Investigating the evolutionary consequences of sexual conflict through pollen and pistil traits in several *Collinsia heterophylla* (Plantaginaceae) populations

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Abstract

Sexual selection is considered to be one of the most important processes influencing the evolution and diversification of species. Sexual conflict, a subset of sexual selection theory, describes how opposing interests in the male and female reproductive systems can lead to one sex increasing its fitness at a cost to the other sex. Traits involved in this conflict may come under selection and evolve, leading to antagonistic coevolution that can increase diversification in separate populations. These forces, traditionally pertaining to animals, have only recently been considered in plants. This study investigates the potential for sexual conflict in *Collinsia heterophylla*, a mixed mating species (utilizing both selfing and outcrossing strategies) with two main pollen and pistil traits thought to be engaged in antagonistic coevolution during pollen competition: 1) the ability of stigmas to delay stigma receptivity in the presence of pollen, and 2) the ability of pollen to force early fertilization in immature stigmas. To explore possible evolutionary outcomes of this conflict, I performed crossing experiments in the greenhouse (within eight natural populations from four regions of California that presumably differ in mating system), as well as measured various floral traits relating to mating system and pollen competition. In the field, the potential for early pollinator visitation was investigated in order to evaluate if the conflict is likely to occur under natural conditions. In the greenhouse, I found that there was a maternal cost of early fertilization in all eight populations, indicating that the cost to the recipient individual is widespread. Across all populations, the timing of stigma receptivity was only influenced by the identity of the recipient, denoting that the female function may generally have more control over onset. Within populations, however, the male and female influence on this trait varied. There were no regional effects on either the cost or the male vs. female influence on onset, but timing of self-pollination appeared positively correlated to timing of stigma receptivity. Interestingly, a positive relationship was found between measures of the magnitude of the cost and the level of female control over onset, potentially suggesting selection on female control when the cost is high. Novel to this study system, I found a negative correlation between donor and recipient influence on onset within individuals; this result hints at trade-offs in sex-allocation and/or a direct genetic link between the male and female traits. In the field, I confirmed that nectar was also produced in early floral developmental stages and that seeds could be produced after pollinators visit flowers in early stages. I conclude that the result of this study is consistent with an influence of antagonistic selection on patterns of timing of stigma receptivity across populations of *C. heterophylla*. Further research in this area is clearly needed to illuminate the intricate forces driving plant evolution, particularly in relation to the impact of pollinators and mating system.

Introduction

One of the reasons that studying biology is so fascinating (and challenging) is the vast multitude of processes that can influence evolution, diversification, and speciation. In modern times it is easy to accept the ideas put forth by Darwin over 150 years ago, "But to the great majority of naturalists and men of science endless difficulties and objections arise, owing to the wonderful variety of animal and vegetable forms, and the intricate relations of the different species and groups of species with each other…"(Wallace 1889). Today, ecologists and evolutionary biologists are still struggling to answer these ever-emerging questions, constantly probing to understand the processes that promote the rich diversity found (past and present) in our world. One of the most important processes thought to drive this diversity in plants and animals is sexual selection, suggested by Darwin as selection in relation to the ability to obtain mates in competition with other individuals (Darwin 1871). Sexual conflict, a more recent development of sexual selection theory, describes the differing interests between male and female reproductive systems, where one sex will increase its own fitness while imposing cost to the other sex (Parker 1979; Arnqvist and Rowe 2005). Specific male and female traits that are engaged in sexual conflict can come under selection and evolve, leading to antagonistic coevolution and therefore increased levels of speciation in geographically separated populations (Gavrilets 2000). Sexual selection and sexual conflict tend to be considered auxiliary forces in the process of speciation, initiating reproductive isolation and allowing genetic drift and local adaptation to drive divergence (Panhuis et al. 2001). More recently, however, sexual selection and conflict have been suggested to have more primary roles in speciation by allowing populations to explore a wide phenotypic range around viability optima (Bonduriansky 2011). There is little empirical evidence for these ideas (Hotzy and Arnqvist 2009), which underscores the importance of future studies on the evolutionary influence of sexual selection.

Though historically considered important only in animals, sexual selection has slowly gained acceptance as an important force in plant evolution as well (Skogsmyr and Lankinen 2002). Likewise, the occurrence of sexual conflict has only recently been investigated in plants (Madjidian and Lankinen 2009). It has been suggested that pollen and pistil traits can coevolve and that sexual selection is particularly responsive to pollen competition (Skogsmyr and Lankinen 2002), making these traits especially interesting in sexual conflict investigations. Pollen competition describes the race that pollen grains engage in to fertilize a limited number of ovules after a simultaneous deposition event (as often occurs by insect pollination) (Mulcahy 1979). While pollen that arrive first tend to have the advantage when it comes to successful fertilization (Burkhardt et al. 2009), there are multiple other factors that can determine the victors of pollen competition. For example, pollen traits such as pollen tube growth rate (Snow and Spira 1991) and chemically facilitated pollen interactions (Varis et al. 2010) can provide a competitive advantage during pollen competition. Analogously, pistil traits such as a larger stigmatic area (Rodrigo et al. 2009) and delayed stigma receptivity (Galen et al. 1986) can increase pollen competition intensity and these traits would therefore

increase the chance of being fertilized by higher quality pollen. This idea parallels cryptic female choice in animals where multiple mating can increase post-copulatory sperm competition, allowing selection of more genetically compatible sperm (Jennions and Petrie 2000).

Collinsia heterophylla, the focal organism of this thesis, shows evidence of sexual conflict during pollen competition, making it an exceptionally useful vector for investigating sexual selection and it's evolutionary consequences (Lankinen et al. 2007; Lankinen and Kiboi 2007; Madjidian and Lankinen 2009). *C. heterophylla* exhibits delayed-stigma receptivity, a trait which delays the stage at which fertilization can occur, thus allowing large amounts of pollen (self and outcrossed) to be deposited on the stigma. This behavior is believed to reduce inbreeding depression (Lankinen and Armbruster 2007) and increase offspring fitness (Lankinen and Madjidian 2011) by increasing pollen competition, providing a clear benefit to the recipient individual. Also, *C. heterophylla* seems to possess traits that allows pollen to force early fertilization upon arriving on the stigma, which ensures male siring success but causes a decrease in maternal seed set (Lankinen and Kiboi 2007). This reduction in seed set after fertilization at early floral developmental stages (Madjidian et al. 2012) is consistent with a cost of sexual conflict (Parker 1979). According to sexual conflict theory, these two opposing pollen and pistils traits are selected to reach their own fitness optima at the cost of the mating partner; the outcome of the interaction between these traits should then influence the timing of onset of stigma receptivity (Parker 1979) .Interestingly, the functionality of these opposing pistil and pollen traits was recently shown to vary across four populations from two geographic regions, with recipient individuals having reduced costs when being fertilized by pollen donors from farther distances compared to local donors (Madjidian and Lankinen 2009). Thus, pollen and pistil traits of *C. heterophylla* may be engaged in antagonistic coevolution (Madjidian and Lankinen 2009). If pistil and pollen traits involved in the conflict lead to assortative mating (i.e. non-random mating of individuals with certain pollen and pistil capacities to influence the timing of stigma receptivity) we would expect a co-dependence between genes causing these effects (e.g. pleiotropic effects (Härdling and Karlsson 2009)). We should then hypothesize that such pollen and pistil influence is not independent, which could be investigated by studying these opposing effects within individual plants (as most angiosperms are hermaphrodites).

Adding another layer of complexity, there is evidence suggesting that there are other factors besides pollen and pistil traits that can influence diversification in *C. heterophylla*. A recent phylogenetic investigation describes the *Collinsia* genus as much more diverse than previously thought, with recent lineages being connected with different habitats, flower sizes, selfing rates, and chromosomal arrangements, as well as loss of interfertility due to divergence (Baldwin et al. 2011). Most *Collinsia* species can be categorized into two groups: 1) large flowered species that tend to retain spatial separation of anthers and stigmas in early floral stages and are characterized by delayed self-pollination and high levels of outcrossing, or 2) small flowered species without spatial separation of sexual parts that tend to have early self-fertilization and high levels of selfing (Armbruster et al. 2002; Kalisz et al.

2012). *C. heterophylla* is a mixed-mating species (employing self-fertilization and outcrossing strategies in the same individual), lying closer to the outcrossing group than the selfing group (Armbruster et al. 2002). Mixed mating systems are somewhat of a mystery because they are not theorized as evolutionarily stable, instead predicted to move towards exclusively selfing or outcrossing strategies; in populations with high levels of inbreeding depression selfing will be selected against, while in populations with low levels of inbreeding depression selfing will be favored (Lande and Schemske 1985). One possible explanation for persisting mixed-mating species is the "best of both worlds" hypothesis, postulating that mixed mating systems may ensure reproductive success by resorting to self-pollination at sites or in seasons where there is an absence of pollinators (Becerra and Lloyd 1992). The mating-system patterns in *Collinsia* are most likely influenced by geographic and environmental factors; for example, species and populations in higher elevations or extreme environments (such as deserts) tend to reach reproductive maturity earlier and are subsequently smaller flowered and more selfing (Elle et al. 2010). It is possible that antagonistic coevolution may be interrelated with mating system evolution, as it is believed that sexual selection is more intense in more outcrossing species (Mazer et al. 2010).

