}, \tag{3.2}$$

and

$$\hat{\theta}_U = \sum_{i=l}^{R} \pi_i \theta_{i,U}. \tag{3.3}$$

Consequently, the parameter estimates are constructed from all of the models and their errors are a composite of the total uncertainty inherent in the models considered.

The candidate models for the field experiment are listed in Table 3.1. Model $D_{f,0}$ is that pollen deposition is a fixed amount ($\mu_{f,d}$). Model $D_{f,a}$ considers the effect of the presence of anthers on pollen deposition, while model $D_{f,p}$ estimates differences between the populations on pollen deposition. The final model, $D_{f,a,p}$ considers both anther and population effects. The parameter estimates indicate the additional amount of pollen deposited as a result of the corresponding effect. For example, the parameter estimate of the anther effect indicates the additional amount of pollen deposition caused by the presence of anthers on the recipient flower. For pollen removal, I only considered two models, the first ($R_{f,0}$) was that removal was a fixed amount and the second ($R_{f,p}$) that the amount was different between populations.

The lab experiment examines eight candidate models for both pollen deposition and removal (Table 3.2). Model $D_{l,0}$ is that pollen deposition is fixed at $\mu_{l,d}$. Model $D_{l,r}$ considers the effect of flower type (donor or recipient) on pollen deposition, while model $D_{l,a}$ estimates the effect of anthers on deposition. Model $D_{l,t}$ estimates the effect of visit duration on pollen deposition. Models $D_{l,r,a}$,$D_{l,r,t}$, and $D_{l,a,t}$ investigate the effects of paired parameters on deposition, and $D_{l,r,a,t}$ considers all three parameters. Similar models were generated for pollen removal. Model $R_{l,0}$ is that pollen removal is fixed at $\mu_{l,r}$. Model $R_{l,r}$ considers the effect of flower type (donor or recipient) on pollen removal, while model $R_{l,s}$ estimates the effect of

Table 3.1: Candidate models of a) pollen deposition and b) pollen removal in *Chamerion angustifolium* after a single *Bombus impatiens* visit for the field experiment. K is the number of parameters in each model and is used in calculating the AIC values of Table 3.3.

Model	K	Description
a) Pollen deposition		
$D_{f,0} = \mu_{f,d}$	2	constant
$D_{f,a} = \mu_{f,d} + A$	4	modified by the presence of anthers
$D_{f,p} = \mu_{f,d} + P$	4	modified by population
$D_{f,a,p} = \mu_{f,d} + A + P$	5	modified by anthers & population
b) Pollen removal		
$R_{f,0} = \mu_{f,r}$	2	constant
$R_{f,p} = \mu_{f,r} + P$	4	modified by population

stigmas on removal. Model $R_{l,t}$ estimates the effect of visit duration on pollen removal. Models $R_{l,r,s}$,$R_{l,r,t}$, and $R_{l,s,t}$ investigate the effects of paired parameters on removal, and $R_{l,r,s,t}$ considers all three parameters.

To place these results in a more traditional context, I also analyzed pollen removal and deposition with ANOVAs using JMP software (ver. 5.0, SAS Institute, 2002). For field pollen deposition, I treated both population and the presence of anthers as fixed effects. I analyzed pollen removal in the field with a one-way ANOVA of population. Pollen deposition in the lab included the identity of the flower as a pollen donor or recipient (i.e., bottom vs. top flower), the presence of anthers, and the time spent foraging as fixed effects. Similar effects were included for lab pollen removal, except the presence of a stigma was used rather than that of anthers.

3.2 Results

3.2.1 Field experiment

Pollen counts were obtained from 88 stigmas, 52 from Clay Butte and 36 from Crazy Creek. On average, a pollinator deposited 159 pollen grains (95% CI 111–207) in a single visit. Model $D_{f,p}$ had the greatest support (Table 3.3). The Akaike weights indicate that $D_{f,p}$ has about three times

Table 3.2: Candidate models of a) pollen deposition and b) pollen removal in *Chamerion angustifolium* after a single *Bombus impatiens* visit for the lab experiment. K is the number of parameters in each model and is used in calculating the AIC values of Table 3.5.

Model	K	Description
a) Pollen deposition		
$D_{l,0} = \mu_{l,d}$	2	constant
$D_{l,r} = \mu_{l,d} + R_d$	4	modified by flower type
$D_{l,a} = \mu_{l,d} + A$	4	modified by the presence of anthers
$D_{l,t} = \mu_{l,d} + T_d$	4	modified by visit duration
$D_{l,r,a} = \mu_{l,d} + R_d + A$	5	modified by flower type & anthers
$D_{l,r,t} = \mu_{l,d} + R_d + T_d$	5	modified by flower type & time
$D_{l,a,t} = \mu_{l,d} + A + T_d$	5	modified by presence of anthers & time
$D_{l,r,a,t} = \mu_{l,d} + R_d + A + T_d$	6	modified by flower type, anthers, & time
b) Pollen removal		
$R_{l,0} = \mu_{l,r}$	2	constant
$R_{l,r} = \mu_{l,r} + R_r$	4	modified by flower type
$R_{l,s} = \mu_{l,r} + A$	4	modified by the presence of stigma
$R_{l,t} = \mu_{l,r} + T_r$	4	modified by visit duration
$R_{l,r,s} = \mu_{l,r} + R_r + A$	5	modified by flower type & stigmas
$R_{l,r,t} = \mu_{l,r} + R_r + T_r$	5	modified by flower type & time
$R_{l,s,t} = \mu_{l,r} + A + T_r$	5	modified by presence of stigma & time
$R_{l,r,s,t} = \mu_{l,r} + R_r + A + T_r$	6	modified by flower type, stigmas, & time

the support of the next closest model $D_{f,a,p}$. However, the scores from the bootstrap datasets show that this support is marginal overall since $D_{f,p}$ was only chosen in ∼48% of the bootstrap datasets. Furthermore, the ANOVA of pollen deposition was not significant (Table 3.4).

Pollen removal was estimated from 19 pairs of flowers, 9 diploids and 10 tetraploids. On average, bees removed 1,058 pollen grains (95% CI 670–1,446) in a single visit. Although the Akaike weights give the most support to the population model ($R_{f,o}$), the bootstrap analysis shows that each model is equally robust. The ANOVA of pollen removal in the field was not significant (Table 3.4).

Table 3.3: Model statistics of candidates for the a) pollen deposition and b) pollen removal in *Chamerion angustifolium* after a single *Bombus impatiens* visit for the field experiment. $\hat{\sigma}^2$ is the estimated mean squared error. $\Delta_i - \text{AIC}$ is the change in support for each model, expressed as the deviation from the best supported model (higher numbers indicate poor support), π_i is the model's Akaike weight, and bootstrap support indicates the number of times the model was chosen in 10,000 bootstraps of the data. The subscript f indicates that these are field models, 0 is the model of mean deposition or removal, a is the effect of anthers, and p of population.

Model	$\hat{\sigma}^2$	$\Delta_i - \text{AIC}$	π_i	Bootstrap support
a) Pollen deposition				
$D_{f,0}$	$4.45\text{x}10^6$	1.40	0.126	0.211
$D_{f,a}$	$4.39\text{x}10^6$	2.31	0.044	0.112
$D_{f,p}$	$4.28\text{x}10^6$	0.00	0.634	0.476
$D_{f,a,p}$	$4.23\text{x}10^6$	1.02	0.195	0.201
b) Pollen removal				
$R_{f,0}$	$4.45\text{x}10^6$	0	0.871	0.458
$R_{f,p}$	$4.39\text{x}10^6$	1.66	0.129	0.542

Table 3.4: ANOVAs of a) pollen deposition and b) pollen removal in *Chamerion angustifolium* after a single *Bombus impatiens* visit for the field experiment.

Source of variation	d.f.	SS	F	P
a) Pollen deposition				
Population	1	$1.61\text{x}10^5$	3.23	>0.05
Presence of anthers	1	$4.72\text{x}10^4$	0.95	>0.30
Residual	85	$4.23\text{x}10^6$		
b) Pollen removal				
Population	1	$2.40\text{x}10^5$	0.31	>0.55
Residual	17	$1.34\text{x}10^7$		

3.2.2 Lab experiment

On average, bees deposited 131 pollen grains (95% CI 88–175, based on 40 stigmas) in a single visit. The model with the greatest Akaike weight included anther and time as effects on pollen deposition ($D_{l,a,t}$, Table 3.5). The bootstrap analysis supported this model well, although the univariate time $D_{l,t}$ and anther $D_{l,a}$ models also had reasonable support. The ANOVA also supported the anther and time model ($D_{l,a,t}$, Table 3.6). Using equations 3.1, 3.2, and 3.3, anthers reduced pollen deposition by 67.6 pollen grains (95% CI 16.8–118.3) and each second of visitation adds 3.0 (95% CI 0.4–5.6, Figure 3.1) pollen grains to the stigma. A one-way ANOVA of the presence of anthers on time spent foraging was significant ($F_{1,38}$=4.22, P<0.01).

Based on 46 pairs of anthers, in the lab a single bee visit removes 2,669 (95% CI 2,133–3,204) pollen grains from a flower. Many of the candidate models had similar Akaike weights (Table 3.5) and four had moderate bootstrap support. Since the mean deposition model had a moderate Akaike weight and the ANOVA of pollen removal was not significant (Table 3.6), there is little justification for making parameter estimates of pollen removal from the lab experiment.

3.3 Discussion

To my knowledge, this study is the first to investigate the role of protandry in reducing within-flower interference. Proximate analyses of pollen dynamics in the protandrous *Chamerion angustifolium* showed that anthers reduce pollen deposition on stigmas of hermaphroditic flowers. However, the presence of anthers also induced pollinators to forage longer on an individual flower, which can increase the amount of pollen deposited through facilitated selfing. Furthermore, the presence of stigmas in hermaphroditic flowers had no detectable effect on pollen removal. These results suggest that male structures are a considerable source of within-flower interference that may have significant effects on both male and female reproductive success.

Table 3.5: Model statistics of candidates for the a) pollen deposition and b) pollen removal in *Chamerion angustifolium* after a single *Bombus impatiens* visit for the lab experiment. $\hat{\sigma}^2$ is the estimated mean squared error. $\Delta_i - \text{AIC}$ is the change in support for each model, expressed as the deviation from the best supported model (higher numbers indicate poor support), π_i is the model's Akaike weight, and bootstrap support indicates the number of times the model was chosen in 10,000 bootstraps of the data. The subscript l indicates that these are lab models, 0 is the model of mean deposition or removal, a is the effect of anthers, s of stigmas, and t of time. The r subscript indicates when pollen donors and recipients are considered.

Model	$\hat{\sigma}^2$	$\Delta_i - \text{AIC}$	π_i	Bootstrap support
a) Pollen deposition				
$D_{l,0}$	1.93x10^4	4.928	0.003	0.000
$D_{l,r}$	1.93x10^4	6.757	0.000	0.046
$D_{l,a}$	1.76x10^4	3.139	0.024	0.164
$D_{l,t}$	1.84x10^4	4.960	0.003	0.407
$D_{l,r,a}$	1.75x10^4	4.895	0.003	0.000
$D_{l,r,t}$	1.81x10^4	6.088	0.000	0.000
$D_{l,a,t}$	1.55x10^4	0	0.878	0.383
$D_{l,r,a,t}$	1.55x10^4	1.999	0.088	0.000
b) Pollen removal				
$R_{l,0}$	3.36x10^6	0.329	0.165	0.000
$R_{l,r}$	3.22x10^6	0.467	0.141	0.158
$R_{l,s}$	3.25x10^6	0.780	0.098	0.235
$R_{l,t}$	3.19x10^6	0	0.241	0.277
$R_{l,r,s}$	3.19x10^6	2.005	0.024	0.000
$R_{l,r,t}$	3.09x10^6	0.572	0.125	0.000
$R_{l,s,t}$	3.07x10^6	0.270	0.177	0.330
$R_{l,r,s,t}$	3.04x10^6	1.860	0.028	0.000

Table 3.6: ANOVAs of a) pollen removal and b) pollen deposition in *Chamerion angustifolium* after a single *Bombus impatiens* visit for the lab experiment.

Source of variation	d.f.	SS	F	P
a) Pollen deposition				
Flower type	1	1.11×10^{1}	0.01	>0.95
Presence of anthers	1	1.02×10^{5}	5.92	<0.05
Visit duration	1	8.07×10^{5}	4.69	<0.05
Residual	36	6.20×10^{5}		
b) Pollen removal				
Flower type	1	1.25×10^{5}	0.37	>0.50
Presence of stigma	1	2.19×10^{6}	0.65	>0.40
Visit duration	1	6.69×10^{6}	2.00	>0.15
Residual	42	1.40×10^{8}		

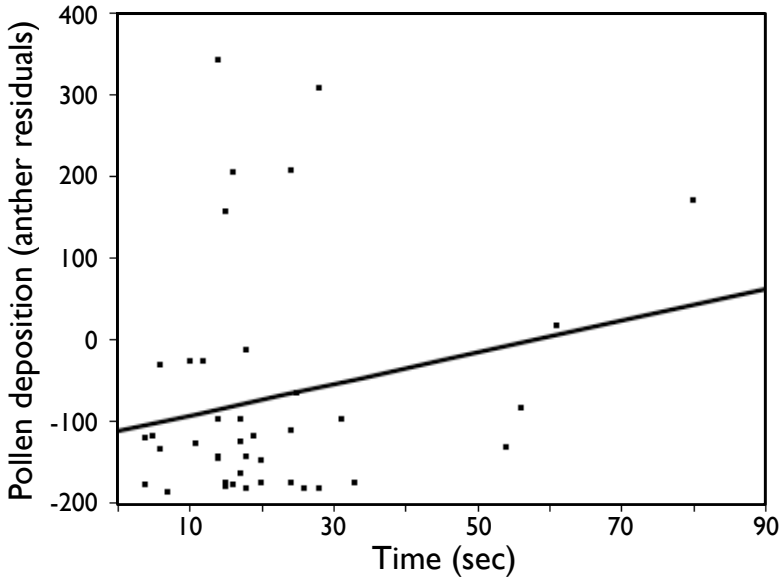


Figure 3.1: The amount of pollen deposited on *Chamerion angustifolium* flowers during a single visit from a *Bombus impatiens* drone as a function of time spent foraging. Pollen deposition is shown as the residuals after the effect of anthers has been removed.

3.3.1 Within-flower interference and protandry

The lab experiment of pollen deposition showed two major consequences of simultaneous hermaphroditism. First, male function reduced the amount of pollen deposited on stigmas. This reduction could occur through female-access interference if anthers prevented pollinators from making contact with the stigma, or through female-action interference if anthers removed incoming pollen from pollinators (Bawa and Opler, 1975; Lloyd and Yates, 1982). If female-action interference was responsible, donor flowers should not have shown an anther effect on pollen deposition since bees carried no pollen when visiting these flowers. Therefore, the anthers appear to restrict a pollinator's contact with the stigma, reducing the opportunity for pollen deposition.

The second consequence of simultaneous hermaphroditism is that pollinators forage longer in the presence of anthers. In the lab experiment, the pollinators exhibited pollen-foraging behaviour, perhaps in response to the small floral displays available. Data presented in Chapter 2 showed that pollinators spent more time foraging on individual flowers in smaller inflorescences. This increase in foraging time is likely in response to the pollinator's assessment of the reward status of the available flowers. When rewards are limiting within a patch, pollinators should spend more time extracting the available resources or move to a new patch (Harder et al., 2001; Morgan, 2003; Waddington, 2001). Since no other patches were available in this experiment (as in Chapter 2), pollinators are forced to spend time extracting rewards from the flowers available. This increase in foraging time caused a significant increase in facilitated self-pollination (Lloyd and Schoen, 1992) through within-flower interference.

Floral interference was initially proposed as a within-flower phenomenon (Lloyd and Webb, 1986; Lloyd and Yates, 1982). Harder and Barrett (1995, 1996) later expanded interference to between-flowers, which is thought to be the dominant form of interference, particularly in large inflorescences where interference can produce significant geitonogamy and pollen discounting (Barrett, 2002; Harder et al., 2000). The results of this chapter and Chapter 2 (in addition to Harder et al. (2000)) show that protandry can reduce both forms of interference and enhance male siring success. In the case of within-flower interference, protandry provides a period of pollen presentation before the onset of stigma presentation. This allows much of the available pollen to be successfully exported while avoiding within-flower

interference. Although the results of the lab study measured pollen deposition on stigmas, this pollen potentially could have been exported. Consequently, within-flower interference may result in pollen discounting (Harder and Wilson, 1998) in addition to facilitated selfing. Such an effect would explain the surprising siring advantage of two-flowered, protandrous plants described in Chapter 2. Unfortunately, facilitated selfing and the influence of within-flower interference on male siring-success are essentially unstudied. These investigations of pollen dynamics in *C. angustifolium* suggest that more attention is warranted for these components of floral biology.

3.3.2 Pollen removal

Pollen removal appears to be unaffected by the presence of a stigma or the amount of time a bee spends foraging on the flower. The insensitivity of pollen removal to the presence of the stigma is surprising in light of the significant changes in the position of the stigma throughout the development of a *C. angustifolium* flower. As described in Chapter 2 and Chapter 4, during male phase the style of *C. angustifolium* is reflexed and the stigma lobes are closed. At the onset of female phase, the stigma opens and the style straightens to position the stigma near the centre of the flower. The functional significance of this herkogamy (spatial separation of gender; Webb and Lloyd (1986)) is unclear. However, the closely related *C. latifolium*(Baum et al., 1994) maintains its stigma in the reflexed position throughout anthesis (pers. obs.). Such a difference suggests an ecological function, but the consequences of such changes in spatial positioning for pollination have not been studied. One possibility is that the reflexed stigma provides a landing platform for pollinators to maximize pollen removal during the male phase. Since the lab experiment compared hermaphroditic flowers with intact stigmas to flowers with their stigmas and styles entirely removed, such a function for the stigma cannot be evaluated with the present data. Nevertheless, a study of pollination in *Echium vulgare* (Boraginaceae) found that the presence of a stigma had little effect on the amount of pollen lost (Rademaker et al., 1997), suggesting that female function is not a source of within-flower interference.

Time spent foraging on a flower is expected to be positively correlated with pollen removal, but as a decelerating function (Harder and Thomson, 1989). A greater range of visit durations might have revealed a relationship between time and pollen removal in *C. angustifolium*. On average, a single

visit removed ∼40% of the pollen available in a *C. angustifolium* flower. Although this represents a substantial portion of the available pollen, the viscine threads and spatially spread presentation of pollen in this plant may limit the amount of pollen available for removal by any single bee. Another possibility for any of these potential effects on pollen removal is that the variance in pollen removal obscures any statistical relationship. Wilson et al. (1994) argued that the vagaries of male success and the general inefficiency of pollination make detecting significant effects on pollen removal exceedingly difficult and, perhaps, biologically irrelevant.

