**Delayed stigma receptivity in Collinsia heterophylla (Plantaginaceae): genetic variation and adaptive significance in relation to pollen competition, delayed self-pollination, and mating-system evolution**

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**Abstract**

To increase our knowledge about mating-system evolution, we need to understand the relationship between specific floral traits and mating system. Species of Collinsia (Plantaginaceae) vary extensively in mating system; this variation is associated with variation in floral morphology and development and with the timing of self-pollination. Counterintuitively, large-flowered, more outcrossing species tend to have delayed stigma receptivity, reducing the amount of time that the stigma is receptive to cross-pollination before autonomous self-pollination. To understand how the timing of stigma receptivity is related to mating-system evolution, we studied in detail the timing of both stigma receptivity and self-pollination (anther–stigma contact) in two greenhouse-grown populations of large-flowered Collinsia heterophylla. Crosses on emasculated flowers at different stages of floral development always produced seeds, suggesting that cross-fertilization can be effected by pollen arriving prior to physiological receptivity. Phenotypic and genetic variation within populations in the timing of stigma receptivity and anther–stigma contact was substantial, although slightly less for the contact. Despite strong interspecific and interpopulation correlations, we did not find an among-genet phenotypic correlation between the traits. This indicates that each trait may respond independently to selection, and the trait association may be the result of correlational selection.

**Keywords**: Collinsia heterophylla; delayed selfing; delayed stigma receptivity; evolvability; flower development; heritability; mixed mating system; Plantaginaceae.

After several decades of research, the patterns and causes of mating-system evolution in plants remain incompletely understood (Goodwillie et al., 2005; Igić and Kohn, 2006). It is clear that evolutionary transitions have occurred repeatedly from outcrossing to selfing (Stebbins, 1950, 1974; Barrett et al., 1996), but it has been difficult to explain the evolution of this transition (Jarne and Charlesworth, 1993; Holsinger, 1991; Takebayashi and Morrell, 2001) and even more difficult to explain the evolution of strategies that combine both outcrossing and selfing (mixed mating systems) (Jarne and Charlesworth, 1993; Goodwillie et al., 2005).

The evolutionary shifts toward self-pollination have been associated with changes in several floral traits, including loss of heterostyly and self-incompatibility, decreased flower size, lower pollen–ovule ratios, and developmental changes affecting the timing of self pollination and the temporal and spatial separation of pollen and stigma (dichogamy and herkogamy) (Grant, 1958; Jain, 1976; Cruden, 1977; Ritland and Ritland, 1989; Fenster et al., 1995; Barrett et al., 1996; Fishman and Wyatt, 1999; Cruden, 2000; Motten and Stone, 2000; Armbruster et al., 2002; Goodwillie and Ness, 2005; Takebayashi et al., 2006). Even though it is important to understand the relationship between specific traits and mating system in order to increase our knowledge about mating-system evolution, the functional significance of these traits is not always clear. Separation of male and female function in time (dichogamy), for example, was traditionally interpreted as a way to avoid selfing (Darwin, 1877; Lloyd and Webb, 1986; Barrett, 2003). If the male function precedes the female function, self pollen will be gone by the time the stigma is receptive (protandry). More recent studies indicate that protandry must have other advantages in some plants (e.g., avoidance of male–female interference; Lloyd and Webb, 1986; Bertin, 1993), however, because many protandrous species are self-incompatible (Routley et al., 2004).

A comparative study of ca. 20 species of the tribe Collinsieae (Plantaginaceae) revealed that mating system and morphological and developmental traits covary continuously (Armbruster et al., 2002). Collinsieae species are self-compatible (no self-incompatibility system is known), and the variation in mating system is caused by differences in timing of anther–stigma contact, i.e., prior, competing, or delayed selfing (see Lloyd, 1992). The loss of herkogamy at the end of anthesis (delayed selfing) promotes outcrossing but provides fail-safe seed set in the event of outcross pollen not arriving early. As expected, more outcrossing species generally have larger flowers, and more selfing species smaller ones. Surprisingly, however, large-flowered species have delayed stigma receptivity, potentially impairing outcrossing by reducing the time that flowers are receptive to outcross pollen prior to self-pollination (Armbruster et al., 2002). Even though the flowers are partially protandrous in the sense that the female function is delayed relative to pollen maturation, the...
anthers continue to dehisce after stigmas have become receptive. Promoting protandry by delaying stigma receptivity would appear to reduce the outcrossing advantages. One hypothesis that resolves the contradiction is that outcross pollen arrives early and stays on the stigma until the stigma becomes receptive, thus promoting outcrossing. Even if this hypothesis turns out to be correct, we must still wonder why delayed receptivity has evolved. The strong correlation of stigma receptivity schedule with mating system across Collinsieae species suggests that understanding the functional significance of this trait would be important for our understanding of mating-system evolution.

Delayed stigma receptivity has been interpreted as a mechanism that will intensify pollen competition (Wilson and Burley, 1983; Galen et al., 1986; Herrero, 2003). Increased competition might be beneficial because compatible or superior pollen donors can be favored (e.g., Mulcahy, 1979, 1983; Marshall and Ellstrand, 1986; Quesada et al., 1993; Paschke et al., 2002; see reviews in Willson, 1994; Skogsmyr and Larkin, 2002). It could also be important because it allows recipients to avoid fertilization by self-pollen of the lowest quality (Armbruster and Rogers, 2004).

