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Sexual antagonism in the pistil varies among populations of a hermaphroditic mixed-mating plant

Evan Hersh[1,2,7], Josefin A. Madjidian[1], Stefan Andersson[1], Maria Strandh[3], W. Scott Armbruster[4,5,6], Åsa Lankinen[3]

[1] Biodiversity, Department of Biology, Lund University, Ecology Building, S-223 62 Lund, Sweden.

[2] Present address: Department of Botany, The University of British Columbia, 6270 University Boulevard, Vancouver, B.C., Canada, V6T 1Z4.

[3] Swedish University of Agricultural Sciences, Plant Protection Biology, P.O. Box 102, S-230 53 Alnarp, Sweden.

[4]School of Biological Science, University of Portsmouth, King Henry Building, King Henry I Street, Portsmouth, PO1 2DY, United Kingdom.

[5]Insitute of Arctic Biology, University of Alaska, Fairbanks, AK 99775 USA

[6]Department of Biology, Norwegian University of Science and Technology, N-7491 Trondheim, Norway

[7] For correspondence. Email: evan.hersh@gmail.com (phone: +1 604 401 6405); Email of other authors: josefinmadjidian@gmail.com; stefan.andersson@biol.lu.se; maria.strandh@slu.se; scott.armbruster@port.ac.uk; asa.lankinen@slu.se.

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ABSTRACT: Sexual conflicts and their evolutionary outcome may be influenced by population-specific features such as ecological context and mating system, but few studies have investigated the link between sexual conflict and mating system. The self-compatible, mixed-mating hermaphrodite *Collinsia heterophylla* (Plantaginaceae) has been proposed to exhibit a sexual conflict over timing of stigma receptivity. This conflict involves 1) delayed stigma receptivity, which intensifies pollen competition, and 2) forced early seed production by pollen, which reduces seed set. We investigated the potential for the conflict to occur under field conditions and performed greenhouse crosses within eight populations to assess its consistency across populations. Flowers were visited and produced seeds after pollination at all developmental stages, suggesting that the conflict can be of significance under natural conditions. In the greenhouse, early pollination imposed costs in all populations. Overall, the timing of first seed set was most strongly affected by the maternal parent, denoting stronger female than male ability to influence onset of stigma receptivity. Crosses also revealed a negative relationship between donor- and recipient-related onset of receptivity within individuals, a novel result hinting at trade-offs in sex-allocation or a history of antagonistic selection. Neither timing of stigma receptivity, timing of first seed set or pollen competitive ability covaried with population outcrossing rate. In conclusion, these results indicate that sexually antagonistic selection may be present in varying degrees in different populations of *C. heterophylla*, but this variation does not appear to be directly related to mating system variability.

Introduction

Sexual conflict describes the differing interests between male and female reproductive systems, expressed either through intralocus conflict when the same trait has different selective optima in males and females, or through interlocus sexual conflict whereby individuals of one sex increase its reproductive competitive success while imposing direct costs on individuals of the other sex (Parker 1979; Arnqvist and Rowe 2005). Interlocus sexual conflict is believed to cause antagonistic coevolutionary arms races (and other dynamic regimes) between male and female traits, potentially accelerating speciation in geographically separated populations (Gavrilets 2000; Gavrilets and Hayashi 2005) or driving temporal cycles of trait exaggeration and trait reduction (Holland and Rice 1998; Rowe et al. 2005). Changing environmental conditions can strongly influence selection in antagonistic coevolution between species (for example, between hosts and parasites; Laine 2009; Mostowy and Engelstädter 2011), raising the possibility of similar environmental effects on sexual conflicts within species.

Experimental support for sexually antagonistic coevolution comes from a diversity of species, including water striders (*Gerris* spp; Rowe and Arnqvist 2002), seed beetles (*Callosobruchus* spp; Rönn et al. 2007), diving beetles (Coleoptera: Dytiscidae; Bergsten et al. 2001), dung flies (*Sepsis cynipsea*; Martin and Hosken 2003), hermaphroditic snails (Stylommatophora spp; Koene and Schulenburg 2005), and hermaphroditic plants (*Dalechampia* spp., Armbruster et al. 1995; *Collinsia heterophylla*, Madjidian and Lankinen 2009; *Arabidopsis lyrata*, Willi 2013). Several studies have used crosses between and within populations to detect signs of sexually antagonistic coevolution (e.g. Andrés and Arnqvist 2001; Jolivet and Bernasconi 2007; Willi 2013, see criticism by Rowe et al. 2003; Pizzari and Snook 2004). However, the influence of ecological

context remains uncertain, as only a few studies have included multiple natural populations of the same species (Bergsten et al. 2001; Edward and Gilburn 2007; Perry and Rowe 2012, Green et al. 2014), although a recent study on *Drosophila melanogaster* demonstrated that populations adapted to different environments (e.g. high ethanol or cadmium levels) differed in the magnitude of male harm and female resistance (Arbuthnott et al. 2014).

Hermaphroditic species can employ mixed-mating strategies, i.e. a combination of outcrossing and selfing in the same individual. In mixed-mating plant species outcrossing rates often vary considerably among populations (Goodwillie et al. 2005). Such variation can be caused by environmental factors (Lloyd 1979; Harder and Aizen 2009) and may represent an evolutionary stable state or a transition to complete selfing or complete outcrossing (Lande and Schemske 1985; Goodwillie et al. 2005; Johnston et al. 2009; Winn et al. 2011). Both interlocus and intralocus sexual conflict is expected to occur in hermaphrodite species (Bedhomme et al. 2009; Abbott 2011; Schärer et al. 2014). A recent model of intralocus sexual conflict showed that sexual antagonism should favor female-based traits in more selfing hermaphrodite populations due to increased similarity between the genetic interests of males and females (Jordan and Connallon 2014). It has also been suggested that the inbreeding depression threshold (benefits of mating with a related individual despite inbreeding depression) is lower for females compared to males, as females also suffer from opportunity costs as the same egg or ovule can only be fertilized once (Parker 1979, 2006). Moreover, it is predicted that sexual selection and sexual conflict is stronger in more outcrossing species as interactions between unrelated individuals increase in magnitude (Brandvain and Haig 2005, Mazer et al. 2010). Crosses between selfing and outcrossing plant populations show some support for the hypothesis of increased sexual conflict in more outcrossing populations (Brandvain and Haig 2005; Willi 2013), but less is known about how sexual conflict

varies among multiple populations that differ in mating system. Previous animal studies investigating sexual conflict in relation to different mating systems have focused on separate-sexed species (e.g. promiscuous and polygynous mating systems; Kokita and Nakazono 2001; Härdling and Karlsson 2009; Dmitriew and Blanckenhorn 2012).

Antagonistic coevolution can lead to assortative mating between individuals with certain male and female conflict traits, causing non-random positive linkage between genes underlying these traits (Härdling and Karlsson 2009). On the other hand, in hermaphrodites male and female traits should covary negatively if they compete for the same resources (Charlesworth and Charlesworth 1981). Previous studies investigating associations between male and female conflict traits in hermaphrodites have focused mainly on intralocus sexual conflicts in animals (see Abbott 2011; Pennell and Morrow 2013).

Sexual conflict in plants has been considered to occur over seed provisioning because when multiple fathers contribute to the brood, paternal genes benefit from increased provisioning of their own offspring, whereas maternal genes favor equal investment in all seeds (Charnov 1979; Queller 1984; Willi 2013), similar to the conflict over parental care in animals (e.g. Szentirmai et al. 2007). The expression of sexual conflict before fertilization has been suggested only recently in plants (Armbruster et al. 1995; Lankinen et al. 2006; Prasad and Bedhomme 2006; Lankinen and Kiboi 2007; Delph et al. 2011; Duffy et al. 2013) and is particularly likely during pollen competition (Bernasconi et al. 2004; Lankinen and Karlsson Green 2015), which parallels sperm competition in animals (Jennions and Petrie 2000). There should be considerable potential for sexual conflict during pollen competition in the pistil compared to before pollination, because at this stage of the life-cycle male and female reproductive structures are in direct contact with each

other. Moreover, pollen (being multicellular gametophytes containing the male gametes) express a large portion of the genome (cf. Arunkumar et al. 2013).