3.3.3 Comparison of lab and field experiments

Estimated rates of pollen deposition were quite similar between the lab and field studies with largely overlapping confidence intervals. Pollen removal, however, was significantly higher in the lab than the field experiment. The relative stability of female success, as measured by pollen deposition, compared to the highly variable pollen removal estimates is consistent with other studies comparing male and female success (Aizen and Basilio, 1998; Bateman, 1948; Harder and Thomson, 1989; Levin and Berube, 1972; Lloyd, 1982; Meagher, 1986; Rademaker et al., 1997). The asymmetry between the pollination effort required to maximize male fitness relative to female fitness has been an important source of research activity (e.g, Charnov, 1982; Cruden, 1977; Morgan, 1993; Morgan and Schoen, 1997; Queller, 1997). Protandry in *C. angustifolium* acts to enhance male reproductive success through reductions in both within- and between-flower interference and their associated facilitated selfing, geitonogamy, and pollen discounting. Consequently, the temporal separation of gender may help reduce this asymmetry between male and female pollination success.

Chapter 4

Genetic architecture of protandry and floral form

In the previous two chapters I investigated the functional significance of protandry using experimental manipulations of flowers and inflorescences. These studies provide insights into the effect of protandry on fitness and the mechanisms by which this advantage is achieved. However, in natural populations the evolutionary response to selection for changes in the degree of protandry or the duration of male phase may be mitigated by several factors (see Chapter 1). A primary consideration for any study of adaptation is the magnitude of heritable variation for the character of interest. In the absence of such variation, selection cannot produce an evolutionary response (Falconer and Mackay, 1996; Fisher, 1930). Furthermore, selection on a trait can cause indirect selection on additional traits through genetic correlations (Antonovics, 1976; Lande, 1979). For example, if two traits are negatively correlated, an increase in the value of one trait cause a decrease in the other trait's value (Lande, 1980; Lande and Arnold, 1983; Roff, 1996). Since natural selection operates on the whole phenotype, rather than the specific trait of interest, the most fit phenotype may represent a compromise among trait values. This leads to a final factor that may mitigate selection, trade-offs between the fitness consequences of traits (Charnov, 1982). Trade-offs are similar to genetic correlations in that they mitigate against unconstrained changes in trait values. However, trade-offs do not necessarily rely on a common genetic architecture. The classic example is the size-number trade-off in reproduction (e.g., Reznick, 1985; Stearns, 1989). In this case an underlying limit on resource availability produces a

negative trade-off between the number of offspring produced and the size of these offspring. Certain circumstances favour many small offspring and others a few large offspring, but the evolution of many large offspring, for example, is not possible.

Essentially no information is available on the genetic architecture of male-phase duration and the potential for the three constraints described above to operate. Presumably, male-phase duration is heritable to some extent. However, the magnitude of heritability has a direct effect on the tempo of evolutionary responses to changes in male-phase duration. Furthermore, correlational selection may be common in flowers due to the requirement that anthers and stigmas are presented in close physical proximity (Armbruster et al., 1999; Armbruster and Schwaegerle, 1996; Berg, 1959, 1960). In particular, pollinators are influenced by floral size and inflorescence display (Bell, 1985; Conner et al., 1996; de Jong and Klinkhamer, 1994; Galen and Newport, 1987; Harder and Barrett, 1996; Strauss et al., 1996). Consequently, any correlation between male-phase duration and these traits may influence pollinator attraction and the fitness of the selected phenotype. For example, if increasing male-phase duration causes a decrease in floral-display size, selection for increased siring-success through increased protandry (e.g., Chapter 2) may be counteracted by the decrease in pollinator activity caused by the decrease in floral display. In the case of trade-offs, if floral longevity is fixed, selection on male-phase duration may cause changes in female-phase duration unassociated with genetic correlations. This reciprocal change can have important fitness consequences if either gender function is time limited. For example, if the frequency of pollinator visits per unit time is fixed, a shortened female phase may result in fruit set becoming pollen limited. Since hermaphrodites must satisfy both male and female function (Charnov, 1982; Morgan and Schoen, 1997), such a reduction in female reproductive success would be selected against and counteract selection for longer male-phase duration.

In this study I investigate constraints on the evolution of protandry by estimating the genetic basis of variation in male-phase duration in the protandrous *Chamerion angustifolium*. Specifically, I address four questions: 1) How variable is male-phase duration within populations?, 2) Is this variation heritable?, 3) Does selection on male-phase cause correlated changes in floral size and display?, and 4) Is there a trade-off between male- and female-phase durations?

4.1 Materials and methods

4.1.1 Study species

Chamerion angustifolium (Onagraceae) is a multi-flowered, herbaceous perennial with protandrous flowers (see Chapter 2 for more details on this species). During the initial male phase, when the anthers have dehisced, the style is strongly deflexed away from the anthers and the four stigma lobes are closed. The stigma lobes then spread apart becoming receptive and the style straightens, positioning the stigma near the centre of the flower. This protandry and the acropetalous (maturing from base to tip) development of the inflorescence, produces an inflorescence with female-phase flowers at the base and male-phase flowers toward the apex (Galen and Plowright, 1988). In this study, pollen remained in the anthers throughout anthesis. Consequently, the female phase is in fact a hermaphroditic phase. However, in the presence of pollinators, pollen is rapidly removed from the anthers (Galen and Plowright, 1988, pers. obs.), so that the genders do not overlap.

4.1.2 Paternal half-sib design and measuring male-phase duration

To estimate additive genetic variation in male-phase duration, paternal half-sibs were created in the summer of 2001 with seed collected from population D2 in Wyoming (Husband and Schemske, 1997). The seed from outcrossed families were germinated and transferred to 4 L pots. From these plants 42 sires were crossed with 53 dams to produce 304 fruits with an average of 7.2 dams per sire. To estimate male- and female-phase durations, anthesis (described above) was divided into stages (Table 4.1). I classified male phase as stages 3–5 and female phase as stages 6–7. These stages were documented for each of the third to seventh opening flowers of one plant per sibship twice daily (at approximately 9:00 and 16:00). This documentation began when the third flower reached stage 3 and ended when all five flowers reached stage 7. Male-phase duration was estimated as the average number of hours elapsed between stages 3 and 6 for the five flowers. My resolution of male-phase duration was limited by the two sampling periods per day. For the purposes of this study, I assumed that if a flower changed stages between sampling periods the change occurred at the end of the earlier period. For example, if at 9:00 a flower was in stage 5 and then in stage 6 at 16:00, male

Table 4.1: The floral stages used to describe anthesis in *Chamerion angustifolium*. Stages 3, 4, and 5 represent male phase while 6 and 7 are female phase.

Stage	Description
1	Closed flower bud
2	Open flower, no dehisced anthers
3	Open flower, $\leqslant 4$ dehisced anthers
4	Open flower, > 4 dehisced anthers
5	Open flower, all anthers dehisced, stigma closed
6	Open flower, all anthers dehisced, stigma open, style reflexed
7	Open flower, all anthers dehisced, stigma open, style fully extended
8	Closed flower

phase ended at 9:00 and eight hours had elapsed in female phase. Alternate assumptions (e.g., the switch occurring just before the second sampling period) would effectively shift all duration estimates by eight hours. Since I am interested in relative allocations and relative changes in phase durations, this assumption does not bias my results.

4.1.3 Selection experiment

I also investigated the genetic relationship between male- and female-phase durations and other floral characters (described below) with an artificial selection experiment. Beginning with the paternal half-sib plants as the base population, I implemented the selection design depicted in Figure 4.1. Plants were ranked according to their male-phase duration and in the parental generation the shortest 20% were assigned to one of two short selection-lines and the longest 20% to one of two long selection-lines. The two control lines were created by randomly selecting plants from the entire distribution of male-phase duration. In the F1 generation the upper and lower 50% of male-phase duration were used for long and short selection-lines, respectively. Each plant within a line was randomly mated to two sires. Pollinations were done by removing two anthers from a sire with forceps and applying the pollen to all four lobes of the recipient stigma. Due to the intense inbreeding depression in *C. angustifolium* (inbred offspring have a relative fitness of 5%, Husband and Schemske 1995) all crosses were made between unrelated paternal half-sibs to generate the first generation,

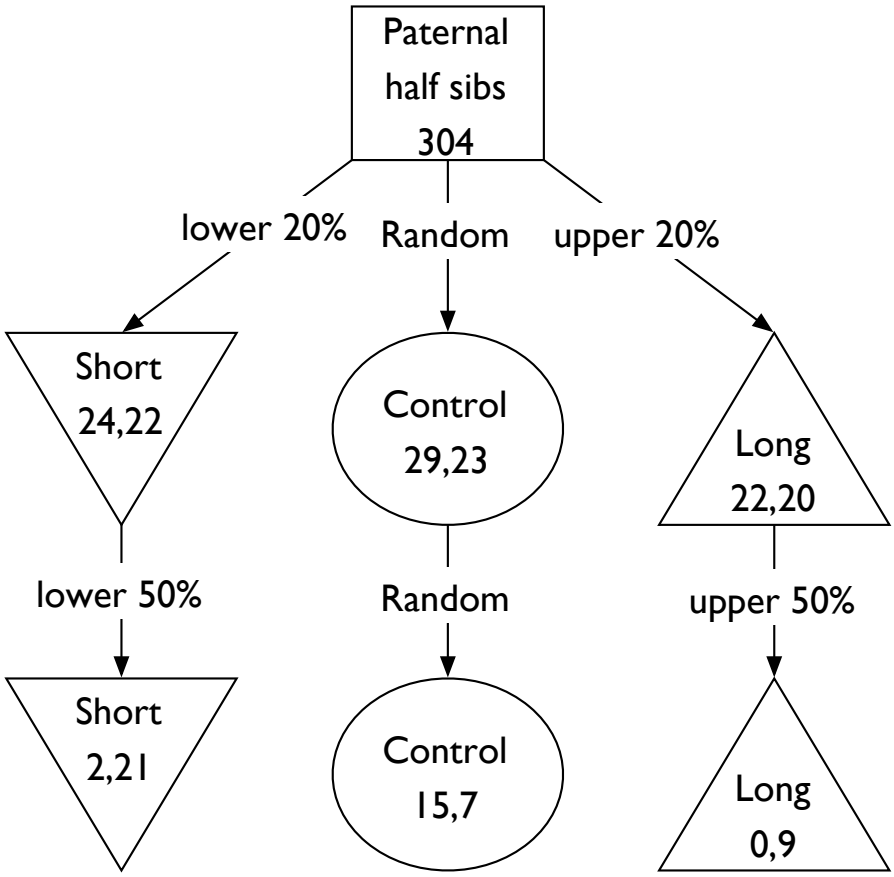


Figure 4.1: A schematic representation of the selection design used. The numbers represent sample sizes. A replicate of the long male-phase duration was lost to greenhouse pests.

and second generation crosses were made between plants that did not share parents. Although this crossing design reduced the influence of inbreeding on my trait measures, the number of suitable mates and population sizes declined with each generation. Consequently, only two generations of selection was possible.

4.1.4 Correlates of male-phase duration

In addition to male- and female-phase durations, several other floral traits were measured for each plant. Floral width, measured as the greatest diameter across the flower, was taken for one randomly chosen flower. Floral separation was measured as the distance from the pedicel of the randomly chosen flower to the pedicel of the next highest flower. The position of the five flowers censused was measured as the distance from the soil surface to the pedicel of the first of the five flowers. Finally, the display length occupied by the censused flowers was measured as the distance from the pedicel of the first flower to the pedicel of the last flower of the five. Display size was measured for the final generation of the selection experiment as the average number of open flowers on a plant for three randomly chosen days the week prior to the census.

4.1.5 Data analysis

The heritability of male-phase duration was estimated from the paternal half-sib design following the methods of Lynch and Walsh (1998). After correcting for unbalanced contributions from each paternal half-sib family, heritability was estimated from the intraclass correlation (t_{PHS}) as:

$$h^2 \simeq 4t_{PHS} = \frac{Var(s)}{Var(s) + Var(e)}, \tag{4.1}$$

where $Var(s)$ is the variation among sires and $Var(e)$ is the error variance. The 95% confidence interval of this heritability estimate was calculated from the F distribution with 41 and 262 df.

I estimated heritabilities and character correlations from all three generations of the selection experiment with restricted maximum-likelihood variance components estimates (REML VCE, ver. 4.2, Neumaier and Groeneveld, 1988). This approach is well suited for unbalanced designs. In addition, since the genealogical relationships of the plants is incorporated into this analysis, the use of selected lines does not bias the genetic estimates obtained. I included replicate lines as an effect in the model. VCE also readily generates standard errors for all heritability and genetic correlation estimates. Based on these standard errors, I constructed 95% confidence intervals for each estimate. If an individual estimate's confidence limit was greater than zero and less than one it was considered statistically significant.

I analyzed the responses of male- and female-phase durations, and total floral longevity to selection on male phase by ANOVA with generation and selection line as fixed effects and replicate as a random effect. I analyzed the responses to selection of floral width, floral separation, inflorescence size, and floral display in the final generation by ANOVA with selection line as a fixed effect. Statistical tests were performed using JMP software (ver. 5.0, SAS Institute, 2002) and I compared all means with Tukey post-hoc tests.

To quantify the magnitude of selection on male-phase duration, I calculated selection differentials for each generation as the weighted average of the difference in male-phase duration of plants chosen for crossing from the population average (Falconer and Mackay, 1996). These selection differentials were then summed to calculate cumulative selection differentials for each selection line across the two generations of selection. The response to selection was measured as the average response of the replicates of each selection line for the first and second generations.

I tested my control lines for evidence of drift using the methods of Lande (1976, 1977). This approach calculates a test statistic:

$$D = \sqrt{\frac{N_e z^2}{h^2 t \sigma_p^2}} \tag{4.2}$$

where N_e is the effective population size (taken as the harmonic mean of the first and second generation), z^2 is the squared divergence from parental to final generation in SDs, h^2 is the heritability (estimated from REML VCE), t is the number of generations (2), and σ_p^2 is the overall phenotypic variance (the MS_{error} from the ANOVA of selection on male-phase duration). D is distributed as a standard normal curve and significant P values show that drift cannot account for the observed changes.

4.2 Results

4.2.1 Paternal half-sib design

I found extensive variation in male- and female-phase durations across the paternal half-sib families. On average, male phase lasted for 17.00±0.48 (mean±SE) hours and female phase for 62.86±0.97 hours. Flowers were 31.5±0.3 mm wide, with an average of 18.0±0.5 mm between flowers. Dis-

Table 4.2: ANOVA of male-phase duration in *Chamerion angustifolium* from the paternal half-sib design. These values were used for estimating heritability and for evaluating whether genetic drift can account for the observed changes.

Source	df	F ratio	P
Sire	41	1.50	<0.03
Error	262	67.0	

play length was 38.2±0.1 cm. The pattern of variation in male-phase duration (Table 4.2) leads to a heritability estimate of h^2=0.27 with a 95% confidence interval of 0.05–0.54.

4.2.2 Selection experiment

Significant responses were generated after two generations of selection on male-phase duration (Table 4.3). Overall, the maximum inbreeding coefficient in my selection experiment was 0.13 and, among the inbred plants, the average inbreeding coefficient was 0.10. 480 plants had inbreeding coefficients of 0. The observed changes in control lines were not due to drift (P<0.001) and are likely due to inadvertent selection or changing environmental conditions. When compared to the control lines, the long and short selection-lines rapidly diverged from each other (Figure 4.2), although the absolute amount of divergence was greater for long selection lines. A comparison of male- and female-phase durations in the final generation (Figure 4.3 and Figure 4.4) shows a reciprocal change in gender durations: selecting for a short male phase produced a longer female phase and vice versa. Consequently, there was no significant difference in total floral longevity across the six selection lines (Table 4.3, $F_{2,492}$=0.20, P>0.80). Plotting the cumulative selection differential against the cumulative response (Figure 4.5) shows that the differentials and responses were very similar for the first generation. One of the short selection replicates then diverged from the remaining lines by showing a reduced response to a high differential.

Despite the large changes in male- and female-phase duration, the ANOVA of the additional floral characters showed no significant differences among selection lines for the final generation (Table 4.4 and Table 4.5). Unfortunately, one of the long selection-line replicates was lost due to greenhouse pests in the second generation of selection.

Table 4.3: ANOVAs of evolutionary responses in a) male-phase duration, b) female-phase duration, and c) total floral longevity after two generations of selection on male-phase duration in *Chamerion angustifolium*. Treatment means for male- and female-phase durations are presented in Figure 4.3 and Figure 4.4, respectively.

Source	df	F ratio	P
a) Male-phase duration			
Generation	2	22.39	<0.0001
Selection line	2	10.35	<0.0001
Replicate	1	0.70	>0.40
Error	492	57.87	
b) Female-phase duration			
Generation	2	15.13	<0.0001
Selection line	2	3.71	<0.05
Replicate	1	3.73	>0.05
Error	492	263.02	
c) Floral longevity			
Generation	2	28.03	<0.0001
Selection line	2	0.20	>0.80
Replicate	1	2.19	>0.10
Error	492	284.18	

4.2.3 Genetic analysis

Based on all three measured generations, each of the floral traits measured had significant heritabilities (Table 4.6). In particular, the narrow-sense heritability of male-phase duration was estimated as 0.23±0.04 (mean±SE). The VCE analysis had a final likelihood of 4,330 and reached status 1 (a unique solution was found) after 103 iterations. I detected significant positive genetic correlations between male-phase duration and floral separation and display length. In addition, floral separation, display length, and display size had positive genetic correlations (Table 4.6).