On the other hand, geitonogamy can be reduced and outcrossing increased when the male function precedes female function in species with vertical inflorescences and flowers maturing from the bottom up (acropetal), because pollinators generally move upward on the inflorescence (Darwin, 1877; Harder et al., 2000). Flowers of *Collinsia* mature acropetally, which should favor delayed stigma receptivity (i.e., protandry). However, if geitonogamous pollen can accumulate on the stigma prior to receptivity and then fertilize the ovules upon stigma receptivity, then delayed stigma receptivity would not reduce geitonogamy.

Thus, delayed stigma receptivity, which has evolved repeatedly in large-flowered *Collinsia* species (Armbruster et al., 2002) and in many other plant species (e.g., Herrero, 1983; Galen et al., 1986; Murdy and Carter, 1987; Ganesaiah and Uma-Shaanker, 1988; Douglas andCrud, 1994; O’Brien, 1996; Stewart et al., 1996; Kingstorn, 1998; Kalinganire et al., 2000; Buide and Guitian, 2002; Bhattacharya and Mandal, 2004; Yi et al., 2006), can be explained by one of two mutually exclusive hypotheses: (1) Delayed stigma receptivity promotes outcrossing through protandry and reduced geitonogamy (the geitonogamy-avoidance hypotheses). (2) Delayed stigma receptivity increases competition among pollen grains (the pollen-accumulation hypothesis).

Although phylogenetic comparison among species is a powerful tool for the study of evolution (Harvey and Pagel, 1991; Armbruster, 1992; Weller and Sakai, 1999), detailed studies of variation within populations are also necessary to reach a deeper understanding of evolutionary mechanisms. For example, it is crucial to estimate heritable variation in order to predict how traits would respond to selection. We have only limited knowledge of such variation in floral developmental traits, such as timing of stigma receptivity or timing of self-compatibility or self-pollination (but see Goodwillie and Ness, 2005, for *Leptosiphon leprous*; Dole, 1992; Kelly and Arathi, 2003; van Kleunen and Ritland, 2004, for *Mimulus guttatus*; and Klips and Snow, 1997, for *Hibiscus laevis*). Furthermore, to evaluate whether delayed stigma receptivity can respond independently to selection or whether variation in this trait merely reflects developmental or genetic correlations with other traits under selection (Lande and Arnold, 1983), we need to investigate whether delayed stigma receptivity and delayed selfing covary within and among individuals.

To better understand the relationships between the timing of stigma receptivity, timing of self-pollination, and mating system evolution, we studied in detail the variation in timing of stigma receptivity and anther–stigma contact across greenhouse-grown individuals of the large-flowered species, *Collinsia heterophylla*. Because our goal was to study variation within rather than between populations, we limited the number of investigated populations to two. To understand better the functional consequences of delaying stigma receptivity, we performed controlled crosses on emasculated flowers at different stages of floral development. This design allowed us to investigate whether pollen deposited before stigma receptivity was able to fertilize ovules upon stigma receptivity and thus provide the opportunity for outcrossing. We also assessed phenotypic and genetic (heritable) variation of timing of both stigma receptivity and anther–stigma contact for an indication of how these traits would respond to selection. Genetic variation was investigated only in the larger of the two populations.

**MATERIALS AND METHODS**

**Study species**—*Collinsia heterophylla* Buist (Plantaginaceae) is a widely distributed diploid annual native of the California Floristic Province (Newsom, 1929; Neese, 1993). Flowering time is between March and June depending on latitude and elevation. Flowers are arranged in whorls on spikes. Outcrossing rates based on allozyme markers of several populations have been estimated to range from 0.32–0.64 (Charlesworth and Mayer, 1995). A variety of native bees, including *Osmia*, *Bombus*, and *Anthophora*, most commonly, serve as pollinators (Armbruster et al., 2002; S. Armbruster, unpublished data). Flowers are zygomorphic, with a five-lobed corolla arranged in one upper and one lower lip. Corolla color is generally white to pale purple on the upper lip and dark purple on the lower lip, although some populations can be dark on parts of the upper lip and others pale purple or off-white on both lips. Flowers have four epipetalous stamens and one pistil, containing up to 16 ovules (Armbruster et al., 2002). When flowers open, all anthers are undehisced and the stigma is not receptive; this makes experimental emasculation of the flowers easy. The anthers usually dehisce one at a time over 3–4 d, i.e., one anther dehisces per day. During this period, the stigma becomes receptive and the style elongates. This eventually places the stigma in contact with the dehisced anthers, and self pollination can occur (see Armbruster et al., 2002 for a more detailed description; see also Kalisz et al., 1999). Ovaries develop into dry, dehiscent seed capsules.

