

Floral Biology of Alabama's *Spigelia* species (Family Loganiaceae)

By

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Abstract

This thesis examines the pollination ecology of Alabama species of *Spigelia*. Alabama currently has two species that are under threat of extinction: *S. gentianoides* and *S. alabamensis*. The U.S. Fish and Wildlife Service has highlighted the need for studies of the reproductive biology of these species. I analyzed reproductive biology of the rare species *S. gentianoides* and *S. alabamensis*, as well as the common species *S. marilandica*. Determination of likely mating strategies of *Spigelia* was based on floral morphology and phenology, and evaluation of floral timing. Controlled pollination experiments were used to determine mating system (if flowers are self-compatible or self-incompatible, as well as the degree to which they can outcross). Additionally, floral visitors were observed to identify potential pollinators. Flowers were diurnal, with two-day to three-day longevity, with highly reduced herkogamy and dichogamy to the point where self-pollination is essentially assured. Controlled pollination experiments showed that *Spigelia* can set fruit via autopollination, selfing, or outcrossing. Floral visitor observations indicated low visitation rates to flowers and few potential pollinators. These results, in addition to prior genetic studies, suggest *Spigelia* species in Alabama are predominantly selfing. Survival of the remaining populations of the rare species, *S. gentianoides* and *S. alabamensis*, is dependent on effective conservation management.

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Introduction

The Earth is currently suffering a wave of anthropogenic biodiversity loss, with rates of extinction estimated to be 100–1,000 times the observed rate between previous mass extinctions in the fossil record (Barnosky et al. 2011, Pimm et al. 2014). For plants, which are the foundation of terrestrial food webs, an estimated 20% of all species are currently threatened with extinction (Brummitt et al. 2015). The International Union for the Conservation of Nature (IUCN) recently updated their Red List, concluding that there are 13,494 threatened species (47.7%) among 28,287 assessed species in major taxonomic plant groups (IUCN 2020). Havens (2014) argues that currently no country has an adequate conservation strategy for plants: they are becoming increasingly rare, their conservation is underfunded, they are not fully protected, and their value is underappreciated.

Plant conservation efforts significantly increased with the passage of the U.S. Endangered Species Act of 1973 (USFWS, 1988). The Act established a legal mandate of unprecedented proportions to promote the collection, analysis, and exchange of biological information. The successful recovery of species requires a significant amount of scientific information, yet the present level of understanding and communication among researchers and resource managers is not sufficient (Shine and Doody 2011). Bridging this gap is a necessity for plant conservation moving forward. While many studies suggest management strategies for particular rare plants, few focus on reproductive biology, and even fewer involve the importance of vectors for pollen transfer (Boyd 1994, Kremen and Ricketts 2000). There is widespread consensus that documentation of the reproductive biology of endangered species may be useful for understanding why they are endangered (Schemske et al. 1994).

Reproduction by seed is essential for plant species migration, promotes adaptation through production of genetic variation, and ultimately allows population persistence (Fenner and Thompson 2005). Two major components of plant reproductive biology are pollination and seed dispersal. These two factors play a critical role in determining genetic diversity and population size (Ellstrand and Elam 1993, Smith et al. 2015) and, despite their importance, they are often overlooked in conservation and management programs (Calviño-Cancela et al. 2012, Neuschulz et al. 2016, Silveira et al. 2016).

Moreover, the successful pollination and seed dispersal of threatened plant species is often dependent upon the abundance of pollinators and seed dispersers (Caughlin et al. 2015, Neuschulz et al. 2016). Lack of pollinators or seed dispersers of threatened species can lead to decreased fitness and eventual extinction (Biesmeijer et al. 2006). Managing these pollinators and seed dispersers is difficult in the field, so botanical gardens often maintain *ex situ* plant collections used for reintroduction and introduction programs (Huang 2011, Faraji and Karimi 2020). Some studies on pollination of threatened plant species in botanical gardens have shown those species are either self-compatible or have generalist pollinators (Chen et al. 2011, Krestova and Nesterova 2013), but this is not always the case. This means it is necessary for researchers in botanical gardens to study the pollination and seed dispersal strategies of threatened plant species.

Information obtained from studies of pollination and seed dispersal can be useful for assessing novel *in situ* and *ex situ* management strategies (Menges 1986). Small and/or isolated fragments of plant populations may be less attractive for pollinators (Delmas et al. 2014), which can lead to a decrease in both pollen quantity (i.e., decrease in pollination events) and pollen quality (i.e., less deposition of conspecific and outcrossed pollen; Eckert et al. 2010). Reintroduction is the quickest and most effective method to augment existing populations for survival and conservation over the long run (Guerrant and Kaye 2007). Management strategies should pay more attention to reproductive biology, and in particular to plant mating strategies, as species that are predominantly selfing will have different conservation needs than those that are predominantly outcrossing (Frankel and Galun 2012, Tang et al. 2020).

Plant Mating Strategies

While some plants can reproduce asexually, sexual reproduction is essential in developing genetically variable offspring that allow a population to adapt to changing environmental conditions. Flowering plants possess various mating strategies that may allow them to mate with themselves, with another individual, or with multiple individuals. For this study, mating strategy will be identified by the following terms: selfing, mixed-mating, and outcrossing. Selfing is self-fertilization and results from self-pollination of a flower (or pollen transfer between flowers on the same plant). Mixed-mating is the ability to produce a mixture of

self-fertilized (selfed) and outbred (outcrossed) seeds. Outcrossing is pollination and fertilization by pollen from a different genetic individual of the same species.

Mating strategy determines the mode of transmission of genetic information from one generation to the next (Brown 1990, Neal and Anderson 2005) and is correlated with life history traits (Brown 1990). Floral traits may either aid in promotion or avoidance of self-fertilization (Darwin 1876). Floral traits encompassing spatial (herkogamy) or temporal (dichogamy) separation of sexual functions in hermaphroditic flower are indicators of mating strategy (Cruden 1977, Barrett and Eckert 1990, Cresswell and Dafni 1995). Floral traits help determine how pollen moves and to what extent plants reproduce via selfing versus outcrossing (Barrett and Harder 1996). Herkogamy determines the distance pollen needs to travel to reach the stigma of the same flower. In angiosperms this can play a crucial role, as the farther a stigma is from an anther, the less likely a plant is to self-pollinate (Barrett et al. 2000, Barrett 2003). Dichogamy is determined by when pollen is released and when a stigma becomes receptive within a flower. Temporal separation of these events reduces the likelihood that pollen will arrive on a receptive stigma of the same flower (Lloyd 1992, Lankinen et al. 2007) and thus decreases ability to self.

Plant mating strategy evolution is a dynamic process, as evidenced by wide variation in outcrossing rates among closely related species and among populations within species (Barrett and Eckert 1990, Takebayashi and Morrell 2001). Understanding the forces governing plant mating system evolution is fundamental to understanding evolution in plant populations because mating system influences numerous evolutionary factors (Barrett and Harder 2017, Shuster and Wade 2019). Mating strategies have diverse genetic effects and play a key role in determining whether populations function as separate evolutionary groups or are part of gene-flow-connected metapopulations (Barrett 2003, Barrett 2010).

Selfing in plants has long been viewed as genetically detrimental to plant species. Selfing lineages suffer from an increased risk of extinction as they have reduced potential for long term adaptation (Wright et al. 2008, Wright et al. 2013). Thus, selfing lineages may be mostly short-lived owing to higher extinction rates (Wright et al. 2013). Selfing has been hypothesized to lead to inbreeding depression but can be useful for adapting locally and for purging genetic load (Husband and Schemske 1996, Kremer and Le Corre 2012). Self-fertile individuals may be at a selective advantage in particular habitats if outcrossing is disfavored because plant density is low

(due to recent colonization or periodic population bottlenecks) or if pollinators are scarce and cross-pollination is inadequate (Schemske and Lande 1985, Wyatt 1988). Self-pollination can ensure sexual reproduction without aid of a pollen vector, and thus can provide reproductive assurance (Takebayashi and Morrell 2001). In addition, in highly self-fertile plants, lethal genes that are expressed early in development may already have been purged (Latta and Ritland 1994, Husband and Schemske, 1996).

Conversely to selfing, plants can outcross. Outcrossing can eliminate deleterious mutations by selection of a more advantageous mutation (Husband and Schemske 1996). High rates of gene flow can increase effective population size (Slatkin 1985). Negative outcomes also can come from outcrossing. Outcrossing populations can experience population bottlenecks, reducing genetic variation, which can quickly lead to depletion of entire populations (Wright et al. 2003).

Mixed mating is a blend of selfing and outcrossing. The mixed mating strategy is a challenge for evolutionary biology. Most models of mating system evolution predict that a mixture of selfed and outcrossed progeny should not exist. Lande and Schemske (1985) and Charlesworth et al. (1990) showed in quantitative genetic models that the balance between the cost of outcrossing and inbreeding depression should result in either complete selfing or complete outcrossing. However, Cheptou and Dieckmann (2002), using an adaptive dynamic modeling approach, predicted that both deterministic and stochastic environmental effects leading to fluctuating populations can produce evolutionarily stable mixed mating systems.

Both outcrossing and mixed-mating plant species depend on pollen vectors for stable mating. Loss of an efficient pollen vector can result in reduced genetic diversity, as this directly affects pollination. Depending on reproductive biology and population history, reduced pollinator service may have several negative impacts on a plant population, including reproductive failure (Jennersten 1988) or decreased effective population size through reduced gene flow and increased selfing (Bawa 1990, Menges 1991, Aizen and Feinsinger 1994).