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 large stigmatic area (Rodrigo et al. 2009) and delayed stigma receptivity (Galen et al. 1986; Ganeshaiah and Shaanker 1998) can increase pollen competition intensity, enhancing the chance of receiving higher quality pollen. According to a model of interlocus sexual conflict over floral receptivity (Lankinen et al. 2006), traits that secure paternity of early arriving pollen (e.g. forced early stigma receptivity) should be selected for, even if they impose a cost to the recipient individual. Similarly, there will be counteracting selection for female traits (e.g. delayed stigma receptivity) that oppose any direct harmful effect that might be inflicted by the male traits (direct female cost, sensu Parker 1979). In this scenario, pistil traits would be selected to avoid pollen that induce costs to the female, a key driver of sexual-conflict dynamics that separates sexual conflict from other evolutionary scenarios, e.g. selection to obtain high quality pollen and improved offspring fitness (Rowe and Day 2006)

Collinsia heterophylla (Plantaginaceae), a mixed-mating hermaphroditic annual herb, belongs to a genus with large variation in mating system (Armbruster et al. 2002; Baldwin et al. 2011) and exhibits considerable interpopulation variation in outcrossing rate (Charlesworth and Mayer 1995; Kalisz et al. 2012). In a recent investigation of *Collinsia*, only the timing of stigma receptivity was positively correlated with outcrossing rate among species (Kalisz et al. 2012), suggesting that this trait is closely connected with the mating system. *C. heterophylla* shows evidence of a sexual conflict over timing of stigma receptivity (Lankinen and Kiboi 2007; Madjidian and Lankinen 2009; Madjidian et al. 2012b). Delayed stigma receptivity favors female

fitness under pollen competition between self pollen by reducing inbreeding depression (Lankinen and Armbruster 2007) and between outcross pollen by increasing offspring quantity (Lankinen and Madjidian 2011). Early-arriving pollen have the potential to force early seed production (e.g. by germinating on partially receptive stigma or growing rapidly in the unripe pistil), which ensures high siring success but reduces seed set, both after single (Lankinen and Kiboi 2007) and multiple pollinations (Madjidian et al. 2012b), indicating a direct female cost. Interestingly, in crosses within and among four populations from two regions foreign donors induced stigma receptivity earlier than local donors, pointing to a history of antagonistic coevolution between pollen and pistil traits (Madjidian and Lankinen 2009). However, it remains to be seen whether pollinators visit and successfully pollinate flowers at early developmental stages, a necessary condition for the expression of the conflict under natural conditions.

In this study we firstly investigated the potential for the sexual conflict to occur under field conditions. Secondly, we assessed how the cost of early seed production and the relative importance of paternal and maternal influence on timing of stigma receptivity vary across eight populations that differ in mating system by conducting one-donor crossing experiments in the greenhouse. We asked: 1) Do flowers produce nectar rewards and get visited at early floral stages and do these visits result in seed set? 2) How common is the cost of early seed production among populations? 3) Do donor and recipient influences on timing of stigma receptivity differ among populations? 4) Do donor- and recipient-influenced onsets of stigma receptivity covary among individuals within populations in a manner that indicates differential sex allocation or other forms of co-dependence between genes influencing male and female onset? 5) Does timing of stigma receptivity covary with population outcrossing rate, pollen competitive ability or general viability (measured as flower production)? It should be noted that significant correlations

do not imply any particular causal relationship between these traits. For example, it is possible that late stigma receptivity results in higher outcrossing rates simply by allowing more time for pollinators to visit the flowers. In order to increase sample size, we included data from four previously studied populations in relevant comparisons (Madjidian and Lankinen 2009).

Materials and methods

Study species and plant material

Collinsia heterophylla Buist (Plantaginaceae) 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 up to 20 ovules in a central pistil that develops into a dry capsule (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. The lower lip is folded to form a keel-like structure, which encloses the fertile parts. As in pea flowers, the anthers and stigma are exposed when a pollinator lands on the lower lobe, but not when non-pollinating insects visit (Armbruster et al. 2002).

When the flowers first open, the style is short, the stigma is unreceptive, and the anthers are undehisced. The anthers dehisce sequentially during 3-4 days, while the style elongates and becomes receptive to incoming pollen (Armbruster et al. 2002). Selfing can occur during later stages of floral development as the style elongates and eventually grows through the dehisced anthers (Kalisz et al. 1999; Armbruster et al. 2002). The mean outcrossing rate of *C.*

heterophylla populations ranges from 0.32 to 0.64 (Charlesworth and Mayer 1995; Kalisz et al. 2012).

Plants used in the greenhouse part of this study were grown in an insect-free greenhouse at Lund University, Sweden, during autumn and winter of 2011-2012. Plants originated from seeds collected by maternal family from eight populations (1, 3, 7, 10, 11, 13, 142, 151) in California during 2008 (Table S1 and Figure S1, available online). To investigate a broad sample of differentiated populations, we selected populations that represent about two thirds of the species' natural range across the California Floristic Province and into northern Baja California (Neese 1993). The two southernmost populations (1, 3) come from the south side of a phylogeographic border in *C. heterophylla*, apparently caused by the uplift of the Transverse Mountain Ranges (Baldwin et al. 2011), whereas the remaining six populations are from north of this border. Plants were grown for one generation in the greenhouse to generate outcrossed progeny for our experimental crosses. These crosses were conducted on emasculated flowers with fully receptive pistils (stage 4, see below). Each plant individual was only used once, either as a donor or a recipient, and up to five replicate crosses were performed for each parent combination. This first generation was also used to estimate the timing of stigma receptivity as indicated by peroxidase activity for each population (see below) as part of a companion study (Å. Lankinen, S. Andersson and J. A. Madjidian, unpublished data).

The previously published data from four additional populations included in population comparisons, was collected in the same greenhouse in 2006 using the same methods as described below (Madjidian and Lankinen 2009; Table S1).

Nectar measurements and pollinator observations

To assess reward availability for pollinators during early floral stages, nectar standing crop was estimated at four floral developmental stages in the field in late April (population 151) and early May 2012 (population 142, Table S1). The floral stages are designated 1-4, corresponding to both the number of open anthers and the number of days after anthesis (Armbruster et al. 2002). We also measured nectar content in the eight greenhouse-grown populations (using the second set of plants, see below) from one flower in each stage 1-4 on a single individual per maternal family. Because nectar was measured in the absence of pollinators, our measurements refer to the nectar volume that had accumulated at each stage. Nectar volume was quantified by inserting a 32 mm long microcapillary tube (1 μl) into the flower's nectar tube and measuring the length of fluid drawn in.

In the two field populations, we also recorded insect visitation (mostly bees and flies) by recording whether *C. heterophylla* flowers received visits by potential pollinators during each stage of flowering, from flower opening (stage 0) to the last stage considered in the present study (stage 4).

Early seed production in the field

To investigate successful seed production after cross pollination during different floral stages under field conditions, we conducted a crossing experiment in one population (151, Table S1) during April 2012. The design included three treatments: i) flowers emasculated at flower opening (stage 0) and having their styles cut during stages 1-4, i.e. day 1-4 after emasculation, allowing fertilization by outcross pollen that arrive naturally up to a particular stage (designated

"natural outcross", N = 8-10 flowers per stage); ii) flowers emasculated at stage 0 and hand-pollinated with supplemental outcross pollen (pollen taken from multiple flowers from another individual in the population) during stages 1-4 (no style removal), allowing fertilization by outcross pollen supplemented at a particular stage (designated "manual outcross", N = 10-12 flowers per stage); and iii) flowers emasculated at stage 0, allowing fertilization by outcross pollen that arrived naturally at all floral stages (designated "control outcross", N = 11 flowers). All flowers included in the experiment came from different individual plants, i.e. we used one flower per plant. Treatments were conducted during 5-day periods (i.e. one day for each stage 0-4) and started sequentially during a two-week span. Seed capsules were collected approximately two weeks after the final crosses. The proportion of flowers that developed into capsules was calculated for each stage and treatment as a measure of successful seed production.