It can be hypothesized that sexually antagonistic coevolution, in combination with geographic and environmental variation in mating system, is partially responsible for the diversity of floral traits occurring in *C. heterophylla* populations. In order to begin to test this hypothesis, it would be informative to study how the cost of early fertilization as well as pistil and pollen influence on the sexual conflict vary across multiple populations from different regions that differ in mating system strategies. Previous studies utilizing one-donor crosses have shown that the identities of both pollen donor and recipient can affect the onset of stigma receptivity in *C. heterophylla* (Lankinen and Kiboi 2007; Madjidian and Lankinen 2009). However, it is not yet known how pistil vs. pollen influence on timing of stigma receptivity vary in relation to differences in outcrossing rates and/or floral traits connected to the mating system (e.g. timing of anther-stigma contact) or other plant traits. Additionally, pistil vs. pollen influence on timing of stigma receptivity may also by affected by the relative magnitude of the cost of early fertilization (i.e. the difference between number of seeds produced at early and late floral stages). Likewise, other pollen and pistil traits of importance during pollen competition (e.g. pollen tube growth rate) may also respond to the level of female influence over timing of stigma receptivity, as more control would enhance pollen competition. Investigating correlations between these traits may help illuminate the processes driving diversification and divergence in *C. heterophylla* populations. In addition, it is remains to be determined whether pollinators visit all stages of floral development, and whether nectar rewards are produced at the earliest stages. Increasing our understanding of *C. heterophylla's* interface with pollinators is crucial to comprehending if the sexual conflict is at all expressed in natural populations.

The overall aim of this study is to further investigate the potential for sexual conflict in *C. heterophylla,* its evolutionary consequences, and how traits involved in the conflict covary with the mating system. Specifically, I will be investigating several questions by conducting one-donor crossing experiments in the greenhouse

using plants from eight populations originating from four regions of California combined with field work in one of these regions:

1) Is there a cost of early fertilization in terms of low seed production at early developmental stages in all populations studied in the greenhouse?

2) Does the timing of stigma receptivity (i.e. the floral developmental stage of first seed set) differ between greenhouse-grown populations? On the population level, is timing of onset influenced by the donor/recipient identities in all populations?

3) Does donor and recipient onset of stigma receptivity covary within individuals? If there is a correlation, is the relationship positive or negative?

4) How does timing of stigma receptivity (as shown through seed set following one-donor crosses in the greenhouse) or relative female influence on this trait covary with other plant traits related to mating system and pollen competition across populations?

5) In the field, is seed set pollen limited in the absence of self pollen? How is fertilization success affected by floral developmental stage (as revealed by stage-specific treatments of different pollen sources)? Is there a cost to early fertilization?

6) Is nectar produced at early stages in the greenhouse and in the field, providing benefits to pollinators at early stages? How often do pollinators visit flowers in the field, and do they visit flowers at early stages?

Materials and Methods

Study Species and Plant Material

Collinsia heterophylla Buist (Plantaginaceae), common name Chinese houses, is a diploid (2n = 14), annual flowering plant native to the California Floristic Province, United States (Newsom 1929; Neese 1993). It flowers between March and June depending on latitude and elevation (Neese 1993). Each flower has four stamens and one pistil, and can contain up to 20 ovules that develop into dry dehiscent capsules (Armbruster et al. 2002; Madjidian and Lankinen 2009). Its corolla is zygomorphic and two-lipped; the upper lip is colored white to light purple, while the lower lip is colored light to dark purple.

When the flowers first open, the pistil is short and unreceptive and the anthers are undehisced. The anthers dehisce one day at a time while the style elongates and becomes receptive to incoming pollen (Lankinen et al. 2007). Selfing can occur in the later stages of development as the style elongates and eventually grows through the anthers (Kalisz et al. 1999; Armbruster et al. 2002). According to allozyme data,

the mean outcrossing rate of *C. heterophylla* populations ranges from 0.32 to 0.64 (Charlesworth and Mayer 1995).

The plants used in the present study were grown in a greenhouse at Lund University, Sweden in autumn and winter of 2011-2012. They originated from seeds that were collected by maternal family from eight natural California populations from four different regions in 2008 (Table 1, Figure. 1). These regions define phylogeographically separated lineages along the Transverse mountain ranges, seen in *C. heterophylla* (Baldwin et al. 2011) as well as many other plant and animal species (Calsbeek et al. 2003). These populations represent around two thirds of the species' natural range, which spreads across the entire California Floristic Province and into northern Baja California(Neese 1993). Seeds were sown and plants grown for one generation in the greenhouse in order to generate outcrossed progeny for my experimental crosses. From this first generation additional population data was also collected on I) floral phenology (measured as start of first flowering), II) floral developmental stage of anther-stigma contact (as an indication of timing of self-pollination(Armbruster et al. 2002)), and III) innate timing of stigma receptivity (measured as the floral developmental stage with stigmatic peroxidase activity (Kearns and Inouye 1993; Lankinen et al. 2007)). I used this population data in trait comparisons among populations.

The field study was conducted in late April and early May 2012 in Napa County using populations 14b and 151 (see Table 1 and Figure 1). Population 151 was located along a roadside with light traffic, and population 14b was located beside a path in a rarely visited nature preserve. Population 151 flowered up to a month earlier than population 14b, and was estimated to have ten times as many individuals (though plants tended to be smaller, with fewer branches and flowers). Both populations grew upon partially shaded slopes and co-occurred with similarly purple-colored *Vicia* sp.

Table 1. Locations of *C. heterophylla* populations from four regions used in greenhouse experiments. Also See Figure 1.

Population	Region	Location	Coordinates
3	1	Riverside county	33.51655 N, 117.33807 W
1	2	Los Angeles County	34.43155 N, 118.62989 W
7	2	Santa Barbara County	34.74107 N, 120.01358 W
10	3	Mariposa County	37.57681 N, 119.94864 W
11	3	Mariposa County	37.50232 N, 120.06873 W
13	3	Madera County	37.17936 N, 119.51235 W
14b	4	Napa County	38.5845 N, 122.37328 W
151	4	Napa County	38.64407 N, 122.37349 W

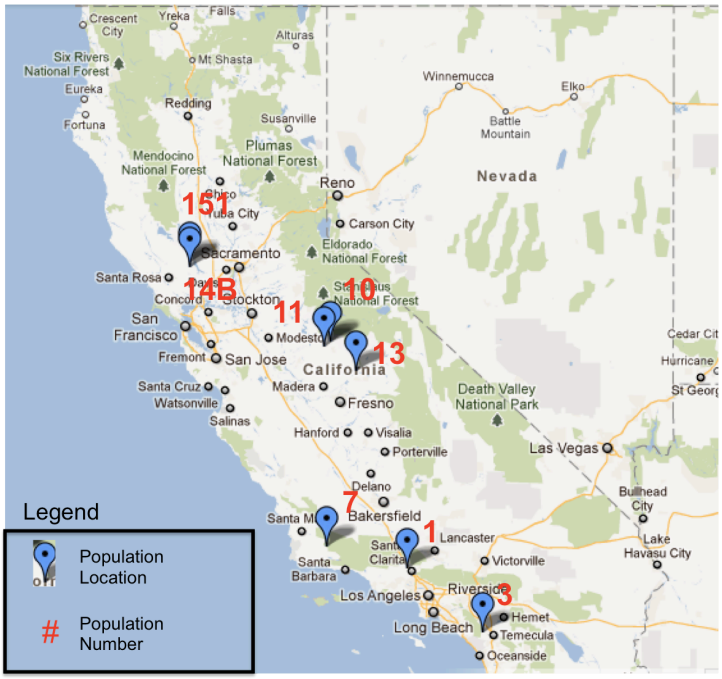


Figure 1. Map of locations of *C. heterophylla* populations used in greenhouse experiments. See Table 1 for details.

Greenhouse Experiments

One-Donor Crosses

In order to investigate costs of early fertilization and timing of stigma receptivity (the floral stage at which flowers can be successfully fertilized, hereafter referred to as realized timing of stigma receptivity) across populations, we conducted one-donor crosses within each population on emasculated flowers at each of the four stages. The floral stages are designated 1-4, corresponding to the number of days after anthesis and the number of open anthers (Armbruster et al. 2002).
Hand pollinations were conducted by depositing pollen from the donor flower on a microscope slide and wiping the slide on the recipient stigma. The pistil was cut after four hours in order to ensure that the flower was fertilized only by the designated pollen at the specific time the hand pollination was performed (Lankinen and Kiboi 2007).

From each of the eight populations used in this experiment, I chose 6-8 unrelated plants (each originating from separate outcrossed sibling groups) to act as pollen donors and recipients in the crosses within each population. Each individual acted as a donor to three unrelated recipients and as a recipient to three unrelated donors (Figure 2). The same individual was used as both donor and recipient because I aimed to investigate both general donor and recipient effects (over three unrelated mates) on the realized timing of stigma receptivity, as well as how male and female components of this trait varied within individuals. Hand pollinations were conducted on two flowers in each of the four stages per donor, equaling 24 pollinated flowers per individual (three donors x four stages x two replicates). With a mean of 6.9 individuals per population and eight populations, the total number of pollinations reached over 1300. Since the populations had different innate flowering times, not all crosses could be performed at the same time. Also, in order to complete the necessary pollinations for each donor-recipient pair, siblings of the main crossing individuals were occasionally used to supplement flowers and were treated as the same donor/recipient individual (Madjidian et al. 2012). The first crosses were conducted in October, and the final crosses were completed in mid December.

Mature seed capsules were collected and allowed to dry, after which the number of seeds was counted and their total weights were measured. We quantified the number of seeds and biomass per capsule as a measure of fecundity, which will give an indication of the maternal cost of early fertilization (Lankinen and Kiboi 2007). For example, a lower number of seeds produced in stages 1-2 compared to stages 3-4 will signify a cost of fertilizing early. Identifying the stage at which seeds were first produced was used to calculate the realized timing of stigma receptivity. In order to investigate male and female influence on timing of stigma receptivity within the same individual, pistil onset was calculated for each recipient as realized timing of stigma receptivity averaged over the three pollen donors used (see (Madjidian et al. 2012). Similarly, pollen onset was calculated by averaging the realized timing of stigma receptivity of the three recipients of the donor's pollen.

