Autonomous Self-Fertilization and Limited Outcrossing in *Linum sulcatum*

A Thesis
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

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May 2015

Acknowledgements

I would like to thank my advisor, Dr. Julie Etterson, for her guidance and support throughout my graduate student experience. Additionally, I would like to thank my committee members, Dr. Nicholas Danz, Dr. David Schimpf, and Dr. Clay Carter for providing valuable feedback on my thesis. Also, I thank Dr. Bryan Bandli from the University of Minnesota Duluth Research Instrumentation Laboratory for his assistance in the use of the confocal microscope and Andrew Olufson for assisting in pollen tube staining. Finally, I gratefully acknowledge the financial support provided by the University of Minnesota Duluth Department of Biology, in the form or teaching assistantships, and the National Science Foundation, for funding the grant which allowed me to travel and collect seeds.

Abstract

The breeding systems employed by wild plant populations have profound effects on their genetic structure and evolution, yet remain unknown or incompletely described for many species. *Linum sulcatum* Riddell (Linaceae) is an herbaceous annual native to eastern North America. This species is thought to be self-compatible, but there has been no experimental evidence to date to support this claim. To assess the breeding system of this species, seeds were collected from wild populations and reared in a controlled environment. Various floral treatments were conducted to determine the self-compatibility of this species and the mode of selfing used. Additional controlled within- and between-population crosses were conducted to determine the relative degree to which this species can outcross. Self-fertilization was highly successful and appears to be achieved autonomously. Outcrossing success was very limited, suggesting this species may exhibit some degree of cross-incompatibility. Furthermore, a separate experiment that examined pollen tube growth found that self-pollination resulted in the formation of more pollen tubes relative to cross-pollination and that complete pollen tube growth can occur less than two hours following self-pollination. This information is relevant to the future persistence of this species, as much of its remaining habitat is distributed among small, highly fragmented patches subjected to current and future environmental change.

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Introduction

Considering the enormous diversity of angiosperms, it is not surprising that the breeding systems of many species have yet to be described. Recent evidence suggests a substantial proportion of angiosperm species are capable of more than one breeding system, and that their relative frequencies may vary among populations (Vogler and Kalisz 2001, Goodwillie et al. 2005, Winn et al. 2011). In particular, self-fertilization is considered common among angiosperm taxa; an estimated 50% of species are self-compatible while 20% use selfing as a primary breeding system (Fryxell 1957, Vogler and Kalisz 2001, Igic and Kohn 2006). The persistence and prevalence of this breeding system was once considered an evolutionary paradox (Darwin 1876, Stebbins 1957) although it is becoming clear that self-fertilization, including that achieved autonomously, can be under positive selection under some conditions (Lloyd 1992). Yet there are few species for which the relative frequency and importance of mixed breeding systems are well understood (Herlihy and Eckert 2004, Kalisz et al. 2012, Dart et al. 2012).

Understanding a species' breeding system, especially rates of outcrossing and self-fertilization, is important because of their profound impact on the genetic structure, and hence the evolutionary potential, of populations (Holsinger 2000). Selfing increases homozygosity, which allows the expression of deleterious recessive alleles, thereby reducing fitness (inbreeding depression) (Charlesworth and Charlesworth 1987). In addition, pollen and ovules that would otherwise be available to form outcrossed progeny are monopolized (pollen and seed "discounting") and this is detrimental if the self-fertilized progeny have lower fitness than the would-be outcrossed progeny (Holsinger et al. 1984, Harder and Wilson 1998). On the other hand, self-pollination provides reproductive assurance which has long been hypothesized to be a major selective force in retaining autonomous selfing in plant populations (Darwin 1877, Jain 1976, Lloyd 1979). Selfing is especially important when potential mates or pollinators are

limited, which is often the case at species' range edges or during the colonization of a previously

uninhabited area (Baker 1955, Kalisz and Vogler 2003). Another advantage of selfing is the two-

fold increase in gene transmission relative to outcrossing (Fisher 1941). Thus, alleles that

facilitate self-fertilization will be selected for as long as the fitness of the progeny is at least half

the fitness of outcrossed progeny (Charlesworth and Charlesworth 1987, Lloyd 1992).

 Recent theoretical studies have suggested that predominantly selfing and outcrossing

breeding systems may in fact represent alternative evolutionary stable states of plant reproduction

(Lande and Schemske 1985, Barrett and Eckert 1990). Furthermore, many have described selfing

as an evolutionary "dead end": that is, the transition from outcrossing to selfing is unidirectional

and that selfing lineages have a negative net diversification rate (Stebbins 1957, Takebayashi and

Morrell 2001). However, others have been critical of these hypotheses and suggest that mixed-

mating systems may also be evolutionarily stabile, particularly in animal-pollinated species

(Vogler and Kalisz 2001, Goodwillie et al. 2005). Among species for which outcrossing rates

have been estimated, 42% exhibit mixed-mating systems (intermediate outcrossing rates)

(Goodwillie et al. 2005). Theoretical models that predict the stability of mixed-mating systems

include factors such as temporal variation in inbreeding depression (Cheptou and Schoen 2002),

biparental inbreeding (Ronfort and Couvet 1995), pollen discounting (Porcher and Lande 2005),

reproductive assurance (Lloyd 1992), and resource allocation (Iwasa 1990). However, only a few

empirical studies have investigated the role these factors play in the evolution of stabile mixed-

mating systems, and these have primarily focused on pollen discounting (Chang and Rausher

1999) and reproductive assurance (Herlihy and Eckert 2002, Kalisz et al. 2004). The selective

pressures underlying the maintenance of mixed-mating systems remain largely unknown.

 Floral traits, such as the distance between anthers and stigmas within a flower

(herkogamy) and the temporal separation in their maturity (dichogamy) influence the propensity

of a species to outcross or self-pollinate (Darwin 1876, Müller 1883). However, many species

have adaptations to increase self-fertilization as well (Kuhn 1867, Darwin 1877, Lord 1981). Self-fertilization can take place in several different forms (Lloyd and Schoen 1992). The most distinct of these, cleistogamy, involves hermaphroditic flowers that remain closed such that pollen from other flowers or plants cannot contact its stigmas. Therefore any seeds produced from cleistogamous flowers can be attributed to self-fertilization. Two additional forms, geitonogamy and facilitated self-fertilization, involve an interaction with a biotic pollinator. Geitonogamy occurs when pollen is transferred between flowers on the same plant and is thought of as an unavoidable consequence of the evolution of cross-fertilization (Lloyd 1992). Facilitated self-fertilization, in contrast, results from self-pollination by a biotic pollinator within an individual hermaphroditic flower. Like geitonogamy, facilitated self-pollination typically occurs when there is also opportunity for cross-pollination. The final form, autonomous self-fertilization, occurs when self-fertilization is achieved within hermaphroditic flowers without the aid of a biotic pollinator and can be further divided into three categories: prior, competing, and delayed (Lloyd and Schoen 1992).

Prior autonomous self-fertilization occurs when the anthers dehisce and are in contact with the stigmas before anthesis (the point at which the flower opens). Competing autonomous self-fertilization occurs in open flowers when dehisced anthers are in contact with the stigmas during the same time interval which cross-pollination can occur. Delayed autonomous self-fertilization occurs when self-fertilization is achieved after the stigmas are available for cross-pollination. This can occur through morphological changes during the lifespan of an individual flower in which the anther-stigma distance is reduced as the flower ages or through a breakdown in self-incompatibility in which self-pollen becomes viable after the opportunity for cross-pollination has passed (Klips and Snow 1997, Vogler et al. 1998, Kalisz et al. 1999).

This study examines the breeding system of *Linum sulcatum* Riddell (Linaceae), an herbaceous annual plant that occurs in North American prairies from Manitoba south to Texas.

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With the exception of its taxonomy and systematics (Rogers 1963, Harris 1968, Rogers 1969), little research has been conducted on this species, especially concerning its breeding systems and evolutionary ecology. It has been suggested that this species may be self-compatible, but to date there has been no published experimental evidence to support this claim (Zaremba 2003). The genus *Linum* is large (~180 species) and contains examples of both self-compatible and self-incompatible species (McDill et al. 2009). The transition from self-incompatibility to self-compatibility is a common evolutionary trajectory in hermaphroditic angiosperms (Charlesworth 2006) and it appears that breeding systems in this genus are evolutionarily labile. Furthermore, annuals are much more strongly associated with self-compatibility then perennials (Stebbins 1970, Barrett et al. 1996), likely due to a reduced cost of reproductive assurance (Lloyd 1992).

Linum sulcatum has several floral attributes that suggest self-compatibility, including homostyly and anther dehiscence prior to anthesis. The corollas, which consist of five petals that are partially fused at the base, abscise from the flower less than one day after anthesis (or after several hours in wild populations subjected to windy conditions, pers. obs.). The corolla abscises over the top of the flower, removing the anthers from the filaments with small claw-like appendages at the base of the petals. This process may increase contact between the anthers and stigmas and may be an adaptation to increase autonomous self-fertilization. "Corolla dragging" has been observed in other angiosperms as a potential adaption to increase autonomous self-fertilization (Dole 1990, Sun et al. 2005, Qu et al. 2007). Specifically, this process suggests delayed autonomous self-fertilization, as corolla abscission would typically follow the opportunity for cross-pollination.

This research investigates the breeding systems of *L. sulcatum* and determining whether they differ among populations across its geographic range. Seeds were collected from wild populations and grown in both greenhouse and growth chamber settings. Several different floral manipulations were conducted to determine if *L. sulcatum* is self-compatible and whether it can

self autonomously. Additionally, both within- and between-population controlled crosses were

conducted to determine the degree to which this species can outcross.

Materials and Methods

Seed collection: During summer 2012, seed was collected from 50 maternal lines within

five populations of *Linum sulcatum* along a latitudinal gradient (Figure 1, 30 maternal lines from

FHK, Table A1). To avoid sampling close relatives, seed was collected from maternal plants

spaced at least 2 m apart along transects. Fruits were dried using silica gel desiccant, after which

the seeds were removed and stored at approximately 4° C until experiments were initiated.

Growth conditions: In fall 2012 approximately 1500 seeds between the five populations

were cool-moist stratified for 30 days at 3.6 °C using coarse, moist sand in petri dishes (separated

by maternal line). Following stratification, the petri dishes were placed in a single growth

chamber (Percival Intellus E36LX4) with fluorescent lighting (11 hours of light per day, 21.1 °C).

Seeds were checked daily for germination and individually transplanted into 4 x 14.5 cm cone-

tainers (60% sand, 40% Pro-Mix, Premier Tech Horticulture) upon emergence of the first true

leaves. Germination rate was approximately 5% and 58 plants survived transplantation. To

mimic natural conditions, photoperiod and temperature were incrementally increased for

approximately six months, reaching a maximum day length of 16 hours and maximum

temperatures of 25.5/23.5 °C (day/night). Approximately two months later the photoperiod was

decreased to 14.25 hours in an attempt to induce flowering on plants that had not yet flowered.

Plants were watered every two weeks and lightly fertilized at six-week intervals (20:20:20 NPK,

0.5 ml/L). 38% of the plants flowered over the course of a year.

In spring 2013, an additional 4500 seeds were stratified as above and germinated in a

greenhouse under white shade cloth to prevent overheating and desiccation. Germination was

assessed daily and seedlings were transplanted into cone-tainers as above upon emergence of the

first true leaves. Germination rate was approximately 10% and 176 plants among the

aforementioned populations were transplanted and grown (atop greenhouse benches without shading). The average daytime temperature within the greenhouse was approximately 27 °C but was subject to fluctuations due to variable external temperature and ventilation system malfunctions. Grow lights (metal halide, 13 hour day length) were not used until September 2013 to account for the decline in the ambient photoperiod. Plants were watered every five days and lightly fertilized every 2 weeks (20:20:20 NPK, 0.5 ml/L). Additionally, the plants were sprayed weekly with a low concentration soap solution to deter mites and other common greenhouse arthropod pests. Approximately 10% of the plants in the greenhouse flowered.