Population outcrossing rates

Population estimates of female outcrossing rates of our eight study populations as well as the four additional populations used for population comparisons (Madjidian and Lankinen 2009) were obtained from analysis of four polymorphic microsatellite loci (A11, A106, A116 and C1) developed for *C. sparsiflora* (J. W. Wright et al., USDA, Forest Service Pacific Southwest Research Station, unpublished data). Field-collected leaves (in 2008 for all 12 populations) from on average 23.2 ± 8.3 (mean ± SD) individuals per population were dried and extracted for DNA using either DNeasy Plant Mini kit (Qiagen) or GeneJET Plant Genomic DNA Purification Kit (Fermentas, Thermo Fisher Scientific) according to the manufacturer's instructions. The microsatellite loci were amplified in one multiplex PCR reaction (containing four primer pairs)

per individual, with fluorophore-labelled (FAM) forward primers and non-labelled reverse primers. Each PCR reaction contained 50 ng DNA, 0.2 µM of each primer, 1x Multiplex PCR Master Mix and 0.6 µl Q-Solution from the Qiagen Multiplex PCR Kit (Qiagen) in a total volume of 10 µl. The following cycling parameters were used: 95°C for 15 min and then 10 cycles of touchdown PCR at 94°C for 30 s, 62°C (-1°/cycle) for 90 s and 72° for 60 s followed by 25 cycles of 94°C for 30 s, 52°C for 90 s and 72° for 60 s. A final extension step at 60° for 30 min was applied. The PCR products were subjected to GeneScan fragment analysis, adding GeneScan 500 ROX Size Standard (Applied Biosystems) to the samples, on an ABI3730XL DNA Analyzer instrument (Applied Biosystems) at Uppsala Genome Center, Sweden. The resulting chromatograms were used for genotyping by size determination with the Microsatellite Plugin in the software Geneious v. 6.1.4 (Biomatters Ltd.).

Population female outcrossing rate (O) was estimated as 1-S where S is the fraction of selfed seeds, calculated from microsatellite genotype frequencies as $S=2F_{is}/(1+F_{is})$, where F_{is} is the population mean inbreeding coefficient (across loci), estimated with the Excel plugin GenAlEx v. 6.5 (Peakall and Smouse 2006, 2012).

One-donor crosses

To investigate costs of early seed production and timing of stigma and floral receptivity in the presence of pollen (the floral stage at which a pollination could lead to seed production, hereafter referred to as "timing of first seed set", table 1), we conducted one-donor crosses within each of our eight focal populations by pollinating emasculated flowers at each of the four stages 1-4. Hand pollinations were conducted by depositing pollen from the donor flower on a microscope

slide and then wiping the slide against the recipient stigma. Half of the style was excised after 4 h, preventing later germination of pollen and ascertaining that seed set only occurred when the stigma had been receptive at the time of the cross (Lankinen and Kiboi 2007); pollen commonly grows to the base of the style (13 mm) within 4 h (Lankinen et al. 2009; Å. Lankinen and W. S. Armbruster, unpublished data).

From each population, we chose 6-8 unrelated plants (each originating from a separate family of outcrossed progeny) to act as pollen donors and recipients in crosses within each population. Each individual acted as a donor to three unrelated recipients and as a recipient to three unrelated donors (figure 1). The same individual was used as both donor and recipient because we not only aimed to investigate general donor and recipient effects (over three mates) on the timing of first seed set, but also how male and female components of this trait covary among individuals. This design will cause some dependence between donors and recipients in the general comparison, but because donor and recipient effects were determined over different mates they can be considered independent. Hand pollinations were conducted on two flowers per donor × stage combination, equaling 24 pollinated flowers per individual (three donors × four stages × two replicates). With a mean of 7 plants per population and eight populations, the total number of pollinations exceeded 1300. We controlled for time and fruit-position effects (which are difficult to separate as whorls mature from bottom to top) by ensuring that every donor served as first, second and third donor on each of its three recipients, respectively. The populations flowered at different times, so all crosses could not be performed at the same time. Also, in order to complete all the necessary pollinations for each donor-recipient pair, siblings of the main crossing individuals were occasionally used to supplement flowers (treated as belonging to the same donor/recipient

as the main individual; Madjidian et al. 2012a). Crosses were performed during October-mid December.

Mature seed capsules were collected and dried for three weeks in room temperature, and the dried seeds were counted and weighed. Fewer seeds and lower capsule weight after pollination at early stages (usually 1-2) compared to later stages (usually 3-4) was presumed to indicate a cost of early seed production (Lankinen and Kiboi 2007). Because we hypothesize that the reduced seed set in partially receptive pistils is a side-effect of a pollen trait that increases reproductive success by forcing early seed production (cf. Morrow et al. 2003), for example, by interfering with pistil signaling (Madjidian et al. 2012b) or by fertilizing the ovules prior to receptivity, we assume this cost to be detectable in single-donor pollinations. It should be noted, however, that this kind of pollen trait only enhances reproductive success of male function if there is subsequent arrival of pollen from other donors.

By identifying the first stage of pollination at which seeds could be produced, the "timing of first seed set" (table 1) could be estimated for each set of four crosses involving the same donor-recipient combination. To study female influences on the timing of first seed set, we estimated "pistil-related timing of first seed set" for each recipient averaged over the three pollen donors used. Similarly, to study male influences, "pollen-related timing of first seed set" for each donor was calculated by averaging values over the three plants used as recipients (table 1, cf. pistil and pollen onset of stigma receptivity in Madjidian et al. 2012a).

Estimates of timing of stigma receptivity, pollen tube growth rate and flower production

The focal populations were also used to determine how timing of first seed set covaries with i) timing of stigma receptivity in the absence of pollen, hereafter referred to "timing of stigma receptivity" (table 1), ii) pollen tube growth rate, a potential determinant of siring ability (Snow and Spira 1991), and iii) flower production, an indicator of general plant vigor (Lankinen and Armbruster 2007). Timing of stigma receptivity was estimated in the first greenhouse generation (see above), whereas the other two traits were measured in a second set of plants from the same 6-8 progeny families used in the crossing experiment, supplemented by 2-4 additional families. Each population was represented by 7-10 families with 5 individuals per family.

Timing of stigma receptivity was measured as stigmatic peroxidase activity 1-4 days after flower opening, using one individual per progeny family (Kearns and Inouye 1993). This trait is associated with the occurrence of pollen tubes in *C. heterophylla* pistils (Lankinen et al. 2007). Intact, pollen-free stigmas collected from emasculated flowers were placed on a microscopic slide in a drop of 3 % hydrogen peroxide (Kearns and Inouye 1993). Vigorous bubbling on the stigmatic surface within a few minutes indicates full stigma receptivity (Lankinen et al. 2007). We used two replicate flowers per stage, i.e. $N = 8$ flowers per plant.

To measure pollen tube growth rate *in vitro*, pollen from two flowers (all four anthers) from one individual per progeny family was mixed. Pollen from this mixture was added to Hoekstra medium (Hoekstra and Bruinsma 1975) at about the same density to avoid lumps of pollen, hence obtaining maximal germination. We germinated pollen 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 using a light microscope. A previous study of *C. heterophylla* confirmed a positive correlation between pollen tube growth rate *in vitro* and *in vivo* (Lankinen et al. 2009). Flower production

was estimated for five individuals per family by multiplying the number of flowers on the main stalk by the number of flowering side branches.