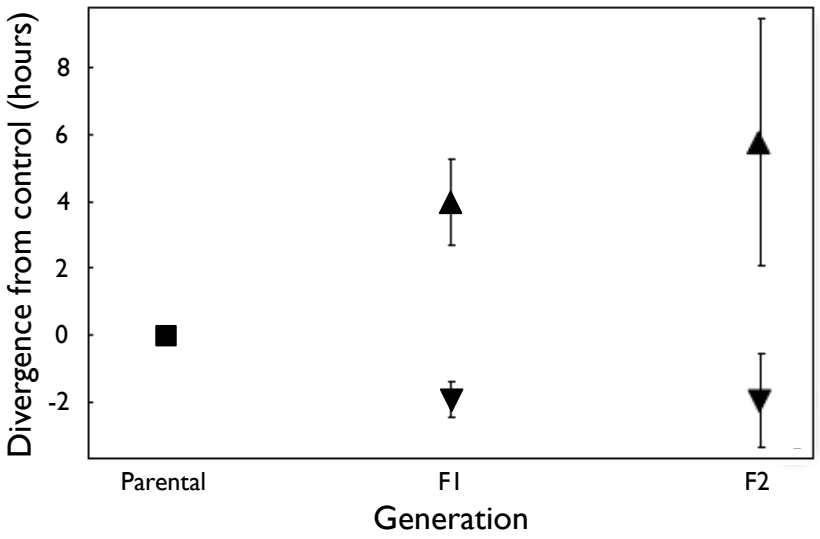


Figure 4.2: The divergence (±SE) from control lines (standardized to 0) for male-phase duration after two generations of selection in *Chamerion angustifolium*. To simplify the presentation, the average of the two replicate lines is plotted. The orientation of triangles indicate the long and short selection-lines. Statistical details are presented in Table 4.3 and the text.

4.3 Discussion

Chamerion angustifolium has significant heritable variation for male-phase duration. The capacity for male-phase duration to respond to selection was demonstrated by the change in mean duration in just two generations of selection. I was able to shorten male phase by approximately 0.75 SD and lengthen it by 2 SDs. Furthermore, despite the genetic correlations estimated, these changes were not associated with any correlated phenotypic changes in other floral traits. However, I did detect a trade-off between allocation in time to male phase and female phase. The consequences of these patterns for the evolution of male-phase duration depend on several considerations.

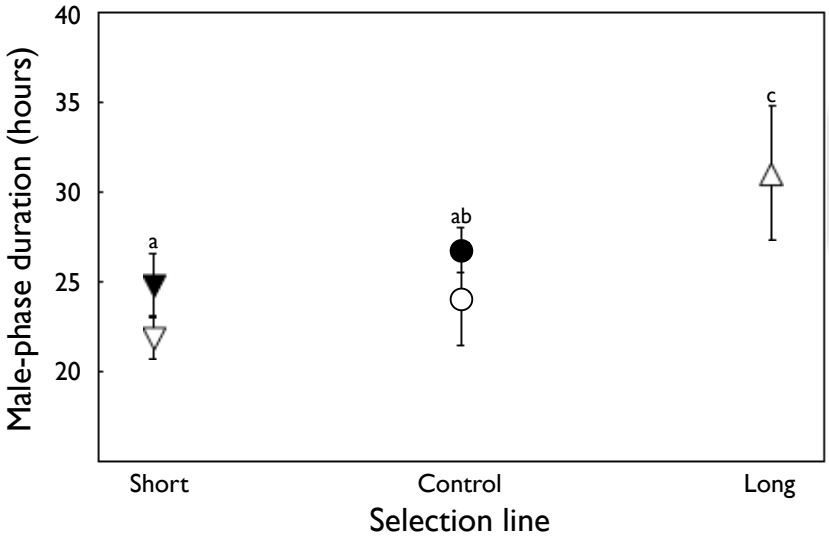


Figure 4.3: Mean (±SE) male-phase duration for the final generation of selected plants in *Chamerion angustifolium*. The orientation of triangles indicate the long and short selection-lines, and circles represent control lines. Closed and open symbols represent the first and second replicate-lines, respectively. Values sharing letters are not significantly different from each other, see Table 4.3a and the text.

4.3.1 Consequences of variation

Cresswell (1998) conducted a literature survey of floral trait coefficients of variation (CVs; $SD/\bar{x}$) for 151 plant species from 48 families. The 365 CVs obtained had a median value of 0.14. Based on the parental generation of my selection experiment, male-phase duration in *Chamerion angustifolium* has a coefficient of variation of 0.5. Consequently, this trait is one of the most variable of floral characters. Unfortunately, I cannot evaluate the functional significance of this variation directly. The two experimental studies of the functional significance of protandry (Harder et al., 2000, Chapter 2) compared dichogamous and adichogamous plants as discrete categories of gender separation. Although both studies demonstrated that dichogamy provides a siring advantage, it is unclear how continuous variation in gender separation would change this advantage. Presumably, the

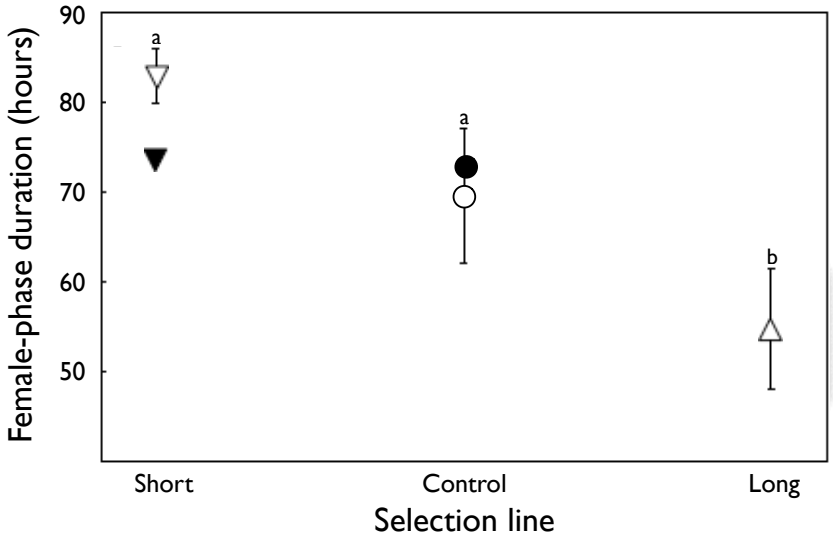


Figure 4.4: Female-phase duration (±SE) for the final generation of selected plants in *Chamerion angustifolium*. The orientation of triangles indicate the long and short selection-lines, and circles represent control lines. Closed and open symbols represent the first and second replicate lines, respectively. Values sharing letters are not significantly different from each other, see Table 4.3b and the text.

frequency of pollinator visits is of considerable importance (e.g., Richardson and Stephenson, 1989). If pollinators visit flowers rapidly and consistently, a short male-phase duration may be sufficient to remove all available pollen and saturate male fitness. However, rare or idiosyncratic pollinator activity may favour longer male phases and pollen packaging and dispensing mechanisms (Harder and Thomson, 1989) to maximize siring success (Devlin and Stephenson, 1984, 1987; Lloyd and Yates, 1982; Nyman, 1993; Preston, 1991; Richardson and Stephenson, 1989; Robertson and Lloyd, 1993).

To my knowledge, this study represents the first estimate of heritability of intra-floral male-phase duration. Schoen (1982) calculated a protandry index (the proportion of male-phase flowers relative to hermaphroditic flowers) in plants grown from open-pollinated fruits of *Gilia achilleifolia* (Polemoniaceae). Based on variation within and among plants from a fruit, Schoen estimated the heritability of the protandry index as 0.47. Camp-

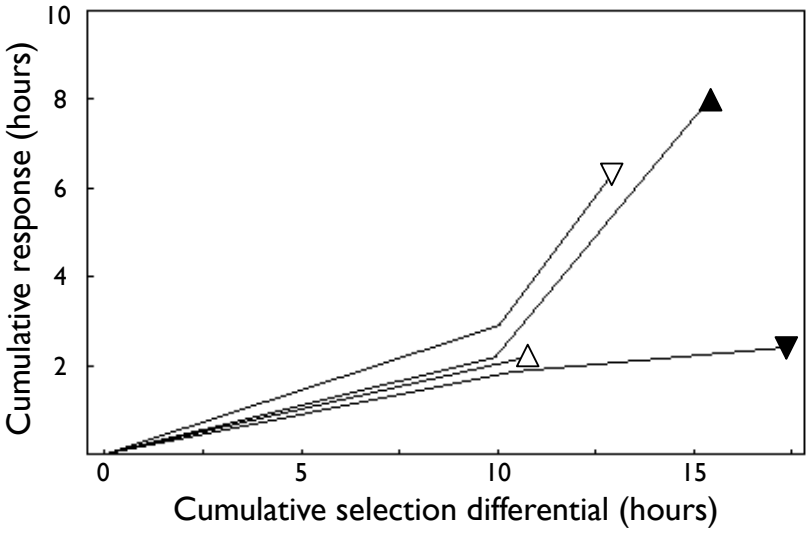


Figure 4.5: The cumulative selection differentials and cumulative responses for male-phase duration in the two generations of short and long selection-lines in *Chamerion angustifolium*. The orientation of triangles indicate the long and short selection-lines. Closed and open symbols represent the first and second replicate lines, respectively. The second long selection-line was lost after the first generation.

Table 4.4: ANOVA of the effect of selection for male-phase duration on four other floral characters in *Chamerion angustifolium*.

Source	df	F ratio	P
Floral width	2, 45	0.71	>0.45
Floral separation	2, 45	0.02	>0.95
Inflorescence size	2, 44	0.73	>0.45
Display size	2, 51	1.31	>0.25

Table 4.5: Summary of floral traits measured in *Chamerion angustifolium* from all three generations. Display size was measured in the second generation only.

Trait	n	Mean	SE
Male-phase duration (hours)	498	18.8	8.1
Female-phase duration (hours)	498	67.6	17.3
Floral width (mm)	453	31.9	4.6
Floral separation (mm)	447	17.4	8.8
Display length (mm)	360	39.2	13.8
Display size (# of flowers)	54	6.9	4.5

bell (1996) estimated the proportion of time spent by *Ipomopsis aggregata* (Polemoniaceae) flowers in the pistillate phase by censusing paternal half-sibs twice a week and estimated heritability as near 0.3. These estimates, although consistent with my results, are based on gender separation between flowers of a plant, rather than within a single flower. Considering surveys of heritabilities in the laboratory and the wild (Mousseau and Roff, 1987; Weigensberg and Roff, 1996), these three estimates of male-phase duration show that protandry is moderately heritable. Consequently, selection for changes in male-phase duration could produce rapid results.

4.3.2 Consequences of correlations

Correlational selection may be the most common form of natural selection (Schluter and Nychka, 1994). In floral biology, 'correlational pleiades' (Berg, 1959, 1960), where selection acts on the variance-covariance structure of flowers rather than individual trait means, may be particularly important. The fitness consequences of changes in male-phase duration need to be balanced against any negative fitness consequences for correlated floral traits. The REML VCE analysis showed significant positive correlations between male-phase duration and several aspects of floral display. The average genetic correlations among morphological traits is 0.47 (Roff, 1996). This places the correlations between male-phase duration and floral display at the higher end of the range of values measured to date.

Due to the importance of floral display for plant reproduction (Fisbein and Venable, 1996; Harder and Barrett, 1995; Morgan, 1993; Schoen and Dubuc, 1990), the correlation with floral display could significantly affect

Table 4.6: Heritabilities (on the diagonal) of and genetic correlations (above diagonal) among male- and female-phase durations, floral width, floral separation, display length, and display size in *Chamerion angustifolium* as estimated from all three generations. Each estimate is ±SE and estimates significantly greater than zero and less than one, based on 95% CI, are indicated in bold.

Male phase	Female phase	Floral width	Floral separation	Display length	Display size
0.23±0.04	-0.17±0.14	-0.13±0.12	**0.49±0.22**	**0.68±0.12**	0.22±0.32
	0.17±0.04	0.04±0.11	-0.26±0.22	-0.18±0.15	0.38±0.29
		0.19±0.04	0.26±0.21	**0.47±0.16**	-0.13±0.10
			0.07±0.03	**0.89±0.13**	**0.68±0.24**
				0.18±0.04	**0.46±0.23**
					0.22±0.32

the evolution of male-phase duration. Selection for increased male-phase duration is predicted to also increase floral-display size. Large displays can increase both male and female reproductive success (Campbell, 1989; Emms and Arnold, 1997). However, geitonogamy and pollen discounting are potential negative fitness consequences of larger displays (Harder and Barrett, 1996; Klinkhamer and de Jong, 1993). Consequently, selection to increase pollen removal in pollinator-limited environments by increasing male-phase duration may also increase pollinator attraction by increasing floral display. The equilibrium male-phase duration would then be a compromise between the gains in siring success due to this increased attraction and the losses due to within-plant pollen transfer (e.g., Harder and Johnson, 2003, Chapter 3).

Despite my estimated genetic correlations from the REML VCE analysis, I detected no correlated response of floral display in my selection experiment. There are several potential ways to reconcile these findings. First, retrospective power analyses of my ANOVA show that display length and size had adjusted powers of 0.16 and 0.27 respectively. Consequently, I cannot rule out the possibility that I am simply unable to detect the changes in display. Second, related to the first, two generations of selection may not have been sufficient to change characteristics of floral display in *C. angustifolium*. Further generations of selection may have produced the predicted responses. Third, the variance-covariance structure of my measured traits may have changed during the selection experiment. However, given the concordance between the pre-selection, paternal half-sib estimate of heritability and the REML VCE estimate from all three generations, this seems unlikely. Furthermore, two generations of selection is not thought to be sufficient to change correlation structures (e.g., Arnold, 1992; Schluter, 1996, but see Stanton and Young, 1994).

4.3.3 Consequences of trade-off

This study detected a significant trade-off between male-phase and female-phase durations. Interestingly, this trade-off did not manifest itself as a genetic correlation. Rather there appears to be a developmental constraint on the duration of anthesis, producing a developmental correlation (e.g, Falconer and Mackay, 1996; Grant, 1975; Lande, 1980) between gender durations. Similarly, Devlin and Stephenson (1984) found that pistillate duration decreased with increased staminate duration in *Lobelia cardinalis* (Campanulaceae). Since hermaphroditic plants must maximize fitness gains

through both genders (Charnov, 1982; Morgan and Schoen, 1997), this trade-off can influence the evolution of male-phase duration.

As discussed earlier, gender function is often limited by the frequency of pollinator visits. Consequently, selection must optimize each gender-phase duration to satisfy both male and female reproductive-success. For example, Aizen and Basilio (1998) found that male function required three times the pollinator activity as female function to maximize reproductive success in *Alstroemeria aurea* (Alstroemeriaceae). In this particular case, nectar production was three times greater during male phase to compensate for this asymmetry in pollinator requirements. In *C. angustifolium* nectar quantity (volume and percent sugar) does not change between male and female phases (Komlos, 1999). Given the extended female phase, this may suggest that male phase is less pollinator limited than female phase. However, Parker et al. (1995) found that *C. angustifolium* allocated 1.5 times the resources (measured as mg of dry mass) to male phase relative to female phase. Further investigation of the relationship between time allocation, pollinator attraction, and fitness benefits of each gender in *C. angustifolium* are required to address these issues.

If selection cannot optimize both genders, this trade-off could favour the evolution of separate sexes (monoecy or dioecy, Bawa, 1980; Charnov, 1982; Richards, 1986). Pellmyr (1987) argued that dichogamy may destabilize hermaphroditism based on the availability of mates and lead to the evolution of separate sexes. However, the relative rarity of separate genders in flowering plants (Lloyd, 1982) suggests that the reproductive contributions of male and female function can often be successfully optimized within a flower.

4.3.4 Evolution of male-phase duration

There is considerable heritable variation for male-phase duration in *Chamerion angustifolium*. Consequently, selection for changes in the degree of protandry should produce a rapid evolutionary response. Although I did not detect any correlated changes in other floral traits, the genetic estimates suggest that attributes of floral display-size are correlated with male-phase duration. Therefore, the evolution of longer male phase might also increase floral display and pollinator attraction. However, the benefits of these changes must be considered with the negative trade-off with female-phase duration. Overall, the evolution of gender duration should represent

a compromise between the time requirements of both male and female function.

Chapter 5

A phylogenetic perspective on the evolution of dichogamy

The previous chapters have been concerned with the evolution of protandry. In this chapter, I explore the evolution of dichogamy within a comparative framework. When Bertin (1993) conducted his analysis of dichogamy in self-incompatible (SI) and self-compatible (SC) species (self-incompatibility, written in full, represents the trait, while SI and SC are the trait states) he did not account for the phylogenetic relationships of the species involved (Felsenstein, 1985). Now that several family-level phylogenies of the angiosperms are available (Chase et al., 1993; Soltis et al., 2000, 1999), Bertin's (1993) data can be analyzed within a phylogenetic context (Harvey and Pagel, 1991). In this study I examined the historical patterns of the evolution of dichogamy in angiosperms. Using an expanded version of Bertin's (1993) database, I mapped the occurrence of dichogamy and self-incompatibility on a phylogeny inferred from 18S rDNA, *rbc*L, and *atpB* sequences (Soltis et al., 2000). Using several statistical procedures for identifying independent evolutionary transitions on the tree, I tested two specific predictions: 1) Protogyny is associated with SC, in support of the inbreeding-avoidance hypothesis, and 2) Protandry is associated with SI, in support of the interference-avoidance hypothesis. In addition, the analyses provide insights into the ancestral states and the evolutionary lability of dichogamy and self-incompatibility in the angiosperms.

5.1 Materials and methods

5.1.1 Source of reproductive and phylogenetic data

The species data on dichogamy and self-incompatibility that were used in this study include those from the original survey of Bertin (1993) and Bertin and Newman (1993), and from 389 additional species for which data were found more recently. In total, the database contains 5,641 species distributed among 244 families. From this database only species with information on both dichogamy and self-incompatibility were used, leaving 906 species for the analyses. To maintain consistency with current angiosperm phylogenies, I used the Angiosperm Phylogeny Group (APG) classification. As a result, some of the family designations in this study differ from the earlier study by Bertin (1993). I converted the original data set by generating a list of genera and their associated families with Stevens (2003) and Watson and Dallwitz (1992) (see also, Dallwitz, 1980; Dallwitz et al., 1993, 1995, 2000) and then re-categorizing each species. The Scrophulariaceae *s. l.* were split into four groups, following Soltis et al. (2000), as follows: Scrophulariaceae1 comprising species of *Phyla* and *Paulowyria*; Scrophulariaceae2 comprising species of *Veronica*; Scrophulariaceae3 comprising species of *Verbascum* and *Scrophularia*; and Scrophulariaceae4 comprising species of *Digitalis*, *Plantago*, *Callitriche*, and *Antirrhinum*.