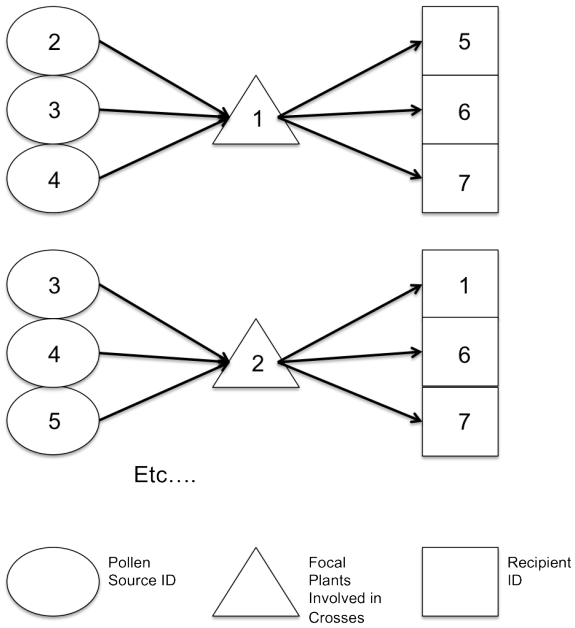


Figure 2. Experimental design for one-donor crossing experiment in order to assess costs of early fertilization and realized timing of stigma receptivity in *Collinsia heterophylla*. One individual (denoted with numbers) is used as both a donor for three and a recipient for three unrelated individuals in other sibling groups. Crosses involving the same donor and recipient combination were performed at each of the four floral developmental stages.

Measurements of additional plant traits

I measured additional plant traits in order to compare how costs of early fertilization and realized timing of stigma receptivity are related to other plant traits of my eight populations (apart from the three traits previously measured, see Study Species and Plant Material), as well as to investigate the potential for pollination visits at early floral stages. For these measurements I used a second set of plants originating from the same 6-8 sibling groups used in the crossing experiment, supplemented by 2-4 additional sibling groups per population (grown a few months later). Each population contained 7-10 sibling groups with 5 individuals per group.

Chosen population traits of interest included pollen tube growth rate, because this trait is often important for siring ability (Snow and Spira 1991), and flower production, an indicator of plant vigor (Lankinen and Armbruster 2007). In order to measure the pollen tube growth rate, pollen from two flowers was mixed from one individual per sibling group and germinated in Hoekstra medium (Hoekstra and Bruinsma 1975) at a constant temperature of 23°C in a dark chamber for 105 minutes. The length of 10 pollen tubes per sample was measured by use of a light microscope. Flower production was determined by multiplying the number of flowers on the main stalk by the number of flowering branches in five individuals per sibling group.

In order to assess the availability of rewards for pollinators, nectar measurements were taken from one flower in each stage from one individual per

sibling group. Nectar amount was quantified by inserting a 32mm long microcapillary tube (1 µl) into the flower's nectar tube and measuring the length of fluid drawn in.

Field Study

Crossing experiment

In order to further explore the costs of early fertilization in natural *C. heterophylla* populations as well as to get an indication of pollen limitation and at which stages pollinators visited flowers, I performed a crossing experiment in a single population (population 151). The experimental design included five treatments: I) a control treatment allowing both self (S) and outcross (X) pollen to arrive across all floral stages (designated control SX); II) a control treatment with flowers emasculated at stage zero, allowing mainly outcross pollen to arrive at all floral stages (designated control X); III) a treatment where the flower's pistils were cut at each stage (1-4), allowing self and outcross pollen to arrive at controlled stages (designated stage SX); IV) a treatment with flowers emasculated at stage zero and pistils cut at each stage (1-4), allowing mainly outcross pollen to arrive at controlled stages (designated stage X); and V) a treatment where flowers were emasculated at stage zero and hand-pollinated with supplemental outcross pollen at each stage (1-4), allowing mainly outcross pollen supplemented at each stage (designated stage X+) (Table 2). Because plants were small in this population (most without side branches), flowers involved in the experiment all came from different individuals. All experimental treatments were started over a period of five days (i.e. I marked flowers at stage zero for all treatments on each of those five days). The experiment was completed four days after the last marking of stage 0 flowers. I returned approximately two weeks later to collect the capsules of the flowers used in the experiment. The seeds were allowed to dry and counted. Due to time constraints, capsules may have been collected before full maturation (which takes about 3 weeks), and were therefore not weighed. Previous field studies have shown that seeds from early-collected seed capsules germinate well in the greenhouse (unpublished data, Å. Lankinen), suggesting that these seeds are functional and would probably not be aborted. The proportion of flowers that developed into capsules was calculated for each stage and treatment as a representation of the success of fertilization. Similarly, the average number of seeds per capsule in each stage and each treatment was used as an indicator of fecundity (cf. greenhouse crosses).

Table 2. Experimental design of field crossing experiment conducted in one natural California population (151) of *C. heterophylla*. All flowers were first labeled at stage 0 in all five treatments. S = self pollen; X = outcross pollen; + = outcross pollen supplement.

Treatment	Manipulations	Type of Pollen Expected	Number of Flowers
Control SX	control	self and outcross	10
Control X	emasculated stage 0	mainly outcross	10
Stage SX	cut pistils stages 1-4	self and outcross, controlled by stage	40 (10 per stage)
Stage X	emasculated stage 0, cut pistils stage 1-4	mainly outcross, controlled by stage	40 (10 per stage)
Stage X+	emasculated, hand pollinated stage 1-4	mainly outcross with outcross pollen supplement	40 (10 per stage)

Field-measurements of plant traits and estimates of pollinator visitation rate

In population 14b and 151, plant traits related to pollinator attraction and mating system were measured and data was collected for pollinator visitation rate and behavior. Measures of plant traits included: I) nectar content, II) timing of anther-stigma contact, III) innate timing of stigma receptivity, IV) presence of pollen on the stigma, and V) flower size. All data was collected from ten flowers per stage 1-4, all belonging to different individuals due to the small size of plants.

In order to assess the availability of rewards for pollinators in the field and to confirm the results found in the greenhouse, nectar was quantified using the same method as in the greenhouse (see above). Anther-stigma contact was estimated by noting the location of the stigma in relation to the open anthers, and innate timing of stigma receptivity was determined by presence of stigmatic peroxidase activity (Kearns and Inouye 1993)(the same methods as in generation 1 in the greenhouse, see Study Species and Plant Material). The presence of pollen on the stigma was estimated by use of a hand lens. Small amounts of pollen are difficult to detect using a hand lens, so if pollen was detected it should have been a fairly large pollen load. Flower size, as an indication of pollinator attraction and possibly pollinator-flower fit, was determined by measuring the lengths of keel, corolla tube, and banner (see Armbruster et al 2002, Figure 3). I measured these traits per floral stage because these traits may change during floral development.

I quantified pollinator visitation by first picking a patch of flowers, and estimating the number of flowers in the patch (1000-1500 flowers). Then I observed pollinators arriving to the patch for a certain time period, and counted how many flowers each pollinator visited before leaving the patch (personal communication, W.S. Armbruster). I also recorded the type of pollinator (bumble bee, honey bee, or fly) and behavior (selective visits, visiting flowers from bottom to top or top to bottom, etc.) whenever possible. Pollinators visiting flowers from bottom to top or top to bottom are likely to transfer pollen to flowers at early floral stages as well as late.

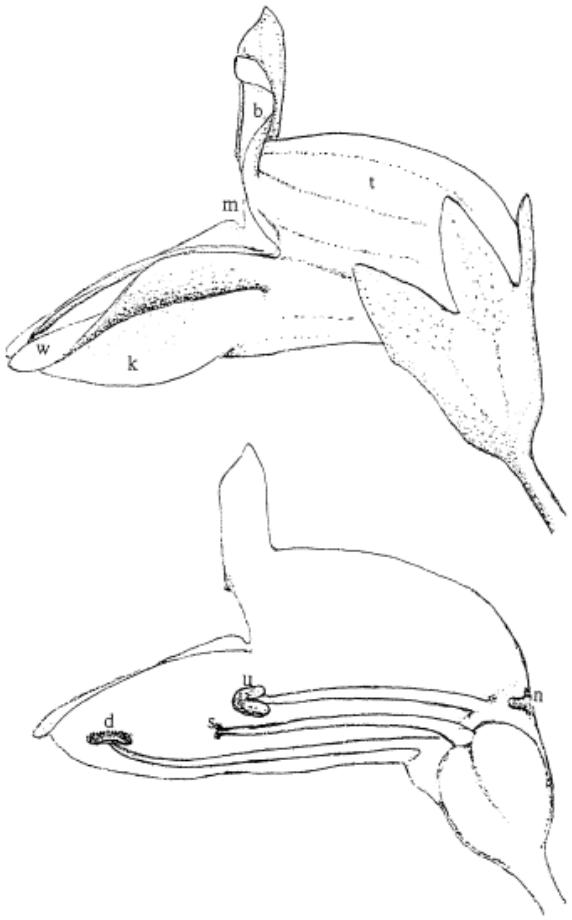


Figure 3. Diagram of *Collinsia torreyi,* which has a similar floral structure to *C. heterophylla*. The lengths of B) banner, K) keel, and T) corolla tube were measured as an indicator of flower size in two natural California populations (14b and 151). Figure taken from Armbruster et al 2002.

Statistical Analysis:

Most statistics were performed using general linear models (GLM) on IBM SPSS 20 for OSX. I used a type III sum of squares in all GLMs. Highly non-significant interactions ($P > 0.2$) were removed from the original model.

For greenhouse crosses, I used a nested, factorial ANOVA to determine the effects of different variables on each trait in question. For mean number of seeds and mean biomass after one-donor crosses (cost of early fertilization), the model tested the effects of region, population (nested within region), stage, donor identity, recipient identity, stage by recipient interaction, and population by stage interaction (the latter two factors nested within population and region). Stage was a fixed factor and all other factors were random. For realized timing of stigma receptivity, I used a similar model that tested for the effects of region, population (nested within region), donor, recipient, and donor by recipient interaction (all nested with population and region). A random-effects ANOVA was also used to test the effects of

donor, recipient, and donor by recipient interaction on realized timing of stigma receptivity for each population individually.