Statistical analysis

The data on successful seed production of flowers pollinated in the field were analyzed in the R environment (R Development Core Team 2014) using a generalized linear model with date of emasculation (stage 0), pollination treatment, stage at treatment and two interactions (treatment × day, treatment × stage) as factors. The original model considered a binomial distribution and a logit function, but was refitted with quasibinomial distribution in order to compensate for overdispersion. Statistical significance ($P < 0.05$) was assessed by testing the change in deviance between successive models with an *F*-test. All non-significant factors or interactions were excluded using backward deletion of higher-order interactions.

Greenhouse data on seed set, seed weight and timing of first seed set following one-donor pollinations were analysed using general linear mixed models in the package lme4 (Bates and Maechler 2010) in the R environment (R Development Core Team 2014). In order to analyze the cost of early seed production across populations, we modeled number of seeds or seed weight per capsule as a function of stage at pollination, population, the population-by-stage interaction, donor identity, recipient identity, and the donor-by-recipient interaction (the latter nested within population). Stage was treated as a fixed factor and all other factors were considered random. For analysis of donor and recipient influence on timing of first seed set we used a random-effects model to test for the effects of population, donor, recipient, and donor-by-recipient interaction (all nested within population). A similar random-effects model was used to determine the effects

of donor, recipient, and donor-by-recipient interaction on timing of first seed set for each population individually. Models were fitted with restricted maximum likelihood (REML). This method provides quantitative estimates of relative importance that can be directly compared among factors, and does so in a manner that is robust to unbalanced data, especially for the type of incomplete factorial design used in the present investigation (Caro et al. 1985). Significance of factors were tested by comparing nested models (refitted with maximum likelihood, ML) in likelihood ratio tests (LR-tests) using the anova-function in R. All factors were tested one by one, under the most complex model. Before testing a simple effect of a factor we removed all interactions involving that factor from the model. It should be noted that LR-tests result in conservative *P*-values for random-effects factor (Zuur et al. 2009). We also compared AIC (Akakie information criterion) of the models fitted with REML, but as the result was always in line with those of the LR-tests AIC is not presented. For models fitted with REML we show estimates of the percentage of variance attributable to the random factors, with special focus on donor identity, recipient identity and interactions involving these factors

Statistical analysis of nectar estimates in the field and other greenhouse data were performed using general linear model ANOVAs in IBM SPSS 20 (IBM Corp. 2011). The relationship between pistil- and pollen-related timing of first seed set (both measured for each individual) was estimated using the Pearson correlation coefficient (r), taking advantage of the fact that each plant was used as both donor and recipient for three mates (see above). This analysis was based on standardized residuals from ANOVA with populations as groups to remove confounding effects of population differences in the overall mean.

Pearson's r was also used to quantify correlations between population outcrossing rate and population means for timing of stigma receptivity and first seed set, pollen tube growth rate and

the index of flower production. Since these analyses also involved data from the four populations sampled in an earlier year, we performed these on the standardized residuals from ANOVA with year as a group variable. Separate analysis only involving the eight focal populations showed very similar results as those from the full analysis (not shown).

Nectar estimates in the field and all greenhouse data were approximately normally distributed, and all model assumptions were validated.

Results

Nectar production and pollinator visitation across floral stages

Nectar was found during all floral stages in the two field populations (142 and 151, Table S1) (stage 1: 0.04 ± 0.01, stage 2: 0.07 ± 0.02, stage 3: 0.14 ± 0.04, stage 4: 0.11 ± 0.03 mean standing crop ± SE [in microliters]), as well as in the eight greenhouse-grown populations (stage 1: 0.09 ± 0.08, stage 2: 0.17 ± 0.13, stage 3: 0.27 ± 0.19, stage 4: 0.41 ± 0.28 (mean secretion rate from anthesis ± SE [in microliters]. Nectar production appeared lower at earlier stages compared to later stages, but this difference was only significant in the greenhouse where nectar was allowed to accumulate across all previous stages ($F_{3,21} = 22.034$, $P < 0.001$ vs. $F_{3,85} = 2.087$, $P = 0.11$ in the field).

Our observational data suggested that flowers received insect visits at least once during each floral stage (including stage 0) in the two populations used for nectar measurements (Table S1).

However, only the bees [primarily species of *Osmia*, *Bombus*, *Anthophora*, and *Habropoda* (*Emphoropsis*)] were able to depress the corolla keel and contact the anthers and stigmas.

Capsule formation across floral stages in the field

Emasculated flowers matured into capsules following both open and manual pollination at all floral stages (population 151, figure 2). The proportion of flowers developing into capsules was not significantly affected by floral stage at pollination ($F_{1,37} = 1.52$, $P = 0.23$), pollination treatment ($P = 0.57$), date of emasculation ($P = 0.81$) or any of the two-way interactions between factors ($P > 0.16$). Thus, we found no evidence for pollen limitation on capsule formation.

Population outcrossing rates

Outcrossing rates of our eight focal populations ranged from 0.29 to 0.82 (mean ± SD, 0.50 ± 0.050) (table 2). The four additional populations also fell within this range (0.32-0.57, table 2), giving a similar mean for all 12 populations (0.49 ± 0.041).

Cost of early seed production

As hypothesized, one-donor pollinations at later stages (3 and 4) resulted in more and heavier seeds compared to earlier stages (1 and 2) (table 3; figure 3). There was no significant stage-by-population interaction, even though some populations (see 7 and 11) appeared to show high seed production after pollination at stage 2 as well as during stage 3 and 4 (figure 3). Both seed traits

were significantly influenced by female identity, but not by population, male identity, and the stage-by-recipient interaction (table 3). The identity of the recipient accounted for more of the variation in seed traits (22-24 %) than population, donor and the investigated interactions involving these factors (0-4 %) (table 3).

Donor and recipient influence on timing of first seed set

The timing of first seed set, evaluated by stage-specific one-donor crosses, differed among populations ($\chi^2 = 11.29$, df = 1, $P < 0.001$; table 2). Although the identity of recipient and donor both affected the timing of stigma receptivity, the effect of recipient was more pronounced ($\chi^2 = 29.76$, df = 1, $P < 0.001$ versus $\chi^2 = 4.72$, df = 1, $P = 0.030$ for the donor effect), indicating stronger female influence on this trait. The donor × recipient interaction was not significant ($\chi^2 = 1.69$, df = 1, $P = 0.19$), suggesting that the donor influence on timing of first seed set was consistent over recipients. According to the overall variance components analysis for this trait, more of the variance was attributed to population (22.0 %) and recipient (19.0 %) than to donor (4.8 %) or donor-by-recipient interactions (7.6 %).

Analyses performed at the population level showed considerable variation across populations in the relative influences of donor and recipient on timing of first seed set, with three populations showing significant recipient effects, one showing both donor and donor-by-recipient interaction, and remaining populations showing no significant effects whatsoever (table 4). Overall, recipient usually accounted for more variance within a population than donor identity or interaction effects.

Relationship between donor- and recipient-related timing of first seed set

Because each individual plant was used as both recipient and pollen donor, we were able to analyze how pollen- and pistil-related timing of first seed set covary among individuals when both traits were measured in the same hermaphrodite individuals. Pearson correlation analysis on standardized residuals (adjusted for significant population differences in the mean phenotype; $F_{7,46} > 6.12$, $P < 0.001$) revealed a significantly negative relationship between pistil- vs. pollen-related timing of first seed set (Pearson $r = -0.360$, $P = 0.008$, df = 52; figure 4).

Relationships among outcrossing rates and plant traits

Contrary to expectation, population outcrossing rate was not significantly positively correlated with population means for timing of first seed set, stigma receptivity or pollen tube growth rate across our total sample of 12 populations (adjusted for study year) (table 5). However, for the two former traits the trend was in the expected direction.

Correlation analyses showed no significant relationship between timing of first seed set and either timing of stigma receptivity (table 5) or flower production (Pearson $r = 0.407$, $P = 0.32$, df = 6); however, timing of stigma receptivity was negatively correlated with pollen tube growth rate (table 5).