For the phylogenetic relationships among families, I used the 'B series' tree and branch lengths of Soltis et al. (2000). This tree, one of 8,000 shortest trees, is based on 567 taxa and three gene sequences (18S rDNA, *rbc*L, and *atpB*), and was found in an heuristic search using the parsimony ratchet (Nixon, 1999). Any phylogeny represents an hypothesis of taxon relationships (Harvey and Pagel, 1991). Although the Soltis et al. (2000) phylogeny represents a current hypothesis, it may be revised as more traits and species are added. Nonetheless, the general structure of the angiosperm phylogeny seems stable (Soltis et al., 1999), and simulations suggest that ignoring phylogenetic relationships altogether produces much greater bias than the use of incomplete phylogenies (e.g., Martins et al., 2002; Purvis et al., 1994). In an attempt to measure the sensitivity of my results to the chosen phylogeny, I performed analyses with three different phylogenies. My main conclusions are based on the original Soltis et al. (2000) phylogeny. I also made two modifications to this tree: 1) Due to the uncertainty of the position of the Lamiales in general and the four Scrophulariaceae taxa

within this group (Stevens, 2003) I excluded the Lamiales; and 2) I set all branch lengths to one.

5.1.2 Trait coding

I used data on intrafloral dichogamy and self-incompatibility from Bertin and Newman (1993). In that study five categories were used to describe dichogamy: 1=protandry, 2=protandry to adichogamy, 3=adichogamy, 4=adichogamy to protogyny, and 5=protogyny. Here, protandry represents categories 1 and 2 and protogyny represents categories 4 and 5. A separate adichogamous category was not included because the analyses used can only compare two traits each with two trait states. In addition, a likely reporting bias against adichogamy means that the sample size for adichogamous taxa is too small for statistical testing. Self-incompatibility designations were based on the original sources, or if self-pollination reduced seed set by more than 50% relative to cross-pollinations the species was coded as self-incompatible.

For this analysis, I used families as phylogenetic units. I summarized the dichogamy and self-incompatibility trait states of species within each family using protandry and SI indices, respectively. The protandry index measures the number of protandrous species relative to the total number of dichogamous species in the family. Consequently it ranges from 0 (exclusive protogyny) to 1 (exclusive protandry). The SI index represents the number of SI species relative to the total number of species with self-incompatibility data. In this case 0 represents complete SC and 1 exclusive SI. I then converted these values into discrete categories. A protandry index greater than 0.8 was classified as protandrous and less than 0.2 as protogynous. Similarly, an SI index greater than 0.8 was classified as SI and less than 0.2 as SC. Since the paired comparison and maximum likelihood analyses cannot accept variable coding, I removed families with variable trait states (i.e., 0.8>index>0.2) from the analysis. Although the 0.8 criterion is somewhat arbitrary, it represents a reasonable compromise between conservative trait-state assignment and sufficient sample size (in terms of the number of families). The use of families as phylogenetic units neglects structure at lower taxonomic levels. However, such structure would add random variation to the family level coding and not bias my demonstrations of trends and correlations. In fact, such variation would reduce the power of my tests (higher Type II error rates) making significant correlations more difficult

to detect. I explore these concerns further with simulations of phylogenetic structure.

5.1.3 Phylogenetic simulations

As a quantitative analysis of trait coding and the consequences of using different criteria, I performed simulations of phylogenetic topology and trait distributions. A family comprised of 20 species was constructed with three different traits. Each trait was coded as a categorical variable with two states: 0 and 1. Trait 1 was 80% trait-state 1, trait 2 65% trait-state 1, and trait 3 50% trait-state 1. The simulations began by generating a random tree-topology for the species of the family. Each species was then randomly assigned a trait state for all three traits. This assignment was repeated 1,000 times to generate 1,000 trees with the same topology, but different trait states at the terminal nodes. Five different tree topologies were generated, each with 1,000 random terminal-node states to yield a total of 5,000 random trees. I then inferred the ancestral state of the family by both the frequency of trait states at the terminal nodes and by maximum-likelihood ancestral-state reconstructions (Schluter et al., 1997, see below for further details) using the 'trace-over-trees' feature of Mesquite (Maddison and Maddison, 2003) with 'uniquely-best states' used as the counting criterion. Comparing the results of these two types of analyses allows me to infer the reliability of my trait-coding criteria. For example, trait 1 corresponds to my 0.8 criterion. If the maximum-likelihood reconstruction estimates trait-state 0 for a high number of the random trees, the 0.8 criterion is biased toward inferring trait-state 1.

5.1.4 Phylogenetic analyses of trait correlation

Initially, I examined the evolution of dichogamy and SI in the angiosperms with maximum-likelihood ancestral-state reconstructions (Schluter et al., 1997) using Mesquite (Maddison and Maddison, 2003). This method calculates the probability distribution of each trait state at every node in the phylogeny based on the distribution of trait states at the terminal taxa. I used the Markov k-state 1 parameter model as the model of evolution for these reconstructions. This model assumes that trait transitions in either direction are equally likely. In the absence of additional data on the evolution of dichogamy and self-incompatibility, this assumption, rather

than a more complex model, is reasonable. Appropriate statistical tests are not currently available to compare the likelihoods of different reconstructions (Schluter et al., 1997). Consequently, I present proportional likelihood scores for each ancestral state and only consider general patterns of trait evolution.

I examined the correlation between dichogamy and SI using two different approaches. Since these approaches use very different techniques, agreement between the two would suggest robust results. Alternatively, if the approaches give different results, these differences may provide insight into the evolutionary processes involved. The first approach was a paired-comparisons analysis (Maddison, 2000, 2003) using Mesquite (Maddison and Maddison, 2003). This approach does not incorporate any branch length information, but has the distinction of relying on very few assumptions. For a given tree, this analysis calculates the maximum number of phylogenetically separate pairs of taxa that have different trait states for the traits of interest (e.g., one taxon is protogynous and SI, while the other is protandrous and SC). For this collection of paired taxa (termed a pairing) the number of positive pairs, those in accordance with the hypothesis, and negative pairs, against the hypothesis, are summed and a probability value is calculated for assessing the null hypothesis of no correlation between traits. In this case, my hypothesis is that SI is associated with protandry and SC with protogyny. If more than one pairing is possible, a range of probability values can be calculated. I implemented this analysis with dichogamy as the independent and self-incompatibility as the dependent trait. This choice is not meant to imply any causation in the correlation between these two traits. Identical results are obtained with the opposite assignment.

For the second approach, I used Pagel's maximum-likelihood method (Pagel, 1994, 1999) as implemented in Discrete (Pagel, 2000). This method compares the likelihoods of two models of trait evolution. The first model is the independent model in which each trait evolves independently of the state of the alternate trait. This independent model calculates four parameters that describe the instantaneous rates of trait evolution (Figure 5.1). α_1 is the rate at which trait 1 (dichogamy) evolves from state 0 (protogyny) to 1 (protandry) and β_1 is the rate for transitions from state 1 to 0. Similar rates are calculated for trait 2 (self incompatibility) as α_2 (SC to SI) and β_2 (SI to SC). The second model is the dependent model in which the rate at which one trait evolves is potentially dependent on the state of the

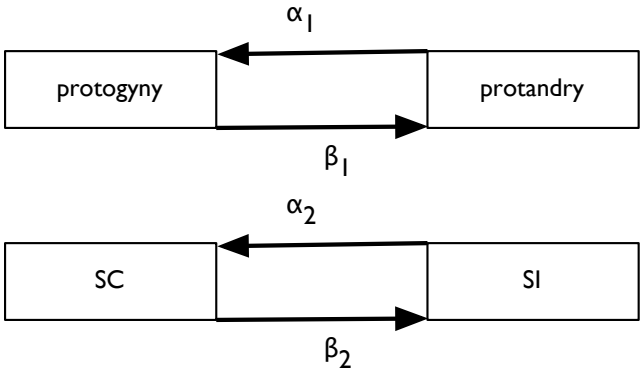


Figure 5.1: Transition-rate parameters from the maximum-likelihood analysis for the independent model of trait evolution. Comparisons of the likelihood of this model to the dependent model of trait evolution (Figure 5.2) tests for a correlation between dichogamy and self-incompatibility.

other trait (i.e., the traits evolve in a correlated fashion). This dependent model calculates eight parameters that can be used to construct a 'flow diagram' of trait evolution (Figure 5.2). For example, q_{13} estimates the transition rate of protogyny to protandry in SC taxa, while q_{31} estimates the change from protandry to protogyny in SC taxa. The dependent model is accepted if it has a significantly higher likelihood of fitting the data than does the independent model using the likelihood-ratio test. The significance of the likelihood ratio was evaluated with 1,000 Monte-Carlo evolutionary simulations of the trait distributions based on the estimated independent model parameters. My analyses also implemented the branch length scaling option (quantified with the κ parameter). This parameter estimates the tempo of trait change (Pagel, 1994). Small values *kappa* <<ß1) indicate that branch lengths have little influence on the rate of traits change. A κ value of 0 means that trait changes are independent of branch lengths (i.e., evolution by punctual change at speciation).

I calculated the statistical significance of the estimated parameters of the dependent model of trait evolution by restricting individual transition parameters to zero and recalculating the likelihood ratio of the model. A comparison of this seven parameter model to the original, unrestricted, dependent model produces a 1df χ^2 test of the likelihood ratio. A significant

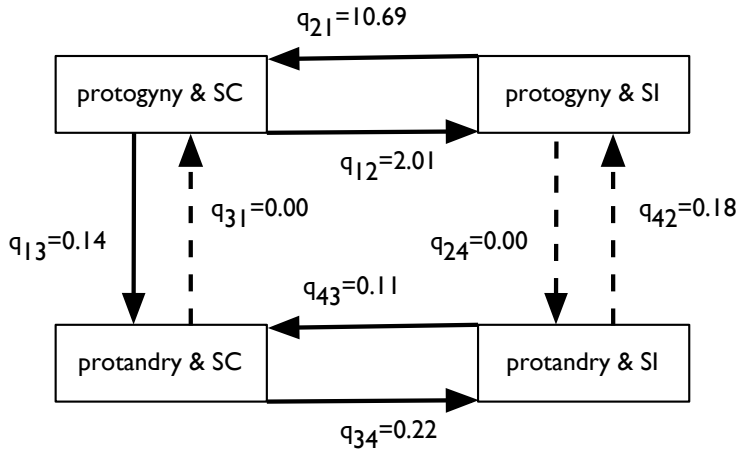


Figure 5.2: Transition-rate parameters from the maximum-likelihood analysis for the dependent model of trait evolution. Solid lines represent significant and broken lines non-significant transitions.

likelihood ratio demonstrates that the parameter is significantly different from zero (see Cézilly et al., 2000, for an example of these tests). Transitions were also compared by restricting paired parameters to be equal to each other. This seven parameter model was also compared to the original dependent model with a 1 df χ^2 test. A significant likelihood ratio indicates that the parameters are significantly different from each other.

5.2 Results

5.2.1 Phylogenetic simulations

Considered across all 5,000 trees, the percentage of trees with trait-state 1 inferred as the most likely ancestral state were 0.981, 0.916, and 0.526 for traits 1, 2, and 3, respectively. The values calculated for each of the five random topologies are presented in Table 5.1. These results suggest that my 0.8 criterion performs well, generating slightly less than 2% different reconstructions from the maximum-likelihood approach. Conversely, a simple majority-rules assignment (i.e., trait 3) performs quite poorly.

Table 5.1: Results of the phylogenetic simulations for trait coding. The proportion of trees for which trait-state 1 was inferred as the most likely ancestor are presented for each tree topology. Trait 1 is equivalent to the criterion used in the phylogenetic analyses. See the text for further details on the traits.

Tree topology	Trait 1	Trait 2	Trait 3
1	1.00	1.00	0.50
2	1.00	1.00	0.50
3	0.95	0.84	0.49
4	1.00	0.96	0.50
5	0.95	0.84	0.59

5.2.2 Ancestral reconstructions

Sufficient data were available to calculate protandry and SI indices (under the 0.8 criterion) for 55 of the families contained in the Soltis et al. (2000) phylogeny. Based on the maximum-likelihood reconstructions, protogyny (Figure 5.3; proportional likelihood 0.98) is the inferred ancestral state for the angiosperms. Both SC and SI (Figure 5.4; proportional likelihood 0.5) are equally likely ancestral trait states. From the basal node, protandry evolves early, after which protogyny re-evolves in several independent cases. Similar results were produced with the exclusion of the Lamiales, and setting branch lengths to 1.

5.2.3 Paired comparisons analysis

The paired comparison analysis of the correlation between dichogamy and self-incompatibility found a significant correlation between traits. The analysis generated 136,680 possible pairings of families. Under the restriction that both traits had to change between the pair, the maximal number of pairs within a particular pairing arrangement was 10. The hypothesis of no correlation between traits was rejected with an average one tail probability of 0.011 and range across all pairings of $0.011<P<0.054$. A random sample of ten pairings, contained 8 pairings with 9 positive (i.e., protandry and SI or protogyny and SC), 1 negative, and 0 neutral pairs, and two pairings with 8 positive, 2 negative, and 0 neutral pairs.

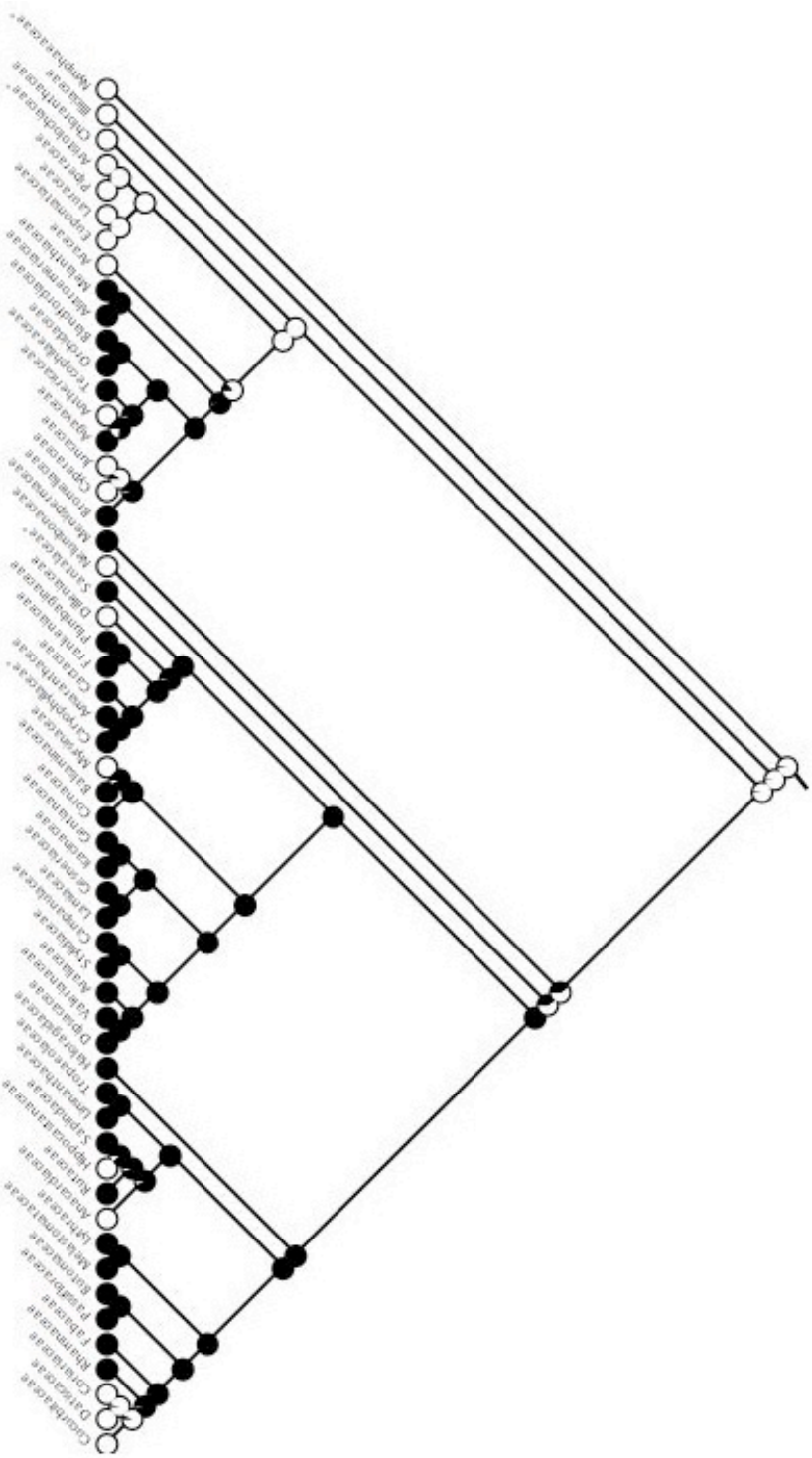


Figure 5.3: Trait-state reconstruction of dichogamy using likelihood ancestral reconstructions. Open circles are protogynous and closed protandrous. The proportional likelihoods of alternate ancestral reconstructions are indicated by the area shaded in each circle.