Population 1 in this study originated from a population in Sisar Canyon, Ventura County, California (approximately 40 maternal families) (population 4 in Armbruster et al., 2002). Population 2 originated from a population in Napa County, California, USA (approximately seven maternal families). The latter greenhouse population was small because the source population had only just begun to fruit at the time of seed collection. Flowers of both populations are off-white (upper lobe)/pale-lavender (lower lobe), but population 2 was slightly darker. Flower sizes in both populations were similar. Plants were raised from seeds and grown in an insect-free greenhouse in the summer of 2001 and in the winter/early spring of 2002 and 2003.

**Crosses on emasculated flowers at different floral developmental stages**—To investigate whether seeds could be produced when pollen was added to the stigma prior to receptivity, we performed controlled crosses on emasculated flowers on five developmental stages in two years (2001 and 2003). Following Armbruster et al. (2002; see also Kalisz et al., 1999), we classified the stages based on number of anthers dehisced (0–4). Because the anther-dehisced stages approximately equals day 0 to 4 after flower opening (Armbruster at al., 2002) and because we worked on emasculated flowers, we used only days since flower opening as an indication of floral developmental stage. We performed all emasculations and crosses at approximately the same time each day.
In 2001, seven plants of population 1 received self or outcross pollen on different flowers. In 2003, five recipients from each of population 1 were crossed with self and outcross pollen as well as with pollen from the other population. In each recipient plant, we used one flower per pollen source for each of the five developmental stages (i.e., totals of 2 × 5 = 10 flowers per plant in 2001 and 3 × 5 = 15 flowers per plant in 2003). Pollen was added to the stigma from a microscopic slide until the stigma was completely covered with pollen. When unrelated pollen was the pollen source, we always mixed pollen from two donors until the microscopic slide was covered at ripening. Seed capsules were also collected from flowers left untreated, i.e., autonomously self-pollinated flowers (= controls), in both years.

**Stigma receptivity**—Timing of stigma receptivity was determined in two ways at each of the five stages of floral development (i.e., roughly the same time on day 0–4 after flower opening). First, in 2002 and 2003 we tested for stigmatic peroxidase activity (SPA) using the method of Kearns and Inouye (1993) in both populations. Intact styles of emasculated flowers of all five developmental stages were placed on a glass slide in a drop of 3% hydrogen peroxide and covered with a cover slip. Stigmas that produced bubbles within 2–3 min were considered receptive. At least two stigmas were tested per individual and developmental stage (i.e., 2 × 5 = 10 flowers per plant). These stigmas were used to calculate one (mean) value of onset of stigma receptivity for each individual plant. The day at which 50% of the plants produced stigmas that transported droplets of peroxidase activity was calculated using logistic regression (using the method in Armbruster et al., 2002), with the value calculated for each individual plant as the sample unit.

Second, to confirm the relationship between SPA and the presence of pollen tubes in pistils, in 2002 we pollinated emasculated flowers of population 1 at each of the five stages of flower development. On the 16 recipients used, 1–4 flowers per stage received outcross pollen from one donor per flower. For total 10 pollen donors were used, with a mean number per recipient of 1.8. Third, in a subset of eight of these 16 recipients we also performed crosses with self-pollen to investigate how pollen source (two outcross donors vs. self) influenced onset of stigma receptivity. Crosses were performed using the method described. Pistils were collected after 3 or 4 h and immediately stored in ethanol (70%). Pollen take between 1 and 1.5 (occasionally 2) h to germinate on the stigma (Å. Lankinen and S. Armbruster, unpublished data). Collected pistils were rinsed under distilled water, and tissues were softened in 1 M NaOH for 2–3 h. Pistils were then thoroughly rinsed under distilled water and stained in a solution of aniline blue in aqueous K3PO4 for at least 3 h. The presence of pollen tubes in the pistil was noted under a UV epifluorescence microscope.

**Anther–stigma contact**—To evaluate stigma receptivity in relation to pollen elongation and anther–stigma contact at each of the five stages of floral development, we measured pistil length in all flowers from 37 of the 50 plants used for testing stigma receptivity in 2002 (population 1; i.e., two flowers per developmental stage for each plant: 2 × 5 = 10 flowers per plant; see previous section). From the measurements of pistil length, we indirectly estimated the age at which anthers and stigma came in contact by comparing, in a subset of the plants, the pistil length at anther–stigma contact with the pistil length at day 4 (both lengths measured in each of two flowers per individual). We observed that the stigma contacted the dehisced anthers when pistil had elongated to 90 ± 5.9% (± SE; Np = 10; Nw = 2 × 10 = 20) of its length at day 4. Pistils >84% of their final length were therefore considered to have stigmas in contact with dehisced anthers. From this indirect estimate based on pistil length, we then calculated the expected flower age at anther–stigma contact for each plant using logistic regression. This was the age at which 50% of the flowers had pistils longer than 84% of their total length (PistilLength50), with the value calculated for each individual plant as the sample unit.