Determining plant mating strategy is critical for conservation management. Because mating strategy affects genetic diversity within and among populations, mating strategy could either limit or promote local adaptation. Endemic species with restricted geographic distributions have concerned biologists confronted with the problem of preserving rare species endangered by

habitat destruction and fragmentation. Efficient conservation initiatives should consider reproductive characteristics and identify mating strategies, or any problems stemming from a particular mating strategy (i.e., limitations in pollen movement or available mates), as reproductive success and gene flow can differ among populations and be influenced by degradation of habitat (Baker 1995, Kwak and Bekker 2006, Saunders and Sipes 2006). Altered mating strategy may cause loss of genetic diversity and/or reduced progeny fitness due to inbreeding depression, losing the genetic variability necessary to adapt to changing environments (Barrett and Kohn 1991, Falk et al. 1991, Menges 1991). Determining population size, structure, and mating strategy is necessary for the creation of management programs and the estimation of minimum viable population sizes (Husband and Barrett 1992, Menges 2000, Wagenius et al. 2007). For rare species, this is crucial when establishing geographic range, habitat specificity, and local population size (Saunders and Sipes 2006, Busch and Schoen 2008, Silva et al. 2015). For example, in small or fragmented populations, a system of self-incompatibility may restrict the supply of mates: this can lead to decreased sexual reproduction and result in a decline in population size (Wilcock and Neiland 2002, Silva et al. 2015).

***Spigelia* of the Southeast**

Spigelia Linnaeus (Loganiaceae) is a neotropical genus of around 60 species, with 7 native to North America, comprising herbs and small shrubs with entire, stipulate leaves (Gould 1996). *Spigelia* grows from a fleshy rhizome and often forms clumps of numerous stems bearing simple, lance-shaped leaves in opposing pairs. Inflorescences are cymose, sometimes reduced to one or three flowers, which are perfect with an entire corolla with five petals and produce fruits (capsules) upon successful fertilization (Gould 1999, Fernández-Casas and Huft 2009).

The genus is endemic to the Americas, with a wide distribution ranging from the southeastern United States to northern Argentina and Chile (Gould 1999, Fernández-Casas and Huft 2009, Popovkin et al. 2011). *Spigelia* has at least three species in Alabama. *Spigelia gentianoides* is associated with sandy longleaf pine ecosystems and areas where burning frequently occurs (Walker 1993, USFWS 2018). *Spigelia gentianoides* is restricted to a few localities, with only five populations in southeastern Alabama and the panhandle of Florida; therefore, it is potentially at risk of extinction. Threats to this species include loss or alteration of

habitat, lack of natural disturbance regimes (i.e., fire), unprotected populations on private lands, and competition from invasive species (Negron-Ortiz 2012). *Spigelia alabamensis* is endemic to 17 glades that have developed over an ancient rock formation known as Ketona Dolomite in Bibb County, Alabama where it is exposed to full sun, with attendant extreme heat and drought, as well as to partial shade in the glade-forest ecotone (Gould 1996, Negron-Ortiz 2012). *Spigelia marilandica* is endemic to bottomland hardwood forests and covers much of the Southeast and some of the Midwest, ranging from Illinois to Florida and from Texas to Virginia (Dunwell 2003).

Conservation planning and long-term survival of a species rely on maintenance of genetic variability and continued natural selection through sexual reproduction and generation turnover (Arrone 2017). Affolter (2005) used allozymes to evaluate genetic variability in both *S. gentianoides* and *S. alabamensis*, as well as in *S. marilandica*. High levels of within-population variation and extremely low levels of among-population variation were observed in *Spigelia*. This finding suggests that there are relatively high levels of gene flow among populations. Further genetic studies on *Spigelia* species by Hershberger (2012) and Hershberger et al. (2015) have revealed introgression between populations and species, although all allele clusters have not entirely introgressed into all populations. Among-species and among-population variation were low, likely the effect of common ancestry as well as relatively frequent introgression among individuals (and populations) of *Spigelia* (Hershberger 2012). Some populations have more allelic diversity between populations in *Spigelia* (Hershberger et al. 2015). With low levels of genetic diversity, gene flow both among populations and among species (introgression) is important in maintaining the heterozygosity of populations and/or species (Hershberger et al. 2015). A better understanding of the pollination ecology of *Spigelia* is needed to develop management strategies that can maintain this gene flow.

Pollination ecology includes flowering time and, often, floral visitors (Willmer 2011). *Spigelia marilandica* sometimes will re-flower after its June bloom period (Darke 2002) and is thought to be pollinated by ruby-throated hummingbirds (Cullina 2000) as well as by insects (Rogers 1988, Affolter 2005). Rogers (1988) proposed that *S. gentianoides* flowers, though remaining nearly closed, are pollinated by moths inserting their proboscises into slits between the petals to find nectar. Additionally, he observed small Halictid bees (sweat bees) entering *S.*

gentianoides flowers by peeling back petals. Affolter (2005) observed 25 visitors on *S. alabamensis*, which included a pipevine swallowtail butterfly (*Battus philenor* L.) and a large beefly (*Bombylius* spp. L.). After flowering, fruits gradually change from green to black as they ripen (Rogers 1988, Gould 1999). The seed capsule will dehisce explosively and so the seeds are locally dispersed (Rogers 1988, Gould 1999).

Spigelia gentianoides and *S. alabamensis* populations have steadily declined over recent decades--a problem compounded by their low annual seed set of 8-14% (Affolter 1995). The U.S. Fish and Wildlife Service listed *Spigelia gentianoides* as endangered under the Endangered Species Act (Act) of 1973 on November 26, 1990 (USFWS 2012). *Spigelia alabamensis* is also of high conservation priority as it is severely limited in geographic range and habitat, with the only known population occurrences in the dolomite limestone glades of Bibb County, Alabama (Gould 1996). *Spigelia gentianoides* and *S. alabamensis* need appropriate conservation management to prevent extinction, while *S. marilandica* has suffered population losses and may be a species of concern in the future (Shotts, personal observation).

This study provides information about the floral biology of *S. gentianoides*, *S. alabamensis*, and *S. marilandica* from Alabama. Specifically, the study was designed to: (1) Examine floral timing of species and any potential differences between species and populations; (2) Describe morphology and phenology of flowers; (3) Explore the relative effectiveness of outcross-, self- and no pollination in producing fruits (using controlled crosses of bagged flowers); and (4) Identify major floral visitor species by observing floral visitors in the field. Results of these experiments can suggest conservation strategies for these three *Spigelia* species.

Methods

Study Species

Spigelia (L.) is a genus of approximately 60 species ranging from the southeastern U.S., along Central America, and into temperate areas of South America (Gould 1999). The genus is endemic to the Americas, with a wide distribution from the southeastern United States to northern Argentina and Chile (Gould 1999, Fernández-Casas and Huft 2009, Popovkin et al. 2011). I observed three species in this study, *S. gentianoides*, *S. alabamensis*, and *S. marilandica*. *Spigelia gentianoides* is critically imperiled in Alabama because of extreme rarity,

with only one known occurrence in Geneva County (Gould 1999). *Spigelia alabamensis* is also rare in Alabama, occurring only in the Bibb County Glades (Gould 1996). In contrast, *S. marilandica* is a relatively common species, ranging across the entirety of the Southeast and found in 62 of 67 counties in Alabama (Fernández-Casas and Huft 2009).

Gould (1999) described *Spigelia gentianoides* as an upright, herbaceous perennial growing 25-40 cm high. Plants can be solitary or clump-forming and rarely branch near the base. *Spigelia alabamensis* differs in most phenotypic traits from *S. gentianoides* (Table 1) and corollas exhibit a color change not noticed in the other two study species. Young buds of *S. alabamensis* are heavily pink pigmented, whereas corollas in open flowers are white-pink to white with darker pink 'nectar guides' within the corolla tube (Affolter 2005).

Traits	<i>S. gentianoides</i>	<i>S. alabamensis</i>	<i>S. marilandica</i>
Height (cm)	10 to 30	10 to 30	30 to 60
Flowers per Inflorescence	3 to 10	1 to 4	3 to 17
Corolla Length (mm)	25 to 30	35 to 60	100 to 150
Flowering Time	Not Known	4 pm-10 pm	9 am-5 pm
Conservation Status	Federally Listed	Critically Endangered, no protection	Common, no protection needed

Table 1. Comparison of phenotypic traits (Hershberger 2012) and conservation status (USFWS 2012) for *Spigelia* study species.

Spigelia marilandica is an upright herbaceous perennial growing from 30 to 60 cm tall. This species has several stems that grow from a large rhizome. It is very distinct from *S. gentianoides* and *S. alabamensis* phenotypically (Table 1) and the glossy, opposite leaves contribute to the plant's ornamental appeal (Cullina 2000).

Study Populations

Five populations from the three study species were chosen within Alabama: three for *S. marilandica* and one population each for *S. gentianoides* and *S. alabamensis*.

The *S. gentianoides* population was located at N31.142, W86.185 in Geneva State Forest of Geneva County (Geneva Forest). The population was located in open longleaf-wiregrass areas within primarily pine and hickory forest. Individuals were scattered within an approximately 200-meter squared area located several hundred meters from the lake at Geneva State Forest.

The *S. alabamensis* population was located at N33.058, W87.0388 in the Bibb County Glades (Bibb County). This population was located on open areas of dolomite limestone glades embedded within a pine-dominated forest. Individuals were scattered between two glades covering in total an approximately 2500-meter squared area.

Three populations for *S. marilandica* were selected. The first population (Park Crossing) was located at N33.454, W85.841 near Park Crossing High School in Montgomery County. This population was located in a mesic hardwood forest with dense understory adjacent to a road. Individuals covered an approximately 1000-meter squared area at the edge of the forest. The second population (Talladega Forest) was located at N32.3089, W86.1712 in Talladega National Forest, Talladega County. This population was located in an open hardwood forest with sparse understory in an approximately 150-meter squared area with individuals extending alongside a creek. The third population (Alley Road) was located at N32.164, W85.531 adjacent to a road near Hurtsboro in Russell County. This population was located in a mesic hardwood forest near a stream at the bottom of a hill. Individuals sparsely populated the forest floor covering an approximate 20,000-meter squared area.