Discussion

In this study on the mixed-mating plant *C. heterophylla*, we confirmed that natural pollination at early floral stages can lead to seed set, suggesting that sexual conflict over timing of receptivity can occur under field conditions. In the greenhouse, we detected costs of early seed production (expressed as reduced fecundity of maternal plants) in eight populations with widely different outcrossing rates. Across all populations the timing of first seed set was more strongly influenced by the identity of the recipient individual than by the pollen donor, indicating stronger female influence on this trait. Donor- and recipient-related timing of first seed set were inversely related across individuals, a novel observation in this study system. Contrary to expectation neither timing of stigma receptivity nor timing of first seed set was positively correlated with outcrossing rate.

Sexual conflict over timing of stigma receptivity across populations

Three lines of evidence — the presence of nectar at early floral stages, our observations of early pollinator visits in the field and the production of seed after natural pollination as early as stage 1, are indicative that the sexual conflict over timing of stigma receptivity can be expressed in natural populations of *C. heterophylla*. Greenhouse experiments involving the eight focal study populations confirmed the presence of a cost of early seed production across all populations, in line with our previous results from one or a few populations (Lankinen et al. 2007; Madjidian and Lankinen 2009; Madjidian et al. 2012b). While we did not confirm whether the reduced early seed set would be evident under more natural conditions (involving uncut styles and arrival

of additional pollen), Madjidian et al (2012b) showed that the cost of early seed production remains when pollen is applied at two consecutive stages.

Also in line with previous studies on *C. heterophylla* (Lankinen and Kiboi 2007; Madjidian and Lankinen 2009), we found that both the identities of the donor and the recipient influence the timing of floral receptivity (measured as timing of first seed set) in the overall analysis of all eight populations. However, the recipient effect was stronger than the donor effect, suggesting that the recipient generally has a greater influence on this trait than the donor. Separate analyses of our eight populations showed different patterns regarding donor and recipient influence, ranging from the lack of any effect to only a recipient effect or a combined effect of both donor and donor by recipient interactions. One might hypothesize that this variation between populations is caused by differences in sexually antagonistic traits, i.e. the ability to induce early receptivity vs. the ability to delay receptivity (cf. Rowe and Arnqvist 2002). In one of the few animal studies that explored sexual conflict across natural populations, the signal of sexually antagonistic coevolution differed in magnitude between populations (Perry and Rowe 2012).

Co-dependence between male and female function in terms of sexual conflict traits

To study the co-dependence between pistil and pollen traits involved in sexual conflict (Härdling and Karlsson 2009), including possible allocation tradeoffs between them (Charlesworth and Charlesworth 1981; Sandmeier and Delph 1997; Mazer et al. 2007), we assessed the correlation between donor- and recipient-related effects on timing of first seed set. Our analyses revealed a significantly negative relationship between the two variables, i.e. individual plants with strong "female" capacity to delay receptivity also had strong "male" capacity to advance it and vice

versa. It is important to note that this correlation can be interpreted as advantageous because, in terms of the conflict, it is favorable to *delay* receptivity when the plant is acting as a recipient (exhibiting high values of timing of stigma receptivity) and *advance* receptivity when it is acting as a donor (exhibiting low values of timing of stigma receptivity). This negative relationship is a novel result both in this study system, and to our knowledge, among hermaphrodites generally. Although the negative relationship may be indicative of a trade-off between the allocation of resources to traits associated with rapid pollen growth vs. early stigma receptivity, we found no correlation between timing of first seed set and overall plant vigour (measured as flower production). In the context of a sexual conflict, the positive combination of conflict traits could also result from selection through assortative mating if donors better able to force early seed production tend to fertilize those recipients that are better at delaying receptivity (Härdling and Karlsson 2009). Another hypothesis, relevant to a mixed-mating species in variable pollination environments (Lloyd 1979), is that less competitive pollen combined with pistils that delay receptivity may reduce seed set via selfing under conditions of low pollinator activity, thus selecting against this trait combination. So far, however, we only have evidence for selectable genetic variation (heritability) in pistil-related onset of receptivity; pollen-related onset shows no or little heritability in this study system (Madjidian et al. 2012a). Further studies are needed to determine how genetic variation in these and other features interacts with selection to generate covariance between sexual conflict traits.

Relationship among outcrossing rates and plant traits

Only a few studies have investigated the evolutionary consequences of sexual conflict in relation to ecological context and mating system. For example, studies on guppies (*Poecilia reticulata*; Magurran and Seghers 1994), seaweed flies (*Coelopa* spp; Edward and Gilburn 2007), a freshwater isopod (*Asellus aquaticus*; Karlsson et al. 2010), and diving beetles (Dytiscidae; Green et al. 2014) showed that the intensity of sexual conflict was affected by environmental condition. Additionally, in the dioecious plant *Silene latifolia* the magnitude of intra-locus sexual conflict (measured in the absence of direct interactions between males and females) varied depending on the amount of precipitation during the flowering season (Delph et al. 2011). Because *C. heterophylla* populations differ in selfing rate (Charlesworth and Mayer 1995; Kalisz et al. 2012) and the strength of sexual selection is expected to increase with increasing outcrossing rate (Brandvain and Haig 2005; Mazer et al. 2010), we hypothesized a link between mating system and traits involved in sexual conflict, with the caveat that such links could also result if late stigma receptivity confers high outcrossing rates.

Estimates of mean outcrossing rates for our eight focal study populations and four additional populations (O = 0.29-0.82, table 2) confirm previous observations of extensive variation among *C. heterophylla* populations (O = 0.32-0.64; Charlesworth and Mayer 1995; Kalisz et al. 2012). However, we were unable to detect a significant association between outcrossing rate and either pollen-tube growth rate, timing of stigma receptivity or timing of first seed set, suggesting that our hypothesis is incorrect or needs to be tested with larger sample sizes. It is quite likely that factors other than outcrossing rate exert a greater influence on these traits, especially timing of receptivity. One possibility is that delaying receptivity is beneficial also for enhancing competition between self-pollen (Lankinen and Armbruster 2007). In this scenario, the ability to delay receptivity would be selectively advantageous regardless of a population's (intermediate)

outcrossing rate, and therefore no correlation between the two variables would be expected. Another possibility is sexual conflict arising from competition between self and outcross pollen; our previous studies show that early arriving self pollen can germinate earlier than outcross pollen and thus inflict female costs by reducing seed set (Madjidian et al. 2012b).

Surprisingly, pollen-tube growth rate showed a significant negative relationship with timing of stigma receptivity among populations. If rapid pollen-tube growth rate confers high male reproductive success under conditions in which the start of pollen germination and growth is highly coordinated (i.e. when receptivity is delayed), we should instead have expected a positive correlation between these two traits (Hove and Mazer 2013). We could, however, hypothesize that this pollen trait is most important for siring success at early floral stages by acting as a male conflict trait, potentially conferring early receptivity and reduced seed set. In fact, preliminary results from sequential two-donor pollinations at stage 1 and stage 2 indicate that rapid pollen tube growth rate is important for siring ability of the first applied donor, and that high early siring success results in lowered seed set (Å. Lankinen and M. Strandh, unpublished data). Unlike timing of stigma receptivity, timing of first seed set was uncorrelated to pollen tube growth rate. Moreover, timing of first seed set and timing of stigma receptivity were not significantly positively associated (though the trend was in the expected direction). We hypothesize that not only receptivity of the stigma but also other pistil mechanisms delay timing of seed set in order to mitigate lowered seed set caused by pollen (e.g. differential pollen tube growth in the stigma vs style; Losada and Herrero 2014), potentially allowing enhanced female influence on when seeds are formed. Further studies are needed to learn more about pistil- and pollen-related timing of first seed set in *C. heterophylla*, as well as the sensitivity of these traits

and early seed reduction to ecological factors related to mating system and other life history attributes.