In 2002, developmental rate of flowers was more or less equal to, or a little faster than one developmental stage per day (Å. Lankinen, personal observation), i.e., more or less agreeing with that found in natural populations (Armbruster et al., 2002). In 2003, floral developmental rate increased to about 12 h per stage, i.e., two anther–dehiscent stages equaled 1 d. This was presumably due to greenhouse conditions that year: the lights were on for 16 h rather than for 13 h per day (which also resulted in higher temperature). In 2003, we estimated, in both populations, the floral stage (number of anthers dehisced) at which anthers and stigmas came into contact. We followed at least two newly opened flowers per individual every 12 until stage 4 was reached. To be able to compare the two methods used to estimate timing of anther–stigma contact, we also measured pistil length in two flowers each day after flower opening in a subset of these plants (i.e., in total 2 × 5 = 10 flowers per plant). Logistic regression was used to estimate the anther-dehiscent stage at which 50% of the flowers had anthers and stigmas in contact (ASC-50) following Armbruster et al. (2002; see also Kalisz et al., 1999 for more details). The sample unit was thus one value for each individual plant.

**Genetic component of stigma receptivity and heritability/evolvability of anther–stigma contact**—To examine the ability of timing of stigma receptivity and anther–stigma contact to respond to selection, we assessed whether there was a genetic component of the variation in these traits. We only used population 1 because we judged the number of maternal families in population 2 to be too small. We estimated broad-sense heritability of stigma receptivity by measuring this trait in six parental plants and in their selfed progeny produced in 2002. Parental plants with as different timing of stigma receptivity as possible were chosen. One to two offspring per mother plant were grown in the greenhouse in 2003.

To calculate narrow-sense heritability and evolvability of the time of anther–stigma contact, we measured this trait in 2003 in half-siblings resulting from one-donor crosses done in 2002. Each of the eight recipients used was crossed with 2–4 of the eight donors. Crosses were performed on emasculated fully receptive flowers (at stage 4 of flower development) using the technique described previously. All crossing combinations were performed twice, and crosses were repeated if the cross failed. This only happened in 3.8% of all crosses. Crosses were repeated if the cross failed. This only happened in 3.8% of all crosses. In each recipient plant, we used one flower per pollen source for all 16 crosses. A few days before seed capsules were ripe, they were bagged to avoid losing any seeds. The mature seeds were collected and sown to assess the time of anther–stigma contact in the offspring. The trait was measured in 2–6 offspring (three from each of the two crosses when available) per half-sib group (using the method described for 2003).

**Statistical analyses**—We analyzed differences in seed set after hand pollinations on emasculated flowers at various floral developmental stages with general linear models (GLMs) using SPSS (SPSS, 1999). Differences within the two populations were tested separately with a mixed model including recipient plant as a random factor, crossing type and stage as fixed factors, and all two-way interactions. To investigate whether unmanipulated flowers (control) differed in seed set compared to hand-pollinated flowers, we made pairwise comparisons between control and hand-pollinated flowers of all developmental stages using Dunnett post-hoc tests (following a significant treatment effect in a mixed model with recipient plant [random factor], treatment [each of the five stages and control = six treatments] [fixed factor], and the interaction). We also analyzed both populations together in a three-way ANOVA including the factors population, cross, and stage (all fixed), and all interactions. We chose to consider population as a fixed factor rather than a random factor because the error degrees of freedom could not be calculated for the three-way ANOVA of the five stages of floral development of one more another–stigma contact.

Logistic regression was performed using the SPSS PROBIT procedure (SPSS, 1999). Variation in onset of stigma receptivity in population 1 did not deviate from normality (Kolmogorov–Smirnov Lilliefors significance correction): \( D = 0.104, df = 60, P = 0.165 \). Variation in anther–stigma contact measured as pistil elongation (method 1) in population 1 deviated significantly from normality (Kolmogorov–Smirnov with Lilliefors significance correction: \( D = 0.399, df = 41, P < 0.001 \)), while the deviation for our second measurement (anther–stigma contact) in this population was smaller and not significant (Kolmogorov–Smirnov Lilliefors significance correction: \( D = 0.190, df = 19, P = 0.070 \)). Because the variation for anther–stigma contact was low in relation to the mean for both measurements of this trait (see Results) and not asymmetrical, we performed ANOVAs and linear regressions on the untransformed data (Falconer and Mackay, 1996). We analyzed the parent–offspring regression of stigma receptivity to estimate the genetic component of this trait (Falconer and McKay, 1996; Lynch and Walsh, 1998). Compared to a standard parent-offspring regression (assuming random mating), using selfed offspring is likely to inflate the estimate of additive genetic variance (because of correlations between the same alleles within individuals) and can also complicate separation of additive genetic variance from other genetic variance (Lynch and Walsh, 1998). The estimate calculated from the parent-offspring regression will thus not be an estimate of narrow-sense heritability (\( h^2 = V_A/V_P \)), where \( V_A \) is the additive genetic variance and \( V_P \) is the phenotypic variance) but rather an estimate of the extent to which phenotypic variance is explained by any genetic component (broad-sense heritability; \( h^2 = V_A/V_P \)). This can be considered the upper limit for narrow-sense heritability. For anther–stigma contact, we estimated heritability in the narrow sense in a half-sib analysis (Falconer and Mackay, 1996).
1996; Kearsey and Pooni, 1996) using the GLM procedure (SPSS, 1999) with father, mother (random effects), and their interaction. We calculated heritability from the variance components as