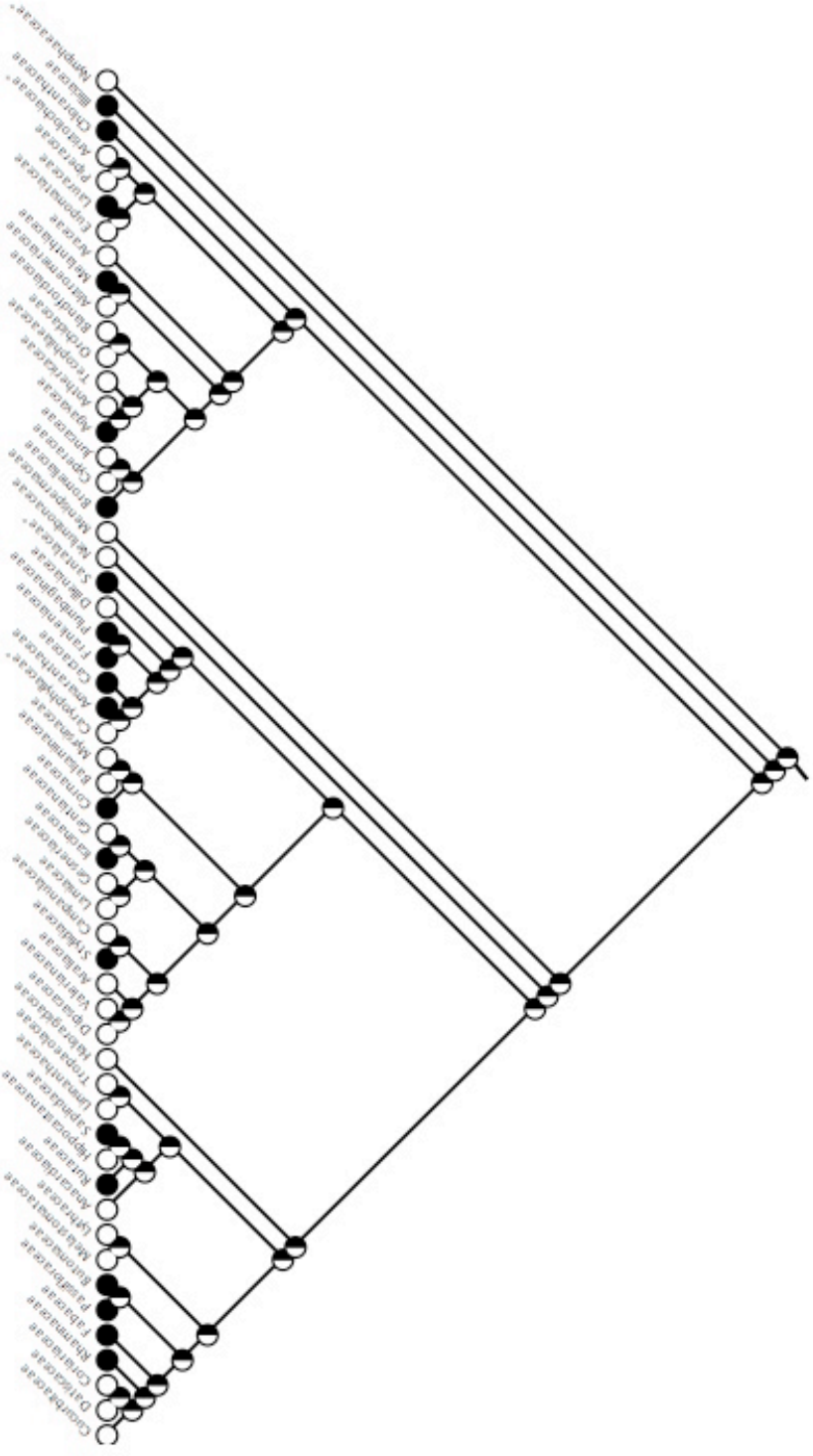


Figure 5.4: Trait-state reconstruction of self-incompatibility using likelihood ancestral reconstructions. Open circles are SC and closed SI. The proportional likelihoods of alternate ancestral reconstructions are indicated by the area shaded in each circle.

Table 5.2: Values and significance of the transition-rate parameters, estimated from the maximum-likelihood analysis, for the dependent model of trait evolution. Likelihood ratios greater than 3.81 indicate significant parameters in the dependent model and are indicated by solid lines in Figure 5.2.

trait transition: from/to	Parameter	LR
Protogyny & SC/protogyny & SI	q_{12}=2.01	4.13
Protogyny & SC/protandry & SC	q_{13}=0.14	3.83
Protogyny & SI/protogyny & SC	q21=10.69	6.56
Protogyny & SC/protogyny & SI	q_{24}=0.00	0.06
Protandry & SC/protogyny & SC	q_{31}=0.00	0.42
Protandry & SC/protandry & SI	q_{34}=0.22	8.41
Protandry & SI/protogyny & SI	q_{42}=0.18	0.35
Protandry & SI/protandry & SC	q_{43}=0.11	6.78
	q_{21}=q_{43}	3.47
	q_{12}=q_{34}	3.53
	q_{43}=q_{34}	0.89
	q_{21}=q_{12}	4.95

5.2.4 Pagel's maximum-likelihood analysis

The dependent model of trait evolution was significantly more likely than the independent model. For the independent model of uncorrelated trait evolution, the final likelihood was -65.66 with α_1=0.22, β_1=0.12, α_2=0.58 and β_2=1.01. The branch length scale (κ) was estimated as 0.003. The dependent model of correlated trait evolution had a final likelihood of -61.2 with κ=0.06 and estimated parameters given in Table 5.2. The likelihood ratio of the two models was 4.5 with P<0.05 as estimated from 1,000 Monte-Carlo simulations. Estimates and statistical tests of each transition rate for the dependent model are presented in Table 5.2 and Figure 5.2. Statistical tests of paired transition rates are given in Table 5.2. Analyses with the exclusion of the Lamiales and branch lengths set to 1 gave qualitatively similar results.

5.3 Discussion

This study is the first to apply current, phylogenetic comparative methods to the correlation between dichogamy and self-incompatibility. Based on the Soltis et al. (2000) phylogeny and an expanded database from Bertin (1993), I inferred ancestral states for both dichogamy and self-incompatibility and detected a significant correlation between these two characters.

5.3.1 Ancestral-state reconstructions

Protogyny was infered as the most likely ancestral state for the angiosperms in this analysis. Other than Willemstein's (1987) suggestion that protandry is the ancestral state of the angiosperms, followed rapidly by the evolution of protogyny, I am not aware of any family-level phylogenetic analysis of dichogamy. In addition, dichogamy appears quite labile with several transitions between protogyny and protandry. These transitions, coupled with the variation below the family level, suggest that dichogamy can respond to changing ecological circumstances.

Although the ancestral state of self-incompatibility was ambiguous in this analysis, SC was inferred as the ancestral state of the angiosperms in a previous phylogenetic analysis of 27 families or higher groups (Weller et al., 1995) using parsimony. Igic and Kohn (2001) inferred self-incompatibility for the majority of dicots based on RNases involved in the self-incompatibility reaction in the Scrophulariaceae *s. l.* , Solanaceae, and Rosaceae. Reconciling the results found here with Igic and Kohn (2001) requires that, although the genetic and molecular basis for self-incompatibility remains static, the phenotypic expression of self-incompatibility evolves to meet the ecological requirements of plant populations.

5.3.2 Character correlations

More important for our understanding of the functional significance of dichogamy, I found significant correlations between dichogamy and self-incompatibility. More specifically, the paired-comparison and maximum-likelihood methods showed that protandry was statistically associated with SI and protogyny with SC more often than expected under a random model. Similar results were found by Willemstein (1987) and Bertin (1993).

The flow diagram produced by the maximum-likelihood analysis (Figure 5.2) partitions the association between dichogamy and self-incompatibility further. The transition rates allow three tests of the hypothesis that protandry serves a function distinct from inbreeding avoidance. 1) The transition rate to SI should be greater in protandrous taxa than in protogynous taxa ($q_{34}>q_{12}$); 2) SI should evolve more frequently than SC in protandrous taxa $q_{34}>q_{43}$; and 3) Protandry should evolve more frequently in SI than in SC taxa ($q_{24}>q_{13}$).

Despite the overall correlation between SI and protandry, none of the three tests are satisfied by the transition-rate estimates. However, the structure of the transition diagram (in particular the q_{31}, q_{24}, and q_{42} parameters) suggests that protandry and SI is a common combination. Protandry may serve an important role in interference avoidance via effects on male function (Bertin, 1993; Harder and Barrett, 1995; Lloyd and Webb, 1986; Wyatt, 1983). To date the two experimental tests of this interference-avoidance hypothesis have also found support for a role of protandry in reducing between-flower interference and, consequently, enhancing male reproductive success (Harder et al., 2000, Chapter 2). Although selection for interference avoidance also explains the evolution of protandry in SC species (q_{13}), the evolution of both protandry and SI offers reductions in both inbreeding and interference. Protandry may also be favoured in SI species relative to SC species for its benefits in reducing the wastage of self pollen deposited within a flower or in other flowers on the same plant. In SI species, such pollen is effectively lost because self-fertilization is impossible, whereas in SC species such pollen is not necessarily lost because it may sire some selfed progeny. Consequently the benefits in reducing the deleterious impacts of interference on male function may be greater in SI than in SC species. The flow diagram also highlights an apparent constraint in dichogamy transitions in SI taxa (q_{24} and q_{42}). The causal factors underlying this constraint are unclear and warrant further investigation.

Analogous tests can be made for the hypothesis that protogyny serves a role in inbreeding avoidance. 1) SC should evolve more frequently in protogynous than in protandrous taxa ($q_{21}>q_{43}$); 2) SC should evolve more frequently than SI in protogynous taxa ($q_{21}>q_{12}$); and 3) Protogyny should evolve more frequently in SC than in SI taxa ($q_{31}>q_{42}$). The loss of SI in protogynous taxa (q_{21}) is the largest transition rate in the model, supporting the first two tests and the inbreeding-avoidance hypothesis for the evolution of protogyny (Lloyd and Webb, 1986) (see Griffin et al. (2000)

for an experimental test of this hypothesis). The loss of SI in protogynous species could be the result of several causes in addition to redundancy of inbreeding avoidance. One possibility is that the detrimental effects of self-pollination are greater in SI than SC species. This could arise as a result of losses to either male or female function. On the male side, the deposition of self pollen in an SI species represents a complete loss of potential male success for this pollen but the same is not true in SC species. On the female side, reproductive losses in SI species could also be greater than losses in SC species if self pollen on the former triggers an SI reaction that disrupts the growth of compatible pollen tubes, the fertilization process for compatible pollen, or the development of ovules or fruits. In fact, several studies have shown that SI species experience a substantial reduction in seed set when cross-pollination is preceded or accompanied by self-pollination (e.g., Ockendon and Currah, 1977; Waser and Price, 1991).

The loss of SI in protandrous families (q_{43}) was unexpected. The detrimental effects of SI discussed for protogynous species may also be relevant for protandrous species. Alternatively, protandry may reduce both inbreeding and interference, as in *Eichhornia paniculata* (Pontederiaceae) (Harder et al., 2000), making SI redundant. This dual function could also explain the lack of support for the three transition-rate parameter tests. However, in *Chamerion angustifolium* protandry had no effect on the frequency of self-fertilization (Chapter 2). An alternate hypothesis for the loss of SI in either protandrous or protogynous taxa is reproductive assurance (Baker, 1955; Darwin, 1876). This is particularly true for protogynous species in which self-pollination is likely to occur after a period of stigma receptivity (delayed selfing; Lloyd and Schoen, 1992). This hypothesis is consistent with the transition from SI to SC in protogynous species (described above). However, experimental tests of reproductive assurance are rare (Herlihy and Eckert, 2002) and this hypothesis cannot be properly judged with current evidence. An association between reproductive assurance and protogyny would be useful for testing this hypothesis.

Much ecological variation and variable selection pressures are contained within the flow diagram of Figure 5.2. For example, life history and floral attributes or pollinator availability and composition can alter the form of dichogamy favoured or the expected relationship between dichogamy and self-incompatibility. Pollinators with negative geotactic foraging behaviour might impose selection for protogyny and SI to reduce interference and inbreeding, respectively. Although these potential ecological correlates are

not included in this analysis, strong associations between dichogamy and self-incompatibility were detected. This does not suggest that ecology is irrelevant. Rather, at broad taxonomic scales I can resolve some general trends in the association between dichogamy and SI. These correlations now require experimental tests conducted at ecological scales (e.g., Griffin et al., 2000; Harder et al., 2000, Chapter 2; see also Baum and Larson 1991).

5.3.3 Comparison with Bertin 1993

Incorporating phylogeny produced a mixture of confirmation and refinement of the earlier results of Bertin (1993). The congruence between this study and the earlier ahistorical ones suggest that these results are biologically significant. The primary conclusion of Bertin (1993) that protandry does not play a strong role in inbreeding avoidance still holds. In addition, the analyses further supported the role of protogyny in inbreeding avoidance. Perhaps more significantly, the rate parameters of the likelihood model suggest specific experimental tests that could be performed on the relationship between dichogamy and self incompatibility. These experiments are important for moving from the correlations uncovered here and in Bertin (1993) to more explicit causal mechanisms for the correlated evolution of dichogamy and self-incompatibility. A final enhancement provided by the likelihood analysis is an estimate of the tempo at which dichogamy and self incompatibility evolve. The low magnitudes of the most-likely branch length scalings and multiple transitions in the phylogeny suggest that these two traits can evolve rapidly and may be associated with speciation events. This is also supported by the fact that many families and genera contain combinations of protandry, protogyny, and adichogamy (Bertin and Newman, 1993). This tempo, coupled with their functions, suggests that dichogamy and self-incompatibility may be capable of responding to changes in ecological circumstances and play an important role in floral function.

Chapter 6

Modelling the evolution of male-phase duration

The previous four chapters have investigated functional, genetic, and phylogenetic aspects of the evolution of dichogamy. In this chapter, I attempt to consolidate the previous discussion into a conceptual framework for considering the evolution of male-phase duration. In particular, I explore the following questions:

1. Are there conditions in which protandry is not adaptive? My results so far strongly demonstrate the adaptive significance of protandry. However, clearly many plants are not protandrous (Bertin and Newman, 1993; Faegri and van der Pijl, 1979; Lloyd and Webb, 1986). Developing a conceptual model allows me to investigate the fitness landscape for different combinations of floral attributes and determine the boundaries of protandry as an adaptation.

2. How does floral display size influence the evolution of dichogamy? Chapter 2 described an unexpected influence of floral display size on the functional significance of protandry. In that chapter I speculated that pollinator behaviour might explain the unexpected siring advantage in two-flowered inflorescences. I incorporate this idea into the model and test its efficacy as an explanatory parameter.

3. How much gender separation is required? In Chapter 4 I suggested treating dichogamy as a continuous trait rather than the dichotomy of protandry or protogyny. Here I model protandry as a continuous

function of male-phase duration and explore the consequences for the adaptive significance of protandry.

4. Does the fixed flower lifespan of *Chamerion angustifolium* constrain or enhance the evolution of protandry? The trade-off between male- and female-phase duration was a surprising result of Chapter 4, but is it significant for explaining the adaptive significance of dichogamy? I construct two variants of the model, one based on a flexible flower lifespan and the other on a fixed lifespan. If the variants differ in their fitness consequences, the flexibility of floral lifespan may be relevant for the evolution of dichogamy.

6.1 The Model

Before describing the details of the model, there are some general considerations to mention. Although my aims are distinct, this model is inspired by work done by Harder and Wilson (1998) and Sato (2002). The model in this chapter is fairly simple. However, I believe that it captures some important aspects of the evolution of protandry without resorting to undue complexities. As a consequence of this simplicity, the model makes some basic assumptions that should be described at the outset. First, I assume that these plants have synchronous dichogamy (e.g., Bhardwaj and Eckert, 2001; McDade, 1986), so that every flower on the plant is simultaneously male or hermaphroditic. Second, all self-fertilization results from prior, non-competing selfing (Lloyd and Schoen, 1992). Third, there is no pollinator limitation (Larson and Barrett, 2000) so that all ovules are sired by either outcross or self pollen. Fourth, no pre-zygotic effects (e.g., pollen-tube competition (Bertin, 1990)) are included. Finally, I assume that protandrous and adichogamous plants differ only in their male-phase duration and have no inherent differences in floral display size, pollinator attraction, or inbreeding depression.

Figure 6.1 is a diagram of the model and its parameters. The model is founded on a common resource limitation for all plants. Plants allocate their fixed resources to flower production and pollen production, resulting in a trade-off between the size of a floral display and the amount of pollen produced by the display. The cost of a flower is weighted by the length of time the flower is open to account for respiration, nectar, and maintenance

costs. The available pollen is then presented during an initial male phase. The amount of pollen removed during this phase is largely a consequence of display size, such that large displays receive more visits. Any pollen remaining after the male phase is then removed during the hermaphroditic phase. In the hermaphroditic phase, pollinator attraction is again a function of display size. However, in the presence of female function, pollen export can be compromised by both within- and between flower interference to produce self-fertilization and pollen discounting. The fitness of any given plant phenotype (i.e., a specific floral display size and male-phase duration) is the sum of its reproductive contributions through outcrossing and selfing. Outcross contributions result from successful pollen export through pollen or import through ovules and must be corrected for the number of gene copies transmitted to the offspring (i.e., 1/2, Fisher, 1930; Morgan and Schoen, 1997). Selfed contributions necessarily occur through both pollen and ovules, but are weighted by the strength of inbreeding depression (Charlesworth and Charlesworth, 1987; Darwin, 1876; Husband and Schemske, 1996). Comparing this fitness measure between plants with different phenotypes estimates the conditions in which a phenotype is favoured. In particular, I compare plants with a separate male phase (protandrous) to plants with only a hermaphroditic phase (adichogamous). There are two variants of the model. The first considers variable flower longevity, such that total floral longevity is not intrinsically constrained. In this variant, only the resource costs of extended flower longevity, and its associated trade-off with pollen production, constrain floral lifespan. The second variant fixes floral longevity so that male-phase duration is negatively correlated with female-phase duration (Chapter 4).