Some of the populations in this study were located in protected areas. As a federally-endangered species (USFWS 2012), *S. gentianoides* is protected by the Federal government under the Endangered Species Act and the population I studied grows on State-owned land. The population of *S. alabamensis* I studied is located at a preserve (Kathy Stiles Freeland Preserve) owned by The Nature Conservancy. One of the *S. marilandica* populations, Talladega Forest, is protected by a federal agency since it grows in Talladega National Forest. For study populations

on protected land, I worked and collaborated with the owning agencies to conduct this study. The Park Crossing and Alley Road *S. marilandica* populations were not in protected areas.

Floral Timing Study

The Floral Timing Study documented peak bloom and duration of flowering for each population to determine differences between populations/species. As *Spigelia* species are rhizomatous, stems at least 1 m apart were selected to increase the likelihood of including genetically different individuals. Plants included were large enough to be recognizable as members of each species prior to flowering. I selected a subset of relatively large plants from each study population and individually marked them before flower buds were produced. I frequently visited populations throughout the flowering season, counting flowers on those individuals that flowered. Populations were visited at 3-5 day intervals from the start to the end of flowering in both 2018 and 2019.

I visited *S. gentianoides* and *S. alabamensis* in May of 2018 and 2019 to tag study plants. In 2018, I selected 100 individuals from over 1400 available *S. gentianoides* plants, and in 2019 I selected 137 individuals from over 1600 available *S. gentianoides* plants. During the same times I tagged plants of *S. alabamensis*. In 2018 I tagged and selected 100 individuals from over 1000 available *S. alabamensis* plants. In 2019, I tagged *S. alabamensis* plants but a drought killed above-ground parts of the plants prior to flowering and as a result this study could not be completed in that year for this species.

Spigelia marilandica began to bloom in April or early June depending on population and year. For the Park Crossing population, in April 2018, I tagged and selected 80 individuals and in 2019 I tagged 60 individuals (for this small population, all individuals present were tagged and selected). Visits to the Talladega Forest population lasted from April to June in both 2018 and 2019. I tagged and selected 50 individuals in 2018 and 45 in 2019 (all tagged *S. marilandica* at Talladega Forest were included for this small population). The third *S. marilandica* population (Alley Road) was not located until 2019, when it was incorporated into the study. I visited that population from April to early June of 2019. I tagged and selected 110 individuals from 200 available plants.

Flower production data were graphed to allow comparisons of flowering timing between populations and years. I determined the census period with the greatest number of flowers for each population, and considered that to be peak bloom for that population. I then expressed the number of flowers at each census period as a percentage of that peak bloom value. These percentage of peak bloom values were graphed from bloom start to bloom end for each population and year. I then visually compared blooming curves for all three species, as well as between populations of *S. marilandica*. I also compared blooming curves between years for populations visited in both 2018 and 2019.

Floral Morphology and Phenology Study

Floral size and flowering timing play important roles in plant reproduction. *Spigelia gentianoides*, *S. alabamensis*, and *S. marilandica* produce hermaphroditic flowers with male and female functions that may vary independently. *Spigelia* has reduced herkogamy, or the spatial separation of male and female organs, yet dichogamy, the temporal separation of male and female maturity, is still undetermined. Unlike most plants, *Spigelia* species deposit pollen directly onto their style. The yellow pollen adheres to short bristles on the style and forms a “pollen pack” (Erbar and Leins 1999). I examined traits associated with dichogamy (timing of anther dehiscence and stigma receptivity) and herkogamy (style length, distance from stigma to pollen pack, and length of pollen pack) from individuals in each population. Plants included in this study were the same individuals used for the Floral Timing Study in 2019.

Dichogamy traits were the timing of pollen presentation on the style and the timing of stigma receptivity. Flowers of these species are open for three days and all five anthers dehisce simultaneously in *Spigelia* flowers, depositing a pollen pack directly onto the style below the stigma. Flowers were selected to include some on their first day of opening (First-day flowers), some after two days of being open (Second-day flowers), and some on their third day of being open (Third-day flowers). Selected flowers were examined to determine when the pollen pack became visible on their styles. To determine timing of stigma receptivity, styles were removed, viewed under a dissecting microscope, and hydrogen peroxide added to determine stigma receptivity. Stigmas were considered receptive when bubbling was observed after hydrogen peroxide was added (Willmer 2011).

Herkogamous traits were style length (Style Length), distance between the stigma to the top of the pollen pack (Distance to Pollen Pack), and the length of the pollen pack (Length of Pollen Pack). As a *Spigelia* flower matures the style elongates and the pollen pack is raised above the anthers, a characteristic referred to as “secondary pollen presentation” (Erbar and Leins 1999). The pollen pack is usually 0.5-2.0 mm below the base of the stigma, but in some cases pollen touches or actually overtops the stigma. Style elongation typically results in pollen being located on the style some 2-4 mm above the top of the anthers. By the time a corolla opens on the first day of anthesis, the elongation of the style is complete (Affolter 2005, Hershberger 2012). Style Length (mm), Distance to Pollen Pack (mm), and Length of Pollen Pack (mm) were measured for the same flowers (First-day, Second-day, and Third-day) used above to determine stigma receptivity.

Flowers from *S. gentianoides* and from *S. marilandica* (Alley Road and Talladega Forest populations) were sampled in 2019. Park Crossing *S. marilandica* was excluded from the study because plants were top-killed by an exceedingly long drought in 2019. *Spigelia alabamensis* was excluded from this study as flowers wilted in the cooler during transportation from the Bibb County Glades to Auburn University.

Six First-day, four Second-day, and three Third-day flowers were selected from *S. gentianoides*. For Alley Road *S. marilandica*, 12 First-day, 10 Second-day, and three Third-day flowers were sampled. For Talladega Forest *S. marilandica*, seven First-day, six Second-day, and two Third-day flowers were selected.

Sample sizes of flowers selected for each day were small, so before comparing herkogamy traits between populations I determined if traits differed by day within each population. If they did not differ, then I could combine data for all days within a population to increase the sample sizes for between-population comparisons. For each population, First-, Second-, and Third-day traits for Style Length, Distance to Pollen Pack, and Length of Pollen Pack were analyzed using one-way ANOVAs using R version 3.3.3 GUI 1.69 Maverick’s build (R Foundation for Statistical Computing 2016). These analyses revealed no significant effect of day on any trait for any population. ANOVA results for Alley Road *S. marilandica* were: Style Length $F_{3,22} = 1.1$, $P = 0.35$, Distance to Pollen Pack $F_{3,22} = 0.3$, $P = 0.76$, and Length of Pollen Pack $F_{3,22} = 1.1$, $P = 0.34$. ANOVA results for Talladega Forest *S. marilandica* were: Style

Length $F_{3,12} = 2.2$, $P = 0.16$, Distance to Pollen Pack $F_{3,10} = 1.7$, $P = 0.24$, and Length of Pollen Pack $F_{3,10} = 0.4$, $P = 0.67$. And for *Spigelia gentianoides* ANOVA results were: Style Length $F_{3,9} = 0.3$, $P = 0.72$, Distance to Pollen Pack $F_{3,7} = 7.7$, $P = 0.17$, and Length of Pollen Pack $F_{3,8} = 6.5$, $P = 0.21$. Thus day could be considered a non-significant factor, and data for all days for each trait were combined from each population for inter-population comparisons.

Herkogamy traits (Style Length, Distance to Pollen Pack, and Length of Pollen Pack) of the three populations were then analyzed using one-way ANOVAs to determine if they differed significantly ($P < 0.05$) among populations. If a significant population difference was detected, that ANOVA was followed by Tukey's multiple comparisons tests to determine which means differed significantly at $P < 0.05$.

Mating System Study

This study was designed to better understand the mating system of *Spigelia*. There is little information in the literature indicating whether *S. gentianoides*, *S. alabamensis*, and *S. marilandica* are self-compatible or self-incompatible, as well as the degree to which they can outcross.

Marked plants from the three populations used for the Floral Morphology and Phenology Study were included. Two *Spigelia* populations (Park Crossing *S. marilandica* and *S. alabamensis*) were excluded from the study because plants were top-killed by drought in 2019.

Flowers were selected in the advanced bud stage; the buds were large enough to suggest they would open in a day or two, but they were too young for the anthers to have dehisced. Non-toxic paint was added to the sepals of buds to mark selected flowers so they could be followed through fruiting. White polyester organza bags (mesh size 0.2 mm by 0.2 mm) were placed over each inflorescence and supported by bamboo stakes so that plants would not be weighed down (especially during rain events). Bags remained on all plants from when the first flower opened to when the last flower dropped from inflorescences. Bags were removed after the last flower matured but were placed back on plants before fruits matured to allow collection of seeds from dehiscing fruits.

The Mating System Study included four treatments. The four treatments were:

Bagged Autogamy: Inflorescences were covered with mesh bags, but no manipulations were performed. The purpose of this treatment was to determine fruit and seed set when pollinators were excluded.

Selfing: Flowers were tagged and hand-selfed with their own pollen. The purpose of this treatment was to determine fruit and seed set when self-pollination was performed.

Xenogamy Near: Flowers were tagged and crossed with pollen from a different individual less than one meter away. The purpose of this treatment was to determine fruit set and seed set in flowers outcrossed with a nearby and possibly closely-related individual.

Xenogamy Far: Flowers were tagged and crossed with pollen from an individual more than five meters away. The purpose of this treatment was to determine fruit and seed set in flowers outcrossed with individuals that were likely less closely-related because they were located farther away than in the Xenogamy Near treatment.