   Floral Manipulations: Six floral treatments were conducted on growth chamber and greenhouse-grown plants to determine the degree to which *Linum sulcatum* outcrosses and/or self-fertilizes, and the extent to which floral morphology contributes to self-pollination. Preliminary analyses showed that rearing conditions did not affect response to pollination treatments and is not considered further (Table A2). First, to determine if *L. sulcatum* can self-fertilize, the anthers were removed on a set of flowers and self pollen was deposited directly on the stigmatic surface (Controlled Self, hereafter). Every effort was made to assure that fertilization was maximized even though it was not possible to apply the same number of pollen grains to each stigma. A second set of flowers were left fully unmanipulated to allow corolla abscission and subsequent basal petal appendage drag to facilitate self-fertilization (w/ Appendages). For comparison, the corolla including petal appendages were removed on a third set of flowers but the anthers remained intact which would allow autonomous selfing during anther dehiscence (w/o Appendages) (Figure 2). Next, to determine the degree to which *L. sulcatum* outcrosses and whether fertilization success differs between populations from different parts of the species range, within-population and between-population controlled crosses were conducted (Within and Between, respectively). Anthers were removed from recipient flowers and, after ensuring the stigmas were pollen-free under a dissecting microscope, donor pollen was

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deposited as above. Finally, to control for the possibility of apomictic seed production, a sixth set

of flowers was emasculated and no pollen was added (No Pollen). The treatments were marked

with colored thread and replicated 22-102 times. Overall, 8-23 maternal lines from 3-5 different

populations were used in each treatment (Table A3). It was not possible to obtain a fully

balanced design with respect to population or maternal line because of asynchronous flower

availability. Flowers were never emasculated in bud as it typically resulted in arrested floral

maturation or damage to floral organs.

Fruit formation was monitored semi-weekly and capsules were collected at the first sign

of dehiscence. Filled seeds per fruit were counted (*L. sulcatum* capsules can contain a maximum

of 10 seeds); those lacking embryos were readily distinguishable by their smaller size, darker

color, and concave morphology. A capsule was considered to have set seed if at least one filled

seed was produced.

*Data Analysis***:** To determine the effect of the floral manipulation treatments on fruit

formation, data was analyzed with logistic regression (JMP Pro 10, SAS Institute 2012).

Population and each of the treatments were included in the model and resulted in stable parameter

estimates. Odds ratios between individual populations and between individual treatments were

used to determine any significant differences in their likelihood of fruit formation. Their

corresponding p values were Bonferroni-corrected to account for the problems associated with

multiple comparisons (α/n, where *n* equals the number of comparisons made).

A separate logistic regression model was fitted to determine the effect treatment had on

seed set. Population was included as an effect in the model; however, the No Pollen treatment

was removed to obtain stable parameter estimates. Odds ratios between individual treatments and

between individual populations were used to determine any significant differences in their

likelihood of seed set. Their corresponding p values were also Bonferroni-corrected.

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A least-squares multiple linear regression model was fitted to determine the effect treatment had on the number of seeds produced per flower (JMP Pro 10, SAS Institute 2012). In addition to Treatment, Maternal Line nested within population and Population were also included as effects in the model to account for variation in seed production both within- and between populations. Tukey tests were used to determine any significant differences in seed production between populations and treatments. The interaction between population and treatment could not be included in the model due to the unbalanced nature of the data set. However, when particular populations and/or treatments were removed in preliminary data analyses to achieve balance, this interaction was never significant.

Results

Fruit Formation: Population and treatment both had a significant effect on fruit formation (Table 1). Populations FHK and FMB were significantly more likely (5x and 10x respectively) to form fruits than ACP (Table 2). All three treatments with the potential for self-fertilization (Controlled Selfing, w/ Appendages, and w/o Appendages) were significantly more likely to form fruits than the No Pollen treatment (7x-15x more likely) (Table 3). Fruits were also 7x more likely to form in the Controlled Self treatment than in the than the Between crossing treatment.

Seed Set: All of the floral manipulations resulted in seed set, with single exception of the No Pollen control treatment which completely failed to set seed even though fruits were sometimes formed. Seed set was not significantly influenced by the population of origin, but did differ between treatments (Table 4). The Controlled Self, w/ Appendages, and w/o Appendages treatments were all significantly more likely to result in seed set than the Within and Between crossing treatments but were not significantly different from each other (Table 5). The Within and Between crossing treatments, while 6x-21x times less likely to result in seed set than the treatments with the potential for selfing, were not significantly different from each other.

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Seed Production: The treatments with the potential for self-fertilization resulted in greater seed production than those with the potential for cross-fertilization. However, Maternal Line (nested within Population), Population, and Treatment all had a significant effect on seed number (Table 6). Populations FHK and TGP both had a significantly higher mean seed number than ACP and KPBS (Figure 3), while FMB did not have a significantly different mean seed number than any of the other four populations. Of the three treatments with the potential for self-fertilization, Controlled Self resulted in the highest least-squares (LS) mean seed number, followed by the w/o Appendages and w/ Appendages treatments respectively (Figure 4). However, their LS mean seed numbers were not significantly different from each other. Of the two floral treatments involving cross-pollination, Within resulted in a higher LS mean seed number than Between. The No Pollen treatment (which did not produce any seeds) resulted in a LS mean seed number that was intermediate of the Within and Between treatments. The LS mean seed number for the Within, Between, and No Pollen treatments were not significantly different from each other but were all significantly lower than the Controlled Self, w/ Appendages, and w/o Appendages treatments.

Discussion

This research has provided a robust test of the breeding system of *Linum sulcatum* and shows that this species has a mixed breeding system and is capable of autonomous self-fertilization. Although the controlled self-pollination treatment resulted in the greatest degree of seed set and seed production, this treatment did not differ significantly from others that permitted autonomous self-fertilization including completely unmanipulated flowers and those that had their corollas and attached floral appendages removed. Seed production did not appear to be a product of apomixis, as none of the emasculated, unpollinated flowers produced a single filled seed. Thus it appears that the physical proximity of the anthers and stigmas within a given flower, as well as the temporal synchrony between anther dehiscence and stigma receptivity,

afford *L. sulcatum* the ability to self-fertilize even in the absence of a pollinator. However, the manual self-pollination process used in this experiment may not have accurately replicated the efficacy of a biotic pollinator in nature. Biotic pollination was not considered in this experiment, and the degree to which facilitated self-fertilization serves as a reproductive strategy for this species in nature, if any, remains unclear (Schoen and Lloyd 1992).

The comb-like basal petal appendages, which were hypothesized to enhance selfing, did not influence seed set in this experiment. However, this may not be a definitive test of the value of floral appendages in facilitating self-fertilization in nature. The absence of strong winds in the growth chamber and greenhouse, which are typical within this species' native range, may have reduced the effect these structures had on self-fertilization. The corollas of experimental flowers remained attached up to several days longer than those observed in nature, which are typically shed within 12 hours post-anthesis in nature (pers. obs.). This may have delayed pollen deposition beyond the point of peak stigma receptivity and obscured the fitness value of this accessory structure. The inclusion of wind, whether artificially or by conducting experiments outdoors, could offer more information on the adaptive nature of these structures (Qu et al. 2007).

The mode of self-fertilization observed in *L. sulcatum* can best be described as autonomous self-fertilization. Of the three modes of autonomous self-fertilization described by Lloyd and Schoen (1992), the results of this experiment suggest either prior or competing, which occur before and after anthesis respectively, as the most appropriate breeding system designation. The third mode, delayed autonomous self-fertilization, seems unlikely for several reasons but was not explicitly tested in this experiment. First, the anthers were in close proximity to the stigmas upon dehiscence, which typically occurred just prior to or just after anthesis. Additionally, cross-pollination rarely resulted in seed set (discussed below) despite the fact that outcrossed pollen was added to recipient stigmas shortly after anthesis. If delayed selfing were occurring, one would expect either changes in morphology that decrease the anther-stigma distance as a flower

ages or a prepotency of outcrossed pollen in the early phases of an individual flower (Vogler et al. 1998, Kalisz et al. 1999). Future studies investigating the rate of pollen tube growth following self-pollination at different time intervals following anthesis could further support the rejection of delayed self-fertilization as the likely mode of autonomous self-fertilization. Furthermore, studies comparing the relative frequency of anther dehiscence prior to and following anthesis could distinguish between prior and competing autonomous self-fertilization.

The plants considered in this experiment exhibited a very limited ability to outcross, regardless of whether the cross occurred within or between members of the different populations that were considered. Nonetheless, cross-pollination occasionally resulted in seed set. Despite a smaller sample size for these two treatments relative to the aforementioned self-pollination treatments, no single population or genotype exhibited proficiency in cross-fertilization. Of the relatively few successful cross-fertilization events, the majority resulted in the production of only a single seed, and none produced more than 3 seeds. Although the stigmas of cross-pollinated flowers were carefully inspected, self-pollen contamination prior to the addition of outcrossed pollen cannot be completely ruled out as a factor responsible for the very limited cross-fertilization observed.

These results suggest that at least some wild populations of *L. sulcatum* may exhibit cross-fertilization incompatibility; that is, some individuals may be obligate selfers. However, this phenomenon may not be universal within the populations considered in this experiment or in other populations across this species' range that were not sampled (Goodwillie et al. 2005). Furthermore, variation in environmental conditions is known to cause changes in self- and cross-fertility (Lloyd and Schoen 1992). The rearing conditions of the plants used in this experiment may have played a role in the receptivity of the stigmas to outcrossed pollen or in the pollen grain's ability to germinate on a foreign stigma. As with the case of facilitating self-pollination, the manual cross-pollination technique used it this experiment may not have been able to match

the efficacy of an insect pollinator in nature. Future studies assessing genetic similarity and inbreeding within wild populations of *L. sulcatum* can determine if the self-compatibility and limited outcrossing observed in this experiment is reflected in wild populations (Schoen and Lloyd 1992). Additionally, studies examining the pollination ecology of this species can determine which role, if any, biotic pollination plays in the reproduction of this species. Although there is no known literature concerning this subject, other species of *Linum* are insect-pollinated (Kearns and Inouye 1994, Armbruster et al. 2006) and the relatively large, sticky pollen grains of *L. sulcatum* suggest the possibility of entomophily.

The cross-incompatibility observed in this experiment could be attributed to pre- or post-zygotic barriers (Levin 1978). The outcrossed pollen may be limited in its ability to form pollen tubes that can descend the length of the style, enter the ovary, and penetrate the ovule. This phenomenon has been well-studied in self-incompatible angiosperms, in which specific loci responsible for pollen tube development and growth have been identified (McClure et al. 1989, Stein et al. 1991). Unsuccessful fertilization could also be attributed to zygotic failure, in which the pollen tube and subsequently the male gametes reach the ovule but fail to develop into a filled seed capable of successful germination. Zygotic failure has also been well-studied in self-incompatible angiosperms (Lipow and Wyatt 2000, Sage and Sampson 2003). This experiment fails to point to a particular mechanism for cross-incompatibility, as some of the cross-pollination events resulted in no fruit formation while others resulted in empty fruits or fruits containing aborted seeds. Future studies involving the assessment of pollen tube growth following outcrossing could elucidate potential mechanisms for cross-incompatibility.

Regardless of floral treatment, the population from which the seeds were collected had a significant effect on seed production. Maternal and Paternal effects could be responsible for the between-population differences observed (Roach and Wulff 1987). Precipitation was well below average at each of the collection locations in 2012 except FMB (Minnesota), and the populations

associated with the lowest seed production (ACP and KPBS, Kansas) were also those that endured the most severe drought (NOAA 2012). However, the between-population differences could be an artifact of the small and unbalanced sample size (and low germination rates in both the greenhouse and growth chamber). For this reason, whole-population inferences from these data would be weak at best.

Although fruit formation was often associated with seed set, the results of this experiment suggest that *L. sulcatum* can produce fruits without seeds as well. Fruit formation may be triggered by pollen tube formation in one or more of the styles and/or the union of gametes within the ovary (Biale 1964). If this is the case, incomplete pollen tube formation or zygote mortality may account for the formation of fruits devoid of filled seeds. Fruit formation is energetically costly, so it seems unlikely that the production of empty fruits would afford any benefit to the plant, though empty fruits appear to play a role in seed predation avoidance in other species (Traveset 1993, Fuentes and Schupp 1998).