\[ h^2 = \frac{r^2_F}{r^2_T} \] for father, 
\[ h^2 = \frac{r^2_M}{r^2_T} \] for mother, and 
\[ h^2 = \frac{2 \times (r^2_F + r^2_M)}{r^2_T} \] for midparent assuming random mating (Falconer and MacKay, 1996), where \( r^2_F \) is the paternal component of offspring variance, \( r^2_M \) the maternal, and \( r^2_T \) the total variance. Heritability may be an inaccurate estimate of the ability of a trait to evolve (see, e.g., Houle, 1992; Hansen et al., 2003 for detailed discussions). For this reason, we also estimated evolvability, \( I_A \), as the additive genetic variance scaled by the square of the trait mean, \( Z \) (\( I_A = 100 \times V_A / Z^2 = 100 \times 4 \times r^2_{F+M} / Z^2 \) (Hansen et al., 2003).

Type III sums of squares were used in all ANOVAs.

**RESULTS**

**Seed set following hand pollinations on emasculated flowers at different developmental stages**—In both populations, seed set was affected by stage of flower development (Fig. 1, Table 1). There was a significant effect of maternal plant in population 1 in 2003, but no other effects of maternal plant or pollen source. Although seed set increased with stage in both populations (Table 2), seed production did not increase much beyond stage 2 in population 1 and beyond stage 1 in population 2 (Fig. 1). In 2001, seed set was already relatively high when flowers were hand pollinated at stage 0 (64% of maximum seed set [stage 4]; Fig. 1). In the same population in 2003, seed set at stage 0 was only 32%. In population 2, seed set at stage 0 was only 6%. The slopes in the three experimental groups were more similar than the intercepts, though somewhat lower for groups with higher intercepts (Table 2). This result suggests that the reduced seed set at stage 0 in 2003 was influenced by the generally lower seed production that year. In addition, fruit abortion occurred only rarely in 2001, whereas in 2003 it was quite common.

There was no difference in seed number between unmanipulated flowers (control) and hand-pollinated flowers at any of the five stages in 2001 (Dunnett post-hoc test; \( P > 0.077 \) for all pairwise comparisons). In this year the number of seeds produced in control flowers equaled seed production by flowers pollinated at stage 4 (Fig. 1). In 2003, number of seeds produced by control flowers was similar to that produced by flowers pollinated at stage 1 (population 1) or between stage 0 and 1 (population 2). However, the relatively lower seed production in controls in 2003 was not significantly different from seed production in hand-pollinated flowers at any of the

![Graph showing seed set following hand pollinations on emasculated flowers at different developmental stages](image-url)
Table 1. Mixed-model ANOVA for seed production per capsule in two populations of Collinsia heterophylla.

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Note: Bold text indicates significant P values (<0.05); total number of flowers per maternal plant = 2 × 5 = 10.

Table 2. Linear regression analyses between number of seeds per five stages (population 1: Dunnett post-hoc test; P > 0.23 for all pairwise comparisons; population 2: Dunnett post-hoc test; P > 0.071 for all pairwise comparisons).

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Note: N = no. of recipient plants.

five stages (population 1: Dunnett post-hoc test; P > 0.23 for all pairwise comparisons; population 2: Dunnett post-hoc test; P > 0.071 for all pairwise comparisons).

Seed production in the two populations was not significantly affected by population origin of either recipient plants or (outcross) pollen source (Fig. 1, Table 3). Although not significant, there was a slight tendency for foreign pollen to produce more seed in late-stage pollinations in both populations. At stage 0, however, foreign pollen produced the least seed in both populations.

Timing of stigma receptivity—The peroxidase test indicated that 50% of plants in the sample had receptive stigmas (stigma peroxidase activity, SPA) at 2.23 (population 1) and 1.95 (population 2) d after flower opening (Table 4). Timing of stigma receptivity did not differ between populations in 2003 (one-way ANOVA; F₁,16 = 0.427, P = 0.523) or between years in population 1 (one-way ANOVA; F₁,59 = 2.42, P = 0.12). Variation in timing of stigma receptivity among individuals was substantial, ranging from day 0 to day 4 in population 1 and from day 1 to day 3 in population 2 (Table 4).

The stage at which pollen tubes first occurred in the pistil varied significantly among recipient plants (population 1: one-way ANOVA; F₁,19 = 2.71, P = 0.027). The maximum variation among the recipients ranged between 1.33 and 3.67 (SD = 0.72), and the mean variation within recipients was 0.58 stages. Pollen type (outcross or self) did not influence the stage at which pollen tubes were present in pistils (Table 5, Wilcoxon signed ranks test; T = 15, N = 7, P = 0.47).

The developmental stage at which stigmas were estimated as receptive according to SPA correlated across plants with the stage at which pollen tubes were detected in the pistil (r = 0.518; Fig. 2). We did find a few cases where SPA indicated that the stigma was receptive slightly before we saw pollen tubes in the pistil, i.e., false positive values. It is, however, not possible to separate these cases from failure of pollen to germinate for reasons unrelated to receptivity. On the other hand, we saw very few false negative results.