Small artist paintbrushes were used for pollen transfer. No more than three flowers were selected per plant and, for each plant, only one treatment was applied per day. Treatments were deliberately selected based on the situation of a flower that day. Pollen for the Selfing treatment was obtained from a mature flower on the same plant. Pollen for Xenogamy Near and Xenogamy Far treatments was obtained from a mature flower from a plant either within one meter or more than five meters distant from the treatment plant. Before use in a second cross, paintbrushes were cleaned with 95% ethanol and dried to avoid accidental transfer of pollen (or disease). After manipulations of flowers were complete, an inflorescence was enclosed in mesh bagging and tied shut with string to exclude pollinators. The stem of a treatment plant was loosely tied to a wooden stake to support and mark the plant.

I visited *S. gentianoides* plants at Geneva Forest from May to June in 2019. The four mating treatments were applied to 218 flowers from 137 study individuals in the following treatment replications: Bagged Autogamy $n = 130$, Selfing $n = 54$, Xenogamy Near $n = 15$, and Xenogamy Far $n = 19$.

I visited Alley Road *S. marilandica* from April to early June of 2019. The four mating treatments were applied to 147 flowers from 110 study individuals in the following treatment

replications: Bagged Autogamy n = 67, Selfing n = 23, Xenogamy Near n = 19, and Xenogamy Far n = 38.

Talladega Forest *S. marilandica* was visited from April to early June 2019. The four mating treatments were applied to 55 flowers from 50 study individuals in the following treatment replications: Bagged Autogamy n = 40, Selfing n = 15, Xenogamy Near n = 10, and Xenogamy Far n = 10.

To determine how fruiting success varied across treatments, I returned to populations 6 weeks after experimental manipulations. Treated flowers were examined and whether or not flowers had set fruit was recorded. When fruits began to mature, bags were examined at daily intervals so that any mature fruit was collected before any other fruits within a bag matured. This ensured that seed counts could be reliably assigned to a particular fruit within a bag.

I compared fruiting success differences among treatments in order to determine the effect of treatment for *S. gentianoides* and the effect of treatment between populations of *S. marilandica* using Generalized Linear Mixed Models (GLMM). Fruiting success per flower was assigned a binary value (0 = failure, no fruit and 1 = success, fruit produced) in the GLMM with a logit link function and binomial error distribution. For *S. gentianoides*, pollination treatment was a fixed categorical variable and individual plant identities were included as random effects to control for non-independence of observing multiple flowers on a single plant. For *S. marilandica*, treatment and population were fixed categorical variables and individual plant identities were included as random effects to control for non-independence of observing multiple flowers on a single plant. Analyses were conducted in R (version 3.3.3, R Foundation for Statistical Computing 2016) using the lme4 package (Bates et al. 2015). I calculated estimated marginal mean treatment probabilities using the emmeans package (Lenth 2020).

Seed set was analyzed by using a one-way ANOVA on each population's dataset to determine if there was a significant effect ($P < 0.05$) of treatments on seed set. If there was, I then used Tukey's multiple comparison tests to determine which treatment means differed ($P < 0.05$) in that population. I used R version 3.3.3 GUI 1.69 Maverick's build (R Foundation for Statistical Computing 2016) for these analyses.

Floral Visitation Observations

Visual floral visitor observations were conducted on all study populations in both 2018 and 2019. Each study population from the Floral Timing Study was included. I made observations of floral visitors throughout the flowering period. Visitations were defined as an insect landing on a flower (or a beak entering a corolla in the case of a hummingbird). Observations lasted approximately one to two hours for each visit. Where possible, insects observed on flowers were immediately captured and identified to family and, if possible, genus.

For *S. gentianoides*, there were approximately 40 hours of observations in 2018 and 50 hours in 2019. In 2018, visitations occurred over seven weeks between April and May. Visits in 2019 took place over six weeks between May and June. Visits to *S. gentianoides* sites started at approximately noon and lasted until approximately 5 pm.

For *S. alabamensis* approximately 50 hours were spent in 2018 and approximately 20 hours in 2019. In 2019 drought drastically reduced the number of flowers, resulting in a low number of observation hours in that year. Visits to *S. alabamensis* sites started at approximately 5 pm and lasted until approximately 8 pm.

For *S. marilandica*, I visited Park Crossing in 2018 and 2019, Talladega Forest in 2018 and 2019, and Alley Road in 2019. For Park Crossing approximately 30 hours in 2018 and 30 hours in 2019 were spent in observations. At Talladega Forest approximately 30 hours in 2018 and 30 hours in 2019 were spent in observations. For Alley Road approximately 40 hours in 2019 were spent in observations. Visitor observations started at flowering, which occurred at approximately 8 am, and lasted until around noon as flowers started to close.

Results

Floral Timing Study

Floral timing of *S. marilandica* populations varied both between years and between populations (Fig. 1). Between years, Park Crossing exhibited similar flowering patterns in both years, with peaks appearing in the same week, but flowering in 2018 had a longer duration (by

14 days) than in 2019. In 2019 the Talladega Forest population had a large delay in flowering, as in 2019 the population flowered 18 days after flowering began in 2018.

When comparing between populations, Alley Road started flowering earlier and demonstrated a similar flowering pattern to Park Crossing in 2019, with peaks appearing 3 days apart, while Talladega Forest started to flower later (11 days in 2019 and 27 days in 2018) than the start of any other population. Unlike Park Crossing, Talladega Forest had a longer bloom time (by 7 days) in 2018 than in 2019.

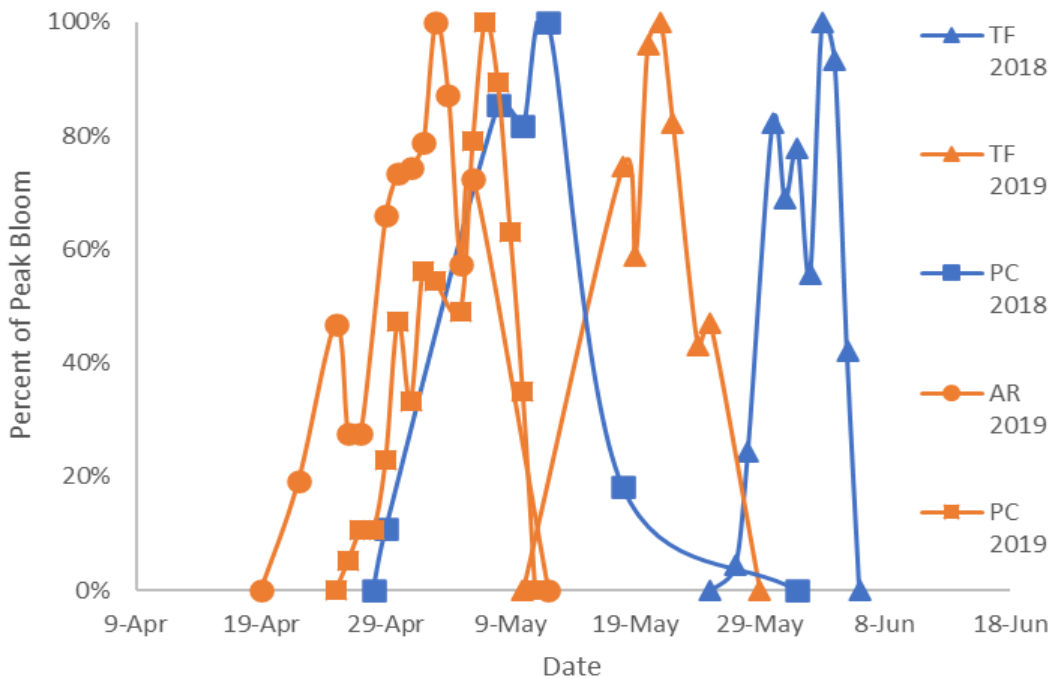


Figure 1. Flowering period and peak bloom in 2018 and 2019 for *S. marilandica* populations. TF = Talladega Forest, PC = Park Crossing, and AR = Alley Road.

Spigelia gentianoides had a 10 day delay in flowering in 2019 versus 2018, and 2019 had an overall shorter bloom period than in 2019 by 9 days (Fig. 2). *Spigelia gentianoides* and *S. alabamensis* had similar peak blooms, peaking around May 9 or 10 and lasting nearly 10 days. *Spigelia gentianoides* started flowering earlier than *S. alabamensis* in 2018 but not in 2019. *Spigelia alabamensis* had a much longer flower duration, lasting 40 days. *Spigelia alabamensis* also had a secondary “peak” in the middle of June that occurred after heavy rainfall in early June

(Fig. 2). While not comparable in size to the May bloom, this secondary bloom provided an additional reproductive opportunity that was not observed for *S. gentianoides* or *S. marilandica*.