In order to assess the effects of donor and recipient on realized timing of stigma receptivity within individuals, I estimated the Pearson correlation coefficient between pistil and pollen onset of stigma receptivity for each individual in all eight populations. I used the residuals from an ANOVA with populations as groups, to remove confounding effects of population differences in the overall means.

In order to compare how pollen and pistil traits covary with other plant traits (as population means), a few new variables were created. The difference between realized timing of stigma receptivity and innate onset of stigma receptivity was calculated and defined as "relative female influence on onset." This variable allows us to compare the relative influence that the female has on onset between populations and between traits, with lower values indicating more female control. "Relative cost" was calculated by subtracting the mean seed biomass of stages 1+2 from the mean biomass of stages 3+4, making it easier to assess and compare the cost of early fertilization. A significant correlation between these two new variables will imply that there is a connection between the amount of female control over stigma receptivity and the relative cost of early fertilization. Correlations were also made between realized timing of stigma receptivity and various traits that may have an influence on it, i.e. innate onset of stigma receptivity, timing of anther-stigma contact, pollen tube growth rate, start of flowering, and flower production. A significant correlation between any of these traits would signify that this trait would have an effect (positive or negative depending on the sign of the correlation) on the realized timing of stigma receptivity.

To get an indication of how fertilization success in the field was influenced by arrival of outcross or mixed pollen, or supplemental outcross pollen, on the presence and absence of seed capsules produced, I used a generalized linear model in the R environment (R Development Core Team 2012), following method suggested by Crawley (2007). In the original model a binomial error structure and a logit link function was used. The model was refitted with quasibinomial errors, in order to compensate for overdispersion. Significance ($P < 0.05$) was assessed by testing the change in deviance with an *F*-test. All non-significant factors or interactions were excluded using backward deletion of highest-order interactions. I first compared the proportion of seed capsules produced for all treatments (stage 4 or equivalent) in order to test for pollen limitation, including the factors day of marking the flower at stage 0, treatment, and their interaction. To investigate whether seeds can be produced at early stages due to pollinator visits and whether there is a cost for fertilization at early stages, I compared stage X and stage X+ (naturally arriving outcross pollen per stage vs. supplemental outcross pollen per stage) with respect to day, treatment, stage and interactions. Because these two treatments did not differ, I then compared treatment stage SX (naturally arriving self and outcross pollen per stage) to the sum of treatments stage X and stage X+ in order to test if pollen source had an effect. While all stage X+ flowers could be considered stage 4 (because the pistils were left uncut), pistils received pollen supplements in each stage and therefore it is still appropriate to test for a stage effect.

In order to investigate whether seed set (when a capsule was formed) is pollen limited in the absence of self pollen in the field, I used ANOVAs to test the effects of day of marking the flower at stage 0, treatment, stage (when appropriate), and interactions on the dependent variable number of seeds produced per capsule. As in the analysis on the proportion of capsules produced, I tested 1) differences between all five treatments, stage 4 only, and 2) differences between treatments stage X and stage X+.I was unable to include stage XS in the latter treatment as no seeds were produced prior to stage 4. Stage and treatment were fixed factors, and day was a random factor.

Logistic regression (SPSS PROBIT procedure, IBM 1998) was used to find the stage when 50% of stigmas came in contact with open anthers (ASC-50) as well as the stage at which 50% of the sampled stigmas were receptive (SR-50), following (Armbruster et al. 2002). Flower size measurements and both greenhouse and field measurements of nectar collections, respectively, were analyzed using ANOVAs to test the effects of population and stage on nectar production. Stage was a fixed factor and population was a random factor. In the model for greenhouse collections of nectar, I included the effect of region (random factor). In this model, population was nested under region.

Results

Greenhouse crosses: Cost of Early Fertilization

As an indication of the cost of early fertilization, I investigated the number of seeds per capsule and seed biomass per capsule at different stages of floral development following one-donor crosses and stage specific removal of the pistil in the greenhouse. As hypothesized, the number of seeds produced and seed biomass per capsule was higher in the later stages of floral development in all populations (Table 3; Figure 4). While this pattern did not differ between populations, there was a stage by population interaction effect, which suggests that the influence of stage on seed production and biomass does differ between populations (Table 3). Seed production and biomass was notably higher in stages 3 and 4 than in stages one and two in all populations except for in population 11, which showed high seed production in stage two as well (Figure 4). Both seed biomass and seed production were influenced by male identity and female identity (Table 3). In addition, seed production and biomass were influenced by a recipient by stage interaction, indicating that recipients show different costs of early fertilization (Table 3).

Table 3. GLMs for number of seeds and mean seed biomass following one donor crosses at floral developmental stages 1-4 and cut pistils within eight populations originating from four regions of California. Population was nested within region, and male and female were nested within population. Significant factors (P < 0.05) are presented in bold. Highly non-significant two-way interactions (P>0.2) were removed from the model.

	# of Seeds per Capsule			Seed Biomass (g)		
Source of variation	df	F	P	df	F	P
Region	3	5.41	0.08	3	4.33	0.11
Population(Region)	4	0.70	0.60	4	0.81	0.52
Stage	3	34.7	**<0.001**	3	4.44	**<0.001**
Donor(Pop(Region))	53	1.82	**<0.001**	53	1.56	**0.01**
Recipient(Pop(Region))	47	3.07	**<0.001**	47	2.93	**<0.001**
Stage*Population(Region)	21	2.48	**<0.001**	21	2.00	**0.01**
Stage*Recipient(Pop(Region))	144	2.21	**<0.001**	144	2.27	**<0.001**
Error	1041			1038		

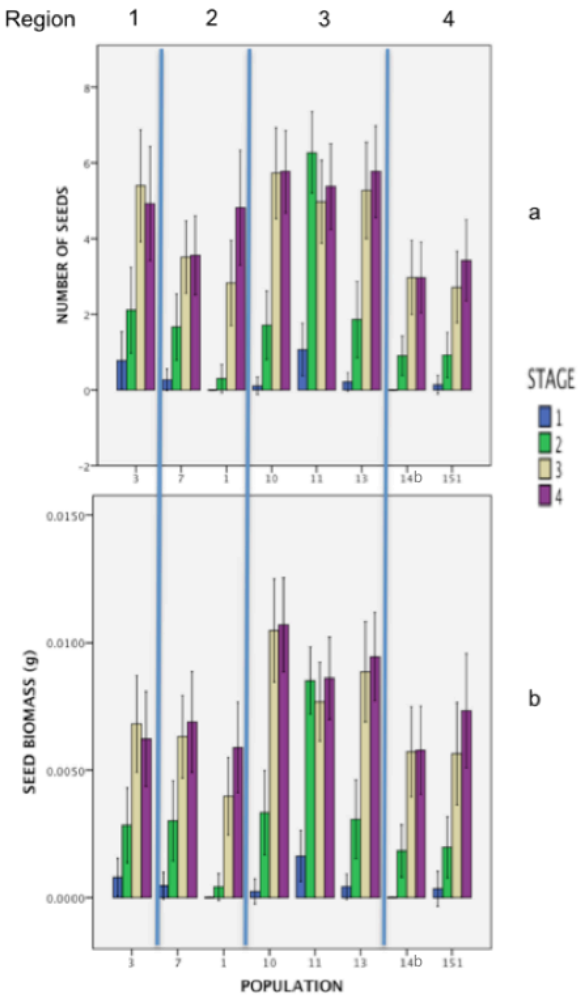


Figure 4. Mean number of seeds produced per capsule (a) and mean seed biomass per capsule (b) after one-donor crosses at stages 1-4 and cut pistils within eight populations originating from four regions of California, presented by floral developmental stage and population. Error bars indicate +/- 2 SE.

Donor and recipient influence on realized timing of stigma receptivity across populations and regions

Realized timing of stigma receptivity, i.e. timing of stigma receptivity evaluated by one-donor crosses and stage-specific cutting of pistils, differed between populations but not between regions (Tables 4 and 5). While the identity of the recipient influenced the timing of stigma receptivity, the identity of the donor showed no significant effect, indicating more female control over onset (Table 4). On the other hand, there was a marginally significant donor x recipient interaction, implying that the identity of the donor may have some influence over onset. Looking within the eight populations, two populations showed both donor and recipient effects on realized timing of stigma receptivity, two populations were instead influenced by the donor by recipient interaction, and one population was only influenced by the identity of the recipient (Table 6). Correcting for multiple testing (Bonferroni 1936)

showed one population with a significant maternal effect and one population with a significant donor x recipient interaction. This result suggests variation in the relative influence of donors and recipients on timing of stigma receptivity across populations.

Table 4. GLMs of realized onset of stigma receptivity assessed by one-donor crosses at stages 1-4 across eight populations from four regions in California. Population was nested within region, and donor and recipient effects were nested within population. Significant values (P<0.05) presented in bold.

Source of Variation	Onset		
	df	F	P
Region	3	1.86	0.279
Population(Region)	4	5.41	**0.007**
Donor(Pop(Region))	50	1.27	0.196
Recipient(Pop(Region))	46	2.06	**0.005**
Donor x Recipient(Pop(Region))	143	1.40	0.059

Table 5. Population means for various floral traits investigated in eight greenhouse-grown populations from four regions of California. Highest four values presented in bold to increase readability, N values in parentheses. P-values from nested ANOVA, with population nested within region.