Conclusion

To our knowledge this is one of very few studies that have investigated sexual conflict in multiple natural populations of the same species. It is also one of the first to consider the link between traits related to sexual conflict and mating system in a mixed-mating species, as well as the link between male- and female-related influences on interlocus sexual conflict in a hermaphrodite. We show in *C. heterophylla* that seeds can be produced after pollination at early floral stages under natural conditions, indicating the possible expression of sexual conflict over timing of stigma receptivity in the field. Both the cost of early seed production and the female influence on timing of first seed set were consistent across the greenhouse-grown populations. The negative relationship between male- and female-related influences on timing of first seed set suggests a genetic link of a kind predicted in a model of sexual conflict dynamics (Härdling and Karlsson 2009); however, we found no evidence for the expected association between outcrossing rate and timing of stigma receptivity or pollen competitive ability. While our results illustrate how plant study systems can be useful for understanding the relation between sexual conflict and mating system, it is clear that we need further studies to unravel how the intricate forces of antagonistic coevolution, ecology, and mating system affect the diversification of taxa.

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Table 1: Measured and derived traits that reflect aspects of floral receptivity in *Collinsia heterophylla*.

Term	Definition/Measure
Timing of stigma receptivity	Timing of stigma receptivity in the absence of pollen, as indicated by stigma peroxidase activity.
Timing of first seed set	The first floral developmental stage (1-4 days after flower opening) at which hand-pollination by pollen from another individual leads to seed production (calculated per set of four crosses of each donor-recipient combination).
Pistil-related timing of first seed set	The first floral developmental stage at which hand-pollination of a given recipient plant leads to seed production (averaged over three donors).
Pollen-related timing of first seed set	The first floral developmental stage at which hand-pollination of a given donor leads to seed production (averaged over three recipient plants).

Table 2: Population means with standard errors for outcrossing rates and plant traits investigated in 12 greenhouse-grown populations of *Collinsia heterophylla*. Data from the last four populations were taken from a previous study (Madjidian and Lankinen 2009).

Pop	Outcrossing rate*	Timing of stigma receptivity (stage)†	Timing of first seed set (stage)	Pollen tube growth rate (mm/105min)	Flower production
1	0.38 ± 0.26 (29)	3.20 ± 0.29 (10)	3.29 ± 0.12 (7)		279 ± 12 (46)
3	0.60 ± 0.10 (27)	3.08 ± 0.25 (10)	2.45 ± 0.13 (8)	0.255 ± 0.006 (12)	222 ± 10 (46)
7	0.47 ± 0.22 (25)	2.73 ± 0.25 (10)	2.79 ± 0.14 (7)	0.263 ± 0.008 (7)	265 ± 8 (33)
10	0.41 ± 0.06 (35)	2.10 ± 0.22 (10)	2.63 ± 0.09 (7)	0.288 ± 0.015 (10)	207 ± 15 (39)
11	0.29 ± 0.20 (29)	3.00 ± 0.22 (10)	1.79 ± 0.08 (7)	0.226 ± 0.006 (11)	242 ± 11 (49)
13	0.49 ± 0.33 (29)	2.14 ± 0.14 (10)	2.51 ± 0.13 (7)	0.335 ± 0.017 (9)	229 ± 12 (43)
142	0.57 ± 0.25 (25)	3.08 ± 0.17 (10)	2.92 ± 0.11 (7)	0.217 ± 0.007 (7)	373 ± 17 (34)
151	0.82 ± 0.13 (17)	3.30 ± 0.20 (10)	2.85 ± 0.15 (6)	0.247 ± 0.007 (11)	228 ± 12 (43)
$P_{Population}$		< 0.001	< 0.001‡	< 0.001	
4	0.57 ± 0.05 (14)	2.41, 2.06-2.67 (8)	2.75 ± 0.07 (7)	17.5 ± 0.86 (6)	
202	0.51 ± 0.15 (9)	2.13, 1.52-2.37 (14)	2.74 ± 0.09 (6)	17.0 ± 1.38 (8)	

203	0.32 ± 0.06 (11)	2.06, 1.33-2.35 (13)	2.21 ± 0.04 (7)	15.9 ± 0.46 (21)
204	0.45 ± 0.03 (27)	2.00, 1.41-2.34 (12)	2.31 ± 0.06 (6)	15.7 ± 1.16 (9)

Note: *N* values in parentheses. *P*-values from one-way ANOVA or when applicable, from nested ANOVA.

*Measured in field-collected material using four polymorphic microsatellite loci.

†Measured in previous generations (see Results section). In the four additional populations used in a previous study, the mean value (and 95% confidence interval) was calculated as the floral developmental stage when 50% of the plants had receptive stigmas (see Madjidian and Lankinen 2009).

‡From a model also including recipient and donor, see Results section.

Table 3: Results from linear mixed model analyses for number of seeds and mean seed weight per capsule following one-donor crosses and subsequent style removal at floral developmental stages 1-4 within eight populations of *Collinsia heterophylla*.

	Seeds per capsule				Seed weight per capsule (mg)			
Source of variation	df	χ^2	*P*	% Var	df	χ^2	*P*	% Var
Stage	**3**	**22.03**	**< 0.001**	-	**3**	**34.85**	**< 0.001**	-
Population	1	3.401	0.065	0	1	0	1	0
Donor(Pop)	1	0.510	0.48	0	1	0	1	0
Recipient(Pop)	**1**	**83.73**	**< 0.001**	**22.4**	**1**	**79.41**	**< 0.001**	**23.8**
Stage × Population	1	0.935	0.33	1.8	1	0	1	0.41
Donor × Recipient(Pop)	1	0.672	0.41	3.2	1	1.627	0.20	3.9

Note: Male and female were nested within population. Significant factors ($P < 0.05$) are presented in bold. % Var denotes the percentage variance accounted for by each random effect.

Table 4: The influence of recipient, donor and their interaction on timing of first seed set after one-donor crosses at floral stages 1-4 in eight populations of *Collinsia heterophylla*, as determined by *P* values from linear random-effects models and the percentage variance accounted for by each factor (% Var).

Population	*N*	Recipient		Donor		Recipient × donor	
		P	% Var	*P*	% Var	*P*	% Var
1	7	**0.042**	**32.2**	0.61	0	1	0
3	8	0.15	18.9	0.72	5.3	1	0
7	7	0.056	18.3	**0.026**	**24.9**	**0.013**	**31.1**
10	7	**0.0032**	**38.0**	0.095	15.0	0.62	6.3
11	7	0.95	4.1	1	0	1	0
13	7	0.090	27.9	0.62	6.3	0.88	0.98
142	7	0.68	7.1	0.56	10.0	1	0
151	6	**0.0070**	**37.5**	0.70	0	0.061	30.9

Note: *N* = number of individual plants, df = 1 for all tests.

Table 5. Correlations between population outcrossing rates and population means for floral traits measured in 12 greenhouse-grown *Collinsia heterophylla* populations, including four populations used in a previous study (Madjidian and Lankinen 2009). Significant correlations are in bold.

	Stigma receptivity (stage)†		Timing of first seed set (stage)		Pollen tube growth rate (mm/105min)	
Trait	*r*	*P*	*r*	*P*	*r*	*P*
Outcrossing rate*	0.381	0.22	0.416	0.18	-0.040	0.91
Stigma receptivity (stage)†			0.258	0.41	**-0.761**	**0.007**
Timing of first seed set (stage)					0.128	0.71
Pollen tube growth rate (mm/105min)						

Note: Analyses controlled for differences between study years (2006, four populations vs. 2011, eight populations) by using standardized residuals from an ANOVA with the factor year.

*Measured in field-collected plant material using four polymorphic microsatellite loci.

†Measured in a previous generation (see Results section).