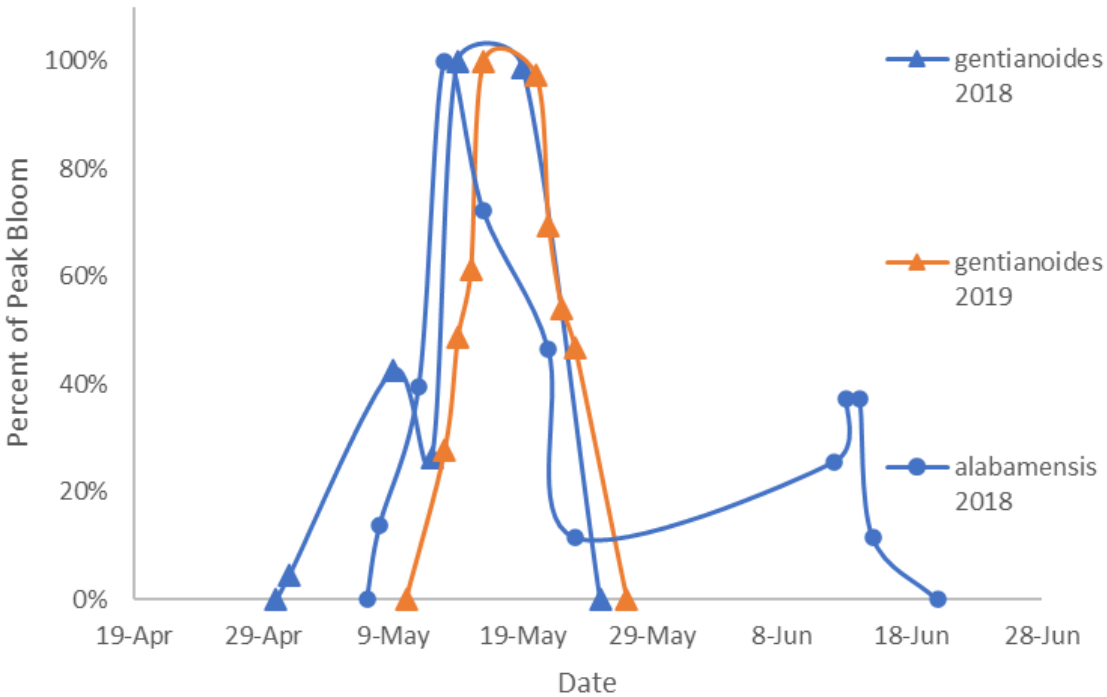


Figure 2. Flowering period and peak bloom in 2018-2019 for *S. gentianoides* and *S. alabamensis*.

Both *S. gentianoides* and *S. alabamensis* displayed longer bloom times (Fig. 2) than the more common *S. marilandica* (Fig. 1). *Spigelia marilandica* Alley Road and Park Crossing populations flowered nearly 2 weeks earlier than either *S. gentianoides* or *S. alabamensis* regardless of year. The *S. marilandica* Talladega Forest population had flowering periods similar to the *S. gentianoides* population. Overall, *S. marilandica* had the shortest average duration for all populations in 2018 and 2019 (20.8 days), while *S. alabamensis* displayed the longest (44 days), and *S. gentianoides*, averaged for both 2018 and 2019, was intermediate (25 days). *Spigelia alabamensis* had the longest bloom of the study species, with a notable second bloom occurring in June 2018 (Fig. 2).

Floral Morphology and Phenology Study

Spigelia flowers generally were consistent in floral longevity between all species in all populations. Flowers typically lasted three days (sometimes two) before wilting. Pollen packs were present upon flower opening for all flowers. Most stigmas were receptive at the time of anther dehiscence and remained receptive through the second day of flowering, with rare cases of receptivity found for Third-day flowers. Receptivity for First-day flowers for Alley Road and Talladega Forest *S. marilandica*, and *S. gentianoides*, was 82%, 86%, and 83%, respectively. Receptivity declined, but was over 50%, for Second-day flowers, at 56%, 60%, and 67%, respectively. The Talladega Forest *S. marilandica* population had one Third-day flower's stigma receptive (33% of Third-day stigmas) while Alley Road *S. marilandica* and *S. gentianoides* did not have any receptive Third-day stigmas.

One-way ANOVA revealed differences between populations in Style Length ($F_{3,49} = 4.9$, $P = 0.001$). *Spigelia marilandica* populations at Alley Road and Talladega Forest had similar Style Length values, while values for *S. gentianoides* were smaller (Tukey's post hoc tests, $P < 0.05$, Table 2). In addition, one-way ANOVA revealed differences between populations for Distance to Pollen Pack ($F_{3,43} = 8.4$, $P = 0.001$). Distance to Pollen Pack for *S. gentianoides* was significantly less than for *S. marilandica*, but the two *S. marilandica* populations did not differ (Tukey's post hoc tests, $P < 0.05$, Table 2). Finally, one-way ANOVA revealed differences between populations for Pollen Pack Length for *S. marilandica* populations were significantly greater than *S. gentianoides* but the two *S. marilandica* populations did not differ (Tukey's post hoc tests, $P < 0.05$, Table 2). Overall flowers of *S. gentianoides* were smaller than flowers of *S. marilandica* and therefore herkogamy was reduced for *S. gentianoides* (Table 2).

Population	Characteristics			
	Day Collected	Style Length (mm)	Stigma to Pollen Pack Distance (mm)	Length of Pollen Pack (mm)
<i>S. marilandica</i>	First (n=11)	50+2.7	3.2+1.5	12+2.8
Alley Road	Second (n=9)	49+1.9	3.3+1.8	10+3.5
	Third (n=4)	50+4.2	2.7+0.8	9.0+2.7
	Mean (n=24)	48+2.8	3.3+1.6	10+3.1
<i>S. marilandica</i>	First (n=7)	48+4.2	2.7+1.5	8.6+1.8
Talladega Forest	Second (n=5)	49+3.1	2.2+2.9	7.2+3.4
	Third (n=3)	52+4.2	2.8+2.7	7.3+3.5
	Mean (n=15)	48+4.9	2.8+2.3	7.8+3.2
<i>S. gentianoides</i>	First (n=6)	17+0.8	0.4+0.3	2.6+1.4
	Second (n=3)	17+1.0	0.3+0.2	2+1.4
	Third (n=3)	17+1.0	1+0	4+0
	Mean (n=12)	17+1.0	1+0	3.4+0.6

Table 2. Herkogamous characteristics (mean \pm SD) in the three populations of *Spigelia*.

Mating System Study

Overall, at least one fruit was produced in all treatments (range: 11-48%) of *S. marilandica* and *S. gentianoides* (Table 3). A generalized linear mixed model was used to examine if proportion of fruit set differed with treatment and if different species/populations had differences in proportion of fruit set by treatment. Likelihood ratio tests indicated fruiting success was significantly affected by treatments for *S. gentianoides* (Chi-square = 14, df = 1, P = 0.003) and for *S. marilandica* (Chi-square = 14, df = 1, P = 0.028).

All treatments where pollen was moved manually (Selfing, Xenogamy Near, and Xenogamy Far) set proportionally higher seed than Autogamy, with Selfing setting the highest proportion of seed among all treatments for all populations (Table 3). By calculating the

estimated marginal means, we created model-predicted probabilities of fruit set (Fig. 4). Model predicted probabilities (Fig. 4) showed the Autogamy treatment had the least probability to set fruit, while all other treatments, Selfing, Xenogamy Near, and Xenogamy Far, had significantly higher probabilities.

Populations	<u>Treatments</u>			
	Bagged			
	Autogamy	Selfing	Xenogamy Near	Xenogamy Far
<hr/> <i>S. marilandica</i>				
Talladega Forest	0.11 (n=40)	0.32 (n=15)	0.2 (n=10)	0.17 (n=10)
emmeans	0.04	0.08	0.08	0.06
CI	[-2.55, -1.12]	[-1.46, -0.01]	[-2.29, -0.45]	[-2.42, -0.75]
<hr/> <i>S. marilandica</i>				
Alley Road	0.19 (n=67)	0.48 (n=23)	0.28 (n=19)	0.24 (n=38)
emmeans	0.04	0.07	0.08	0.05
CI	[-1.9, -0.88]	[-0.86, -0.28]	[-1.73, -0.55]	[-1.73, -0.12]
<hr/> <i>Spigelia</i>				
gentianoides	0.14 (n=130)	0.27 (n=54)	0.17 (n=15)	0.14 (n=19)
emmeans	0.03	0.05	0.0588	0.04
CI	[-2.59, -1.58]	[-1.51, -1.13]	[-2.46, -0.78]	[-2.54, -1.13]

Table 3. Proportions of treated flowers that developed a fruit, and the estimated marginal mean probability of fruiting success (“emmeans”) by population and treatment (95% confidence intervals inside brackets).

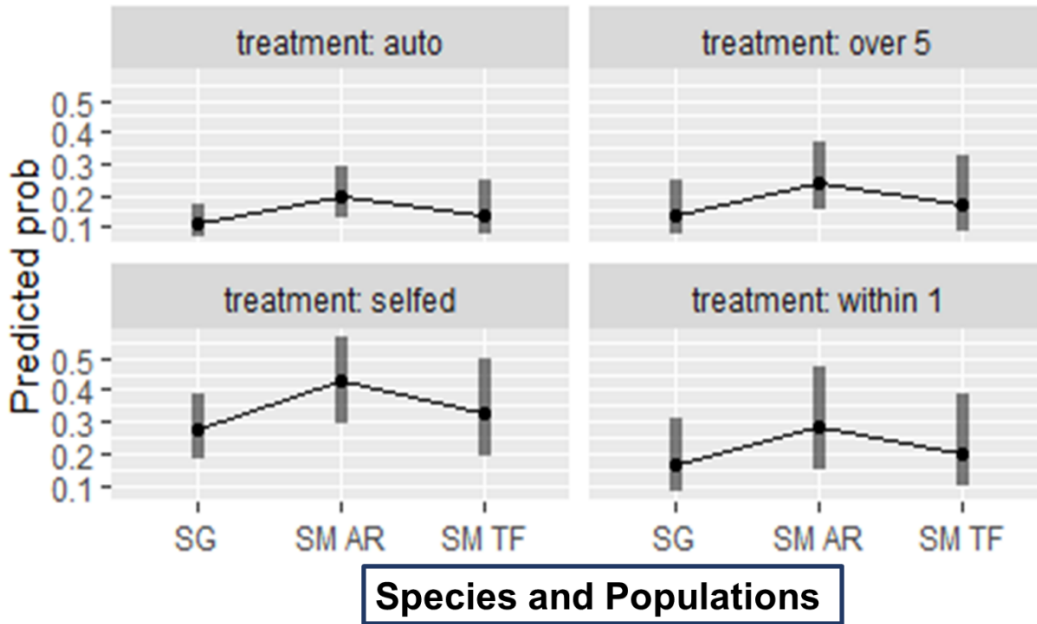


Figure 3. Predicted probability of fruiting success from treatments in the Mating System Study.