Region	**Population**	**Start of Flowering (days)[1]**	**Anther-Stigma contact (stage)[1]**	**Innate onset of stigma receptivity (stage)[1]**	**Realized onset of stigma receptivity (stage)**	**PTGR ($1.6e^{-9}$m/s)**	**Flower Production (# of flowers)**
1	3	20.16(64)	2.79(29)	**3.08(20)**	2.45(8)	**25.48(12)**	222.33(46)
2	1	23.25(64)	**3.52(28)**	**3.16(19)**	**3.29(7)**	14.64(9)	**279.46(46)**
2	7	**23.69(49)**	2.67(29)	2.73(20)	**2.79(7)**	**26.27(7)**	**265.33(33)**
3	10	**26.77(47)**	2.83(24)	2.10(20)	2.63(7)	**28.84(10)**	207.08(39)
3	11	14.95(57)	2.50(20)	3.03(20)	1.79(7)	22.56(11)	**241.73(49)**
3	13	**25.69(35)**	**3.04(24)**	2.18(22)	2.51(7)	**33.47(9)**	229.49(43)
4	14b	**23.88(43)**	**3.06(24)**	**3.13(24)**	**2.92(7)**	21.69(7)	**373.15(34)**
4	151	17.80(60)	**3.00(31)**	**3.30(20)**	**2.85(6)**	24.70(11)	227.77(43)
$P_{Population\ (Region)}$					0.007^2	>0.001	>0.001
P_{Region}					0.28^2	0.56	0.47

[1]Measured in a previous generation in the greenhouse.
[2]Test also included recipient and donors effects, see Table 4.

Table 6. GLMs of realized onset of stigma receptivity after one-donor crosses and subsequent removal of pistils at stages 1-4 for each population. Significant values (P<0.00625 after Bonferroni correction) presented in bold. When two-way interactions were highly non-significant (P>0.2), they were removed from the model.

Region	Population	N	FEMALE (p)	MALE (p)	FEMALE*MALE (p)
1	3	8	.250	.500	-
2	1	7	.036	.614	-
2	7	7	.499	.343	**0.006**
3	10	7	**.001**	.027	-
3	11	7	.300	.913	-
3	13	7	.008	.046	-
4	14b	7	.286	.214	-
4	151	6	.234	.682	.025

Greenhouse crosses: Relationship between donor and recipient influence on realized onset of stigma receptivity within individual plants

Because each individual plant was used as both recipient and pollen donor, I was able to analyze how recipient vs donor effects on realized timing of stigma receptivity (as averaged over three unrelated mates) were correlated within individuals. Combining all populations, I found a negative relationship between the standardized residuals of donor and recipient realized onset of stigma receptivity (Figure 5). This pattern indicates that donor and recipient influence on timing of stigma receptivity is not independent within an individual plant.

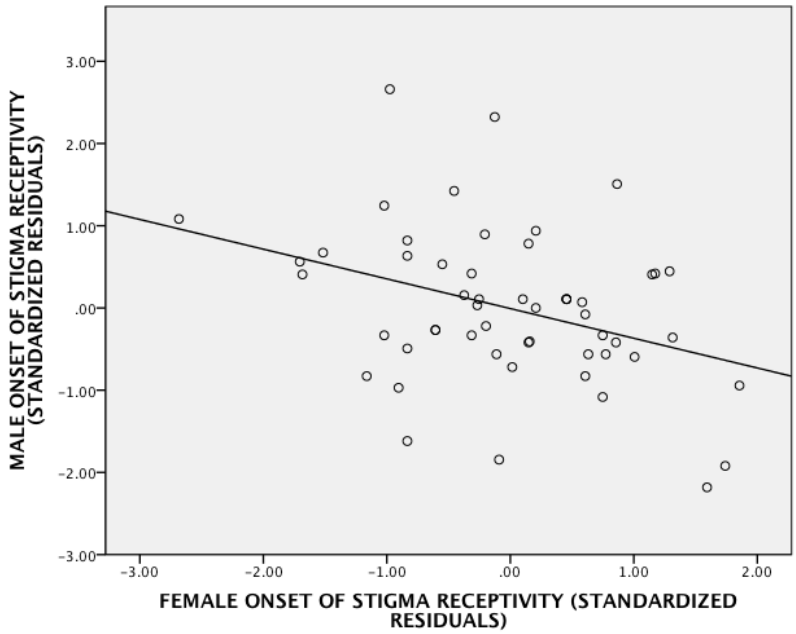


Figure 5. Relationship between standardized residuals of realized onset of stigma receptivity of male vs female function (each averaged over three mates) within a plant individual, following one-donor crosses and subsequent pistil removal. Pearson correlation: R = -0.360, P = 0.008, df = 52.

Relationships between realized onset and floral traits among greenhouse-grown populations

Pollen tube growth rate and flower production differed between populations but not within regions (Table 5). Comparing realized onset of stigma receptivity to pollen tube growth rate and flower production as well as to the other three traits investigated in a previous greenhouse generation (start of flowering, anther-stigma contact, innate timing of stigma receptivity), showed a positive relationship between realized onset and stage of anther-stigma contact (Table 7). No other correlations were significant. It should be noted that the correlation between realized onset of stigma receptivity and timing of anther-stigma contact was not strong enough to still be significant after controlling for multiple testing (Bonferroni).

The relative female influence on onset (where low values indicate stronger female control over stigma receptivity) showed a negative relationship with the relative cost (mean biomass in stages (3+4)-(1+2)), possibly suggesting that populations with higher relative costs have greater female control on stigma receptivity (Figure 6a). We also hypothesized that higher female control on stigma receptivity would select for more intense pollen competition and faster pollen tube growth rate, thus leading to a negative relationship between pollen tube growth rate and relative female influence on onset. However, no significant relationship was found between relative female influence on onset and pollen tube growth rate, but the trend was negative (Figure 6b).

Interestingly, population 11 stands out as having the earliest start of flowering, anther-stigma contact, and realized onset of stigma receptivity despite its close proximity to populations 10 and 13, which had developmental traits that were generally slower (Table 5). Furthermore, population 11 vs population 10 and 13 had the opposite combination of the two values relative cost and relative female influence on onset (Figure 6).

Table 7. Pearson correlations between realized onset and other plant traits. No significant values after Bonferroni correction (P<0.01)

Trait 1	Trait 2	R	P	DF
Realized onset	Innate Onset of Stigma Receptivity	0.183	0.665	6
	Anther-Stigma Contact	0.822	0.012	6
	Pollen Tube Growth Rate	-0.395	0.333	6
	Start of Flowering	0.525	0.181	6
	Flower Production	0.407	0.317	6

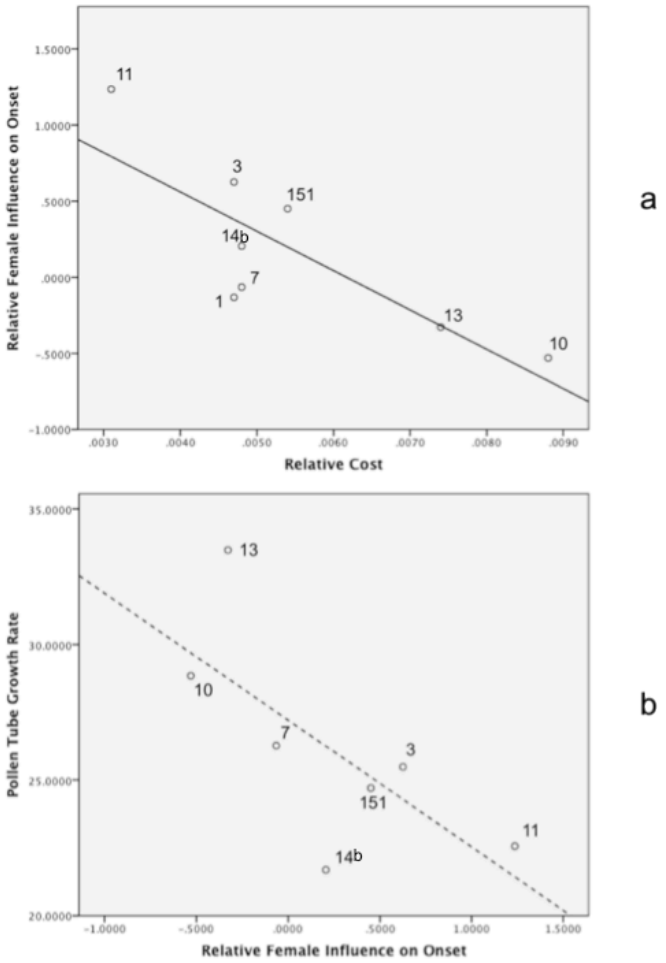


Figure 6. a) Relationship between relative female influence on onset (difference between crosses onset and innate onset of stigma receptivity) and relative cost (seed biomass stages (3+4) – (1+2). Pearson correlation: R = -0.808, P = 0.015, df = 6. b) Relationship between relative female influence on onset and pollen tube growth rate. Pearson Correlation: R = -0.706, P = 0.076, df = 5. Population 1 was removed as an outlier. Numbers refer to population identity.

Seed production in the field following experimental treatments

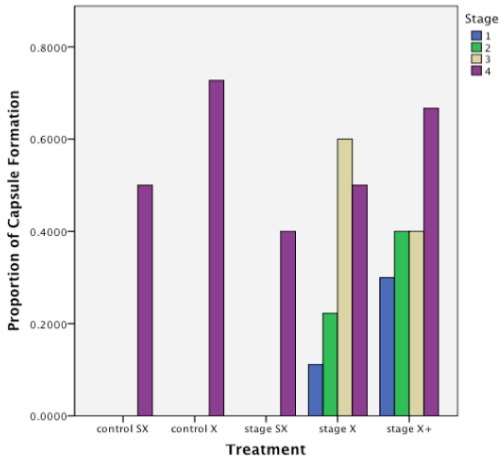
To get an indication of how fertilization success was influenced by arrival of outcross or mixed pollen, or supplemental outcross pollen, I first compared the proportion of seed capsules produced for all treatments (stage 4 or equivalent). There were no effects of treatment (df = 22, F = 0.058, p = 0.81) or day (df = 21, F = 3.862, p = 0.06), and therefore with this limited number of crosses no evidence suggesting that the success of fertilization differs between treatments receiving pollen from different sources (Figure 7a). To investigate whether seeds can be produced at early stages due to pollinator visits and whether there is a cost for fertilization at early stages, I compared outcross treatments stage X and stage X+. Seeds were formed in early floral stages in both treatments stage X and stage X+

(Figure 7a). The fact that seeds were produced in early stage flowers that were emasculated and had pistils cut (see stage X, Figure 7a) indicates that pollinators do visit flowers at early stages. There was higher fertilization success in later stages (df = 37, F = 6.092, p = 0.018), which suggests that fertilization success varies with stage independent of whether the stigma was cut (and prevented from pollen arriving later) or received a large outcross pollen load (Figure 7a). Cutting pistils per stage, but allowing both self and outcross pollen on the stigma (stage SX) did not result in any seeds prior to stage 4 (Figure 7a). Comparing treatment SX to treatments stage X and stage X+ showed both a treatment effect (df = 57, F = 12.886, p = <0.001) and a stage effect (df = 56, F = 13.495, p = <0.000).