6.1.1 Variable flower longevity

I begin by modelling the trade-off between resource allocation to male-phase duration (ψ: the proportion of time spent in male phase) and to pollen production (P_0: the total number of pollen grains produced) as a linear function of floral display size (F: the number of open flowers):

$$P_0 = 100 - F(\psi + 1) \tag{6.1}$$

In this equation, 100 represents the total resources available for flower and pollen production. Hermaphroditic phase duration is fixed as 1 and male-phase duration is a fraction of female phase. Consequently, ψ is bounded

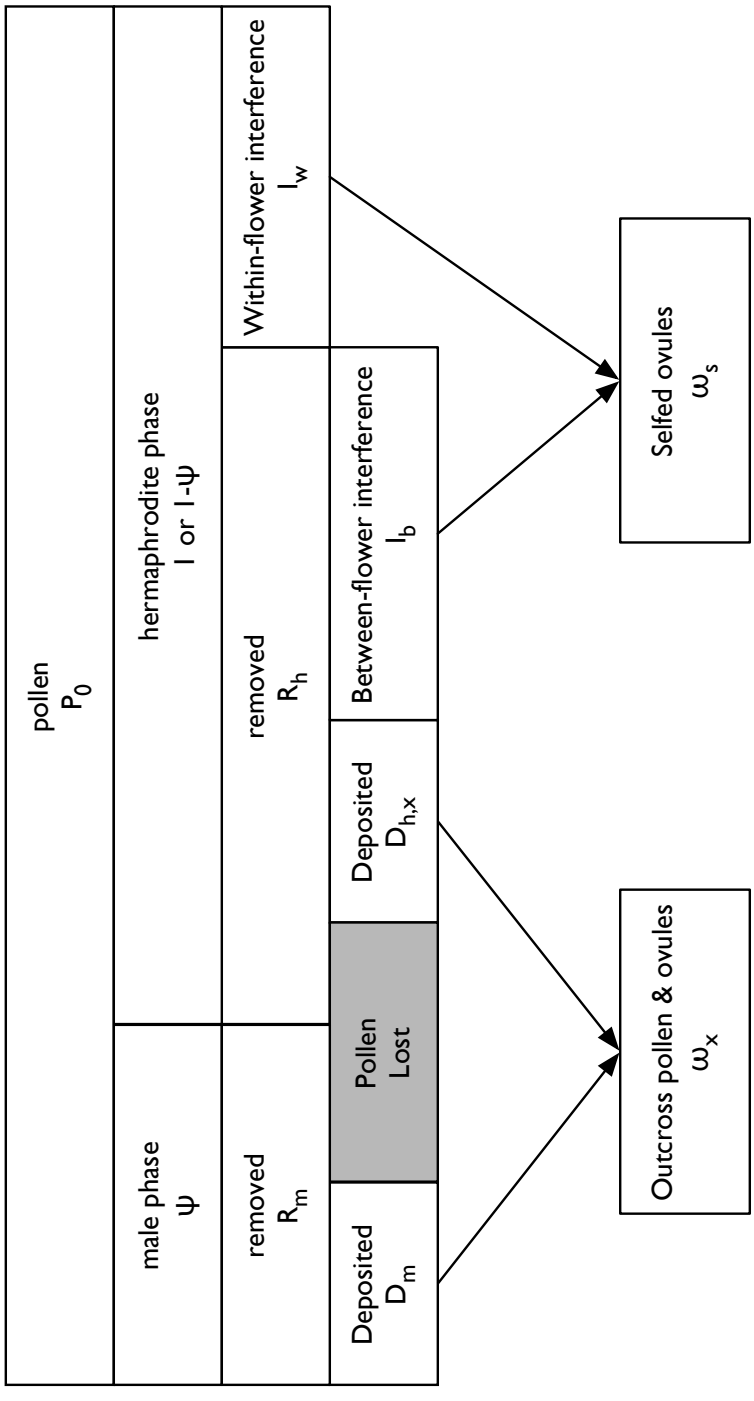


Figure 6.1: A diagrammatic representation of the model. Hermaphroditic-phase duration is dependent on which variant is considered. Although the pollen lost (shaded area) is not explicitly included in the model, it is a consequence of the difference between pollen removal and deposition. The fitness of a phenotype is the sum of its contributions through outcrossing and selfing.

by 0 and 1, and total flower longevity is 1+ ψ. A parameter for converting resource allocation to the number of pollen grains produced could be incorporated into equation 6.1. However, since I will be comparing relative fitness, such a parameter would cancel out of the fitness calculations and I do not include it here. Rather proportional allocation to pollen production is used as a proxy for pollen number.

Analogous to the empirical results in Figure 2.1 of Chapter 2, pollinators visit each plant as a logarithmic function of floral display size (Figure 6.2):

$$V = \log F \tag{6.2}$$

An alternative approach to considering inflorescence-level visitation is to model the visitation frequency of an individual flower. Such an approach would add additional complexity to the model without a corresponding increase in insights. The important aspect of visitation is that it is related to total floral display size, but as a negatively exponential function. Considering pollination at the inflorescence level provides a level of abstraction that focuses on the plant's total reproductive success, rather than its component flowers considered individually.

Male phase

Since male phase begins anthesis, all of the pollen produced (P_0) is available at the beginning of male phase. Pollen removal during male phase (R_m) is then calculated as:

$$R_m = xV\psi \tag{6.3}$$

where x is the portion of pollen removed during a single pollinator visit and is set to 0.1. Pollen deposition during male phase (D_m) is then Equation 6.3 multiplied by the fraction of this pollen deposited on stigmas (z), also set to 0.1:

$$D_m = zR_m \tag{6.4}$$

Consequently, pollen removal during male phase is correlated with both the duration of male phase and with floral display size, through its effect on pollinator attraction. Large floral displays with extended male phase will export much of their pollen before the hermaphroditic phase begins.

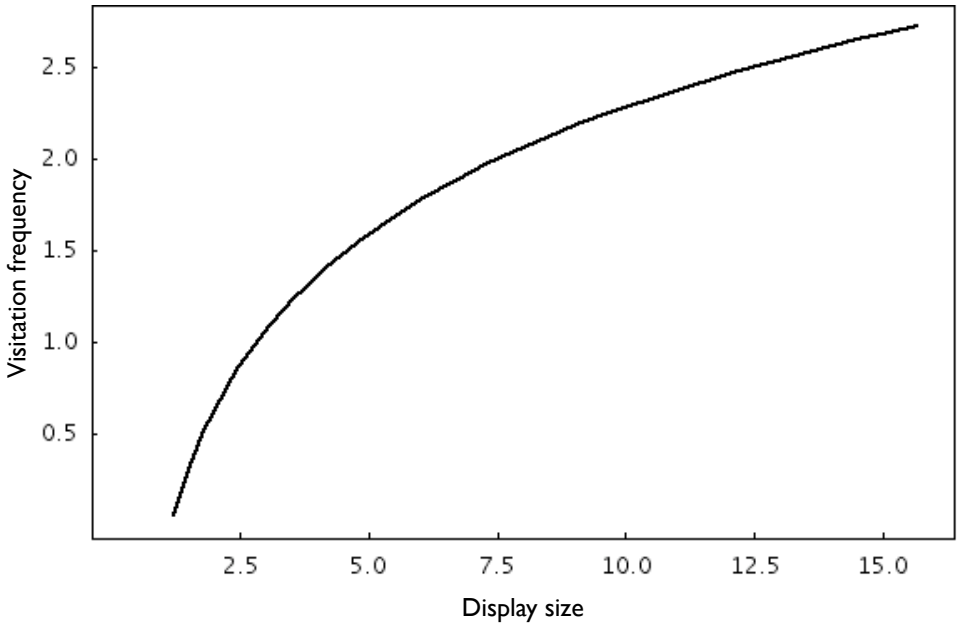


Figure 6.2: The relationship between floral display size and pollinator visitation used to model pollen removal.

Hermaphroditic phase

During the hermaphroditic phase, any pollen removed may be redeposited within a flower (within-flower interference), deposited within the plant (between-flower interference), exported to another plant (outcrossing), or lost from the system (e.g., through pollinator grooming). After male phase, some proportion of the initial pollen has been removed. I calculated the pollen available during hermaphroditic phase as:

$$P_h = 1 - R_m, \tag{6.5}$$

and created two interference parameters, within-flower interference (I_w, Figure 6.3) and between-flower interference (I_b, Figure 6.4) as:

$$I_w = zV, \tag{6.6}$$

and

$$I_b = xzVF. \tag{6.7}$$

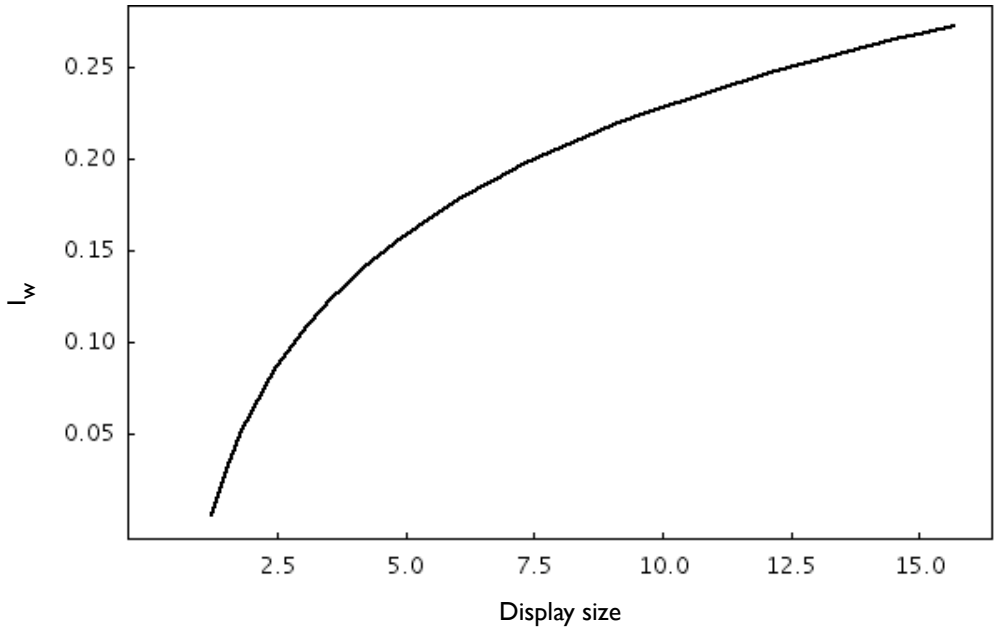


Figure 6.3: Within-flower interference increases as a decelerating function of floral display size.

Consequently, within-flower interference increases as a declining function with floral display size as discussed in Chapter 2. Between-flower interference is similar to within flower, except that the deposition fraction (z) is included and the interference increases with floral display size (F).

The removal of pollen during the hermaphroditic phase is partitioned into outcross and selfed components. The removal of pollen during hermaphroditic phase that is apportioned to outcrossing ($R_{h,x}$) is:

$$R_{h,x} = P_h(1 - I_w - I_b) \tag{6.8}$$

In words, it is the amount of pollen remaining after male phase that is not lost due to either interference or pollinator grooming. This pollen removed is then multiplied by the deposition fraction z to calculate the proportion of pollen deposited on outcross stigmas during the hermaphroditic stage ($D_{h,x}$).

Self-pollen deposition during the hermaphroditic stage ($D_{h,s}$) is:

$$D_{h,s} = P_h(I_w + I_b), \tag{6.9}$$

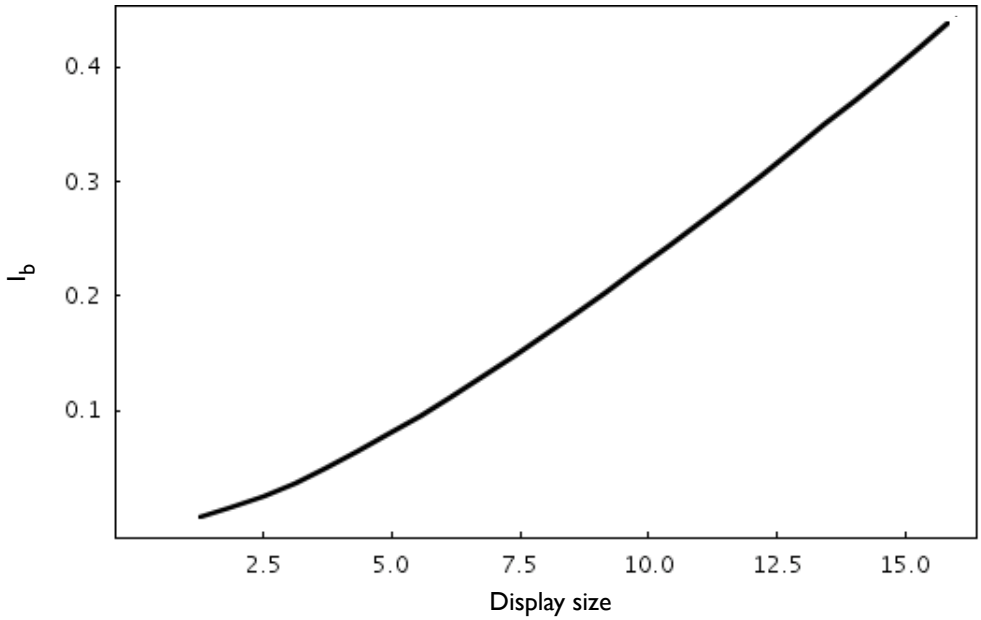


Figure 6.4: Between-flower interference increases with floral display size.

which is the pollen deposited by both forms of interference.

Fitness calculations

Next, I tally the pollen and ovule contributions from selfing and outcrossing and weight them by their fitness consequences. For fitness contributions from selfing this is:

$$\omega_s = (1 - \delta) D_{h,s}, \tag{6.10}$$

where δ is the relative fitness of selfed vs. outcross progeny (inbreeding depression; Charlesworth and Charlesworth, 1987; Darwin, 1876; Husband and Schemske, 1996).

When an individual plant successfully outcrosses through either pollen grains or ovules, it contributes only half of the genetic material of the resulting offspring (Fisher, 1930; Morgan and Schoen, 1997), which must be considered in the fitness calculations. Consequently, fitness contributions

through outcrossing are:

$$\omega_x = \frac{D_m + D_{h,x} + (1 - D_{h,s})}{2} \tag{6.11}$$

The total fitness of an individual is then the sum of these two contributions, $\omega_s + \omega_x$. I then calculate a fitness measure of a protandrous plant (ω_p) with $\psi > 0$ relative to an adichogamous plant (ω_a) with $\psi = 0$ as:

$$W = \frac{\omega_p}{\omega_a} \tag{6.12}$$

This relative fitness measure is then used to explore the circumstances under which protandry is favoured over adichogamy (i.e., when $W > 1$).

6.1.2 Fixed flower longevity

The fixed-anthesis model is very similar to the flexible-anthesis model, so I will only describe the changes. Hermaphroditic-phase duration in equation 6.1 is changed to:

$$P_0 = 100 - F(\psi + 1 - \psi), \tag{6.13}$$

so that male- and female-phase durations are perfectly, negatively correlated (as in Chapter 4) and total anthesis sums to 1.

Removal during hermaphroditic phase (6.8) becomes:

$$R_{h,x} = (1 - \psi)P_h(1 - I_w - I_b), \tag{6.14}$$

and deposition (6.9) becomes:

$$D_{h,s} = (1 - \psi)P_h(I_w + I_b), \tag{6.15}$$

6.2 Results

6.2.1 Variable flower longevity

Due to fixed resource availability, resource allocation to pollen production declines with increases in either floral display size or male-phase duration (Figure 6.5) to reach a minimum of ~70% at 16 flowers and $\psi = 1$. Although extended male phase reduces total pollen production, when male phase is

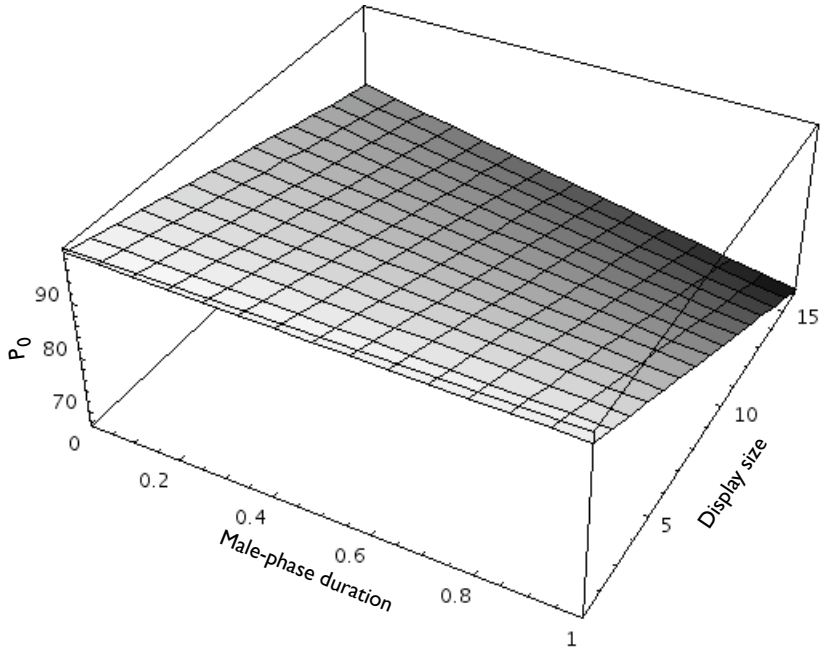


Figure 6.5: The proportion of resources allocated to pollen production as a function of floral display size and male-phase duration for variable flower longevity.

extended it receives much of the pollinator activity and pollen is successfully exported without interference from female function (Figure 6.6a). This concentration of pollination during the male phase is associated with a reciprocal change in the amount of pollen available during the hermaphroditic stage (Figure 6.7) and, consequently, pollen deposition during the hermaphroditic phase (Figure 6.6b). These effects cause a decrease in selfed ovule production (Figure 6.6c) with increasing male-phase duration and a corresponding increase in outcross ovule production (Figure 6.6d).

The fitness consequences of shifts in pollinator activity between male and hermaphroditic phases is largely determined by the strength of inbreeding depression (Figure 6.8). Reproduction through outcrossing avoids the fitness reductions produced by inbreeding depression. However, more pollen is lost through this process due to the deposition fraction (z). Although pollen removal during the hermaphroditic phase may be involved

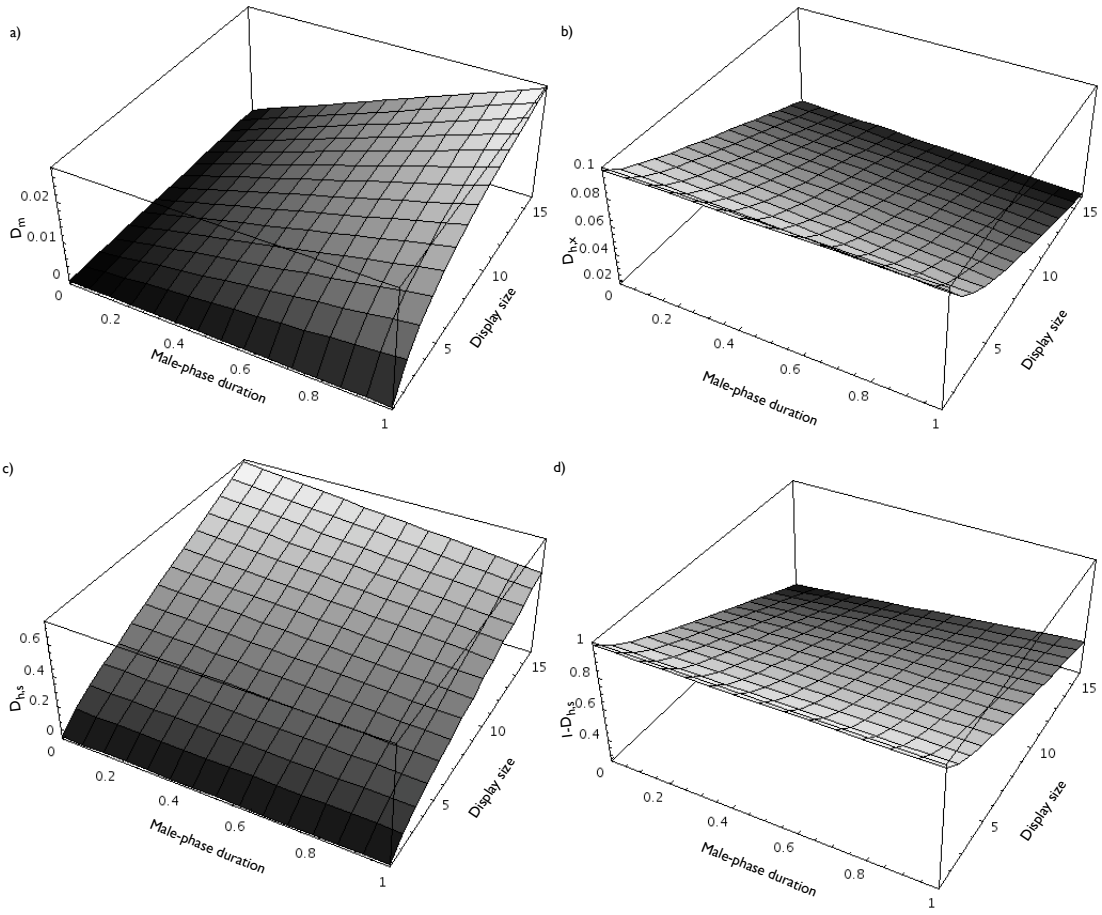


Figure 6.6: Deposition of pollen in male and hermaphroditic phases at different display sizes and male-phase durations for variable flower longevity. a) The portion of pollen successfully exported during male phase, b) Pollen export during hermaphroditic phase, c) The production of selfed ovules, and d) The production of outcrossed ovules.