Overall, flowers of all *Spigelia* species were able to set some seeds regardless of treatment (Table 4). ANOVAs demonstrated that treatments did not significantly affect seed set for either the Talladega Forest *S. marilandica* population ($F_{3,10} = 2.2$, $P = 0.20$) or the *S. gentianoides* population ($F_{3,34} = 0.5$, $P = 0.72$). In contrast, there was a significant treatment effect on seed set for the Alley Road *S. marilandica* population ($F_{3,40} = 3.1$, $P = 0.04$). In that case, Tukey's multiple comparison tests showed that seed set for the Xenogamy Near treatment was significantly less than all other treatments ($P < 0.05$).

Populations	<u>Treatments</u>			
	Bagged			
	Autogamy	Selfing	Xenogamy Near	Xenogamy Far
<i>S. marilandica</i> Talladega Forest	5.5±4.5 (n=4)	5.8±3.1 (n=5)	5.7±2.1 (n=3)	3.8±2.9 (n=4)
<i>S. marilandica</i> Alley Road	7.4±4.5 ^a (n=13)	6.2±3.5 ^a (n=12)	2±0 ^b (n=4)	7.4±3.1 ^a (n=9)
<i>Spigelia</i> <i>gentianoides</i>	3.1±1.8 (n=18)	4.2±3.5 (n=11)	3.2±2.7 (n=5)	2.3±2.4 (n=3)

Table 4. Mean seed set per fruit (\pm SD) for each treatment in the *Spigelia* populations. Different superscripts for the Alley Road *S. marilandica* population denote significantly different means by Tukey's multiple comparison tests ($P < 0.05$).

Floral Visitation Observations

Visual studies showed exceptionally low flower visitation for all *Spigelia* species, with numerous field days resulting in either solitary insect visits or none whatsoever. Of documented visitors, *Bombus* species were the most prevalent for all *Spigelia* (Table 5). Hummingbirds, Syrphids, and Lepidoptera were observed visiting *S. marilandica* at very low rates.

For *S. gentianoides* and *S. alabamensis* I attempted to observe flower visitors at all hours of the day, from dawn to night. Visitations typically occurred between noon and 7 pm as *S. gentianoides* has not been reported to open during any specific time (Gould 1999). For *S. gentianoides*, after hundreds of hours of field observations, insect visitors were scarce. I did happen to observe bees, possibly *Bombus*, visit on extremely rare occasions (12 occurrences over 122 visits) but was unable to capture those visitors. Bees visiting appeared to reflex petals and stick heads in to access nectar.

Flowers of *S. alabamensis* opened during the late afternoon, suggesting possible nocturnal pollination. I stationed myself at dusk in locations where hundreds of open flowers were visible and often did not see visitors. However, I found a few *Bombus pensylvanicus* visiting in the middle of July. All species were collected and vouchered for deposit in the Auburn University Natural History Museum.

Order	Family	Species	<i>S. marilandica</i>	<i>S. alabamensis</i>	<i>S. gentianoides</i>
Coleoptera	Lampyridae	ND*	1	-	-
	Scarabaeidae	<i>Canthon viridis</i>	-	1	-
Hymenoptera			-	-	-
	Apidae	<i>Bombus impatiens</i>	-	2	-
	Apidae	<i>Bombus</i> sp.	-	1	-
	Apidae	<i>Bombus pensylvanicus</i>	-	1	-
	Halictidae		1	-	-
	Megachilidae	<i>Megachile campanulae</i>	2	-	-
	Formicidae	<i>Brachymyrmex patagonicus</i>	-	3	-
<i>Solenopsis invicta</i>			5	-	-
Hemiptera	Miridae	ND*	1	-	-
	Cicadellidae	ND*	1	-	-
	Coreidae	ND*	1	-	-
Diptera	Syrphidae	ND*	1	-	2
Lepidoptera	ND*		-	-	1

Table 5. Observed insect floral visitors to *Spigelia* flowers in 2019 and the numbers of each insect species visiting flowers during observations. (ND* for Not Determined).

Discussion

Secondary Pollen Presentation

Secondary pollen presentation, in which pollen grains from anthers are transferred onto other floral structures, is a potential response to selection and has evolved in 16 families (Howell et al. 1993). It is hypothesized to increase the accuracy of pollen capture and subsequent transfer

by pollinators (Fan et al. 2015). Some species of *Spigelia* (*S. anthelmia* and *S. splendens*) reproduce via autogamy and secondary pollen presentation has a significant role (Erbar and Leins 1999). My experiments with Alabama *Spigelia* showed that a great amount of pollen was released from the anthers onto the style in the bud stage, occasionally when the stigma was receptive. I also documented limited temporal separation between pollen release and stigmatic receptivity. This limitation in dichogamy and herkogamy could lead to the high selfing rate observed for *S. gentianoides* and *S. marilandica*.

My observations indicated that potential pollinators of *Spigelia* are bees. For instance, *Megachile campanulae* and *Bombus impatiens* could be considered as good vectors of pollen, since I observed them contacting both stigma and pollen pack when visiting *Spigelia* flowers. During visitation, self-pollen is expected to be transmitted onto the stigma, likely causing a deposition of heterologous and homologous pollen during consecutive visits. This may cause pollen discounting, which according to some (Harder and Wilson 1998, Galloway et al. 2002) may reduce male fitness. Moreover, self-interference of pollen grains (Broyles and Wyatt 1993) also may cause fitness reduction (Cesaro et al. 2004).

My study contributed to a better understanding of secondary pollen presentation in the genus, also establishing the bases for new investigations on this phenomenon, such as its reproductive cost determined by homologous pollen deposition on the stigma during the flower-insect interaction and pollinator efficiency. Research on reproductive costs for secondary pollen presentation is still scarce and represents a field of investigation that needs developing. In conclusion, my results demonstrated that sexual functions, in relation to secondary pollen presentation, are spatially and temporally related in *Spigelia*.

Cleistogamy in *S. gentianoides*

Spigelia gentianoides has an odd flowering morphology, in that its flowers have never been documented opening and therefore can be considered cleistogamous. Cleistogamy is a pollination system in which unopened flowers are capable of autogamy resulting in fruit production (Wang et al. 2019). My results support *S. gentianoides* as having cleistogamous flowers, as flowers were not observed to open and my experiments showed that fruits and seeds

are produced by bagged and unmanipulated flowers. In a majority of cleistogamous species, closed (cleistogamous) and open (chasmogamous) flowers usually occur on the same individual during a single flowering season (Morinaga et al. 2008). *Spigelia gentianoides* is unusual in that there are no open flowers on an inflorescence.

Another oddity is that while flowers remain closed, *S. gentianoides* still produces nectar (Shotts, personal observations 2018-2019). Rogers (1988) proposed that moths and “big bees” had methods to deal with cleistogamy and reach this floral reward. *Spigelia gentianoides* may be a viable nectar source for insects, as it is one of very few flowers available in its habitat in late April (Shotts, personal observations 2018-2019). Another federally endangered plant species, *Xyris tennesseensis*, also has closed flowers that can be forced open (Wall et al. 2002). In that case, *Lasioglossum zephyrum* manipulates *Xyris tennesseensis* flowers to open prematurely, ensuring first access to the floral rewards (Wall et al. 2002). Rogers (1988) stated that he did observe Halictid bees (sweat bees) entering *S. gentianoides* flowers by peeling back petals. Additionally, while I was unable to capture any insects, individuals of *Bombus* sp. were observed peeling petals back and inserting their bodies into the flowers (Shotts, personal observations 2018-2019). By forcing their way in, these insects come in contact with both pollen pack and stigma, as they are within one millimeter of each other (Table 2). This can ensure pollen comes in contact with insects reaching for nectar at the base of a flower, promoting pollen movement both to its own stigma and potentially to other *S. gentianoides* flowers.

This study provides evidence for autonomous self-pollination in *S. gentianoides*. Outside of my observations supporting autogamy in *S. gentianoides* (Table 3), insects have been reported forcing their way to the floral reward. Cleistogamy has typically been thought of as a way to ensure autogamy, but there likely is some level of pollen transmission in *S. gentianoides* when insect floral visitations occur. In conclusion, my results demonstrated that *S. gentianoides* flowers ensure that some pollen transfer occurs, at least to their own stigmas, when insects visit.

Mating Strategy in Alabama *Spigelia*

Historically, species that are predominantly selfing risk extinction (Husband and Schemske 1996) because selfing can lead to inbreeding depression and eventual extinction

(Barrett 2000). Recently, some studies have shown that there are benefits to selfing, as it is useful in adapting locally and purging genetic load (Le Corre and Kramer 2012). Furthermore, autogamy can help ensure sexual reproduction without dependence on pollen vectors, also known as reproductive assurance (Takebayashi and Morrell 2001). Selfing flowers have floral characteristics, such as herkogamy and dichogamy, that are highly reduced to the point where self-pollination is very likely (Barrett and Harder 2017). Since selfing species are highly adaptable to their environment (Zhang et al. 2019), they are best suited for conservation strategies that involve habitat maintenance, or translocation to areas that are more suitable than what current conditions permit (i.e., climate change) (Oostermeijer et al. 2003, Beaumont et al. 2019).

The floral morphology and phenology of *Spigelia* is as expected for self-compatible species. An important feature of the floral biology of *Spigelia* is the secondary pollen presentation, which causes close stigma–pollen pack proximity and lack of temporal separation between pollen grain release and stigma receptivity, so that (as I found in the pollen transfer experiments) self-pollination is enabled, providing reproductive assurance in the absence of pollinators. Selfing ability likely is reinforced by the advantages of reproductive assurance, but these advantages are countered by increased risk of inbreeding depression (Charlesworth et al. 1990, Lloyd 1992, Lande et al. 1994). *Spigelia* is self-pollinating in the wild. Flowers that have not received pollen from other flowers can pollinate themselves: this may be facilitated by breaks in the style where the pollen pack is deposited (Erbar and Leins 1999). Greenhouse mating studies determined that, in the absence of floral visitors, *S. marilandica* and *S. alabamensis* would not set seed (Affolter 2005). Since floral visitors were excluded in both studies (Affolter 2005, Shotts personal observation), in the absence of animal visits, wind may increase pollination success by moving pollen to stigmas of the same flower.