In order to investigate whether seed set was pollen limited in the absence of self pollen, I first compared the mean number of seeds produced per capsule (stage 4 or equivalent) for all treatments. These treatments included outcross pollen, self and outcross pollen, and supplemental outcross pollen sources. There was no treatment effect (Table 8, Figure 7b) and therefore no evidence to suggest that the absence of self pollen is limiting the amount of seeds produced. Nor was there evidence to support that supplemental outcross pollen increased seed set. When comparing the number of seeds produced (in all stages) between treatments stage X[emasculated with pistils cut stage 1-4] and stage X+[emasculated with supplemental outcross pollen], there was no stage effect but a treatment effect (Table 8, Figure 7b), suggesting that seed production decreased when stigmas received supplemental outcross pollen. Treatment stage SX was removed from this test because seeds were only produced in stage 4. While there was no significant stage by treatment effect, there was a trend suggesting that the number of seeds produced in early stages differed more between the two treatments(Figure 7b) compared to in late stages.

Table 8.GLMs for number of seeds following a crossing experiment at floral developmental stages 1-4, utilizing five treatments with different pollen sources (naturally pollinated with self (S), outcross (X), or hand-pollinated with supplemental outcross (X+) in one native California population. Control SX = untreated, control X = emasculated, stage SX =pistils cut stage 1-4, stage X = emasculated, pistils cut stage 1-4, and stage X+ = emasculated, supplemented with outcross pollen stage 1-4. See Table 2 for more details. Significant factors (P < 0.05) presented in bold. Non-significant interactions were removed from the model.

	# of seeds per capsule (all treatments, stage 4 only)			# of seeds per capsule (treatments stage X and stage X+ only)		
Source of variation	df	F	P	df	F	P
Day	4	0.558	0.70	4	2.124	0.11
Treatment	4	1.213	0.34	1	7.859	**0.01**
Stage	-	-	-	3	1.469	0.25
Error	20			23		



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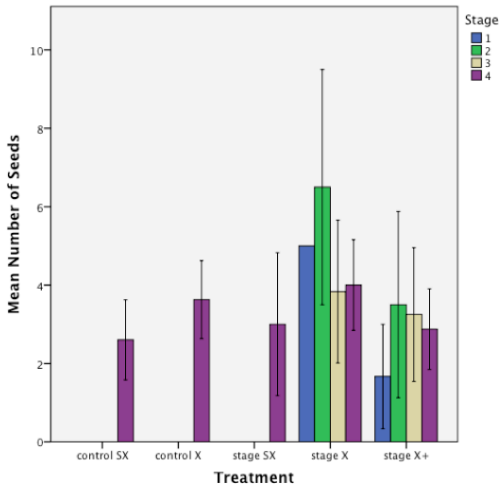

b

Figure 7. (a) Proportion of capsule formation and (b) mean number of seeds produced per capsule following a crossing experiment at floral developmental stages 1-4, utilizing five treatments with different pollen sources (naturally pollinated with self (S), outcross (X), or hand-pollinated with supplemental outcross (X+) in one native California population. Control SX = untreated, control X = emasculated, stage SX =pistils cut stage 1-4, stage X = emasculated, pistils cut stage 1-4, and stage X+ = emasculated, supplemented with outcross pollen stage 1-4. See Table 2 for more details.. Error bars represent +/- 2 SE.

Nectar production in the greenhouse and the field

Nectar was produced at all stages in all eight greenhouse-grown populations, though in much lower quantities at earlier stages than at later stages (Table 9a, Figure 8a). Nectar production also differed between populations, and there was a population by stage interaction (Table 9a), indicating a more pronounced difference between stages in some populations compared to others (Figure 9a).

In general, less nectar was produced in the field compared to in the greenhouse (Figure 8). In line with the greenhouse results, nectar was produced at all stages and there was a trend suggesting increased nectar production in the later stages in the field (Figure 8b). This trend, however, was non-significant (Table 9b). Different amounts of nectar were produced in the two populations, but it should be noted that

nectar collections were performed several weeks apart due to differences in phenology.

Table 9. A) GLMs of nectar production in floral developmental stages 1-4 from eight populations and four regions in California. Population is nested within region. B) GLMs of nectar production in floral developmental stages 1-4 from two natural California populations. Significant values (P<0.05) presented in bold.

A)Greenhouse			
Source of Variation	df	F	P
Region	3	1.33	0.382
Population(Region)	4	6.21	**0.002**
Stage	3	22.03	**<0.001**
Population x Stage(Region)	21	3.67	**<0.001**
Error	280		
B) Field			
Source of Variation	df	F	P
Population	1	6.45	**0.013**
Stage	3	2.07	0.111
Error	88		

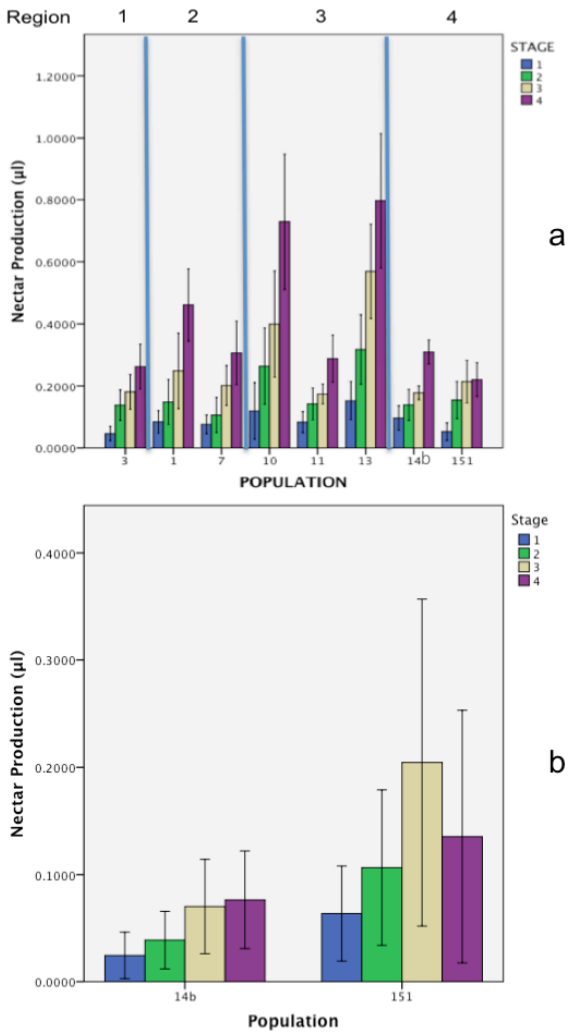

Figure 8. A) Mean nectar production in the greenhouse, presented by floral developmental stage and population. Populations organized by region. B) Mean nectar production in the field, presented by stage and population. Error bars indicate +/- 2 SE.

Field measured floral traits and pollinator visitation rate

I measured various floral traits in the field in order to compare them to results from the greenhouse. ASC-50 in both populations (p14b = 3.16, p151 = 2.92) was similar to what had previously been found when these populations were grown in the greenhouse (see Table 5). SR-50, on the other hand, was at a much earlier stage in both populations (p14b = 2.15, p151 = 1.67) compared to what was found in the greenhouse (see Table 5).

The additional measurements taken on the presence of pollen on the stigma showed a trend indicating that there was an increase in the number of stigmas with pollen present as the flowers increased in stage (Table 10). Measurements of flower size (Table 11) showed that keel length increased with stage ($F_{(3, 77)} = 4.01$, p =

0.01) and banner length was shown to differ between populations ($F_{(1, 77)} = 1.58$, p = 0.000).

In order to investigate pollinator activity, I quantified pollinator visitation in the field for six one-hour sessions. Three of these sessions I did not see any pollinators, most likely due to non-sunny weather conditions. While there was not enough data to perform statistical analyses, the observational data shows that multiple pollinator functional types (bumble bees, honey bees, and flies) visit *C. heterophylla* in natural populations (Figure 9). I noticed that 6 out of 13 bumble bees observed in population 151 and 5 out of 5 bumblebees observed in population 14b visited flowers from the bottom up. All other visits were selective on one or two flowers on the stalk.

Table 10. Proportion of stigmas with pollen present (enough to be visible with a hand lens) in floral developmental stages 1-4 in two California populations. N=10 flowers per stage.

Population	Stage	Pollen
151	1	0
	2	0
	3	0.6
	4	0.8
14b	1	0
	2	0.1
	3	0.2
	4	0.5

Table 11. Mean measurements (mm) of three flower characteristics relating to size in floral developmental stages 1-4 in two California populations. N=10 flowers per stage.