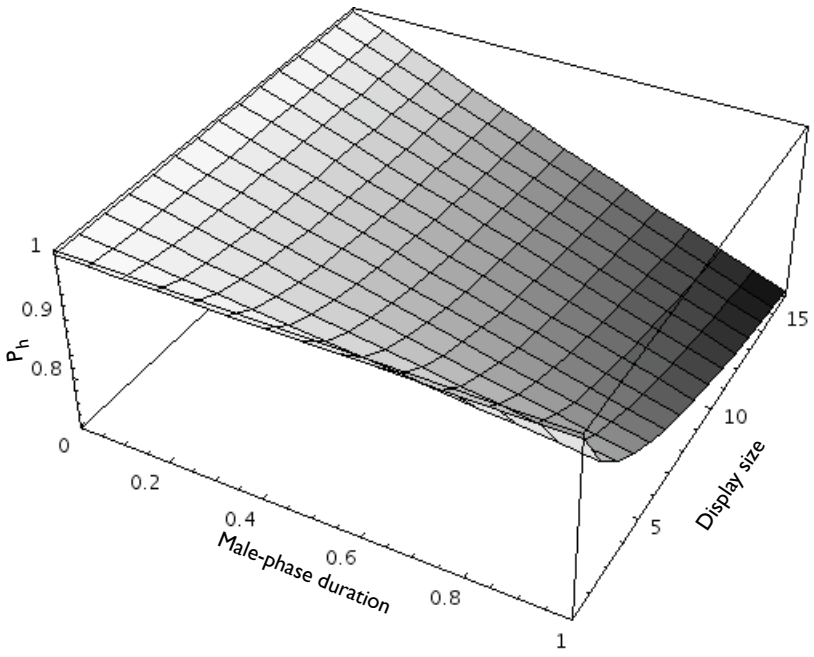


Figure 6.7: Pollen remaining after male phase for variable flower longevity.

in interference, if inbreeding depression is sufficiently low, more pollen may successfully fertilize ovules.

As a consequence of these pollination dynamics, protandrous plants have a higher relative fitness at large floral display sizes and with high inbreeding depression (Figure 6.8). Large floral displays maximize the frequency of interference (either within- or between-flower) and high inbreeding depression maximizes the negative consequences of interference. Exporting pollen during the male phase in protandrous plants avoids interference and maximizes outcross siring success.

6.2.2 Fixed flower longevity

Unlike with variable flower longevity, pollen production declines only with floral display size when flower longevity is fixed (Figure 6.9) to reach a minimum of ∼80% at 16 flowers. As with variable flower longevity, when flower longevity is fixed, pollen deposition during male phase increases with both

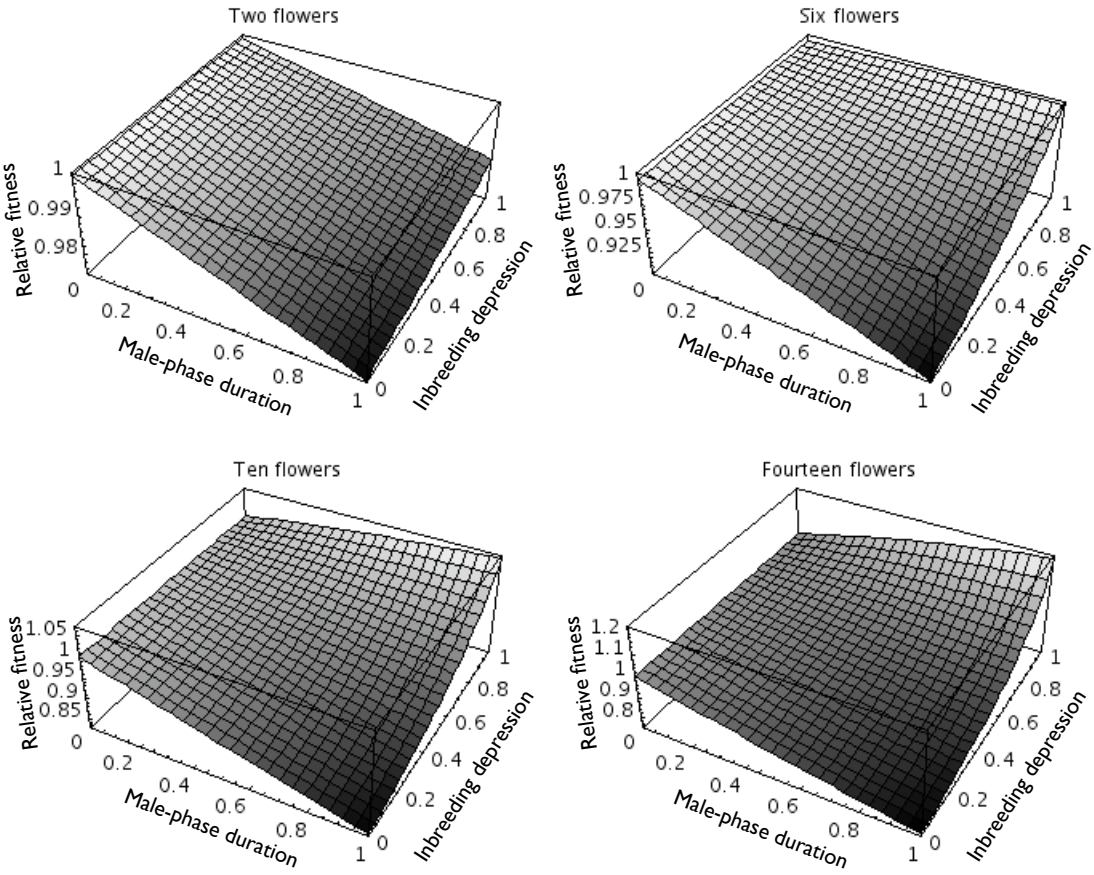


Figure 6.8: Relative fitness landscapes of protandry for variable flower longevity. Each panel shows the fitness of protandrous relative to adichogamous plants at different combinations of inbreeding depression and male-phase duration. The effect of increasing display size is shown across the panels from two to fourteen flowers.

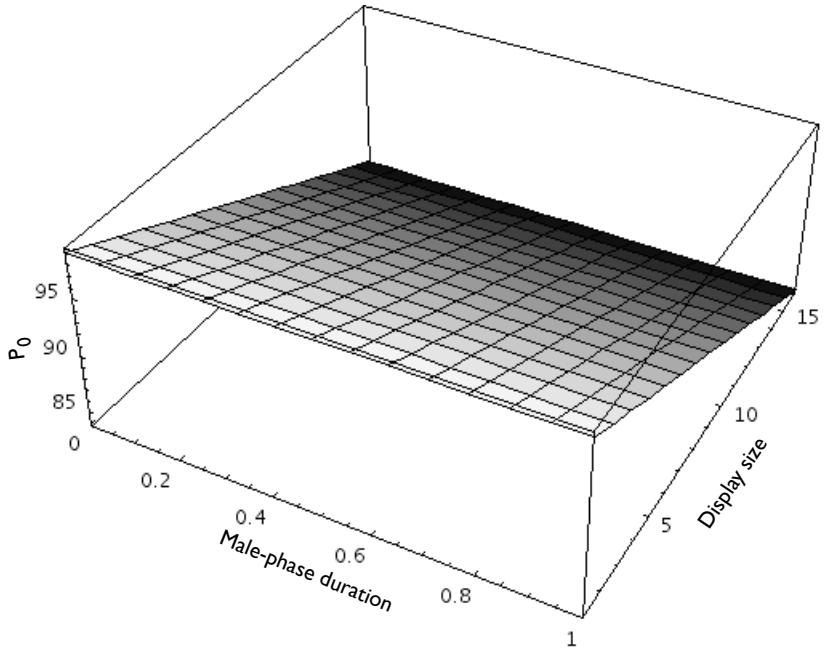


Figure 6.9: Pollen production as a function of floral display size and male-phase duration for fixed flower longevity.

floral display size and male-phase duration (Figure 6.10a) which is also associated with a reciprocal decrease in the amount of pollen remaining for hermaphroditic phase (Figure 6.11). Outcross, hermaphroditic pollen deposition decreases with floral display size (Figure 6.6b) as pollen is removed during male phase. Outcross ovule production (Figure 6.6c) is high over much of the parameter space, except for large displays with short male-phase duration. Hermaphroditic, self-pollen deposition is reciprocal to outcross-ovule production (Figure 6.6d).

When flower longevity is fixed, there is a much wider range of parameters at which protandrous plants have a higher fitness than adichogamous plants (Figure 6.12). This is a consequence of the removal of the reduction in pollen production with increasing male-phase duration.

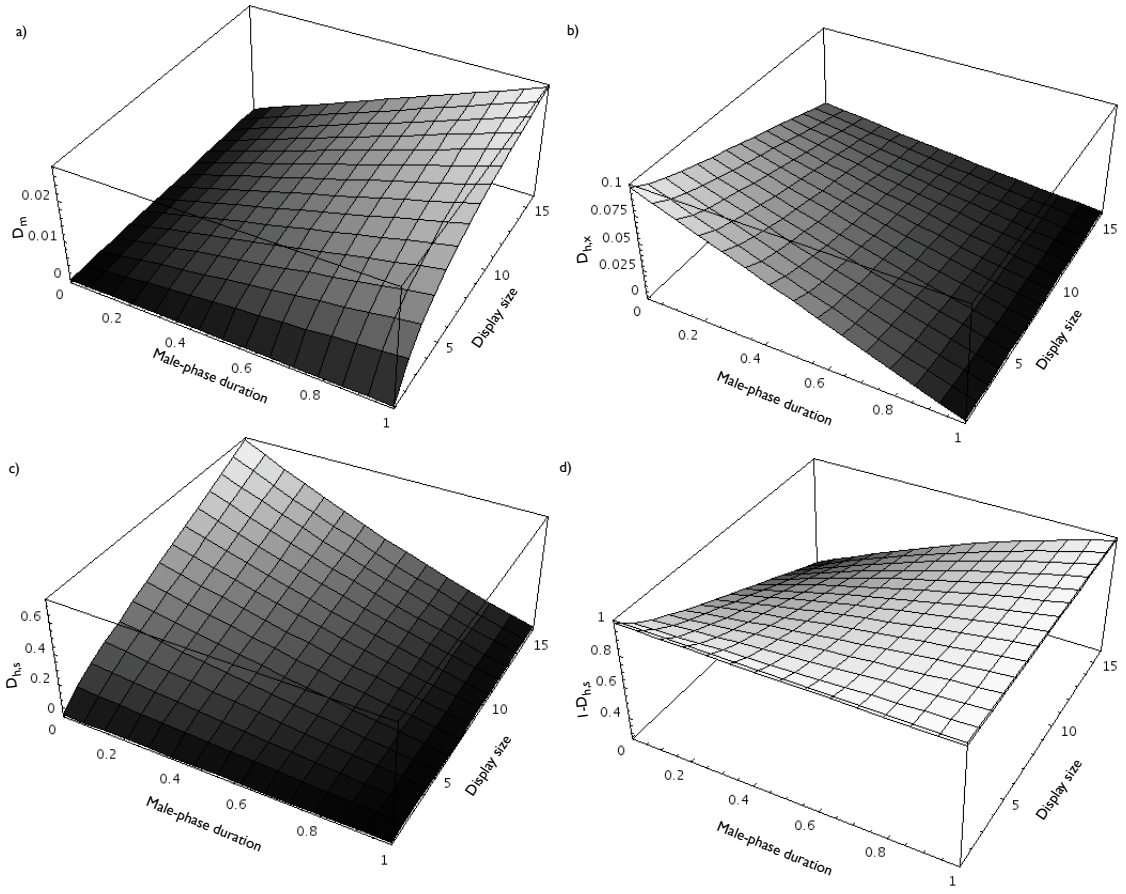


Figure 6.10: Deposition of pollen in male and hermaphroditic phases at different display sizes and male-phase durations for fixed flower longevity. a) The portion of pollen successfully exported during male phase, b) Pollen export during hermaphroditic phase, c) The production of selfed ovules, and d) The production of outcrossed ovules.

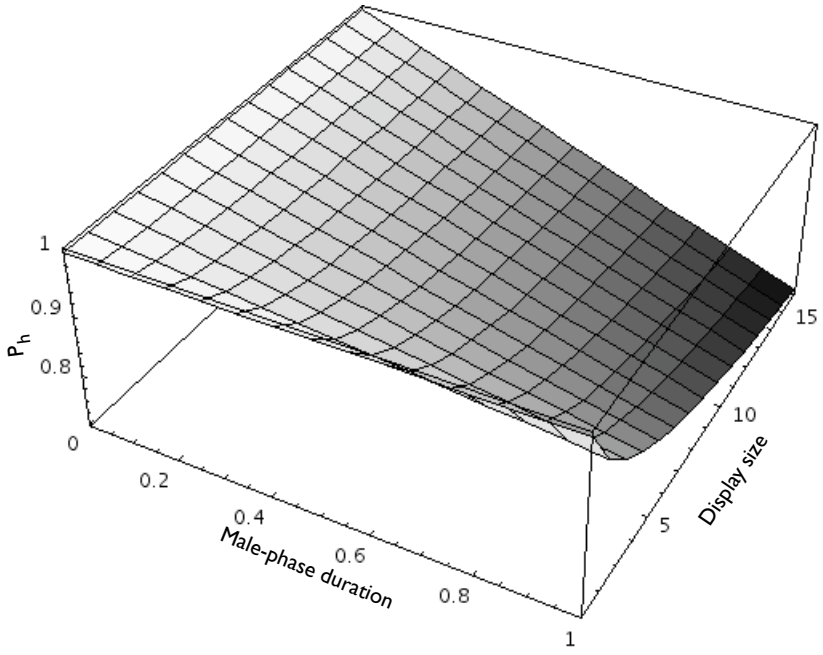


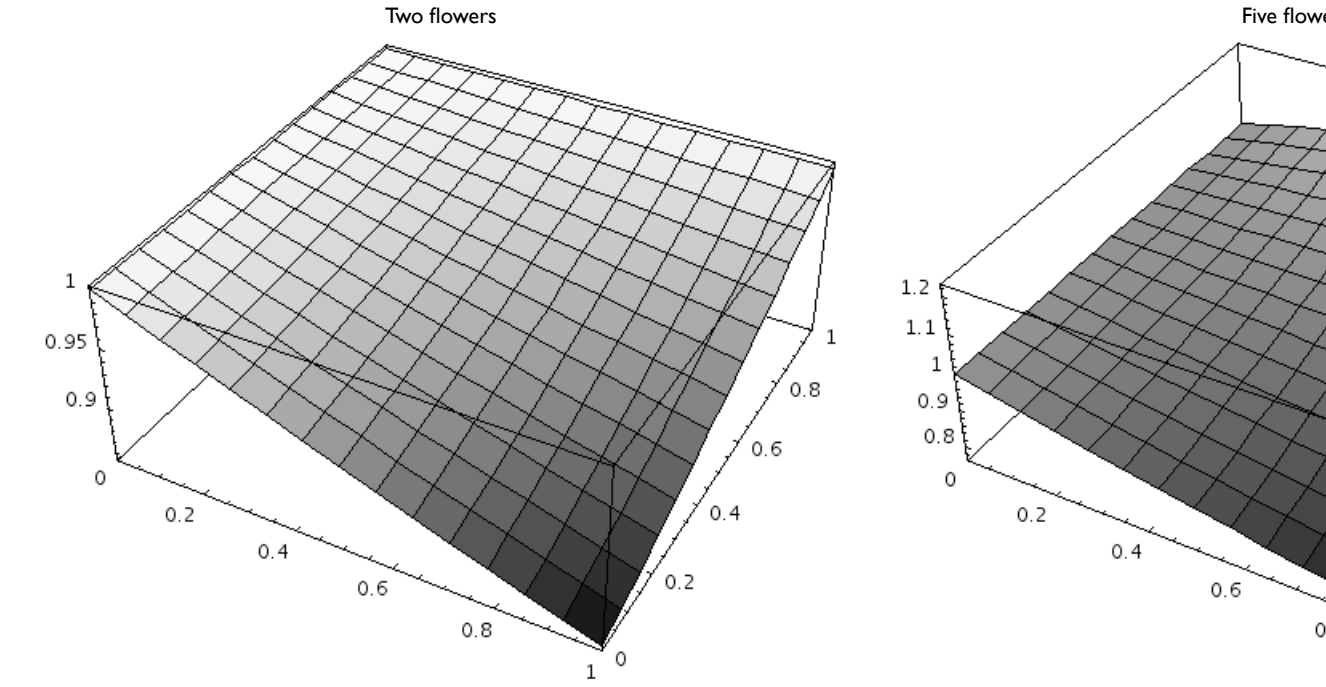
Figure 6.11: Pollen remaining after male phase for fixed flower longevity.