Conservation Management of *Spigelia*

Spigelia flowers set fruit in the Bagged Autogamy treatment by all species (Table 3). These results indicate that *Spigelia* can reproduce sexually in the absence of pollinators. The rarity of *S. gentianoides* and *S. alabamensis* may be attributable to short seed dispersal distances and to low habitat availability (Pulliam 2000). However, given their high rates of fruit set and

seed germination, and their ability to produce seeds in the absence of pollinators, *S. gentianoides* and *S. alabamensis* may in fact be rare but not intrinsically endangered (Schemske et al. 1994, Kearns and Inouye 1998, Guerrant Jr and Kaye 2007). Selfing species contain most of their genetic diversity within populations, and their genetic diversity will be less affected by reduced gene flow (Honnay and Jacquemyn 2007). Nevertheless, their narrow distributions make them vulnerable to habitat destruction.

My results suggest certain management strategies for *Spigelia*. The survival of the remaining *S. gentianoides* and *S. alabamensis* populations is dependent on the commitment and effective responses of the relevant local, regional, and national authorities. In the case of *S. alabamensis*, habitat protection is necessary because of the specificity of the species' habitat (Ketona Glades), and the limited occurrences of that habitat mean there are few chances to establish additional populations. For *S. gentianoides*, habitat management is needed, especially with appropriate application of fire to keep habitat in the current site relatively open. Unlike *S. alabamensis*, new populations of *S. gentianoides* might be established in other longleaf pine sites using habitat suitability modeling. Management should include a Habitat Suitability Model (HSM) for *S. gentianoides* to identify these potential new sites.

Taxonomic ranks of *S. alabamensis* and *S. gentianoides*

Spigelia alabamensis and *S. gentianoides* were formerly classified as subspecies within the same species, *S. gentianoides*. In 1992 and 1993, *S. alabamensis* was found in 17 Ketona Glades, nearly 200 miles from the nearest populations of *S. gentianoides* in Florida (Allison 1994, Allison and Stevens 2001). Subsequent assessment of *S. gentianoides* and *S. alabamensis* suggested that *S. alabamensis* populations warranted specific rank (USFWS 2009). Gould (1996) listed several morphological trait differences between *S. gentianoides* and *S. alabamensis* and additional molecular and morphological studies further corroborated this rank change (Weakley et al. 2011, Hershberger 2012, Hershberger et al. 2015). Primary points for taxonomic differences of the two species are differences in morphological traits, such as plant size, leaf shape, flower size, and number of flowers produced, along with habitat type, with *S. alabamensis* found on dolomite limestone glades whereas *S. gentianoides* is found in longleaf pine savannas (Gould 1996). Further differences between species are highlighted in blooming period as shown

in my data: while having very similar times in peak bloom, *S. alabamensis* has a significantly longer bloom time than *S. gentianoides*, with flowers produced two to three months longer (Fig. 2). Furthermore, peak blooms for *S. alabamensis*, and possibly other *Spigelia* species (Dunwell 2003), may be stimulated by rain events, with additional blooming events were observed after heavy rainfall.

The taxonomic separation of *S. alabamensis* from *S. gentianoides* led to uncertainty in *S. alabamensis*' conservation status because only *S. gentianoides* is protected under the U.S. Endangered Species Act (Weakley et al. 2011, USFWS 2012). Such taxonomic uncertainty can negatively impact species conservation, with the worst scenario being extinction (Ceballos et al. 2015, Pelletier et al. 2018, Humphreys et al. 2019, Le Roux et al. 2019). Ultimately, *S. alabamensis* still needs protection status comparable to *S. gentianoides*. The solution for better protection is to list *S. alabamensis* under the Endangered Species Act.

Literature Cited

- Aizen MA, Feinsinger P. 1994. Habitat fragmentation, native insect pollinators, and feral honeybees in Argentine 'Chaco Serrano'. *Ecological Applications*. 2:378-392.
- Allison J, Stevens TE. 2001. Vascular flora of Ketona dolomite outcrops in Bibb County, Alabama. *Castanea*. 66:154-205.
- Affolter JM. 2005. Conservation biology of *Spigelia gentianoides* and *S. marilandica*: Genetic variation, reproduction biology, and propagation. Final Project Report, Georgia Cooperative Fish and Wildlife Research Unit, University of Georgia, Athens, Georgia.
- Baker HG. 1955. Self-compatibility and establishment after long-distance dispersal. *Evolution*. 9:347-349.
- Baker WL. 1995. Longterm response of disturbance landscapes to human intervention and global change. *Landscape Ecology*. 3:143-159.
- Barnosky AD, Matzke N, Tomiya S, Wogan GO, Swartz B, Quental TB, Marshall C, McGuire JL, Lindsey EL, Maguire KC, Mersey B. 2011. Has the Earth's sixth mass extinction already arrived? *Nature*. 471:51-57.
- Barrett SC. 1989. Mating system evolution and speciation in heterostylous plants. In: Otte D, Endler J, eds. *Speciation and its consequences*. Sinauer, Sunderland, Massachusetts: pp. 257-283.
- Barrett SC, Harder LD. 1996. Ecology and evolution of plant mating. *Trends in Ecology & Evolution*. 2:73-79.
- Barrett SCH, Kohn JR. 1991. Genetic and evolutionary consequences of small population size. In: Falk DA, Holsinger KE, eds. *Genetics and conservation of rare plants*. Oxford University Press, New York: pp. 3-30.
- Bates D, Kliegl R, Vasishth S, Baayen H. 2015. Parsimonious mixed models. arXiv preprint arXiv:1506.04967.
- Bawa KS. 1990. Plant-pollinator interactions in tropical rain forests. *Annual Review of Ecology and Systematics*. 21:399-422.

- Beaumont LJ, Esperón-Rodríguez, M, Nipperess, DA, Wauchope-Drumm, M, Baumgartner JB. 2019. Incorporating future climate uncertainty into the identification of climate change refugia for threatened species. *Biological Conservation*. 237:230-237.
- Biesmeijer JC, Roberts SP, Reemer M, Ohlemüller R, Edwards M, Peeters T, Schaffers AP, Potts SG, Kleukers RJMC, Thomas CD, Settele J. 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science*. 313:351-354.
- Boyd RS, 1994. Pollination biology of the rare shrub *Fremontodendron decumbens* (Sterculiaceae). *Madrono*. 41:277–289.
- Brown AH. 1989. Genetic characterization of plant mating systems. In: Brown AH, Clegg MT, Kahler A, Weir B, eds. *Plant population genetics, breeding, and genetic resources*. Sinauer, Sunderland, Massachusetts: pp. 145–162.
- Broyles SB, Wyatt R. 1993. The consequences of self-pollination in *Asclepias exaltata*, a self-incompatible milkweed. *American Journal of Botany*. 80:41-44.
- Brummitt NA, Bachman SP, Griffiths-Lee J, Lutz M, Moat JF, Farjon A, Donaldson JS, Hilton-Taylor C, Meagher TR, Albuquerque S, Aletrari E. 2015. Green plants in the red: A baseline global assessment for the IUCN sampled Red List Index for plants. *PLOS one*. 10:0135152.
- Busch JW, Schoen DJ. 2008. The evolution of self-incompatibility when mates are limiting. *Trends in Plant Science*. 13:128-136.
- Calviño-Cancela M, de Silanes MEL, Rubido-Bará M, Uribarri J. 2013. The potential role of tree plantations in providing habitat for lichen epiphytes. *Forest Ecology and Management*. 291:386-395.
- Caughlin TT, Ferguson JM, Lichstein JW, Zuidema PA, Bunyavejchewin S, Levey DJ. 2015. Loss of animal seed dispersal increases extinction risk in a tropical tree species due to pervasive negative density dependence across life stages. *Proceedings of the Royal Society B: Biological Sciences*. 282:20142095.

- Ceballos G, Ehrlich PR, Barnosky AD, García A, Pringle RM, Palmer TM. 2015. Accelerated modern human-induced species losses: Entering the sixth mass extinction. *Science Advances*. 1:1400253.
- Cesaro AC, Barrett SH, Maurice S, Vaissiere BE, Thompson JD. 2004. An experimental evaluation of self-interference in *Narcissus assoanus*: functional and evolutionary implications. *Journal of Evolutionary Biology*. 17:1367-1376.
- Charlesworth D, Morgan, MT, Charlesworth B. 1990. Inbreeding depression, genetic load, and the evolution of outcrossing rates in a multilocus system with no linkage. *Evolution*. 44:1469-1489.
- Chen IC, Hill JK, Ohlemüller R, Roy DB, Thomas CD. 2011. Rapid range shifts of species associated with high levels of climate warming. *Science*. 333:1024-1026.
- Cheptou PO, Dieckmann U. 2002. The evolution of self-fertilization in density-regulated populations. *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 269:1177-1186.
- Cruden RW. 1977. Pollen-ovule ratios: A conservative indicator of breeding systems in flowering plants. *Evolution*. 31:32-46.
- Cullina W. 2000. *The New England Wild Flower Society guide to growing and propagating wildflowers of the United States and Canada*. Houghton Mifflin Harcourt Co., Boston and New York.
- Dafni A 1992. *Pollination ecology: A practical approach*. Oxford University Press, Oxford.
- Darke R. 2002. *American woodland garden*. Timber Press, Portland, Oregon.
- Darwin C. *Means of fertilisation*. 1876. *The effects of cross and self fertilisation in the vegetable kingdom: By Charles Darwin*. John Murray, London.
- Delmas CE, Escaravage N, Pornon A. 2014. Massive floral display affects insect visits but not pollinator-mediated pollen transfer in *Rhododendron ferrugineum*. *Plant Biology*. 1:234-243.