		Stage			
Population	Flower part	1	2	3	4
14b	Keel	16.7	16.8	17	16.7
151		16	17.9	17.8	17.4
14b	Corolla tube	6.17	6.6	6.4	5.9
151		5.6	6.4	6.6	6.3
14b	Banner	7.25	7.5	7.3	7.2
151		8.1	9.2	8.8	8.8

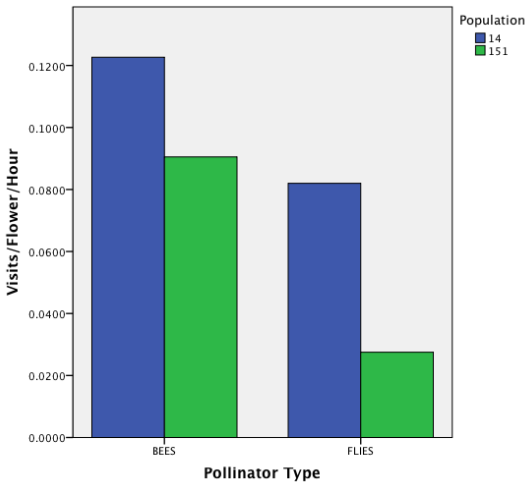

Figure 9. Recorded visits/flower/hour from pollinator observations in two natural California populations.

Discussion

In this study I have been continuing an investigation of the existence and effects of a sexual conflict surrounding the timing of stigma receptivity in *Collinsia heterophylla*. This conflict is realized through the interaction between two pistil and pollen traits: 1) the ability of certain individuals to enhance offspring fitness and increase pollen competition by delaying stigma receptivity (sometimes referred to as the female trait) (Lankinen and Armbruster 2007; Lankinen et al. 2007; Lankinen and Madjidian 2011), and 2) the ability of particular pollen donors to fertilize ovules at early floral developmental stages, resulting in a reduction in seed set for the recipient individual (sometimes referred to as the male trait) (Lankinen and Kiboi 2007; Madjidian and Lankinen 2009). In order to further examine the prevalence and evolutionary outcome of this conflict across many *C. heterophylla* populations, I performed crossing experiments in the greenhouse and in the field, as well as measured various other floral traits. I found that there was a cost of early fertilization in all populations investigated in the greenhouse, indicating that the recipient cost is widespread. Interestingly, across all populations the timing of stigma receptivity was only affected by the identity of the recipient individual and not the donor, possibly denoting more female control over onset. Timing of anther-stigma contact was positively correlated with timing of stigma receptivity. In addition, there was a positive relationship between relative cost and the level of female control over onset, potentially suggesting selection for more female control when the cost of early fertilization is high. While there were mixed effects of donor or donor-recipient interaction on onset within populations, donor and recipient onset was negatively correlated within individuals. This novel finding hints at a possible trade-offs in sexual resource allocation and a genetic link between the two male and female traits involved in the conflict. In the field, I confirmed that nectar can be produced at early as well as late stages, and that seeds can be produced after pollinators visit flowers at early stages. These results are consistent with a potential

for sexually antagonistic coevolution between the male and female traits in *C. heterophylla*.

Negative impact imposed by the conflict over timing of stigma receptivity across populations

Traits involved in sexual conflict should be expected to negatively impact the fitness of the opposite sex (Parker 1979). In plants, it is possible that a pollen trait can impose a direct cost to the female (or recipient individual) (Bernasconi et al. 2004). As found in previous studies, *C. heterophylla* has exhibited reduced seed set following fertilization in early floral stages (Lankinen and Armbruster 2007; Lankinen et al. 2007; Madjidian and Lankinen 2009). My results confirm these findings, as the cost of early fertilization was apparent in both mean number of seeds per capsule and mean biomass per capsule. This cost has not been measured in as many populations or regions before indicating that the pattern is widespread. The magnitude of the cost did not differ between populations, but the population by stage effect suggests that the cost of early fertilization will become apparent at different stages in different populations.

Fecundity was affected by both donor and recipient identity, suggesting that the size and number of seeds produced is variably influenced by the combination of different donors and recipients. In addition, the recipient by stage effect indicates that different recipients are more or less susceptible to the costs of early fertilization. Looking at Figure 3, it becomes quite clear that the difference in seed production between stages varies greatly between populations. For example, population 10 has a much greater increase in seed biomass in later stages than population 3, while population 11 does not seem to incur any cost at all for being fertilized in stage 2 (Figure 4). These results support the hypothesis that the traits involved in the sexual conflict are occurring and evolving at varying levels in different *C. heterophylla* populations (Madjidian and Lankinen 2009).

One drawback to this study is that we cannot confirm whether these costs would still be evident if the pistils were left uncut (as would be occurring in the field). It is possible that the cost would disappear if uncut pistils were allowed to receive subsequent pollen depositions. However, Madjidian et al (2012) showed that the cost of early fertilization was still present after an additional pollination. This result suggests that the cost of early fertilization found in my greenhouse study would still be present, even if the pistil was left uncut and additional pollinations were performed.

Male and female influence on realized timing of stigma receptivity across populations

Because *C. heterophylla* populations differ so much in selfing rates (Charlesworth and Mayer 1995), and selfing rates tend to be correlated with timing of stigma receptivity and other floral traits in *Collinsia* (Kalisz et al. 2012), it may be expected that *C. heterophylla* populations would differ in realized timing of stigma receptivity

as well. In line with this expectation, the realized timing of stigma receptivity differed between all eight populations. While previous studies have found that both the identities of the donor and the recipient can influence the timing of stigma receptivity (Lankinen and Kiboi 2007; Madjidian and Lankinen 2009), I only found a recipient effect. This may be due to the fact that I used eight populations; perhaps the female trait is more widespread while the male trait is only present in certain populations. In line with this, it was found in Madjidian and Lankinen's (2009) study that pollen donors were better able to induce stigma receptivity when crossed with recipients from another population compared to recipients within their own. This may explain my findings, as I only performed within-population crosses and therefore did not find a donor effect. This pattern may denote that the recipient has more control over the timing of stigma receptivity than the donor, particularly within populations. With that in mind, I found a marginally significant donor by recipient interaction effect across all populations. This suggests that the identity of the donor may have some effect on onset, depending on the recipient individual. For example, population 7 had a significant donor by recipient effect (Table 6), indicating that the realized timing of stigma receptivity depends on both donor and recipient identity in that population. This may be a chance finding due to relatively low sample size in each population, but if not it could indicate that rapid runaway evolution is occurring in some populations due to a combination of sexual selection, natural selection, and genetic drift (Lande 1981; Uyeda et al. 2009)

Co-dependence between male and female function in terms of realized timing of stigma receptivity

Sexual allocation theory predicts that functions sharing the same resources (such as male and female reproduction in hermaphrodites) should be negatively correlated (Charlesworth and Charlesworth 1981). However, there is little evidence supporting trade-offs between allocation to male and female function in strict hermaphrodites (Sandmeier and Delph 1997), and existing investigations have documented a mix of both positive and negative correlations (Mazer et al. 2007). In order to investigate a possible trade-off between the male and female traits involved in sexual conflict in *C. heterophylla*, I correlated the donor and recipient effects on onset within individuals. These traits were found to have a negative relationship, a result that is novel to this study system. The relationship indicates that if an individual has an earlier timing of stigma receptivity as a recipient, it will have a later timing of stigma receptivity as a donor and vice versa. This finding is both exciting and perplexing. For example, Madjidian et al (2012) found a marginally significant positive relationship ($p=0.056$) when comparing parental donor timing of stigma receptivity (male) with innate timing of stigma receptivity in offspring (female). Though these correlations are not directly comparable (my experiment did not use multiple generations, and I was comparing recipient realized timing of stigma receptivity instead of innate timing of stigma receptivity), they are similar in that they both show relationships between pollen and pistil traits involved in the conflict. These opposing results are puzzling. It is possible that Madjidian et al (2012) did not find a negative relationship because they were comparing offspring traits and not traits

within the same individuals. In addition, they were only investing a single population. As suggested by Mazer et al (2007), mating system can influence the genetic covariance between traits, and the basic assumption of negative correlation between these traits may not be true of autogamously selfing species. This may offer some explanation for the contradicting correlations, especially if the population used by Madjidian et al (2012) is a more selfing population.

The negative relationship found in this study could be suggestive of a trade-off involving differential resource allocation to either male or female function. This is interesting when viewing within the context of sexual conflict, because it implies that individuals may have to "choose" between forced early fertilization and delayed stigma receptivity. Alternatively, it may fit perfectly into the theory of sexual conflict if the male and female trait are genetically linked (Härdling and Karlsson 2009). Individuals that are more "tough" in terms of the conflict will be able to both delay stigma receptivity *and* force early fertilization depending on whether it is acting as a recipient or a donor. On the other hand, individuals that are "weak" or less "tough" in terms of the conflict will not be able to delay stigma receptivity *or* force early receptivity (personal communication, W.S. Armbruster). In this case, donors better at forcing early fertilization would be able to fertilize more recipients that are better at delaying receptivity, which can lead to assortative mating and eventually reproductive isolation(Härdling and Karlsson 2009). These ideas are based on many assumptions, and as with anything would benefit from further research. It would be interesting to perform a similar experiment where individuals were used as donors and recipients for more than three other individuals within the population, as well as multiple individuals from other populations.

Expression of the conflict over timing of stigma receptivity in the field?

While evidence for sexual conflict in *C. heterophylla* has been found in the greenhouse, it is not yet known whether this conflict will be expressed in naturally occurring populations. In order for results found in the greenhouse to be relevant, we must confirm that the costs of early fertilization exist in the field. Likewise, it is important to verify that pollinators visit flowers at early stages, because if not there is little chance that the costs seen in the greenhouse will be realized in natural growing populations. In the field crossing experiment, fertilization success was not shown to differ between the stage 4 (or equivalent) flowers from the different treatments, providing no evidence that successful capsule production differs with various sources of pollen. This result implies that flowers are not pollen-limited in the absence of self-pollen.