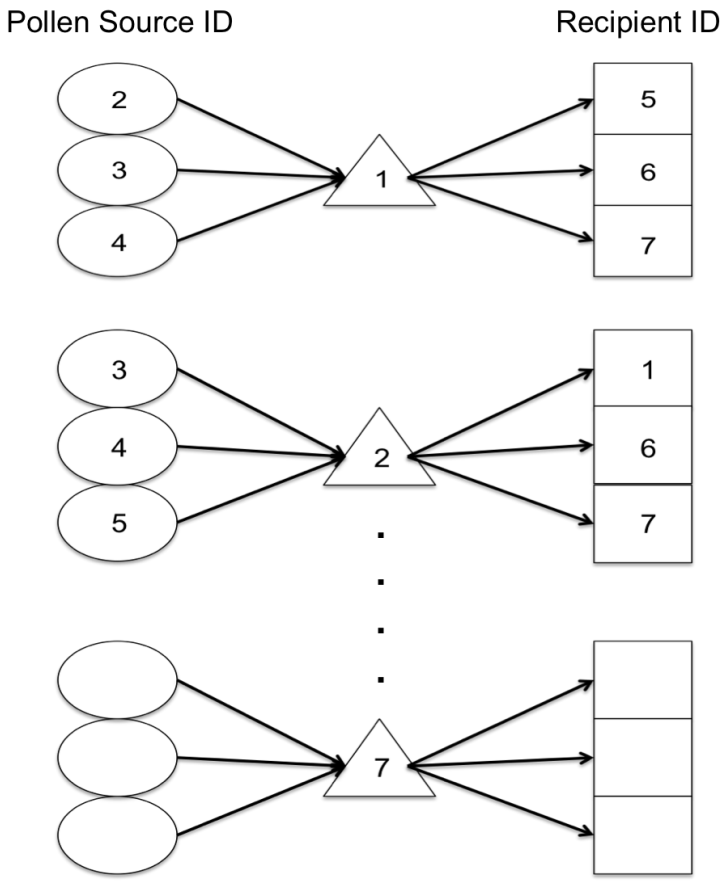

Figure 1: Experimental design for controlled one-donor hand pollinations in order to assess costs of early seed production and timing of first seed set across populations of *Collinsia heterophylla*. Each experimental plant individual (denoted by triangles) received pollen from three unrelated plants (denoted by ovals). The focal plant was also used as a pollen donor for three unrelated recipients (denoted by squares). Number of plants used in crosses per population (mean ± SD) = 7 ± 0.53. Crosses involving the same donor and recipient combination were repeated twice at each of the four floral developmental stages.

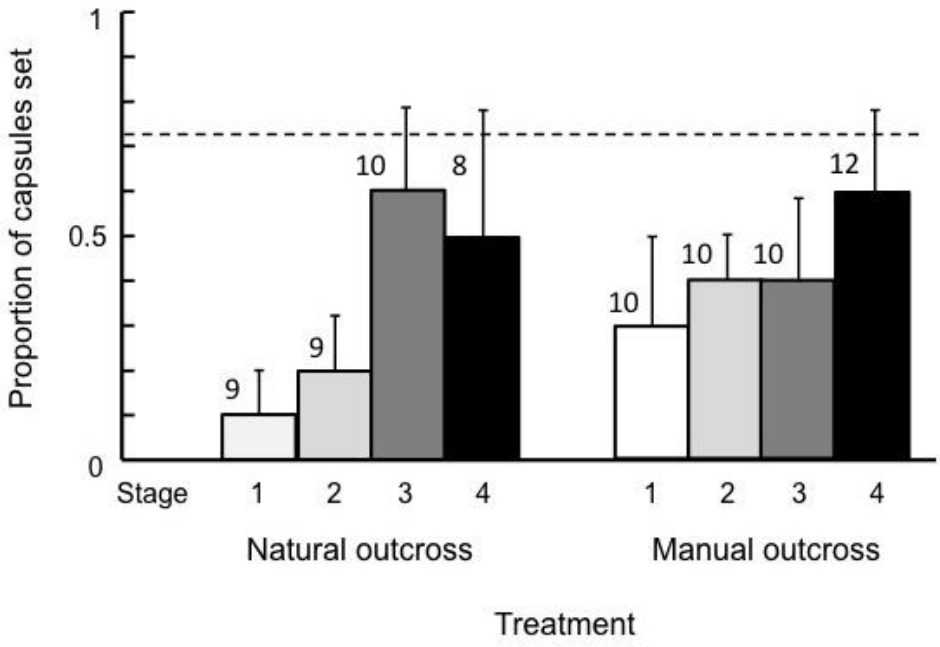

Figure 2: Proportion of flowers maturing into a capsule following natural or manual outcross pollinations of emasculated flowers at different floral stages in a natural population (no 151) of *Collinsia heterophylla*. Stage 1-4 represent the stage at which the style was cut off (naturally pollinated flowers) to prevent further pollination or the stage at which supplemental outcross pollen was added to the stigma (manually outrossed flowers). The dashed line represents the mean fruit set for emasculated control flowers subjected to open pollination at all stages (N = 11 flowers). Numbers shown above bars = number of flowers. Error bars indicate standard error.

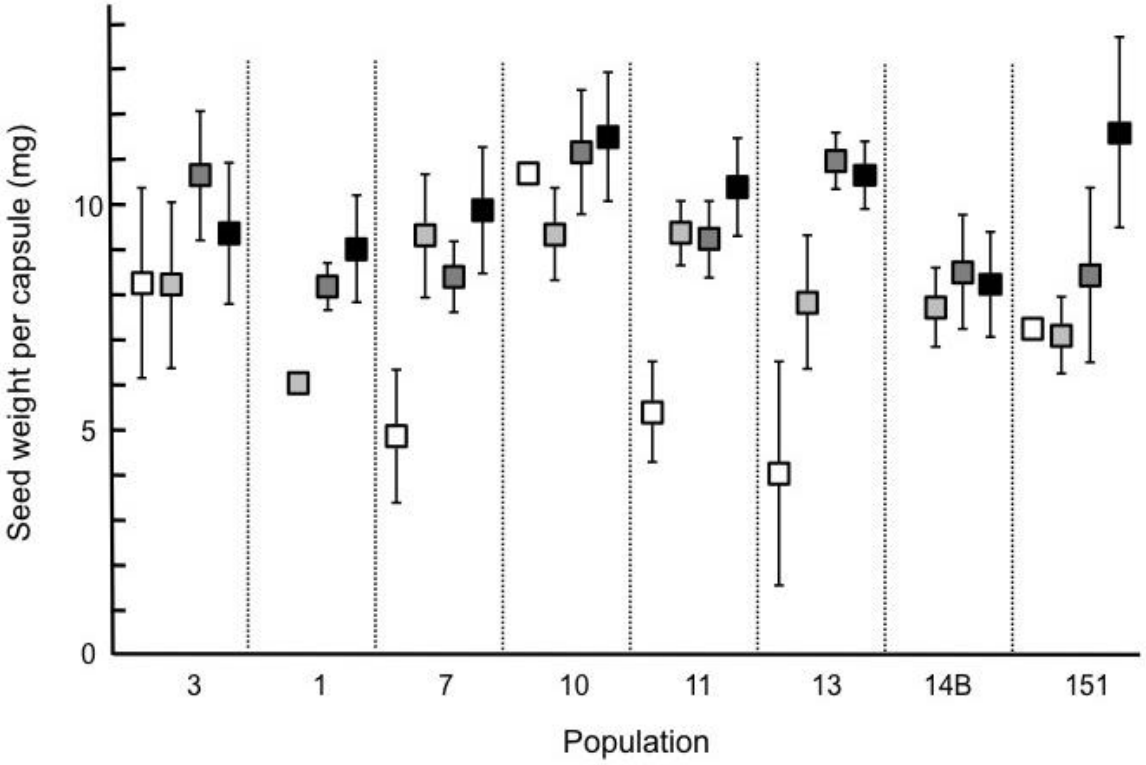

Figure 3: Mean seed weight per capsule after one-donor crosses at floral developmental stages 1-4 within eight populations of *Collinsia heterophylla*. White bars = stage 1, light gray bars = stage 2, dark grey bars = stage 3, black bars = stage 4. Error bars indicate standard error (averaged over recipient plants).