The self-compatibility and limited cross-compatibility observed in *L. sulcatum* in these experiments has significant evolutionary and ecological implications. For self-compatibility to have evolved in this species, the costs of seed and pollen discounting, as well as inbreeding depression, must have been lower than the benefits of increased gene transmission and/or reproductive assurance (Lloyd 1992). Nonetheless, the selective pressures that shaped the breeding systems of *L. sulcatum* in the past may not be relevant today. The tallgrass prairie of the Midwestern United States, which occupies the core of the species range of *Linum sulcatum*, is one of the most destroyed ecosystems in the world, with an estimated 1-18% of its original land cover remaining (Samson and Knopf 1994). Furthermore, much of the intact native tallgrass prairie is distributed among relatively small, highly fragmented patches. This is a stark contrast to the millions of continuous acres of tallgrass prairie that occupied this region prior to European settlement. These factors may reduce the ability for this species to disperse into previously

unoccupied regions that may be more suitable in the future or, may prevent more favorable genotypes from moving in (Kramer and Havens 2009). Fragmentation typically reduces genetic variation (Young et al. 1996), which would only be exacerbated in a highly selfing population. Cross-fertilization, even in relatively low frequency, could restore genetic diversity, but is also likely to be affected by fragmentation by reduced pollinator abundance and diversity (Rathcke and Jules 1993).

Climate change is expected to proceed at a rate that may exceed the ability of most plants to respond through adaptation (Davis and Shaw 2001). Cross-fertilization could create novel genetic variation that may be adaptive under future climate scenarios, but the degree to which *L. sulcatum* populations can outcross, if at all, remains unclear. Self-fertilizing populations subjected to fragmentation and climate change may be severely limited in their ability to respond through adaptive evolution and it is unlikely that this could be compensated by phenotypic plasticity alone. The future persistence of this species may be in jeopardy. Future research that considers how breeding systems affect the response of plant populations to both habitat fragmentation and climate change could aid in determining the extinction risk of *L. sulcatum* (Leimu et al. 2010).

Table 1: Likelihood ratio test statistics for logistic regression of fruit formation (yes/no) for five populations of *Linum sulcatum* subjected to the following pollination treatments: controlled self-pollination, no manipulation, basal petal appendages removed, within-population cross-pollination, between-population cross-pollination, and anthers removed prior to dehiscence.

Source	DF	X^2	p
Population	4	13.91	**0.008**
Treatment	5	37.85	**<0.0001**

Table 2: Odds ratios (95% confidence intervals) of fruit formation (yes/no, columns (numerator) / rows (denominator)) for five populations of *Linum sulcatum* (Frenchman's Bluff, MN (FMB), Freda Haffner Kettlehole, IA (FHK), Konza Prairie Biological Station, KS (KPBS), Anderson County Prairie, KS (ACP), and Tallgrass Prairie Preserve, OK (TGP)) subjected to five pollination treatments. Tests and confidence intervals on odds ratios are likelihood ratio based. Significant odds ratios following Bonferroni correction are in bold.

	ACP	FHK	FMB	KPBS	TGP
ACP	-	-	-	-	-
FHK	**0.11 (0.02-0.49)***	-	-	-	-
FMB	**0.22 (0.08-0.60)***	2.00 (0.53-9.92)	-	-	-
KPBS	0.49 (0.17-1.39)	4.39 (1.12-22.39)	2.20 (0.93-5.22)	-	-
TGP	0.42 (0.16-1.09)	3.80 (1.05-18.59)	1.90 (0.96-3.86)	0.87 (0.39-1.97)	-

*p<0.05

Table 3: Odds ratios (95% confidence intervals) of fruit formation (yes/no, columns (numerator) / rows (denominator)) for *Linum sulcatum* from five populations subjected to the following pollination treatments: controlled self-pollination (Controlled Self), no manipulation (w/ Appendages), basal petal appendages removed (w/o Appendages), within-population cross-pollination (Within), between-population cross-pollination (Between), and anthers removed prior to dehiscence (No Pollen). Tests and confidence intervals on odds ratios are likelihood ratio based. Significant odds ratios following Bonferroni correction are in bold.

	Controlled Self	w/ Appendages	w/o Appendages	Within	Between
Controlled Self	-	-	-	-	-
w/ Appendages	2.10 (0.97-4.55)	-	-	-	-
w/o Appendages	2.13 (0.91-4.98)	1.01 (0.41-2.47)	-	-	-
Within	4.20 (1.50-11.75)	2.00 (0.69-5.77)	1.97 (0.68-5.73)	-	-
Between	**7.27 (2.90-19.13)****	3.46 (1.33-9.36)	3.41 (1.22-9.96)	1.73 (0.54-5.71)	-
No Pollen	**15.48 (5.22-51.85)*****	**7.36 (2.40-25.27)***	**7.27 (2.37-24.95)***	3.69 (1.09-13.74)	2.13 (0.61-7.98)

*p<0.01, **p<0.001, ***p<0.0001

Table 4: Likelihood ratio test statistics for logistic regression of seed set (yes/no) for five populations of *Linum sulcatum* subjected to the following pollination treatments: controlled self-pollination, no manipulation, basal petal appendages removed, within-population cross-pollination, and between-population cross-pollination.

Source	DF	X^2	p
Population	4	7.55	0.11
Treatment	4	50.85	**<0.0001**

Table 5: Odds ratios (95% confidence intervals) of seed set (yes/no, columns (numerator) / rows (denominator)) for *Linum sulcatum* from five

populations subjected to the following pollination treatments: controlled self-pollination (Controlled Self), no manipulation (w/ Appendages),

basal petal appendages removed (w/o Appendages), within-population cross-pollination (Within), and between-population cross-pollination

(Between). Tests and confidence intervals on odds ratios are likelihood ratio based. Significant odds ratios following Bonferroni correction are in

bold.

	Controlled Self	w/ Appendages	w/o Appendages	Within
Controlled Self	-	-	-	-
w/ Appendages	1.84 (0.90-3.78)	-	-	-
w/o Appendages	2.29 (1.05-5.01)	1.24 (0.54-2.85)	-	-
Within	**20.63 (6.18-95.40)***	**11.22 (3.23-53.11)**	**9.02 (2.62-42.35)***	-
Between	**14.59 (5.20-48.93)***	**7.94 (2.75-27.00)**	**6.38 (2.09-22.77)***	0.71 (0.13-3.42)

*p<0.01, **p<0.001, ***p<0.0001

Table 6: Effects tests for linear regression of seed number per fruit produced by maternal lines sampled from five *Linum sulcatum* populations subjected to five pollination treatments: controlled self-pollination, no manipulation, basal petal appendages removed, within-population cross-pollination, between-population cross-pollination, and anthers removed prior to dehiscence.

Source	DF	F	p
Maternal Line (Population)	21	3.14	**<0.0001**
Population	4	6.27	**<0.0001**
Treatment	5	12.52	**<0.0001**
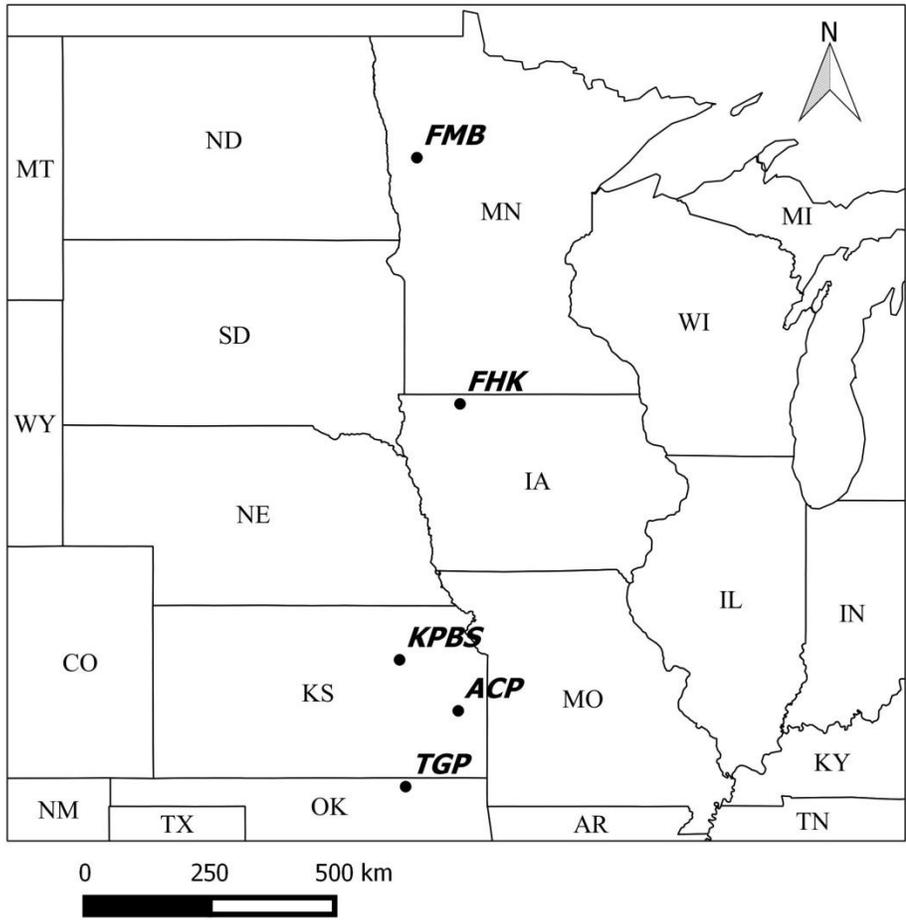
Figure 1: *Linum sulcatum* seed collection locations in the Midwestern United States including:
Frenchman's Bluff (FMB), Freda Haffner Kettlehole (FHK), Konza Prairie Biological Station
(KPBS), Anderson County Prairie (ACP), and Tallgrass Prairie Preserve (TGP).
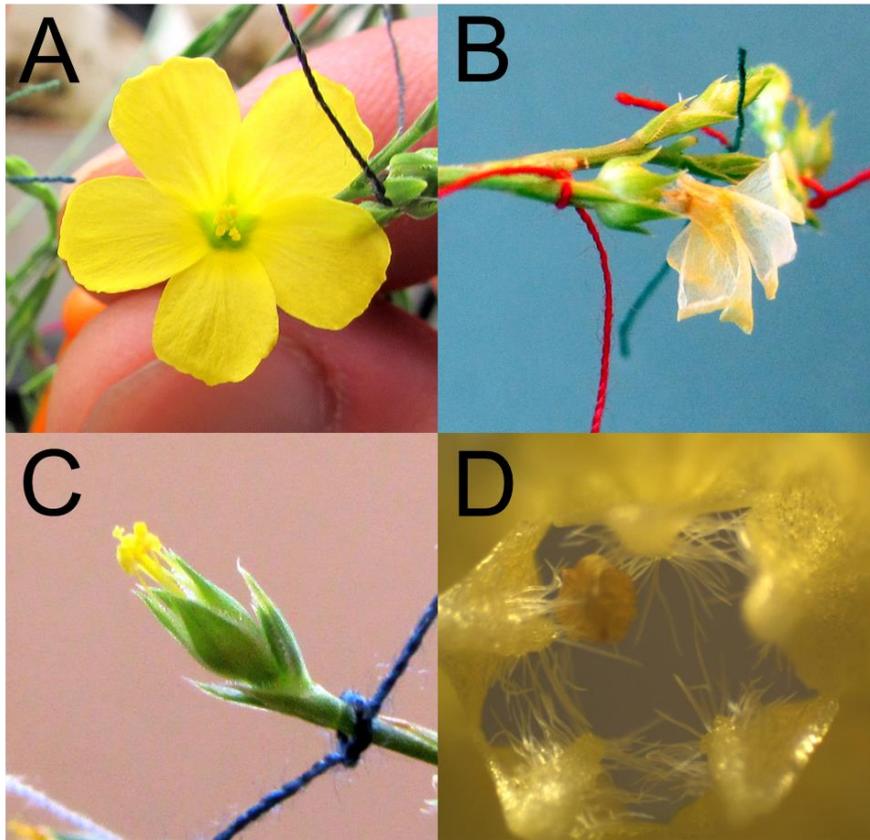
Figure 2: *Linum sulcatum* experimental flowers: A) immediately following anthesis, B) several days after anthesis showing the withered corolla still attached at its base to the anthers and stigmas, C) a flower where the corolla has been removed, D) a view from underneath an intact, partially fused corolla showing the comb-like basal petal appendages holding a removed anther.
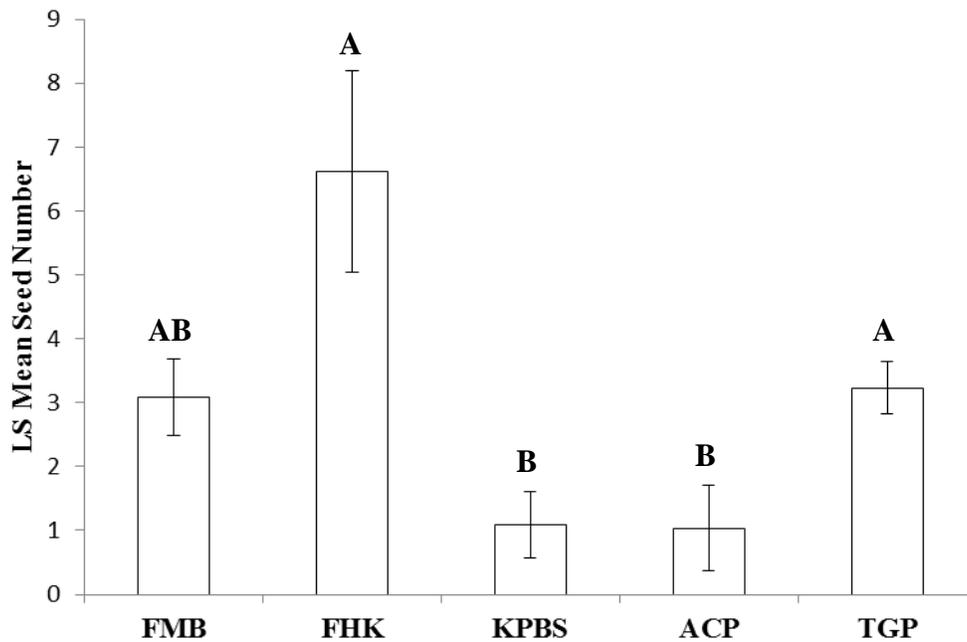
Figure 3: Least-squares means (se) of seed number produced by individual *Linum sulcatum* flowers subjected to six pollination treatments sampled from the following populations: Frenchman's Bluff, MN (FMB), Freda Haffner Kettlehole, IA (FHK), Konza Prairie Biological Station, KS (KPBS), Anderson County Prairie, KS (ACP), and Tallgrass Prairie Preserve, OK (TGP). Letters indicating significant differences are from Tukey's honest significant difference test, $p < 0.05$.
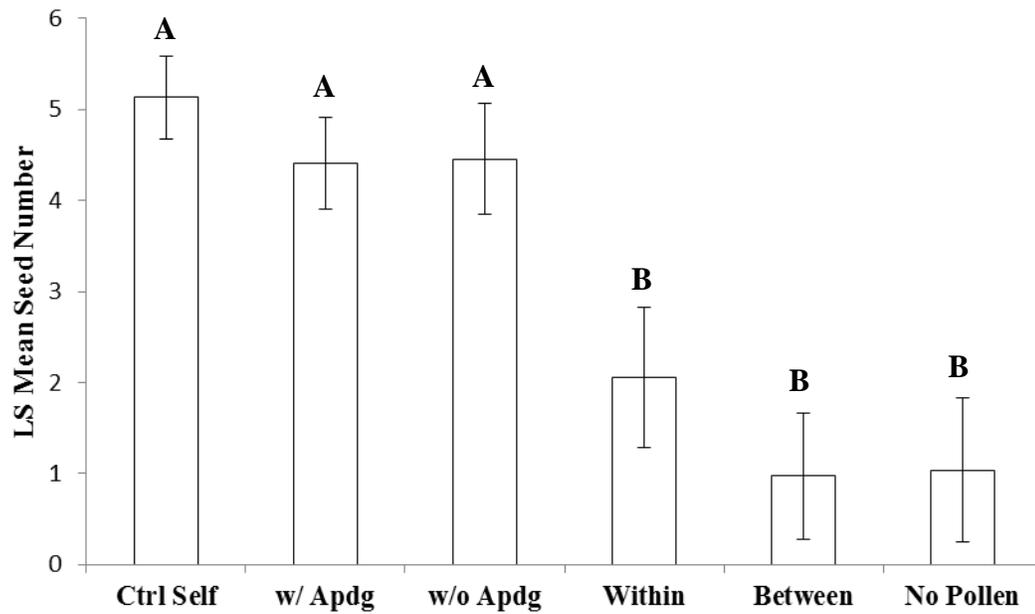
Figure 4: Least-squares means (se) of seed number produced by individual *Linum sulcatum* flowers from five populations subjected to the following pollination treatments: controlled self-pollination (Ctrl Self), no manipulation (w/ Apdg), basal petal appendages removed (w/o Apdg), within-population cross-pollination (Within), between-population cross-pollination (Between) and anthers removed prior to dehiscence (No Pollen). Letters indicating significant differences are from Tukey's honest significant difference test, $p < 0.05$.