Anther–stigma contact—In 2002, anther–stigma contact (calculated as pistil elongation to >84% of pistil length at day 4 in 50% of all plants) occurred 1.70 d after flower opening (population 1, Table 4). No phenotypic relationship between timing of stigma receptivity and anther–stigma contact was detected (both populations and years: partial correlation, correcting for population and yr; rₚ = −0.041, df = 44, P = 0.79).

In 2003, anther–stigma contact (ASC-50) occurred at anther–dehiscence stage 2.85 in population 1 (Table 4). This equaled 1.43 d after flower opening. In population 2, ASC-50 was estimated as stage 2.01, or 1.01 d (Table 4). This difference was marginally significant (one-way ANOVA; F₁,24 = 3.56, P = 0.072).

The two measures we used to estimate the time of anther–stigma contact were correlated (Pearson correlation; r = 0.668, N = 10, P = 0.035). We therefore recalculated estimates from 2003 to equal “days” and found that the stigmas of population 1 contacted their anthers earlier in 2003 than in 2002 (one-way ANOVA; F₁,52 = 4.24, P = 0.044, Table 4). For both methods used to calculate anther–stigma contact, the magnitude of variation among plants was lower for anther–stigma contact than for stigma receptivity (Table 4).

Genetic components of timing of stigma receptivity and anther–stigma contact—The parent–offspring regression of timing of stigma receptivity in selfed progeny was similar between generations (b = 0.887; Fig. 3); i.e., time of stigma receptivity had a high broad-sense heritability. This suggests a major genetic component of variance in this trait.

Narrow-sense heritability and IA evolvability for timing of anther–stigma contact were substantial in the half-sib analysis (Table 6; values for midparent: h² = 0.558, IA = 4.69%). Because the values for father and mother were similar, maternal effects were probably not substantial. The estimate for midparent should thus be the most appropriate.

DISCUSSION

Timed hand pollinations conducted on Collinsia heterophylla, a species with a mixed mating system, showed that...
pollen arriving on the stigma before receptivity can survive and fertilize the ovules upon stigma receptivity. Accumulation and survival of pollen on unreceptive stigmas will thus promote outcrossing despite delayed stigma receptivity reducing the time period for cross fertilization prior to autonomous self-pollination. It seems unlikely, however, that delayed stigma receptivity could prevent geitonogamous self-fertilization, because geitonogamous pollen arriving early can also fertilize the ovules upon receptivity.

Variation in timing of stigma receptivity among individuals within the two populations studied was substantial, while variation in anther–stigma contact was considerably less. Variation in both traits had substantial genetic components.

Functional consequences of delaying stigma receptivity—
Stigma receptivity is delayed in all species of Collinsia with larger flowers, even though this reduces or completely eliminates the time period for cross-fertilization prior to autonomous self-pollination. It appears unlikely, however, that delayed stigma receptivity could prevent geitonogamous self-fertilization, because geitonogamous pollen arriving early can also fertilize the ovules upon receptivity.

Variation in timing of stigma receptivity among individuals within the two populations studied was substantial, while variation in anther–stigma contact was considerably less. Variation in both traits had substantial genetic components. The two traits were not correlated among plants.

Table 3. Three-way ANOVA for seed production per capsule in two populations of Collinsia heterophylla (2003). Flowers were hand pollinated with either outcross pollen from the same population or pollen from the other population at stages 0–4 of flower development (day 0–4 after flower opening). Hand-pollinated flowers were emasculated at flower opening.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population</td>
<td>1</td>
<td>0.600</td>
<td>0.440</td>
</tr>
<tr>
<td>Cross</td>
<td>1</td>
<td>1.45</td>
<td>0.232</td>
</tr>
<tr>
<td>Stage</td>
<td>4</td>
<td>8.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Population × cross</td>
<td>1</td>
<td>0.255</td>
<td>0.615</td>
</tr>
<tr>
<td>Population × stage</td>
<td>4</td>
<td>0.575</td>
<td>0.681</td>
</tr>
<tr>
<td>Cross × stage</td>
<td>4</td>
<td>0.632</td>
<td>0.641</td>
</tr>
<tr>
<td>Population × cross × stage</td>
<td>4</td>
<td>0.979</td>
<td>0.422</td>
</tr>
</tbody>
</table>

Note: Bold text indicates significant P values (<0.05). Five recipient plants per population were used and 2 × 5 = 10 flowers per individual plant.

Table 4. Floral developmental stage when 50% of flowers (calculated as mean values for individual plants) had receptive stigmas (SPA-50) or anthers and stigma in contact (Pistil-34%/50 and ASC-50) estimated during 2 yr in two populations of Collinsia heterophylla. The mean value for each plant individual was based on 10 flowers per plant for SPA-50 and Pistil-34%/50 and on two flowers per plant for ASC-50. CV, maximum (max), and minimum (min) values indicate the magnitude and range of variation among individual plants. Stage of floral development was estimated as day 0–4 after flower opening for stigma receptivity and the anther–stigma contact-measurement in 2002. In 2003 the developmental stage when anther–stigma contact occurred was estimated as anther dehiscence stage (see methods). Also in 2003, two anther dehiscence stages equaled approximately 1 d rather than 2 d in 2002. The 95% CI in column 3 could not be calculated for ASC-50 in population 2 (2003) because of small sample size.