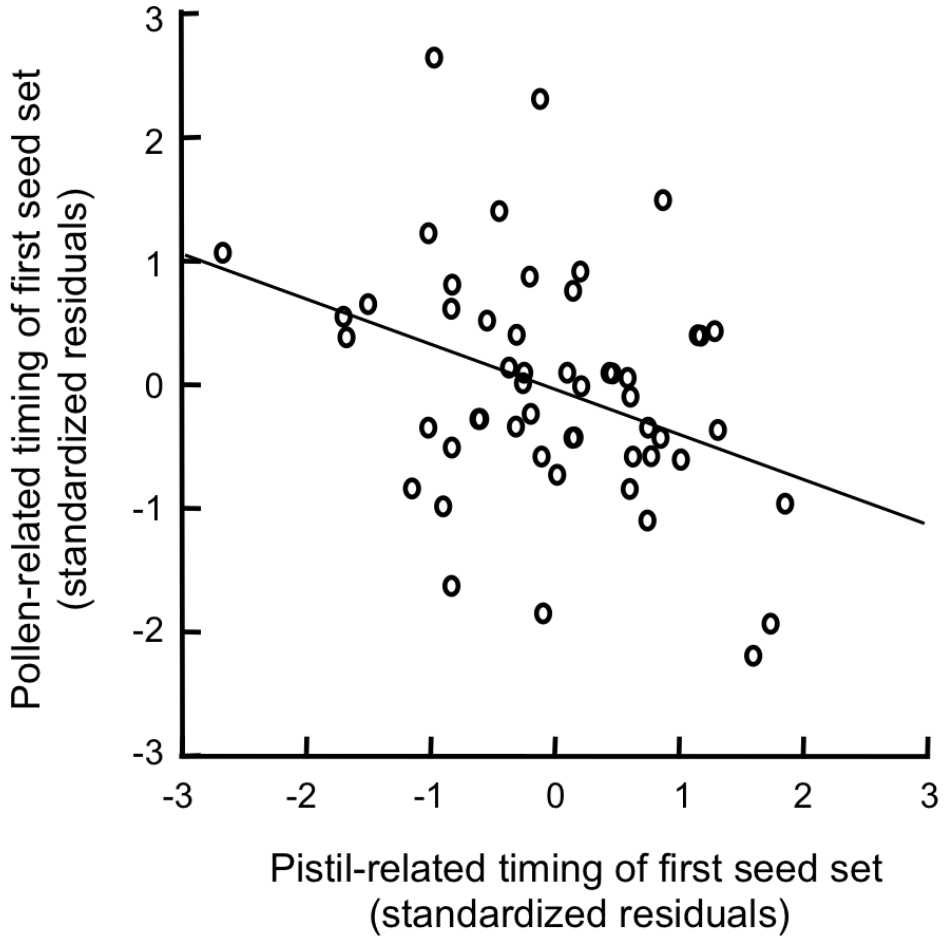

Figure 4

Figure 4: Relationship between standardized residuals for pollen- and pistil-related timing of first seed set (each averaged over three mates and estimated in the same hermaphrodite plant individuals), following one-donor crosses and subsequent style removal.

Supporting Information

Table S1. Locations of *Collinsia heterophylla* populations used in the field study (F), in the greenhouse crossing experiment (G) and in a previous greenhouse experiment (P, Madjidian and Lankinen 2009).

Population	Type	County	Coordinates
1	G	Los Angeles	34.43155 N, 118.62989 W
3	G	Riverside	33.51655 N, 117.33807 W
7	G	Santa Barbara	34.74107 N, 120.01358 W
10	G	Mariposa	37.57681 N, 119.94864 W
11	G	Mariposa	37.50232 N, 120.06873 W
13	G	Madera	37.17936 N, 119.51235 W
142	F* + G	Napa	38.58450 N, 122.37328 W
151	F + G	Napa	38.64407 N, 122.37349 W
4†	P	Ventura	34.42650 N, 119.10570 W
202†	P	Ventura	34.12175 N, 118.87294 W
203†	P	Mariposa	37.65482 N, 119.88640 W
204†	P	Mariposa	37.50196 N, 120.12360 W

*This population was only used in the field for estimating nectar.

†Numbered differently in the previous study: population 4 = 1, 202 = 2, 203 = 3, 204 = 4.

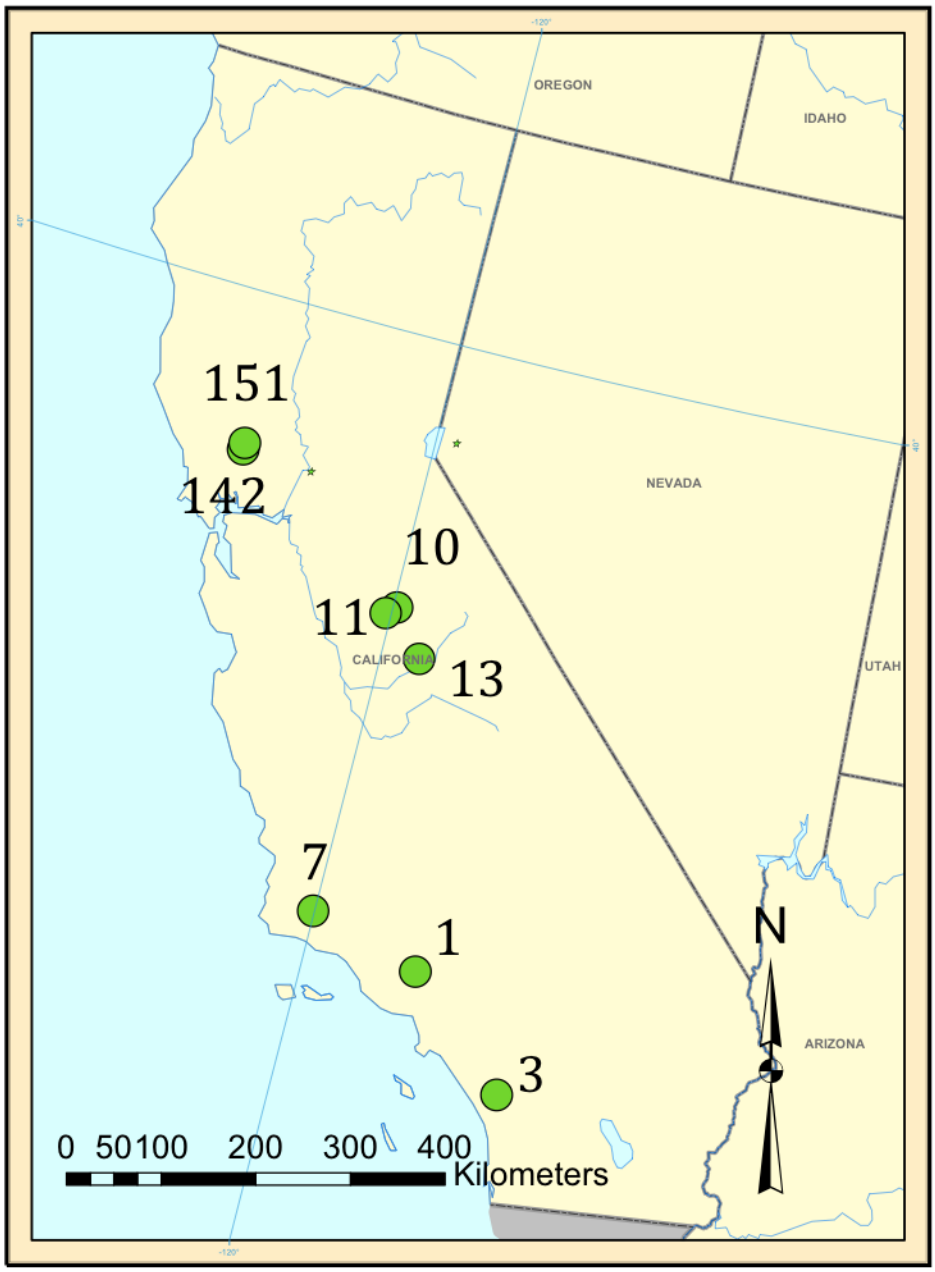

Figure S1: Locations of *Collinsia heterophylla* populations in California