Chapter 2: Differences in Pollen Tube Growth in *Linum sulcatum* Following Self-

and Cross-Pollination

Introduction

Successful pollen tube formation and growth is an essential component of sexual

reproduction in angiosperms. This process begins when a mature pollen grain contacts a

receptive stigma. The pollen grain germinates, during which its vegetative cell forms a pollen

tube that grows through the style tissue and carries the sperm to the embryo sac within an ovule in

the ovary. Here the sperm cells can double-fertilize the megagametophyte and, if successful,

form a viable seed. The route to successful fertilization involves an extremely complex series of

interactions between the male gametophyte (the pollen grain) and both the sporophyte (stigma,

style, and ovary) and megagametophyte (embryo sac) of the female (Dresselhaus and Franklin-

Tong 2013). Although major advances in understanding the mechanisms underlying pollen

germination (Taylor and Hepler 1997, Edlund et al. 2004) and pollen tube growth and guidance

(Mascarenhas 1993, Franklin-Tong 1999, Steinhorst and Kudla 2013) have been made, many

aspects remain unknown (Johnson and Preuss 2002, Kessler and Grossniklaus 2011). Given the

complexities associated with sexual reproduction in angiosperms, it is unsurprising that there are

many opportunities for fertilization to be impeded from the time a pollen grain contacts a stigma

to the fusion of gametes within the embryo sac. Genetic determination of pollen tube formation

and growth can result in pollen incompatibility, which is especially relevant among

hermaphroditic angiosperms that exhibit the potential for both self- and cross-pollination.

Mechanisms underlying self-incompatibility (SI), in which an individual genotype is

unable to fertilize its own ovules, have been identified in several angiosperm families and include

three general categories: gametophytic, sporophytic, and late-acting SI (Pandey 1960, Gibbs

2014). Gametophytic SI is a function of haploid genotype of the male gametophyte (the pollen

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grain) which typically arrests pollen tube development within the style (Newbigin et al. 1993). In contrast, sporophytic SI is determined by the diploid genotype of the parental anther in which the pollen was produced (the sporophyte) (Nasrallah and Nasrallah 1993). Sporophytic SI typically results from the interaction between the pollen grain and stigma in which pollen tubes are unable to form and/or penetrate into the style. Late-acting SI occurs within the ovary and is poorly understood but has been identified in several species (Seavey and Bawa 1986, Lipow and Wyatt 2000). It can result from the inability of the pollen tube to penetrate the ovule, complications within the ovule that prevent fertilization (both prezygotic failures), or complications following gamete fusion that prevent the development of a viable seed (postzygotic failure) (Gibbs 2014).

Regardless of the form, SI is generally thought to be an adaptation to avoid the deleterious effects of inbreeding (Takayama and Isogai 2005). However, not all angiosperm species are self-incompatible; approximately 50% are capable of self-fertilization while 20% utilize selfing exclusively (Fryxell 1957, Vogler and Kalisz 2001, Igic and Kohn 2006). In predominantly self-fertilizing species, it is thought that the deleterious effects of inbreeding are offset by the selective advantage it can provide, particularly in the form of increased gene transmission (Fisher 1941) and reproductive assurance, especially after the opportunity for cross-fertilization has passed (Lloyd 1979). This situation, known as delayed autonomous self-fertilization, can be facilitated by changes in floral morphology that reduce the distance between the anthers and stigmas (Kalisz et al. 1999) or may occur because of a temporal breakdown in SI (Vogler et al. 1998). Alternatively, autonomous self-fertilization can occur prior to anthesis before the opportunity for cross-pollination exists (prior autonomous self-fertilization), or after anthesis during the same time that cross-pollination can occur (competing autonomous self-fertilization) (Lloyd and Schoen 1992).

Although SI has been researched extensively it appears that few, if any, studies have attempted to describe intraspecific incompatibility of outcrossed pollen in primarily selfing wild

plant populations. Cross-incompatibility (CI) has been described interspecifically (Camadro and Peloquin 1981, Heslop-Harrison 1982) and intraspecifically; however, studies investigating intraspecific CI appear to be limited to domesticated species (Kermicle and Allen 1990, Egea and Burgos 1996). Thus, it is unclear the degree to which predominantly selfing plant species exhibit CI, and it is possible that similar mechanisms that prevent selfing in SI species may underlie CI in predominantly selfing species (Dresselhaus et al. 2011).

The herbaceous annual species *Linum sulcatum* provides an opportunity to assess both delayed autonomous self-fertilization and CI, as the breeding system of this species has yet to be fully described. It has been postulated to be self-compatible (Zaremba 2003), and recent evidence suggests it may utilize autonomous self-fertilization (Lloyd and Schoen 1992) as its primary reproductive strategy and may be limited in its ability to outcross (Jahnke 2014, Chapter 1). To test this hypothesis, experiments were conducted to determine: 1) if delayed autonomous self-fertilization occurs in this species, and 2) the degree to which it is cross compatible. If self-fertilization is delayed as a result of a temporal breakdown in SI, one would expect an increase in pollen tube growth over time. If this species can cross-fertilize, pollen tube growth will occur but it may differ between pollinations conducted within versus between populations. Finally, if self pollen germinates and forms complete pollen tubes more readily than cross pollen, CI may exist and be under similar genetic control as that found in gametophytic and sporophytic SI systems. However, if both self and cross pollen are equally able to form and grow pollen tubes, then CI, if occurring at all, may be analogous to the late-action or ovarian SI systems.

Materials and Methods

Study species: Linum sulcatum Riddell (Linaceae) is an herbaceous annual angiosperm native to eastern North America in the United States and Canada (Zaremba 2003). Previous researchers hypothesized this species may be self-compatible and further support for this was found in recent experimental evidence suggesting it has an autonomous self-fertilizing breeding

system and that it may be limited in its outcrossing ability (Jahnke 2014, Chapter 1). In that experiment, plants reared from wild-collected seeds were grown in both growth chamber and greenhouse settings and seed set was assessed following various floral treatments and controlled self- and cross-pollinations. The treatments with the potential for self-fertilization resulted in significantly greater seed set than cross-pollination treatments, although some cross-pollinations were successful. Seed set was not significantly different between manually self-pollinated and unmanipulated flowers, suggesting autonomous self-fertilization. Furthermore, within- and between-population cross pollination rarely resulted in seed set, suggesting a limited ability to outcross.

Seed collection and cultivation: In summer of 2012, *Linum sulcatum* seeds were collected from 50 maternal plants spaced at least 2 m apart within each of three wild populations (Figure 5, Table A7). Seeds were stored by maternal line for three months at 4 ˚C, after which approximately 900 seeds between the three populations were cool-moist stratified at 3.6 ˚C for 30 d in coarse sand and germinated (7% success rate) in a growth chamber (Percival Intellus E36LX4) at 21.1 ˚C (light/dark) with an 11-hour photoperiod. Upon emergence of the first true leaves, seedlings were transplanted into 4 x 14.5 cm cone-tainers (60% sand, 40% Pro-Mix, Premier Tech Horticulture). To mimic natural conditions, photoperiod and temperature were incrementally increased for six months, reaching a maximum day length of 16 h and maximum temperatures of 25.5/23.5 ˚C (light/dark) after which the photoperiod was reduced by 1.75 h but the temperature remained constant.   Plants were watered bimonthly and fertilized at six week intervals (20:20:20 NPK, 0.5 ml/L).

Pollination treatments: To determine whether *Linum sulcatum* exhibits delayed self-fertilization, styles were manually self-pollinated and removed at increments of 2, 5, and 8 hours after pollination. These time increments were chosen arbitrarily as pollen tube growth rates were unknown for this species. Self-pollination was conducted at anthesis, which coincided with the

initiation of the daily photoperiod. Candidate flowers on 11 maternal lines between the three populations were observed for the presence of dehisced anthers and pollen-free stigmas. Fine-tipped forceps were used to first emasculate and then manually self-pollinate each of the five stigmas within a flower. It was not possible to deliver a consistent number of pollen grains per flower but each stigma received at least two pollen grains. The flowers were marked with colored thread and revisited 2, 5, and 8 hours after self-pollination, during which one or more styles were carefully removed at the base (Figure 6) and fixed within individually labeled microcentrifuge tubes containing 0.5 ml of 3:1 95% EtOH:glacial acetic acid.