Flowers were successfully fertilized in early stages by pollinators (Figure 7a, notice treatment stage X), indicating that there is potential for this conflict to be expressed in the field. A higher proportion of seed capsules were formed in later stages than in earlier stages in both treatment with stage-specific removal of pistils and treatment with stage-specific supplemental hand-pollinations, which is congruent with a cost of early fertilization in the field. However, no stage effect could be detected for seed set, even in treatments with stage-specific removal of pistils. In the greenhouse, where seed production is strongly affected by floral

developmental stage, seed capsules rarely abort after stage 1 (Madjidian et al. 2012). These results could indicate that resources are limited in the field, leading to higher abortion rate of seed capsules with few seeds. On the other hand, this field study had a small sample size, making it difficult to compare to greenhouse studies using hundreds of flowers per stage. Interestingly, a recent study by Madjidian et al (2012) found that flowers given large pollen loads in early stages of floral development had reduced seed set compared to flowers given small pollen loads. This result is consistent with dose-dependent harm related to a trait involved in sexual antagonism (Johnstone and Keller 2000). When viewing the number of seeds produced in the field, my results may agree with the theory of dose-dependent harm. Seed set in the treatment with stage-specific supplemental pollen added tended to be lower in stages 1+2 when compared to the treatment with stage-specific cutting of emasculated pollen where seeds could only be formed following natural pollination (and pollen loads were presumably smaller).

It is interesting to see that while anther-stigma contact field measurements were similar to what was found in the greenhouse, innate stigma receptivity was found to be much earlier in the field. This may indicate that anther-stigma contact is genetically controlled, while innate stigma receptivity is more physiologically controlled and dependent on resource availability and environmental cues (cf. Lankinen et al. 2007). It is strange that no seeds were produced in treatment with cut pistils and no emasculation until stage 4, even though flowers had access to both self and outcross pollen. It is possible that there is a large amount of abortion in the field due to lack of resources, and the small sample size allowed for this result through chance. On the other hand, ASC-50 in this population (151) was close to stage 3, which implies that most flowers would only have access to outcross pollen until then. Perhaps no pollinators visited these flowers in early stages, so no flowers were produced until after self-pollen was made available (in which case flowers may actually be pollen limited in the absence of self-pollen!).

Floral reward and shape, and pollinator behavior in relation to floral development

Nectar is one of the main rewards that insects and animals receive when visiting (and subsequently pollinating) flowers (Waser et al. 1996). It has been shown in a previous study that nectar can be produced in increasing amounts as the flower matures (Durkee et al. 1981). However, as nectar production has never formally been investigated in *C. heterophylla*, it is important to determine whether nectar is produced in all floral stages, both in the greenhouse and in the field. My results confirm that nectar was produced at all stages in all eight populations in the greenhouse, though in much lower amounts in the early stages. Nectar was produced in all stages in both field populations as well, though in much lower amounts than in the greenhouse. This difference in total nectar production between the greenhouse and the field is most likely due to evaporation and pollinators depleting nectar reserves before I had a chance to measure them. While not statistically significant, nectar production in the field showed a similar trend as in the greenhouse, with an incremental increase in production as stage increases. In the greenhouse, it was interesting to see that nectar production differed between

the populations. It seems obvious that differing nectar production in *C. heterophylla* populations may have repercussions on outcrossing rates, particularly in populations that tend to be more outcrossing than selfing. On the other hand, the reduced nectar quantities in early stages may deter pollinators from visiting less mature flowers, which would result in more visits to fully receptive flowers and decreased cost to the recipient plant. Varying nectar production in different *C. heterophylla* populations may be another selective force influencing the species' diversification, as reduction in rewards for pollinators may lead to less pollinator visits, thus selecting for more selfing genotypes. However, the population by stage effect indicates that the amount of nectar produced in each stage also differs between populations; this provides some evidence that the evolution of the mating system may be influencing nectar production, not the other way around!

When viewing the results on flower size it is no surprise that the length of the keel increased with stage, as the keel encases the pistil and stamens, which are also elongating as the flower matures. It is possible that the rapidity of this elongation may influence how effective pollination is at early stages. However, it is interesting to note that the length of the banner differed between the two populations. It would be informative to measure the flower size in more *C. heterophylla* populations in order to investigate whether it has some relationship with outcrossing rates or pollinator visitation.

The results on pollinator visitation provided some insight into the ecology of the *C. heterophylla* mating system; I was able to confirm that multiple pollinator types visit *C. heterophylla* in the field, and that certain pollinators will visit flowers in all floral stages. The bumblebees, which seemed to be the main pollinator type visiting *C. heterophylla* in the field, would often visit every flower on a single plant from the bottom-up. This indicates that, though they visit the more mature flowers first (the lower half of the whorl tends to have later-stage flowers), they eventually visit flowers in stage 0, 1, and 2 as well. Honey bees and flies, the other two pollinator types I observed visiting *C. heterophylla*, tended to visit only a few flowers on each individual and without any discernible pattern. In addition, it is questionable whether the latter types were successfully facilitating pollination. For example, the bumblebees were depressing the keel and gathering pollen from the exposed stamens with their hind legs, behavior which can be considered as actively pollinating. Alternatively, the flies would often enter the corolla tube (presumably in search of nectar) without depressing the keel or exposing any of the flower's sexual parts, behavior I would consider as nectar thieving. Similarly, honeybees would enter the corolla tube without actively touching the flower's sexual parts, though they tended to accidentally depress the keel due to their size. This is all rather basic observational data, and it is obvious that it would be beneficial to perform a formal study investigating the pollination ecology of *C. heterophylla* in order to illuminate this dimension of the species' mating system and its influence on sexual conflict.

Realized timing of stigma receptivity in relation to other floral traits across populations

Collinsia species are highly divergent throughout their natural range due to being geographically isolated by the Transverse mountain ranges (Baldwin et al. 2011). In addition, *Collinsia* species tend to differ in selfing rate and timing of floral maturation depending on various habitat types (Elle et al. 2010), falling into predominantly selfing or outcrossing groups (Armbruster et al. 2002; Kalisz et al. 2012). My results confirm this variation across populations. For example, populations 1, 14b, and 151 had later anther-stigma contact, innate onset of stigma receptivity, and realized onset compared to other populations (Table 5). These populations would therefore be categorized into the more outcrossing group. Similarly, populations 10 and 11 had low anther-stigma contact, innate onset of stigma receptivity, and realized onset (Table 5) and can therefore be grouped into the more selfing category. Population 7 on the other hand was intermediate when looking at start of flowering, timing of anther stigma contact, innate and realized timing of stigma receptivity (Table 5), which could indicate that it lays somewhere in the middle of the self-outcross spectrum. This pattern can be confirmed by correlating these pollen and pistil traits with molecular data that illuminates relatedness and levels of inbreeding within populations.

According to sexual selection theory, females incurring high levels of cost from sexually antagonistic male traits will evolve a defense to this cost (Gavrilets 2000). This may explain why I found a negative relationship between relative female influence on onset and relative cost. This result suggests that populations with a higher cost of early fertilization have more female control. In terms of sexual conflict this makes sense, as it could indicate that high costs of early fertilization select for strong female control over the timing of stigma receptivity (thus allowing a release from the costs of the male trait).

Theory predicts that there should be a positive relationship between a mate choice trait and a trait conferring high reproductive success (as with cryptic mate choice in animals, see (Eberhard 1996). I found a (non-significant) negative trend between the relative female influence on onset and pollen tube growth rate (Figure 6b). Though this is somewhat counter-intuitive, this trend is in the expected direction (as lower values of relative female influence means more female control); thus, populations with higher pollen tube growth rates have higher female control, and vice versa. These results may not be totally accurate, as taking a representative measurement of pollen tube growth rate proved to be difficult. There were thousands of pollen tubes grown through the medium on each microscope slide, and only ten measurements were taken per slide. Most slides had pollen tubes lengths ranging from very short to very long, with short tubes being rather easy to measure and long tubes extremely difficult to measure (due to curvy growth trajectories and constant overlap with other pollen tubes). For this reason, it may be better to evaluate *in vivo* pollen tube growth rate in the future (as opposed to *in vitro* as with this study), as it more accurately measures pollen performance (Mazer et al. 2010). With that in mind, a previous study on *C. heterophylla* confirmed a positive

relationship between pollen tube growth rate in germination medium and in the pistil (Lankinen et al. 2009), which supports the accuracy of my methods.

Conclusion

This study provides further evidence that sexual conflict and antagonistic coevolution can significantly impact floral evolution and plant species diversification. By investigating more populations than have previously been studied in the greenhouse, I have confirmed that both the cost of early fertilization and the female influence on stigma receptivity is present in a large portion of *C. heterophylla*'s natural range. The positive relationship between relative cost of early fertilization and level of female control is in line with sexual conflict theory, which predicts that selection will favor increased female control when mating costs are high (Gavrilets 2000). Furthermore, the negative relationship found between male and female influence on onset within individuals alludes to a genetic link between the two traits involved in the conflict, as recently predicted in sexually antagonistic coevolution dynamics (Härdling and Karlsson 2009). These greenhouse results are coupled with the results from my field work, which confirm that nectar is produced at early stages and that seeds can be produced after pollinators visit early stage flowers; this helps verify that the sexual conflict perceived in the greenhouse is relevant to what is occurring in natural *C. heterophylla* populations.

A recurring theme in this thesis is the complex variety of forces that are involved in the coevolution of the sexes (Arnqvist and Rowe 2005), made all the more complicated when investigating an organism that contains both in the same individuals (as exemplified in animal hermaphrodites, see (Michiels and Koene 2006). The outcome of the sexual conflict depends on the multifaceted interactions between all of the pollen and pistil traits involved, compounded by eons of geographic, environmental, and ecological history. This study barely scratched the surface of the ecological element (particularly pollination ecology), an aspect of this system that is essential to unveiling the evolutionary history of *Collinsia heterophylla*. Further research should focus on combining ecology, DNA sequencing, and the extensive evolutionary work already done in this field. Sexual selection and antagonistic coevolution are becoming more accepted as important processes shaping plant evolution, and continued research in this area may help increase our understanding of how both plants and animals speciate and diversify into the profusion of forms we see today.

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