<table>
<thead>
<tr>
<th>Population</th>
<th>Year</th>
<th>Stage when 50% of stigmas receptive/in contact (95% CI)</th>
<th>CV</th>
<th>Min</th>
<th>Max</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stigma receptivity, SPA (day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2002</td>
<td>2.31 (2.23–2.39)</td>
<td>0.37</td>
<td>0.5</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>1</td>
<td>2003</td>
<td>1.72 (1.36–2.03)</td>
<td>0.50</td>
<td>0</td>
<td>3.5</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>2002</td>
<td>2.23 (2.16–2.31)</td>
<td>0.39</td>
<td>0</td>
<td>4</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>2003</td>
<td>1.94 (1.53–2.33)</td>
<td>0.33</td>
<td>1</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

| Anther–stigma contact, Pistil-34%/ (day) | | | | | | |
| 1          | 2002 | 1.70 (1.37–2.08)                                     | 0.23 | 1   | 3   | 37 |

| Anther–stigma contact, ASC (anther developmental stage)/ (day) | | | | | | |
| 1          | 2003 | 2.85 (2.64–3.07)/1.43                                 | 0.23 | 1.5/0.75 | 4/2 | 19 |
| 2          | 2003 | 2.01/1.01                                            | 0.22 | 2/1 | 3.5/1.75 | 6 |

Note: Bold text indicates an estimate for both 2002 and 2003. 95% CI = 95% confidence interval; CV = standard deviation/mean; N = number of plant individuals.
This is likely to be important in bee-pollinated flowers that are arranged in whorls on spikes that flower from the bottom up, such as in *C. heterophylla*. Our observation that both self- and outcross pollen arriving prior to receptivity were able to survive and fertilize the ovules upon receptivity does not, however, support delayed stigma receptivity as a mechanism to avoid geitonogamy in *C. heterophylla*. Hence, geitonogamy is not reduced in *Collinsia*, despite delayed stigma receptivity relative to pollen release, because pollen from flowers located lower on the same spike will also arrive early and be able to “wait” on the stigma until receptivity.

**Timing of stigma receptivity and anther–stigma contact within and between populations**—The similarity in times of stigma receptivity (about 2 d after flower opening) and anther–stigma contact (slightly earlier than stigma receptivity) between our two greenhouse populations was expected from their similarity in flower size and from the results of the Armbruster et al. (2002) study of variation among populations and species. Intriguingly, the range of variation among individuals within each population was greater for timing of stigma receptivity than for timing of anther–stigma contact.

Previous measurements in the field of one of the same and one different population of this species indicated that stigma receptivity slightly preceded anther–stigma contact rather than the opposite (Armbruster et al., 2002). While timing of anther–stigma contact in the field was similar to our result (in the first year), stigmas became receptive relatively later in the greenhouse. This discrepancy might be caused by environmental differences between the field and the greenhouse. Stigma receptivity was, however, not significantly affected by the difference in day length and temperature between years in the greenhouse. The inconsistency could also to some extent be influenced by how stigma receptivity was measured in combination with the large variation for this trait. In the greenhouse, it was possible to make estimates for all developmental stages on the same individual for a large number of plants (*N* = 60, based on 2 × 5 flowers per individual = 600 flowers), while in the field most collected flowers were from different individuals for practical reasons (and only a total of 23 and 25 flowers [1–2 per plant] were used, respectively) (Armbruster et al., 2002).

The slightly earlier anther–stigma contact in the second year was presumably due to the environmental influence on floral developmental rate. Interestingly, although the anther-dehiscence developmental stage at which anthers and stigma touched was strongly influenced by environmental condition in the greenhouse, the day of anther–stigma contact (after flower opening) was not greatly affected. This suggests that anther development is more influenced by environmental condition than pistil elongation.

Our finding that stigma receptivity occurs after anther–stigma contact in greenhouse populations of *C. heterophylla* was consistent with the pattern seen in other large-flowered...
Collinsia species (C. verna, C. tinctoria, C. multiflora, and C. corymbosa; Kalisz et al., 1999; Armbruster et al., 2002). Delaying stigma receptivity to after anther–stigma contact does not necessarily impair outcrossing, as long as early arriving pollen can survive on the stigma until receptivity occurs. For example, cross pollen arriving in the first pollination may grow tubes down the style shortly before the arrival of most self pollen, or self pollen may grow more slowly (cryptic self-incompatibility, e.g., Bertin and Sullivan, 1988; Cruzan and Barrett, 1993; but see Snow and Spira, 1993; Melser et al., 1997), at least at earlier developmental stages (Ascher and Peloquin, 1966; Goodwillie et al., 2004). In a preliminary study of C. heterophylla pistils, we could not find a difference in average pollen performance between self and outcross pollen (applied in separate pollinations) in any of the developmental stages (A. Lankinen et al., unpublished data). It is, however, possible that outcross pollen has an advantage when outcross and self pollen grow simultaneously in the pistil (Aizen et al., 1990; Nemeth and Smith-Huerta, 2002; Kruszewski and Galloway, 2006).