To determine if self-pollination is more likely to result in pollen tube formation than cross-pollination, a number of within- and between-population cross-pollination events were conducted in addition to the aforementioned self-pollination events. A total of 15 maternal lines were considered. Plants were observed at the onset of anthesis for flowers with stigmas void of self-pollen contamination. Sires for these flowers were chosen opportunistically depending on the available plants with dehisced anthers to serve as pollen donors. Sire pollen was added to the recipient stigmas manually using a fine-tipped forceps to place the sire anthers in contact with the stigmatic surface of the dam. At least two pollen grains were applied per stigma. Cross-pollinated flowers were labeled and their respective styles were removed 8 hours after pollination (in a similar manner as the self-pollination styles) and fixed in 0.5 ml of 3:1 95% EtOH:glacial acetic acid for at least 24 hrs.

Pollen tube growth: To visualize pollen tube growth, styles were stained using aniline blue, which takes advantage of the fluorescent properties of the callose plug deposition associated with pollen tube formation (Martin 1959). Following fixation, the styles were softened by adding 0.5 ml of 2.5 M NaOH for 24 hours, washed 3-5 times with deionized water, and stained with 0.2 ml of decolorized aniline blue (0.1 mg aniline blue/ml DI water diluted 1:20 in a 0.1 M K_2HPO_4 buffer) for 24 hours. Styles were mounted with one drop of 50% glycerin, sealed under a

coverslip with clear nail polish, and the stored at room temperature in dark conditions until imaging with a confocal microscope. Images were captured using a Zeiss LSM 710 microscope with a 405 nm diode laser and an excitation wavelength range of 410-585 nm with digital gain adjusted to acquire maximum contrast between pollen tube and style tissue. For each style, three images were captured using ZEN software (Carl Zeiss AG) including images of the stigma, the bottom of the style, and a composite image ("tile scan") that included the stigma and the entire style (Figure 7). Pollen tubes were considered to be successfully formed if the tube descended the entire length of the style.

*Data analysis***:** Because 98% of the visualized styles contained either complete (descending the entire length of the style) or no pollen tube formation at the time of fixation, data were analyzed as a categorical factor with two levels (pollen tube formation/no pollen tube formation). The effect of style removal time on pollen tube formation was analyzed using a logistic regression model with removal time and population of origin as factors (JMP Pro 10, SAS Institute 2012). It was not possible to include maternal line nested within population in the model because of insufficient balance of the treatments. Odds ratios were calculated to determine any significant differences in the likelihood of pollen tube formation between populations and between style removal intervals. Their corresponding p values were Bonferroni-corrected to account for the problems associated with multiple comparisons (α/n, where n equals the number of comparisons made).

A separate logistic regression model was used to determine whether self-pollinated stigmas were more likely to result in pollen tube formation than cross-pollinated stigmas. Treatment (self-pollination, within-population cross-pollination, and between-population cross-pollination) and population of origin were included as effects in the model. Maternal line nested with population was again excluded as an effect as it yielded unstable parameter estimates. Odds ratios were calculated to determine any significant differences in the likelihood of pollen tube

formation between populations and between treatments. Their corresponding p values were Bonferroni-corrected.

Results

   ***Delayed self-fertilization*:** Of the 131 styles successfully imaged, 91 showed complete pollen tube formation (a pollen tube extending through the stigma and the entire length of the style). Two styles showed evidence of partial pollen tube formation; however, neither of these pollen tubes descended beyond the stigma (into the style tissue) and were therefore pooled with the remaining 40 styles which contained no pollen tubes. The population from which the seeds were collected had a significant effect on the likelihood of pollen tube formation, while style removal time (hours after pollination) was insignificant (Table 7). Plants originating from Tallgrass Prairie Preserve (TGP) were significantly more likely to form pollen tubes following a self-pollination event than those from both Anderson County Prairie (ACP) and Frenchman's Bluff (FMB) (Table 8). Styles removed 8 hours after pollination contained the highest proportion of pollen tubes, followed by 2 and 5 hours, respectively (Figure 8). However, pairwise comparisons between the different style-removal time intervals yielded insignificant odds-ratios (Table 9).

   ***Self- vs. cross-pollination*:** Between the three treatments (including all of the successfully-imaged self-pollinated styles), 121 of the 209 imaged styles exhibited complete pollen tube formation while four styles exhibited partial pollen tube formation (none of which descended beyond the stigma, again pooled with styles without pollen tube formation). Both the population from which the seeds originated and the pollination treatment had a significant effect on pollen tube formation (Table 10). Plants originating from TGP were significantly more likely to result in pollen tube formation than those from both ACP and FMB (Table 11). Self-pollination resulted in the highest proportion of pollen tube formation, followed by within-

population and between-population cross-pollination, respectively (Figure 9). Self-pollinated styles were significantly more likely to result in pollen tube formation than between-population cross-pollinated styles but not within-population cross-pollinated styles (Table 12).

Discussion

The frequency of pollen tube (PT) formation following the removal of self-pollinated styles at different time intervals suggests self-fertilization in *Linum sulcatum* can occur just after or perhaps even prior to anthesis. Self-pollinated styles removed 2 hours after pollination were equally as likely to contain PTs as those removed 5 and 8 hours after pollination. If delayed autonomous self-fertilization was occurring as a result of a temporal breakdown in self-incompatibility, one would expect that the styles removed later would contain more PTs than those removed earlier (Vogler et al. 1998). No evidence to support this pattern was found here. Therefore, either prior or competing autonomous self-fertilization (Lloyd and Schoen 1992) appear to be more accurate descriptions of the self-fertilization observed in *L. sulcatum*. Prior autonomous self-fertilization occurs before anthesis and, while *L. sulcatum* flowers have been observed to contain self pollen on their stigmas prior to anthesis (pers. obs.), the degree to which pollen tubes can reach the ovule prior to anthesis remains unknown. Removing styles prior to anthesis and assessing pollen tube growth could determine if prior autonomous self-fertilization is possible.

The results of this experiment suggest PT formation can occur in less than two hours post self-pollination. Considering the average style length is approximately 4 mm, *L. sulcatum* pollen tubes can grow at least 2000 μm/h. A growth rate that exceeds 2000 μm/h would not be unusual for a derived angiosperm lineage, as rates greater than 10,000 μm/h have been reported (Williams 2008). Removing styles at time intervals less than 2 hours following self-pollination could result in a much more precise estimate of pollen tube growth rate for this species.

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The likelihood of PT formation differed between the pollination treatments, but not as strongly as seed set differed between similar treatments (Jahnke 2014, Chapter 1). Relative to self-pollinated styles, only the between-population cross-pollinated styles were significantly limited in their ability to form PTs. Thus, the previously observed limited seed set following between-population crosses may be influenced by the inability of an outcrossed pollen grain to form a pollen tube. This type of incompatibility may be analogous to sporophytic SI systems (Nasrallah and Nasrallah 1993) in which the diploid genotype of the pollen donor may render a pollen grain unable to successfully germinate. In contrast, within-population cross-pollination was equally as likely to result in PT formation relative to self-pollination. This suggests the previously observed limited seed set following within-population crosses may not be limited by pollen tube growth alone, and rather may be due to incompatibility within the ovarian tissue. The mechanisms underlying this type of incompatibility may be analogous to the late-acting or ovarian SI systems that have been described in other species (Seavey and Bawa 1986).

Differences in PT formation were apparent between populations for all pollination treatments. Plants originating from Tallgrass Prairie Preserve (OK) were significantly more proficient at PT formation than those from Anderson County Prairie (KS) or Frenchman's Bluff (MN). It is possible that this difference could be attributed to fixed genetic differences between populations that underlie pollen tube formation. Alternatively, maternally-derived carryover effects could have affected pollen tube formation and contributed to the differences observed (Roach and Wulff 1987). However, the populations were represented by relatively few numbers of maternal lines and were unbalanced in this respect. Thus, between-population differences in PT formation may instead be an artifact of the small and unbalanced sample size.

Interestingly, regardless of pollination treatment and population of origin, the styles without completely formed PTs never contained a partially formed tube with arrested growth. In contrast, styles with completely formed PTs occasionally contained other, partially formed tubes

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within the same style. Two conclusions can be drawn from this. First, it appears that the inability of a pollen grain to form a PT may be determined by the interaction of the pollen grain and stigma, as opposed to interactions between the developing pollen tube and the style tissue. If the latter were true, one would expect to see partially formed tubes within styles without completely formed PTs. Also, competition may occur between pollen tubes in styles that contained at least one completely formed PT, as some tubes were able to descend the full length of the style while the growth in others was arrested. Pollen tube competition between self and cross pollen within individual flowers has identified in several species, and in general pollen tubes that grow faster are more likely to fertilize ovules (Spira et al. 1992, Williams 2012). However, any pollen tube competition that occurred in this experiment could not have been genetically based, as only one pollen genotype was added to any given flower.

In conclusion, the study suggests that self-fertilization in *L. sulcatum* occurs shortly after anthesis, which is uncharacteristic of the type of delayed autonomous self-fertilization that results from a temporal breakdown of SI. This experiment, in conjunction with that which assessed seed set following opportunities for self-pollination, suggests either prior or competing autonomous self-fertilization as appropriate breeding system designations. The limited cross-fertilization success that has been observed in this species may be due in part to limits in pollen tube formation once cross pollen is placed on a stigma.   Nonetheless, both cross-pollination treatments resulted in styles that contained PTs. Therefore, interactions within the ovary, including postzygotic failure following gametic fusion, may also account for limits in cross-fertilization. Future studies investigating activities within the ovary may further explain the mechanisms associated with self- and cross-fertilization in *L. sulcatum*.

Table 7: Likelihood ratio test statistics for logistic regression of pollen tube formation (yes/no) for three populations of *Linum sulcatum* with styles removed at intervals of 2, 5, and 8 hours after self-pollination.

Source	DF	X^2	p
Population	2	23.24	**<0.0001**
Removal Time	2	1.60	0.45

Table 8: Odds ratios (95% confidence intervals) of pollen tube formation (yes/no, columns (numerator) / rows (denominator)) between three populations of self-pollinated *Linum sulcatum* styles including: Anderson Country Prairie, KS (ACP), Frenchman's Bluff, MN (FMB), and Tallgrass Prairie Preserve, OK (TGP). Tests and confidence intervals on odds ratios are likelihood ratio based. Significant odds ratios following Bonferroni correction are in bold.

	ACP	FMB	TGP
ACP	-	-	-
FMB	0.81 (0.31-2.16)	-	-
TGP	**0.11 (0.03-0.33)***	**0.13 (0.04-0.35)***	-

*p<0.001

Table 9: Odds ratios (95% confidence intervals) of pollen tube formation (yes/no, columns (numerator) / rows (denominator)) between three populations of *Linum sulcatum* styles removed 2, 5, and 8 hours after self-pollination (HAP). Confidence intervals on odds ratios are likelihood ratio based.

	2 HAP	5 HAP	8 HAP
2 HAP	-	-	-
5 HAP	1.56 (0.56-4.44)	-	-
8 HAP	0.83 (0.30-2.30)	0.54 (0.20-1.43)	-

Table 10: Test statistics for nominal logistic regression of pollen tube formation (yes/no) for three populations of *Linum sulcatum* subjected to three pollination treatments (self-pollination, within-population cross-pollination, and between-population cross-pollination).

Source	DF	X^2	p
Population	2	20.25	**<0.0001**
Treatment	2	9.79	**0.008**

Table 11: Odds ratios (95% confidence intervals) of pollen tube formation (yes/no, columns (numerator) / rows (denominator)) between three populations of *Linum sulcatum* styles subjected to self- and cross-pollination treatments including: Anderson Country Prairie, KS (ACP), Frenchman's Bluff, MN (FMB), and Tallgrass Prairie Preserve, OK (TGP). Confidence intervals on odds ratios are likelihood ratio based. Significant odds ratios following Bonferroni correction are in bold.