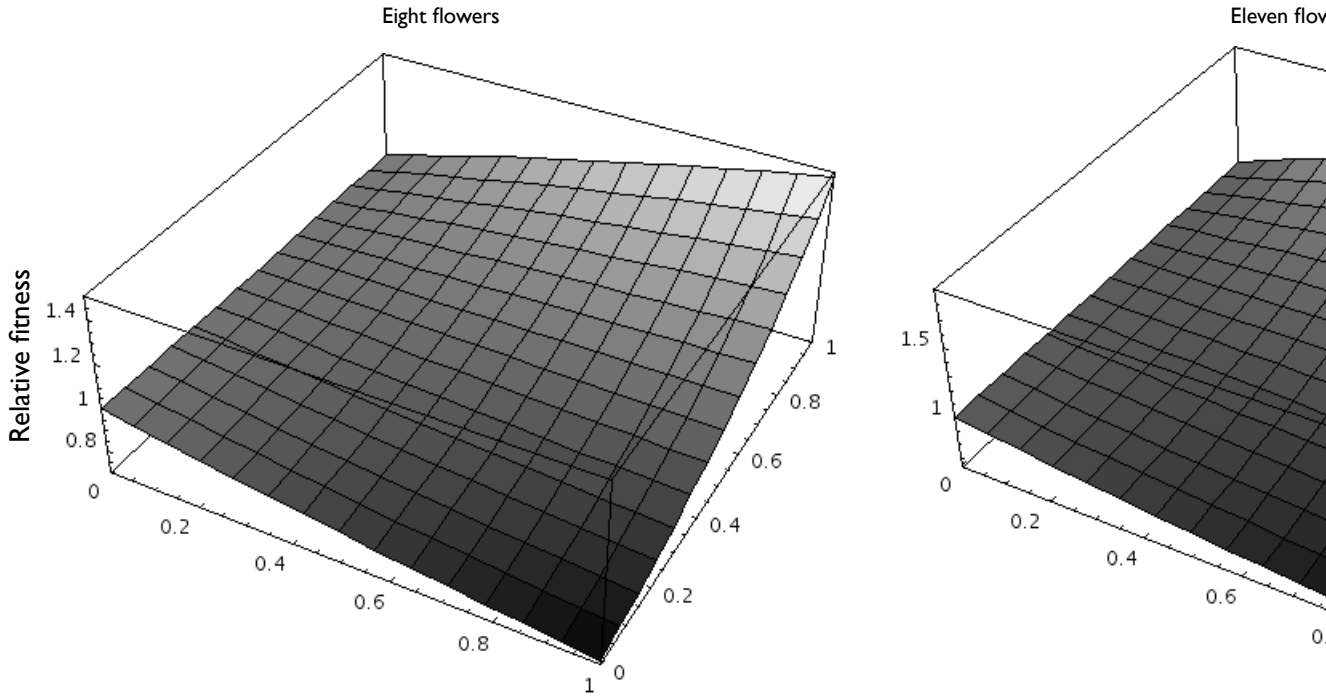
6.3 Discussion

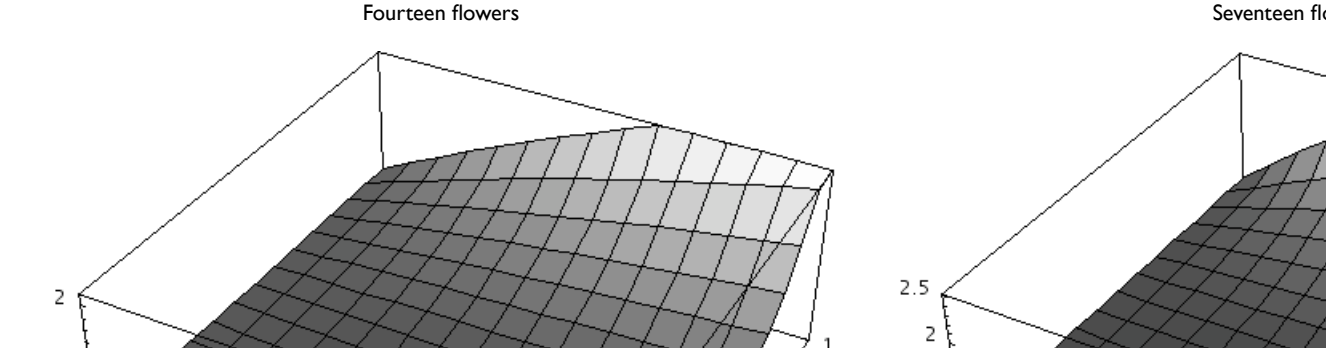
Relatively simple considerations of pollen movement produced a dynamic fitness landscape for male-phase duration. The results of this conceptual modelling are relevant to the questions posed in the Introduction of this chapter, which I consider below.

6.3.1 Relative fitness of protandry

The fitness of any particular phenotype in this model is a combination of reproduction through selfing and outcrossing. Selfing has two primary benefits. The first is the well known transmission advantage of selfers (Fisher, 1930; Morgan and Schoen, 1997). In this model, a self-fertilization event contributes two genomes to the next generation, one through pollen and the other through seed. However, an outcrosser only contributes a single genome to any fertilization event, either through pollen or seed. The second benefit of selfing is that within-flower interference can maximize pollen


Two flowers
Five flowe
1
0.95
0.9
0
0.2
0.4
0.6
0.8
1
0
0.2
0.4
0.6
0.8
1
1.2
1.1
1
0.9
0.8
0
0.2
0.4
0.6


Eight flowers
Eleven flow
Relative fitness
1.4
1.2
1
0.8
0
0.2
0.4
0.6
0.8
1
0
0.2
0.4
0.6
0.8
1
1.5
1
0
0.2
0.4
0.6


Fourteen flowers
Seventeen flo
2
2.5
2

deposition, relative to outcrossing or geitonogamy, by avoiding pollen loss (expressed by z, the deposition fraction). Counteracting these two benefits of selfing is inbreeding depression, a classic determinant of plant fitness (Darwin, 1876; Fisher, 1930; Husband and Schemske, 1996; Lande and Schemske, 1985; Schemske and Lande, 1985). In comparison to selfing, reproduction through outcrossing does not suffer from inbreeding depression. However, more pollen may be required for outcrossing to realize the same number of fertilizations as selfing, due to the increased possibility of pollen loss. Considering these fitness effects, I can address the relative fitness of protandry for various plant phenotypes.

Clearly there are many areas of parameter space in which protandry is not advantageous. In particular, the relative fitness of protandry is largely a function of inbreeding depression and floral display size. When inbreeding depression is high, long male-phase duration is favoured because it provides greater opportunity for pollen to be removed in the absence of female function. This maximizes the amount of pollen contributed to outcrossing. However, when inbreeding depression is low, both the transmission bias of selfing and, to a lesser extent, pollination efficiency favour adichogamous plants.

Large floral displays also favour the evolution of protandry, which is consistent with suggestions by Harder and Barrett (1995, 1996) and Harder et al. (2000), as well as the results of Chapter 2. Large inflorescences attract more pollinators (Bell, 1985; Geber, 1985; Klinkhamer and de Jong, 1990; Queller, 1983; Schemske, 1980; Schmid-Hempel and Speiser, 1988) which can rapidly remove most of the available pollen during the male phase and increase outcross siring-success. Furthermore, during the hermaphroditic phase large inflorescences experience greater between-flower interference which reduces outcross siring-success due to pollen discounting even in the absence of inbreeding (Harder et al., 2000, Chapter 2). This can be particularly acute in adichogamous plants which have no capacity for interference avoidance. However, the siring advantage of protandrous plants begins to asymptote at large floral display sizes (e.g., 17 flowers, Figure 6.12 bottom-right panel). This occurs once most of the pollen is removed during male phase and no benefits are realized by further extensions of male phase. Unlike the fixed pollinator attraction and pollen removal functions of this model, Harder and Thomson (1989) explored the consequences of modifying these parameters for pollen export. They found that plants should modify the amount of pollen available at any given time to match pollinator activ-

ity. Incorporating this capability into this model would enhance the siring success of protandrous plants, since the plants would allocate more pollen to male phase to enhance pollen export.

The interaction between the effects of inbreeding depression and floral display size produces several fitness peaks and valleys for the evolution of protandry (Figure 6.8 and Figure 6.12). Regardless of the costs of inbreeding, protandry is not favoured in small inflorescences. Small inflorescences have more resources for pollen production and, in this model, suffer from modest within- and between-flower interference (but see Chapter 3). Although protandrous plants with small floral displays export more pollen than adichogamous plants, much of this exported pollen is lost and does not contribute to reproductive success. With between five and eight flowers (depending on which model variant is considered) protandry offers enhanced fitness when inbreeding depression is high. At these floral display sizes, between-flower interference begins to accumulate and, coupled with the high inbreeding depression, begins to discount both ovules and pollen. This siring advantage of protandry continues to increase with floral display size. However, even at large floral display sizes (i.e., 17 flowers), adichogamy is still favoured with low inbreeding depression. As described earlier, there is an efficiency to within-flower interference that can favour selfing in the absence of counteracting inbreeding depression.

6.3.2 Floral display size and pollinator behaviour

Modelling pollinator behaviour so that within-flower interference increases rapidly initially with floral display size did not produce the parabolic siring success curve of Figure 2.3. This may be a consequence of the synchronous dichogamy assumption. In Chapter 2, I argued that within-flower interference declined with floral display size in adichogamous plants, while remaining constant in protandrous plants. In addition, both protandrous and adichogamous plants experienced increasing between-flower interference with increasing floral display size. In this model, protandrous plants only experience interference during the hermaphroditic phase. This issue would best be addressed through empirical studies of the influence of display size on within-flower interference. Although several studies have explored the influence of floral display size on within-plant pollen transfer (e.g., Klinkhamer and de Jong, 1990; Rademaker and de Jong, 1998; Robertson, 1992), I am

not aware of any studies of the relationship between floral display size and within-*flower* pollen transfer.

6.3.3 Protandry as a continuous trait

Treating protandry as a continuous trait demonstrated that the duration of male phase can affect the fitness of protandry. In particular, long male phases imposed a greater resource cost with variable flower longevity which reduced the plant's pollen production. As discussed above, this resource cost was readily compensated for by benefits of long male phase with high inbreeding depression and large display sizes. Overall, the resource costs imposed by extended male phase were relatively modest. This is almost exclusively a consequence of the model parameters. Raising the cost of maintaining a flower would reduce the range of parameters in which the relative fitness of protandry is greater than adichogamy. True allocation costs may be much greater and asymmetrical between genders in natural populations. Morgan (1997) considered the ideal allocation of resources to male and female function and found that plants should allocate attraction to satisfy both genders. If male function, for example, is limited by time, allocation to attraction should increase to compensate. Aizen and Basilio (1998), for example, found that *Alstroemeria aurea* (Alstroemeriaceae) produces three times more sugar in its nectar during male phase than female phase. Interestingly, this change in allocation matched the requirement of three times the pollinator visitation to satisfy male function relative to female function. Furthermore, Parker et al. (1995) found that *Chamerion angustifolium* allocates 1.5 times the resources (measured as dry weight) to male function relative to female. These findings exemplify the fact that although time can be considered a resource to be exploited and optimized, plants have many ways of satisfying the resource requirements of gender function.

6.3.4 Variable vs. fixed flower longevity

Incorporating fixed flower longevity into the model to mimic the findings in Chapter 4 for *Chamerion angustifolium* created a much wider area of parameter space favouring protandry over adichogamy. This change is largely due to the removal of the resource cost associated with extended anthesis when flower longevity is variable. In this case, inbreeding and interference

are sufficient to favour the evolution of protandry. Although this comparison is relevant for *C. angustifolium* it is unclear how widespread fixed flower longevity is in the angiosperms.

Chapter 7

The evolutionary significance of dichogamy

Given the previous chapters, I can now discuss the evolutionary significance of protandry in *Chamerion angustifolium* and, by extension, the evolutionary significance of dichogamy in general. I begin with a discussion of the beneficial aspects of the temporal separation of gender. This is followed by a description of constraints and limitations on dichogamy. Throughout this discussion I will highlight outstanding questions and make suggestions for further research.

7.1 Why are flowers dichogamous?

To review, there are two primary hypotheses for the evolution of dichogamy. The traditional explanation is that the separation of gender reduces the frequency of inbreeding (Bertin, 1993; Darwin, 1862; Lloyd and Webb, 1986). Given the pervasive fitness cost of inbreeding depression (Charlesworth and Charlesworth, 1987; Darwin, 1876; Husband and Schemske, 1996) such a reduction would be strongly favoured by natural selection. However, following work of Lloyd and Webb (1986), Bertin (1993) found, in a broad survey of the angiosperms, that self-incompatible (SI) plants, physiologically incapable of inbreeding, were as likely to be dichogamous as self-compatible (SC) plants. These observations led to the interference-avoidance hypothesis for the evolution of dichogamy (Barrett, 2002; Bertin, 1993; Lloyd and Webb, 1986). This hypothesis considers that the simultaneous presentation

of both genders reduces the effectiveness of either gender's function. Interference was originally considered to be a within-flower effect, such as stigma clogging where the deposition of self pollen impedes the pollen-tube growth of outcross pollen (Howlett et al., 1975; van der Pijl, 1978). However, in a series of papers, Harder and Barrett (Harder and Barrett, 1995, 1996; Harder et al., 2000) extended interference to between flowers. As pollinators move through an inflorescence, female function may interfere with male function when stigmas remove pollen from a pollinator's body. This reduction in pollen export results in geitonogamy (the transfer of self-pollen between flowers of an inflorscence; de Jong et al. (1993); Lloyd and Schoen (1992)) and pollen discounting (the reduction in the number of pollen grains exported by a plant; Harder and Wilson (1998)). Between-flower interference accumulates as floral display size increases. Therefore, large floral displays should suffer from both geitonogamy and pollen discounting. However, large floral displays also attract more pollinators (Bell, 1985; Geber, 1985; Klinkhamer and de Jong, 1990; Queller, 1983; Schemske, 1980; Schmid-Hempel and Speiser, 1988), creating a fitness trade-off between pollination efficiency and pollinator attraction. Protandry may mitigate this trade-off by reducing interference between flowers and allowing both large floral displays and reduced pollen discounting and geitonogamy.

Chapter 2 demonstrated that protandry enhanced male outcross siring-success in *Chamerion angustifolium*. Although this result is generally consistent with between-flower interference avoidance, the enhanced siring success of protandrous, two-flowered plants was unexpected. If between-flower interference increases with inflorscence size, two-flowered plants should have shown the smallest siring advantage. Chapter 3 showed that within-flower interference also occured in *C. angustifolium* through male function interfering with a pollinator's access to the stigma. Furthermore, the presence of anthers increased foraging time on single flowers, causing increased facilitated selfing which could reduce pollen export. However, the relationship between display size and within-flower interference requires an experimental analysis to fully understand the influence of within-flower interference on male and female reproductive success.

These results demonstrate that protandry reduces both within- and between-flower interference between gender functions and enhances male outcross siring success in *Chamerion angustifolium*. Similar results for between-flower interference were found with a single inflorescence-size class in *Eichhornia paniculata* (Pontederiaceae), except that dichogamy reduced

both pollen discounting and geitonogamy. Extending these results to angiosperms in general is supported by the correlation between protandry and SI described in Chapter 5. Consequently, considerable evidence exists to show that protandry functions to reduce floral interference and is selected for to increase male reproductive-success with large floral displays. However, the link between display size and protandry would benefit from further, experimental investigation.

Protogyny provides a period of stigma receptivity in the absence of pollen availability. Therefore, protogyny is expected to be effective at reducing inbreeding (Bertin, 1993; Lloyd and Webb, 1986). Support for this hypothesis was provided by the correlation between protogyny and SC in Chapter 5. However, the single experimental test of this hypothesis found that protogyny did not reduce inbreeding in *Aquilegia canadensis* (Ranunculaceae) (Griffin et al., 2000). Clearly more research into the functional significance of protogyny is required. Moreover, the potential for a correlation between protogyny and reproductive assurance (Chapter 5) warrants further investigation.

7.2 Why are many flowers not dichogamous?

Given these benefits of the temporal separation of gender, for a complete evolutionary explanation of dichogamy it is important to consider why all plants are not dichogamous. The substantial genetic variation present in *Chamerion angustifolium* for male-phase duration (Chapter 4) and the presence of dichogamy in over 250 families (Chapter 5) indicate that dichogamy is not constrained by a lack of genetic variation. The only genetic correlation of any significance found with male-phase duration in *C. angustifolium* was a positive correlation with floral display size (Chapter 4). In this case, the correlation may be favoured by natural selection. Since protandry is particularly beneficial to large floral displays (Chapter 2), this genetic correlation couples display size and temporal separation to maximize pollen export and pollinator attraction. Of course, the generality of the genetic architecture for *C. angustifolium* is likely quite restricted. Any particular species may have a unique **G** matrix for floral characters. Whether such genetic correlations are common among dichogamous plants requires much

more research.

The trade-off between male- and female-phase durations (Chapter 4) may represent a common constraint on the evolution of dichogamy. Although the specific number of hours required to saturate either gender function will vary among species, all hermaphroditic species must satisfy both male and female function (Charnov, 1982). Considerable research has focussed on resource trade-offs between male and female function, particularly in the literature pertaining to the evolution of dioecy (separate sexes; Bawa (1980); Cruden (1988); Vamosi et al. (2003)). However, considering time as a resource has rarely been explored. In this context, time provides opportunities for flowers to attract pollinators and both import and export pollen. The fitness benefit accrued for a given unit of time is likely a function of pollinator abundance and floral attractiveness. For example, highly attractive foral displays with a high pollinator abundance would require only a short amount of time to saturate both male and female function through pollen removal and deposition. A key piece of missing information is the fitness consequences of quantitative variation in the duration of gender function. How much dichogamy is enough? For example, placing the selected lines generated in Chapter 4 into the field and monitoring pollen removal and deposition might demonstrate that short and long-duration male phases are equally superior to adichogamous plants in terms of pollen export.

A further potential constraint on the evolution of dichogamy was detected in Chapter 4. Neither of the transition-rate parameters between protandry and protogyny for SI taxa were statistically distinguishable from 0. The basis of this constraint could be physiological, genetic, or ecological, and may prevent the evolution of dichogamy in a variety of circumstances. In addition, the transition from protandry to protogyny in SC taxa was estimated as 0. The coupling of these phylogenetic constraints may act as a 'gate' to the evolution of protandry. Once a taxon evolves protandry, the subsequent evolution of protogyny may be rare and could explain the relative preponderance of protandry relative to protogyny among biotically pollinated plants (Bertin, 1993; Lloyd and Webb, 1986).

7.3 Conclusion

The widespread occurence of dichogamy in the angiosperms and the significant siring advantage offered by protandry demonstrate that interference avoidance can have a profound effect on floral evolution. After the long and prosperous use of inbreeding avoidance as an explanation for floral evolution, interference avoidance provides an excellent opportunity to reinterpret the evolution of floral form. When applied in an adaptive context, as described in this thesis, such an approach is certain to be fruitful.

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