**Genetic variation of timing of stigma receptivity and anther–stigma contact**—Data on genetic or heritable variation in timing of stigma receptivity and selfing are limited. Dole (1992) found genetic variation in anther–stigma distance, a trait influencing the delayed selfing mechanism in *Mimulus guttatus*. This result was confirmed by Kelly and Arathi (2003; $V_A = 36\%$ of total variance), although estimates from field data did not show significant heritability for this trait (van Kleunen and Ritland, 2004). In *Hibiscus laevis*, anther–stigma distance, and thus the potential for autonomous selfing, differed among populations (Klips and Snow, 1997). In *Leptosiphon jeponsi*, plants differed substantially both within and among populations in the timing of selfing due to breakdown of the self-incompatibility system (Goodwillie and Ness, 2005). In *C. heterophylla*, genetic variation for timing of stigma receptivity (89% of total variance) was high, though the small sample sizes makes this estimate less certain. Heritable variation for anther–stigma contact (56% of total variance for midparent) was also substantial. The estimated evolvability indicates that the trait will change 5% per generation in response to a standardized selection gradient (i.e., as strong as selection on fitness itself). In *Dalechampia*, evolvabilities ranged between 0.01 and 1.71% for 20 floral characters (Hansen et al., 2003), suggesting that our value is relatively high. It should be noted, however, that because anther–stigma contact is influenced by environmental factors and our estimate is based on data from the greenhouse, we might have overestimated heritability. On the other hand, because evolvability is independent of environmental variance (unlike heritability, it is calculated independent of the phenotypic variance), this estimate should also be representative of natural conditions (Houle, 1992; Hansen et al., 2003).

Because we did not find any phenotypic relationship between these two genetically based traits in a nearly uniform greenhouse environment (including data for both populations and years), it is unlikely that stigma receptivity is delayed as a consequence of developmental changes connected with delaying anther–stigma contact (i.e., because of a genetic correlation caused by linkage or pleiotropy). We cannot rule out a genetic correlation, but the lack of a phenotypic correlation makes it hard to imagine a genetic correlation strong enough to significantly limit an independent response to selection. The strong association between the two traits across populations and species (Armbruster et al., 2002) may thus be the result of correlated selection pressures or correlated selection, i.e., selection on the functional interaction of the two traits (see Endler, 1995; Armbruster and Schwagerle, 1996). This idea, however, needs to be tested by more extensive studies involving more populations and species across a wider range of environmental conditions.

**Final remarks**—In this study of *Collinsia heterophylla* we showed that delayed stigma receptivity does not prevent either outcrossing or geitonogamous selfing, even though the stigma becomes receptive slightly later than anther–stigma contact. We found heritable variation in timing of both stigma receptivity and anther–stigma contact. Because delayed stigma receptivity was not correlated with timing of anther–stigma contact among individuals, each of these traits may respond independently to correlational selection. At this point, the selective advantage of delaying stigma receptivity is unclear, and the large phenotypic and genetic variation found for this trait is puzzling. It can be hypothesized that the selective advantage of delaying stigma receptivity in the outcrossing species of *Collinsia* is intensified pollen competition either between pollen from different donors (e.g., Mulcahy, 1979; Marshall and Ellstrand, 1986; Quesada et al., 1993; Paschke et al., 2002; see reviews in Willson, 1994; Skogsmyr and Lankinen, 2002) or between related pollen (geitonogamous self or kin) (Armbruster and Rogers, 2004; Lankinen and Armbruster, 2007). If delayed stigma receptivity also can increase pollen load size of self pollen in the case of autonomous selfing (e.g., by allowing a time period during which self pollen can accumulate on the stigma), this might reduce inbreeding depression when pollinators are scarce and plants are forced to self (Lankinen and Armbruster, 2007). This, in turn, can influence the stability of mixed mating systems (Armbruster and Rogers, 2004).

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**Table 6.** Components of variance ($\sigma^2$) and estimates of heritability ($h^2$) and $I_A$-evolvability for timing of anther–stigma contact in *Collinsia heterophylla* calculated using a two-way ANOVA (half-sib analysis).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>$F$</th>
<th>$P$</th>
<th>$\sigma^2$</th>
<th>$h^2$</th>
<th>$I_A$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>7</td>
<td>3.26</td>
<td>0.027</td>
<td>0.111</td>
<td>0.684</td>
<td>5.75</td>
</tr>
<tr>
<td>Mother</td>
<td>7</td>
<td>3.37</td>
<td>0.029</td>
<td>0.0702</td>
<td>0.432</td>
<td>3.63</td>
</tr>
<tr>
<td>Father × mother</td>
<td>12</td>
<td>1.36</td>
<td>0.197</td>
<td>0.0757</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>100</td>
<td></td>
<td>0.392</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>0.649</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:** The father × mother interaction indicates nonadditive genetic variance in the absence of epistasis and shared environmental effects. Because the interaction was not significant, this genetic variance was presumably unimportant.


SPSS. 1999. SPSS 11.0, Syntax reference guide. SPSS, Chicago, Illinois, USA.