	ACP	FMB	TGP
ACP	-	-	-
FMB	1.33 (0.60-3.00)	-	-
TGP	**0.26 (0.10-0.64)***	**0.19 (0.09-0.41)****	-

*p<0.05, **p<0.0001

Table 12: Odds ratios (95% confidence intervals) of pollen tube formation (yes/no, columns (numerator) / rows (denominator)) between three populations of *Linum sulcatum* styles subjected to the following pollination treatments: between-population cross-pollination (Between), within-population cross-pollination (Within), and self-pollination (Self). Confidence intervals on odds ratios are likelihood ratio based. Significant odds ratios following Bonferroni correction are in bold.

	Between	Within	Self
Between	-	-	-
Within	0.57 (0.21-1.55)	-	-
Self	**0.31 (0.14-0.66)***	0.54 (0.23-1.28)	-

 *p<0.01
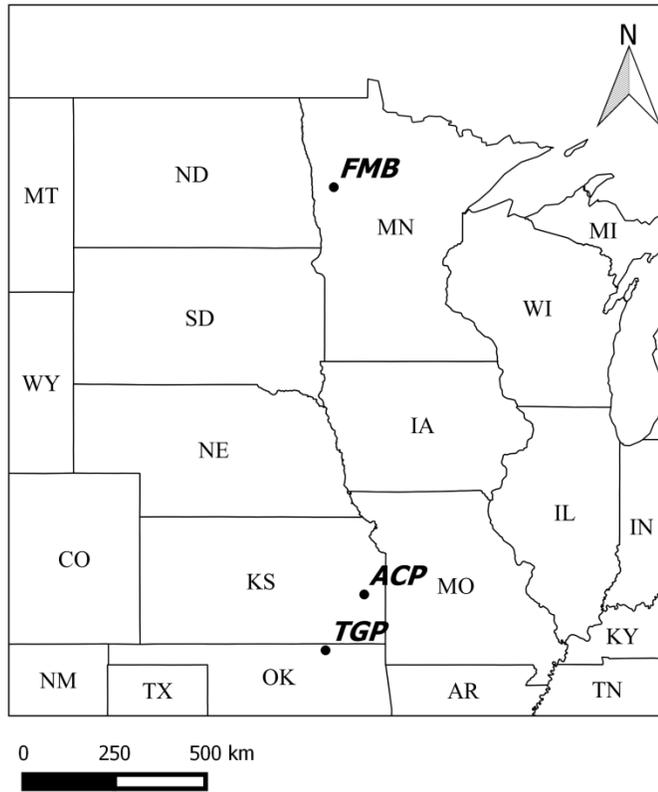
Figure 5: *Linum sulcatum* seed collection locations in the Midwestern United States including: Frenchman's Bluff (FMB), Anderson County Prairie (ACP), and Tallgrass Prairie Preserve (TGP).

Figure 6: An excised *Linum sulcatum* gynoecium with an arrow showing the approximate location where styles were cut to examine pollen tube growth.
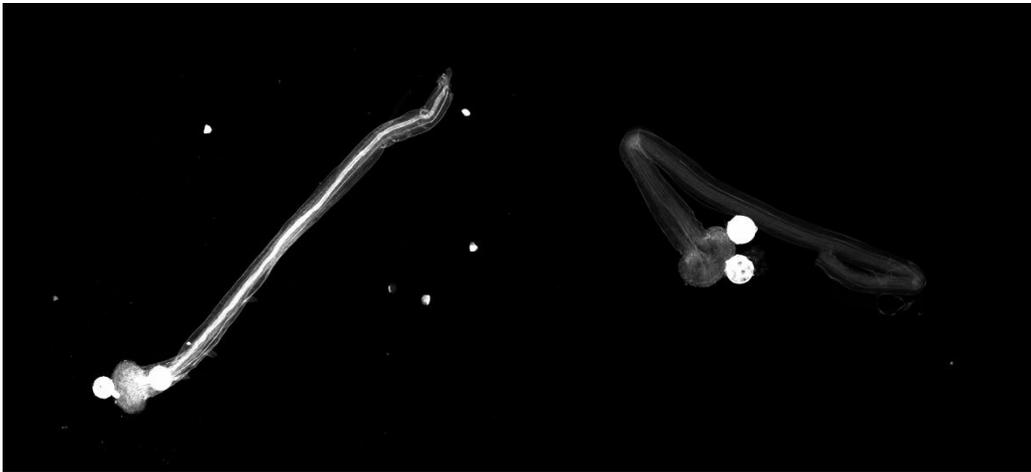
Figure 7: Confocal micrographs of two pollinated and stained *Linum sulcatum* styles showing successful (left) and unsuccessful (right) pollen tube formation.
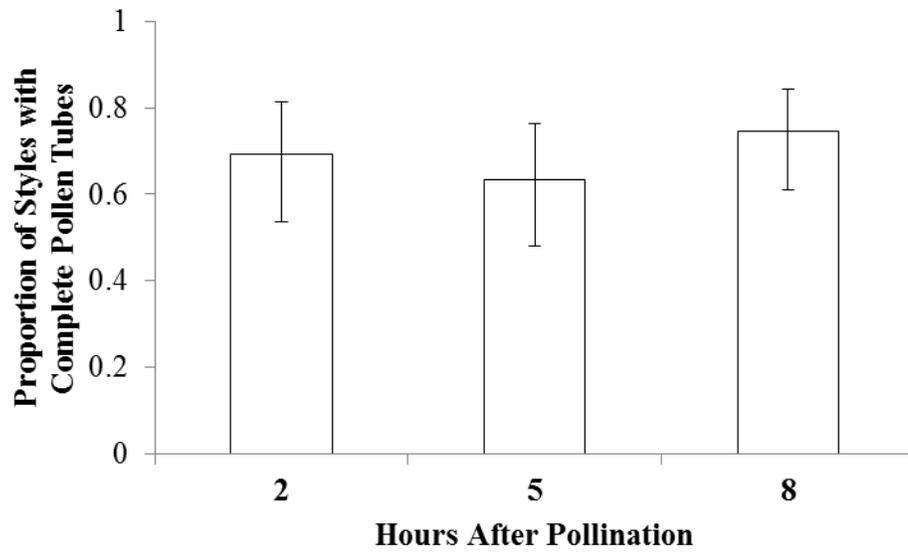
Figure 8: Proportion of *Linum sulcatum* styles with pollen tube formation removed 2 ($n = 39$), 5 ($n = 41$), and 8 ($n = 51$) hours after self-pollination. Error bars represent 95% Wilson score intervals.
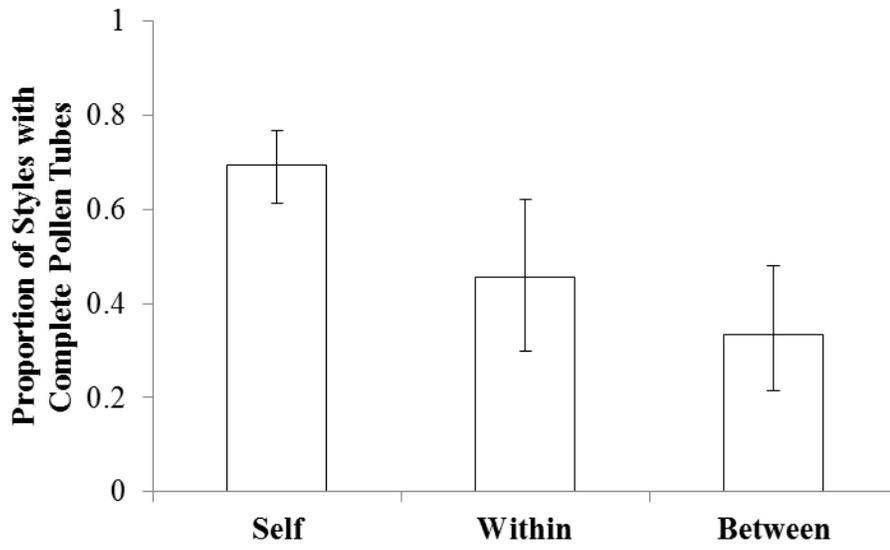
Figure 9: Proportion of *Linum sulcatum* styles with pollen tube formation following three pollination treatments (self-pollination (Self, *n* = 131), within-population cross-pollination (Within, *n* = 33), and between-population cross-pollination (Between, *n* = 45). Error bars represent 95% Wilson score intervals.

Bibliography

Armbruster, W. S., Pérez-Barrales, R., Arroyo, J., Edwards, M. E., and Vargas, P. (2006).
Three-dimensional reciprocity of floral morphs in wild flax (*Linum suffruticosum*): a new
twist on heterostyly. *New Phytologist* 171(3): 581-590.

Baker, H. G. (1955). Self-compatibility and establishment after 'long-distance'
dispersal. *Evolution* 9(3): 347-349.

Barrett, S. C. H., and Eckert, C. G. (1990). Variation and evolution of mating systems in seed
plants. Pp. 229-254 in S. Kawano ed. Biological approaches and evolutionary trends in
plants. Academic Press Limited.

Barrett, S. C., Harder, L. D., and Worley, A. C. (1996). The comparative biology of pollination
and mating in flowering plants. *Philosophical Transactions: Biological Sciences*
351(1345): 1271-1280.

Biale, J. B. (1964). Growth, maturation, and senescence in fruits: recent knowledge on growth
regulation and on biological oxidations has been applied to studies with
fruits. *Science* 146(3646): 880-888.

Camadro, E. L., and Peloquin, S. J. (1981). Cross-incompatibility between two sympatric
polyploid *Solanum* species. *Theoretical and Applied Genetics* 60(2):65-70.

Chang, S. M., and Rausher, M. D. (1999). The role of inbreeding depression in maintaining the
mixed mating system of the common morning glory, *Ipomoea purpurea*. *Evolution*
53(5):1366-1376.

Charlesworth, D. (2006). Evolution of plant breeding systems. *Current Biology* 16(17): R726-
R735.

Charlesworth, D., and Charlesworth, B. (1987). Inbreeding depression and its evolutionary
consequences. *Annual Review of Ecology and Systematics* 18:237-268.

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Cheptou, P. O., and Schoen, D. J. (2002). The cost of fluctuating inbreeding depression. *Evolution* 56(5):1059-1062.

Dart, S. R., Samis, K. E., Austen, E., and Eckert, C. G. (2012). Broad geographic covariation between floral traits and the mating system in *Camissoniopsis cheiranthifolia* (Onagraceae): multiple stable mixed mating systems across the species' range? *Annals of Botany* 109(3):599-611.

Darwin, C. (1876). *The effects of cross and self fertilisation in the vegetable kingdom*. John Murray.

Darwin, C. (1877). *The different forms of flowers on plants of the same species*. John Murray.

Davis, M. B., and Shaw, R. G. (2001). Range shifts and adaptive responses to Quaternary climate change. *Science* 292(5517): 673-679.

Dole, J. A. (1990). Role of corolla abscission in delayed self-pollination of *Mimulus guttatus* (Scrophulariaceae). *American Journal of Botany* 77(11):1505-1507.

Dresselhaus, T., and Franklin-Tong, N. (2013). Male–female crosstalk during pollen germination, tube growth and guidance, and double fertilization. *Molecular Plant* 6(4):1018-1036.

Dresselhaus, T., Lausser, A., and Márton, M. L. (2011). Using maize as a model to study pollen tube growth and guidance, cross-incompatibility and sperm delivery in grasses. *Annals of Botany* 108(4):727-737.

Edlund, A. F., Swanson, R., and Preuss, D. (2004). Pollen and stigma structure and function: the role of diversity in pollination. *Plant Cell Online* 16:S84-S97.

Egea, J., and Burgos, L. (1996). Detecting cross-incompatibility of three North American apricot cultivars and establishing the first incompatibility group in apricot. *Journal of the American Society for Horticultural Science* 121(6):1002-1005.

Fisher, R. A. (1941). Average excess and average effect of a gene substitution. *Annals of Eugenics* 11(1): 53-63.

Franklin-Tong, V. E. (1999). Signaling and the modulation of pollen tube growth. *Plant Cell Online* 11(4):727-738.

Fryxell, P. A. (1957). Mode of reproduction of higher plants. *Botanical Review* 23(3):135-233.

Fuentes, M., and Schupp, E. W. (1998). Empty seeds reduce seed predation by birds in *Juniperus osteosperma*. *Evolutionary Ecology* 12(7):823-827.

Gibbs, P. E. (2014). Late-acting self-incompatibility–the pariah breeding system in flowering plants. *New Phytologist* 203:717-734.

Goodwillie, C., Kalisz, S., and Eckert, C. G. (2005). The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology, Evolution, and Systematics* 36:47-79.

Harder, L. D., and Wilson, W. G. (1998). A clarification of pollen discounting and its joint effects with inbreeding depression on mating system evolution. *American Naturalist* 152(5):684-695.

Harris, B. D. (1968). Chromosome numbers and evolution in North American species of Linum. *American Journal of Botany* 55(10):1197-1204.

Herlihy, C. R., and Eckert, C. G. (2002). Genetic cost of reproductive assurance in a self-fertilizing plant. *Nature* 416(6878):320-323.

Herlihy, C. R., and Eckert, C. G. (2004). Experimental dissection of inbreeding and its adaptive significance in a flowering plant, *Aquilegia canadensis* (Ranunculaceae). *Evolution* 58(12): 2693-2703.

Heslop-Harrison, J. (1982). Pollen-stigma interaction and cross-incompatibility in the grasses. *Science* 215(4538):1358-1364.

Holsinger, K. E. (2000). Reproductive systems and evolution in vascular plants. *Proceedings of the National Academy of Sciences, U.S.A.* 97(13):7037-7042.

Holsinger, K. E., Feldman, M. W., and Christiansen, F. B. (1984). The evolution of self-fertilization in plants: a population genetic model. *American Naturalist* 124(3):446-453.

Igic, B., and Kohn, J. R. (2006). The distribution of plant mating systems: study bias against obligately outcrossing species. *Evolution* 60(5):1098-1103.

Iwasa, Y. (1990). Evolution of the selfing rate and resource allocation models. *Plant Species Biology* 5(1):19-30.

Jain, S. K. (1976). The evolution of inbreeding in plants. *Annual Review of Ecology and Systematics* 7:469-495.

JMP® Pro, Version *10*, SAS Institute Inc., Cary, NC, 1989-2012

Johnson, M. A., and Preuss, D. (2002). Plotting a course: multiple signals guide pollen tubes to their targets. *Developmental cell* 2(3):273-281.

Kalisz, S., and Vogler, D. W. (2003). Benefits of autonomous selfing under unpredictable pollinator environments. *Ecology* 84(11):2928-2942.

Kalisz, S., Vogler, D. W., and Hanley, K. M. (2004). Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature* 430(7002):884-887.

Kalisz, S., Randle, A., Chaiffetz, D., Faigeles, M., Butera, A., and Beight, C. (2012). Dichogamy correlates with outcrossing rate and defines the selfing syndrome in the mixed-mating genus *Collinsia*. *Annals of Botany* 109(3):571-582.

Kalisz, S., Vogler, D., Fails, B., Finer, M., Shepard, E., Herman, T., and Gonzales, R. (1999). The mechanism of delayed selfing in *Collinsia verna* (Scrophulariaceae). *American Journal of Botany* 86(9):1239-1247.

Kearns, C. A., and Inouye, D. W. (1994). Fly pollination of *Linum lewisii* (Linaceae). *American Journal of Botany* 81(9):1091-1095.

Kermicle, J. L., and Allen, J. P. (1990). Cross-incompatibility between maize and teosinte. *Maydica* 35(4):399-408.

Kessler, S. A., and Grossniklaus, U. (2011). She's the boss: signaling in pollen tube reception. *Current Opinion in Plant Biology* 14(5):622-627.

Klips, R. A., and Snow, A. A. (1997). Delayed autonomous self-pollination in *Hibiscus laevis* (Malvaceae). *American Journal of Botany* 84(1):48-53.

Kramer, A. T., and Havens, K. (2009). Plant conservation genetics in a changing world. *Trends in Plant Science* 14(11):599-607.

Kuhn, M. (1867). Einige Bemerkungen über *Vandellia* und den Blüten dimorphismus. *Botanische Zeitung* 25: 65-67.

Lande, R., and Schemske, D. W. (1985). The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* 39(1):24-40.

Leimu, R., Vergeer, P., Angeloni, F., and Ouborg, N. (2010). Habitat fragmentation, climate change, and inbreeding in plants. *Annals of the New York Academy of Sciences* 1195(1):84-98.

Levin, D. A. (1978). *The origin of isolating mechanisms in flowering plants* Springer US.

Lipow, S. R., and Wyatt, R. (2000). Single gene control of postzygotic self-incompatibility in poke milkweed, *Asclepias exaltata* L. *Genetics* 154(2):893-907.

Lloyd, D. G. (1979). Some reproductive factors affecting the selection of self-fertilization in plants. *American Naturalist* 113(1):67-79.

Lloyd, D. G. (1992). Self- and cross-fertilization in plants. II. The selection of self-fertilization. *International Journal of Plant Sciences* 153(3):370-380.

Lloyd, D. G., and Schoen, D. J. (1992). Self-and cross-fertilization in plants. I. Functional dimensions. *International Journal of Plant Sciences* 153(3):358-369.

Lord, E. M. (1981). Cleistogamy: a tool for the study of floral morphogenesis, function and evolution. *Botanical Review* 47(4): 421-449.

Martin, F. W. 1959. Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technology* 34:125-128.

Mascarenhas, J. P. (1993). Molecular mechanisms of pollen tube growth and differentiation. *Plant Cell* 5(10):1303-1314.

McClure, B. A., Haring, V., Ebert, P. R., Anderson, M. A., Simpson, R. J., Sakiyama, F., and Clarke, A. E. (1989). Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. *Nature* 342(6252):955-957.

McDill, J., Repplinger, M., Simpson, B. B., and Kadereit, J. W. (2009). The phylogeny of *Linum* and Linaceae subfamily Linoideae, with implications for their systematics, biogeography, and evolution of heterostyly. *Systematic Botany* 34(2):386-405.

Müller, H. (1883). *The fertilisation of flowers*. Macmillan.

Nasrallah, J. B., and Nasrallah, M. E. (1993). Pollen-stigma signaling in the sporophytic self-incompatibility response. *Plant Cell* 5(10):1325-1335.

Newbigin, E., Anderson, M. A., and Clarke, A. E. (1993). Gametophytic self-incompatibility systems. *Plant Cell* 5(10): 1315-1324.

NOAA National Climatic Data Center, State of the Climate: National Overview for Annual 2012, published online December 2012, retrieved on November 18, 2014 from http://www.ncdc.noaa.gov/sotc/national/2012/13.

Pandey, K. K. (1960). Evolution of gametophytic and sporophytic systems of self-incompatibility in angiosperms. *Evolution* 14(1):98-115.

Porcher, E., and Lande, R. (2005). The evolution of self-fertilization and inbreeding depression under pollen discounting and pollen limitation. *Journal of Evolutionary Biology* 18(3):497-508.

Qu, R., Li, X., Luo, Y., Dong, M., Xu, H., Chen, X., and Dafni, A. (2007). Wind-dragged corolla enhances self-pollination: a new mechanism of delayed self-pollination. *Annals of Botany* 100(6):1155-1164.

Rathcke, B., and Jules, E. (1993). Habitat fragmentation and plant-pollinator interactions. *Current Science* 65(3):273-277.

Roach, D. A., and Wulff, R. D. (1987). Maternal effects in plants. *Annual Review of Ecology and Systematics* 18:209-235.

Rogers, C. M. (1963). Yellow flowered species of *Linum* in eastern North America. *Brittonia* 15(2):97-122.

Rogers, C. M. (1969). Relationships of the North American species of *Linum* (flax). *Bulletin of the Torrey Botanical Club* 96(2):176-190.

Ronfort, J., and Couvet, D. (1995). A stochastic model of selection on selfing rates in structured populations. *Genetical research* 65(03):209-222.

Sage, T. L., and Sampson, F. B. (2003). Evidence for ovarian self-incompatibility as a cause of self-sterility in the relictual woody angiosperm, *Pseudowintera axillaris* (Winteraceae). *Annals of Botany* 91(7):807-816.

Samson, F., and Knopf, F. (1994). Prairie conservation in North America. *BioScience* 44(6):418-421.

Schoen, D. J., and Lloyd, D. G. (1992). Self-and cross-fertilization in plants. III. Methods for studying modes and functional aspects of self-fertilization.*International Journal of Plant Sciences* 153(3):381-393.

Seavey, S. R., and Bawa, K. S. (1986). Late-acting self-incompatibility in angiosperms. *Botanical Review* 52(2):195-219.

Spira, T. P., Snow, A. A., Whigham, D. F., and Leak, J. (1992). Flower visitation, pollen

> deposition, and pollen-tube competition in *Hibiscus moscheutos* (Malvaceae). *American*
>
> *Journal of Botany* 79(4):428-433.

Stebbins, G. L. (1957). Self fertilization and population variability in the higher plants. *American*

> *Naturalist* 91(861):337-354.

Stebbins, G. L. (1970). Adaptive radiation of reproductive characteristics in angiosperms, I:

> pollination mechanisms. *Annual Review of Ecology and Systematics* 1:307-326.

Stein, J. C., Howlett, B., Boyes, D. C., Nasrallah, M. E., and Nasrallah, J. B. (1991). Molecular

> cloning of a putative receptor protein kinase gene encoded at the self-incompatibility
>
> locus of *Brassica oleracea*. *Proceedings of the National Academy of*
>
> *Sciences* 88(19):8816-8820.

Steinhorst, L., and Kudla, J. (2013). Calcium-a central regulator of pollen germination and tube

> growth. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1833(7):1573-
>
> 1581.

Sun, S. G., Guo, Y. H., Gituru, R. W., and Huang, S. Q. (2005). Corolla wilting facilitates

> delayed autonomous self-pollination in *Pedicularis dunniana* (Orobanchaceae). *Plant*
>
> *Systematics and Evolution* 251(2-4):229-237.

Takayama, S., and Isogai, A. (2005). Self-incompatibility in plants. *Annual Review of Plant*

> *Biology* 56:467-489.

Takebayashi, N., and Morrell, P. L. (2001). Is self-fertilization an evolutionary dead end?

> Revisiting an old hypothesis with genetic theories and a macroevolutionary
>
> approach. *American Journal of Botany* 88(7):1143-1150.

Taylor, L. P., and Hepler, P. K. (1997). Pollen germination and tube growth. *Annual Review of*

> *Plant Biology* 48(1):461-491.

Traveset, A. (1993). Deceptive fruits reduce seed predation by insects in *Pistacia terebinthus* L. (Anacardiaceae). *Evolutionary Ecology* 7(4):357-361.

Vogler, D. W., and Kalisz, S. (2001). Sex among the flowers: the distribution of plant mating systems. *Evolution* 55(1):202-204.

Vogler, D. W., Das, C., and Stephenson, A. G. (1998). Phenotypic plasticity in the expression of self-incompatibility in *Campanula rapunculoides*. *Heredity* 81(5):546-555.

Williams, J. H. (2008). Novelties of the flowering plant pollen tube underlie diversification of a key life history stage. *Proceedings of the National Academy of Sciences, U.S.A.* 105(32):11259-11263.

Williams, J. H. (2012). Pollen tube growth rates and the diversification of flowering plant reproductive cycles. *International Journal of Plant Sciences* 173(6):649-661.

Winn, A. A., Elle, E., Kalisz, S., Cheptou, P. O., Eckert, C. G., Goodwillie, C., Johnston, M. O., Moeller, D. A., Ree, R. H., Sargent, R. D., and Vallejo-Marín, M. (2011). Analysis of inbreeding depression in mixed-mating plants provides evidence for selective interference and stable mixed mating. *Evolution* 65(12):3339-3359.

Young, A., Boyle, T., and Brown, T. (1996). The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology and Evolution* 11(10):413-418.

Zaremba, R. E. (2003). *Linum sulcatum* Riddell grooved flax. *New England Plant Conservation Program.*

Appendix

Table A1: Geographic coordinates and seed collection dates for *Linum sulcatum* populations sampled in 2012. 50 maternal lines (30 from FHK) were sampled per population at a distance of at least 2 m.

Population Code	Name	State	Latitude	Longitude	Collection Date
FMB	Frenchman's Bluff	MN	47.197487	-96.181439	7/21/2012
FHK	Freda Haffner Kettlehole	IA	43.346944	-95.221739	7/19/2012
KPBS	Konza Prairie Biological Station	KS	39.068194	-96.566201	7/4/2012
ACP	Anderson County Prairie	KS	38.182305	-95.261605	7/1/2012
TGP	Tallgrass Prairie Preserve	OK	36.845066	-96.431313	7/7/2012

Table A2: Test statistics for three models considering the effect *Linum sulcatum* rearing conditions (growth chamber or greenhouse) and pollination treatment had on (a.) fruit formation (logistic regression), (b.) fertilization success (logistic regression), and (c.) seed number (least-squares multiple linear regression).

a.

Source	DF	ChiSquare	p
Rearing Condition	1	1.77	0.18
Treatment	5	31.60	**<0.0001**

b.

Source	DF	ChiSquare	p
Rearing Condition	1	0.26	0.61
Treatment	5	49.75	**<0.0001**

c.

Source	DF	F	p
Rearing Condition	1	0.07	0.80
Treatment	5	16.22	**<0.0001**

Table A3: Number of experimental units (*Linum sulcatum* flowers) sampled per population (columns) in each treatment (rows). The number of maternal lines per population represented by each treatment is in parentheses. Treatments include: controlled self-pollination (Controlled Self), no manipulation (w/ Appendages), basal petal appendages removed (w/o Appendages), within-population cross-pollination (Within), between-population cross-pollination (Between), and anthers removed prior to dehiscence (No Pollen).

Treatment	Population					
	ACP	FHK	FMB	KPBS	TGP	Total
Controlled Self	14 (3)	2 (1)	24 (8)	25 (3)	37 (8)	102 (23)
w/ Appendages	6 (2)	10 (2)	15 (5)	9 (3)	18 (7)	58 (19)
w/o Appendages	4 (2)	2 (1)	25 (6)	0 (0)	16 (4)	47 (13)
Within	0 (0)	0 (0)	17 (3)	2 (2)	5 (3)	24 (8)
Between	0 (0)	4 (1)	7 (1)	10 (3)	8 (5)	29 (10)
No Pollen	1 (1)	0 (0)	15 (4)	2 (2)	4 (2)	22 (9)

Table A4: Pairwise odds ratios (95% confidence intervals) of fruit formation (yes/no, Population 1 / Population 2) for five populations of *Linum sulcatum* (Frenchman's Bluff, MN (FMB), Freda Haffner Kettlehole, IA (FHK), Konza Prairie Biological Station, KS (KPBS), Anderson County Prairie, KS (ACP), and Tallgrass Prairie Preserve, OK (TGP)) subjected to five pollination treatments. Tests and confidence intervals on odds ratios are likelihood ratio based. Significant p values following Bonferroni correction are in bold.

Population 1	/ Population 2	Odds Ratio	p
ACP	FHK	0.11 (0.02-0.49)	**0.03**
ACP	FMB	0.22 (0.08-0.60)	**0.031**
FHK	FMB	2.00 (0.53-9.92)	1.00
ACP	KPBS	0.49 (0.17-1.39)	1.00
FHK	KPBS	4.39 (1.12-22.39)	0.33
FMB	KPBS	2.20 (0.93-5.22)	0.71
ACP	TGP	0.42 (0.16-1.09)	0.75
FHK	TGP	3.80 (1.05-18.59)	0.42
FMB	TGP	1.90 (0.96-3.86)	0.66
KPBS	TGP	0.87 (0.39-1.97)	1.00
FHK	ACP	9.04 (2.05-50.86)	**0.03**
FMB	ACP	4.52 (1.67-12.47)	**0.031**
FMB	FHK	0.50 (0.10-1.87)	1.00
KPBS	ACP	2.06 (0.72-5.97)	1.00
KPBS	FHK	0.23 (0.04-0.89)	0.33
KPBS	FMB	0.46 (0.19-1.07)	0.71
TGP	ACP	2.38 (0.92-6.19)	0.75
TGP	FHK	0.26 (0.05-0.95)	0.42
TGP	FMB	0.53 (0.26-1.04)	0.66
TGP	KPBS	1.15 (0.51-2.59)	1.00

Table A5: Pairwise odds ratios (95% confidence intervals) of fruit formation (yes/no, Treatment 1 / Treatment 2) for *Linum sulcatum* from five populations subjected to the following pollination treatments: controlled self-pollination (Controlled Self), no manipulation (w/ Appendages), basal petal appendages removed (w/o Appendages), within-population cross-pollination (Within), between-population cross-pollination (Between), and anthers removed prior to dehiscence (No Pollen). Tests and confidence intervals on odds ratios are likelihood ratio based. Significant p values following Bonferroni correction are in bold.

Treatment 1	/ Treatment 2	Odds Ratio	p
Between	w/ Appendages	0.29 (0.11-0.75)	0.16
Between	No Pollen	2.13 (0.61-7.98)	1.00
w/ Appendages	No Pollen	7.36 (2.40-25.27)	**0.005**
Between	w/o Appendages	0.29 (0.10-0.82)	0.29
w/ Appendages	w/o Appendages	1.01 (0.41-2.47)	1.00
No Pollen	w/o Appendages	0.14 (0.04-0.42)	**0.006**
Between	Controlled Self	0.14 (0.05-0.34)	**<0.001**
w/ Appendages	Controlled Self	0.48 (0.22-1.03)	0.88
No Pollen	Controlled Self	0.06 (0.02-0.19)	**<0.0001**
w/o Appendages	Controlled Self	0.47 (0.20-1.10)	1.00
Between	Within	0.58 (0.18-1.85)	1.00
w/ Appendages	Within	2.00 (0.69-5.77)	1.00
No Pollen	Within	0.27 (0.07-0.92)	0.54
w/o Appendages	Within	1.97 (0.68-5.73)	1.00
Controlled Self	Within	4.20 (1.50-11.75)	0.10
w/ Appendages	Between	3.46 (1.33-9.36)	0.16
No Pollen	Between	0.47 (0.13-1.64)	1.00
No Pollen	w/ Appendages	0.14 (0.04-0.42)	**0.005**
w/o Appendages	Between	3.41 (1.22-9.96)	0.29
w/o Appendages	w/ Appendages	0.99 (0.40-2.42)	1.00
w/o Appendages	No Pollen	7.27 (2.37-24.95)	**0.006**
Controlled Self	Between	7.27 (2.90-19.13)	**<0.001**
Controlled Self	w/ Appendages	2.10 (0.97-4.55)	0.88
Controlled Self	No Pollen	15.48 (5.22-51.85)	**<0.0001**
Controlled Self	w/o Appendages	2.13 (0.91-4.98)	1.00
Within	Between	1.73 (0.54-5.71)	1.00
Within	w/ Appendages	0.50 (0.17-1.45)	1.00
Within	No Pollen	3.69 (1.09-13.74)	0.54
Within	w/o Appendages	0.51 (0.17-1.47)	1.00

| Within | Controlled Self | 0.24 (0.09-0.66) | 0.10 |

Table A6: Pairwise odds ratios (95% confidence intervals) of fertilization success (yes/no, Treatment 1 / Treatment 2) for *Linum sulcatum* from five populations subjected to the following pollination treatments: controlled self-pollination (Controlled Self), no manipulation (w/ Appendages), basal petal appendages removed (w/o Appendages), within-population cross-pollination (Within), and between-population cross-pollination (Between). Tests and confidence intervals on odds ratios are likelihood ratio based. Significant p values following Bonferroni correction are in bold.

Treatment 1	/ Treatment 2	Odds Ratio	p
Between	w/ Appendages	0.13 (0.04-0.36)	**0.001**
Between	w/o Appendages	0.16 (0.04-0.48)	**0.008**
w/ Appendages	w/o Appendages	1.24 (0.54-2.85)	1.00
Between	Controlled Self	0.07 (0.02-0.19)	**<0.0001**
w/ Appendages	Controlled Self	0.54 (0.26-1.12)	0.97
w/o Appendages	Controlled Self	0.44 (0.20-0.95)	0.37
Between	Within	1.41 (0.29-7.87)	1.00
w/ Appendages	Within	11.22 (3.23-53.11)	**0.001**
w/o Appendages	Within	9.02 (2.62-42.35)	**0.002**
Controlled Self	Within	20.63 (6.18-95.4)	**<0.0001**
w/ Appendages	Between	7.94 (2.75-27.00)	**0.001**
w/o Appendages	Between	6.38 (2.09-22.77)	**0.008**
w/o Appendages	w/ Appendages	0.8 (0.35-1.84)	1.00
Controlled Self	Between	14.59 (5.20-48.93)	**<0.0001**
Controlled Self	w/ Appendages	1.84 (0.90-3.78)	0.97
Controlled Self	w/o Appendages	2.29 (1.05-5.01)	0.37
Within	Between	0.71 (0.13-3.42)	1.00
Within	w/ Appendages	0.09 (0.02-0.31)	**0.001**
Within	w/o Appendages	0.11 (0.02-0.38)	**0.002**
Within	Controlled Self	0.05 (0.01-0.16)	**<0.0001**

Table A7: Geographic coordinates and seed collection dates for *Linum sulcatum* populations sampled in 2012. Fifty maternal lines were sampled per population at a distance of at least 2 m.

Population Code	Name	Collection Date	State	Latitude	Longitude
FMB	Frenchman's Bluff	7/21/2012	MN	47.197487	-96.181439
ACP	Anderson County Prairie	7/1/2012	KS	38.182305	-95.261605
TGP	Tallgrass Prairie Preserve	7/7/2012	OK	36.845066	-96.431313

Table A8: Pairwise odds ratios (95% confidence intervals) of complete pollen tube formation (yes/no, Population 1 / Population 2) for three populations of self-pollinated *Linum sulcatum* styles: Anderson County Prairie (ACP), Frenchman's Bluff (FMB), and Tallgrass Prairie Preserve (TGP). Tests and confidence intervals on odds ratios are likelihood ratio based. Significant p values following Bonferroni correction are in bold.

Population 1	/ Population 2	Odds Ratio	p
ACP	FMB	0.81 (0.31-2.16)	1.00
ACP	TGP	0.11 (0.03-0.33)	**0.0002**
FMB	TGP	0.13 (0.04-0.35)	**0.0001**
FMB	ACP	1.23 (0.46-3.28)	1.00
TGP	ACP	9.28 (3.04-31.57)	**0.0002**
TGP	FMB	7.56 (2.83-22.96)	**0.0001**

Table A9: Pairwise odds ratios (95% confidence intervals) of complete pollen tube formation (yes/no, HAP 1 / HAP 2) for self-pollinated *Linum sulcatum* styles from three populations removed 2, 5, and 8 hours after pollination (HAP). Tests and confidence intervals on odds ratios are likelihood ratio based. Significant p values following Bonferroni correction are in bold.

HAP 1	/ HAP 2	Odds Ratio	p
2	5	1.56 (0.56-4.44)	1.00
2	8	0.83 (0.30-2.30)	1.00
5	8	0.54 (0.20-1.43)	0.64
5	2	0.64 (0.23-1.79)	1.00
8	2	1.20 (0.44-3.30)	1.00
8	5	1.87 (0.70-5.10)	0.64

Table A10: Pairwise odds ratios (95% confidence intervals) of complete pollen tube formation (yes/no, Population 1 / Population 2) for three populations of self- and cross-pollinated *Linum sulcatum* styles: Anderson County Prairie (ACP), Frenchman's Bluff (FMB), and Tallgrass Prairie Preserve (TGP). Tests and confidence intervals on odds ratios are likelihood ratio based. Significant p values following Bonferroni correction are in bold.

Population 1	/ Population 2	Odds Ratio	p
ACP	FMB	1.33 (0.60-3.00)	1.00
ACP	TGP	0.26 (0.10-0.64)	**0.01**
FMB	TGP	0.19 (0.09-0.41)	**<0.0001**
FMB	ACP	0.75 (0.33-1.68)	1.00
TGP	ACP	3.86 (1.56-9.85)	**0.01**
TGP	FMB	5.14 (2.44-11.46)	**<0.0001**

Table A11: Pairwise odds ratios (95% confidence intervals) of complete pollen tube formation (yes/no, Treatment 1 / Treatment 2) for *Linum sulcatum* styles from three populations subjected to three pollination treatments: self-pollination (Self), within-population cross-pollination (Within), and between-population cross-pollination (Between). Tests and confidence intervals on odds ratios are likelihood ratio based. Significant p values following Bonferroni correction are in bold.

Treatment 1	/ Treatment 2	Odds Ratio	p
Between	Within	0.57 (0.21-1.55)	0.82
Between	Self	0.31 (0.14-0.66)	**0.006**
Within	Self	0.54 (0.23-1.28)	0.48
Within	Between	1.74 (0.65-4.77)	0.82
Self	Between	3.22 (1.52-7.01)	**0.006**
Self	Within	1.84 (0.78-4.38)	0.48