- Dunwell W. 2003. *Spigelia marilandica* propagation: A review. Combined Proceedings of the International Plant Propagators Society. 53:510-512.
- Ellstrand NC, Elam DR. 1993. Population genetic consequences of small population size: implications for plant conservation. Annual Review of Ecology and Systematics. 1:217-242.
- Erbar C, Leins P. 1999. Secondary pollen presentation and a curious rupture of the style in *Spigelia* (Spigeliaceae, Gentianales). Plant Biology. 4:389-402.
- Falk DA, Holsinger KE. 1991. Genetics and conservation of rare plants. Oxford University Press, New York.
- Fan YL, Kress WJ, Li QJ. 2015. A new secondary pollen presentation mechanism from a wild ginger (*Zingiber densissimum*) and its functional roles in pollination process. PLOS ONE. 10:0143812.
- Faraji L, Karimi M. 2020. Botanical gardens as valuable resources in plant sciences. Biodiversity and Conservation. 2:1-22.
- Fenner MK, Thompson K. 2005. The ecology of seeds. Cambridge University Press, Cambridge.
- Fernández-Casas FJ, Huft MF. 2009. *Spigelia*. Flora Mesoamericana. 1:633-634.
- Frankel R, Galun E. 2012. Pollination mechanisms, reproduction and plant breeding. Springer Science & Business Media, New York.
- Galloway LF, Cirigliano T, Gremski K. 2002. The contribution of display size and dichogamy to potential geitonogamy in *Campanula americana*. International Journal of Plant Sciences. 163:133-9.
- Gould KR. 1999. Three new species of *Spigelia* (Strychnaceae) from Mexico. Brittonia. 4:407-414.
- Gould KR. 1996. A new, disjunct variety of *Spigelia gentianoides* (Loganiaceae) from Bibb County, Alabama. SIDA, Contributions to Botany. 1:417-421.
- Guerrant Jr EO, Kaye TN. 2007. Reintroduction of rare and endangered plants: Common factors, questions and approaches. Australian Journal of Botany. 55:362-370.

- Harder LD, Wilson WG. 1998. A clarification of pollen discounting and its joint effects with inbreeding depression on mating system evolution. *The American Naturalist*. 152:684-95.
- Havens K, Kramer AT, Guerrant Jr EO. 2014. Getting plant conservation right (or not): the case of the United States. *International Journal of Plant Sciences*. 175:3-10.
- Hershberger AJ. 2012. Assessment of genetic variability of *Spigelia marilandica* and *S. gentianoides* using amplified fragment length polymorphism (AFLP) markers and clonal propagation of stem cuttings of *S. marilandica*, *S. gentianoides* var. *alabamensis*, and *S. marilandica* X *S. gentianoides* var. *alabamensis* F2 and F3 hybrids. Ph.D. dissertation, University of Georgia.
- Hershberger AJ, Jenkins TM, Robacker C. 2015. Molecular genetic variability of *Spigelia marilandica* and *S. gentianoides*. *Journal of the American Society for Horticultural Science*. 140:120-128.
- Honnay O, Jacquemyn H. 2007. Susceptibility of common and rare plant species to the genetic consequences of habitat fragmentation. *Conservation Biology*. 3:823-831.
- Howell GJ, Slater AT, Knox RB. 1993. Secondary pollen presentation in angiosperms and its biological significance. *Australian Journal of Botany*. 41:417-438.
- Huang H. 2011. Plant diversity and conservation in China: Planning a strategic bioresource for a sustainable future. *Botanical Journal of the Linnean Society*. 166:282-300.
- Husband BC, Barrett SC. 1992. Effective population size and genetic drift in tristylous *Eichhornia paniculata* (Pontederiaceae). *Evolution*. 46:1875-1890.
- Husband BC, Schemske DW. 1996. Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution*. 50:54-70.
- IUCN 2019. The IUCN red list of threatened species. IUCN, Version 2019–2). <http://www.iucnredlist.org/>
- Jennersten OL. 1988. Pollination in *Dianthus deltoides* (Caryophyllaceae): Effects of habitat fragmentation on visitation and seed set. *Conservation Biology*. 2:359-366.

- Kremen C, Ricketts T. 2000. Global perspectives on pollination disruptions. *Conservation Biology*. 14:1226-1228.
- Kremer A, Le Corre V. 2012. Decoupling of differentiation between traits and their underlying genes in response to divergent selection. *Heredity*. 108:375-385.
- Krestova IN, Nesterova SV. 2013. Flowering and pollination of the native daylily species (*Hemerocallis*) in the Botanical garden Institute, Far East branch, Russian Academy of Sciences. *Contemporary Problems of Ecology*. 6:448-454.
- Kwak MM, Bekker RM. 2006. Ecology of plant reproduction: extinction risks and restoration perspectives of rare plant species. In: Wasser NM, Ollerton J, eds. *Plant-pollinator interactions: From specialization to generalization*. University of Chicago Press, Chicago: pp. 362-386.
- Lande R, Schemske DW, Schultz ST. 1994. High inbreeding depression, selective interference among loci, and the threshold selfing rate for purging recessive lethal mutations. *Evolution*. 48:965-978.
- Lankinen Å, Kiboi S. 2007. Pollen donor identity affects timing of stigma receptivity in *Collinsia heterophylla* (Plantaginaceae): a sexual conflict during pollen competition? *The American Naturalist*. 170:854-863.
- Latta R, Ritland K. 1994. The relationship between inbreeding depression and prior inbreeding among populations of four *Mimulus* taxa. *Evolution*. 48:806.
- Lenth R, Singmann H, Love J, Buerkner P, Herve M. 2020. emmeans: estimated marginal means. R package version 1.4. 4. American Statistical Association. 34:216-220.
- Lloyd DG. 1992. Self-and cross-fertilization in plants. II. The selection of self-fertilization. *International Journal of Plant Sciences*. 153:370-380.
- Loveless MD, Hamrick JL. 1984. Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics*. 15:65-95.
- Menges ES. 1986. Predicting the future of rare plant populations: Demographic monitoring and modeling. *Natural Areas Journal*. 6:13-25.

- Menges ES. 1991. Seed germination percentage increases with population size in a fragmented prairie species. *Conservation Biology*. 5:158-164.
- Menges ES. 2000. Applications of population viability analyses in plant conservation. *Ecological Bulletins*. 1:73-84.
- Morinaga SI, Nagano AJ, Miyazaki S, Kubo M, Demura T, Fukuda H, Sakai S, Hasebe M. 2008. Ecogenomics of cleistogamous and chasmogamous flowering: genome-wide gene expression patterns from cross-species microarray analysis in *Cardamine kokaiensis* (Brassicaceae). *Journal of Ecology*. 96:1086-1097.
- Neal PR, Anderson GJ. 2005. Are 'mating systems' 'breeding systems' of inconsistent and confusing terminology in plant reproductive biology? Or is it the other way around? *Plant Systematics and Evolution*. 250:173-185.
- Negron-Ortiz, V. 2012 Recovery plan for *Spigelia gentianoides* (gentian pinkroot).
<<http://www.fws.gov/panamacity/resources/Spigelia%20gentianoides%20Recovery%20Plan.pdf>>
- Neuschulz EL, Mueller T, Schleuning M, Böhning-Gaese K. 2016. Pollination and seed dispersal are the most threatened processes of plant regeneration. *Scientific Reports*. 6:1-6.
- Oostermeijer JG, Luijten SH, Den Nijs JC. 2003. Integrating demographic and genetic approaches in plant conservation. *Biological Conservation*. 113:389-398.
- Pimm SL, Jenkins CN, Abell R, Brooks TM, Gittleman JL, Joppa LN, Raven PH, Roberts CM, Sexton JO. 2014. The biodiversity of species and their rates of extinction, distribution, and protection. *Science*. 344:1246752
- Popovkin AV, Mathews KG, Santos JC, Molina MC, Struwe L. 2011. *Spigelia genuflexa* (Loganiaceae), a new geocarpic species from the Atlantic forest of northeastern Bahia, Brazil. *PhytoKeys*. 6:47.
- Rogers GK. 1988. *Spigelia gentianoides*-a species on the brink of extinction. *Plant Conservation*. 3:1-8.

- Saunders NE, Sipes SD. 2006. Reproductive biology and pollination ecology of the rare Yellowstone Park endemic *Abronia ammophila* (Nyctaginaceae). *Plant Species Biology*. 21:75-84.
- Schemske DW, Lande R. 1985. The evolution of self-fertilization and inbreeding depression in plants. II. Empirical observations. *Evolution*. 39:41-52.
- Schemske DW, Husband BC, Ruckelshaus MH, Goodwillie C, Parker IM, Bishop JG. 1994. Evaluating approaches to the conservation of rare and endangered plants. *Ecology*. 75:584-606.
- Shine R, Doody JS. 2011. Invasive species control: understanding conflicts between researchers and the general community. *Frontiers in Ecology and the Environment*. 9:400-406.
- Silva JL, Souza AF, Jardim JG, Goto BT. 2017. Community assembly in harsh environments: the prevalence of ecological drift in the heath vegetation of South America. *Ecosphere*. 6:1-8.
- Slatkin M. 1985. Gene flow in natural populations. *Annual Review of Ecology and Systematics*. 16:393-430.
- Smith S, Bubeck D, Nelson B, Stanek J, Gerke J. 2015. Genetic diversity and modern plant breeding. In: Ahuja MR, Jain SM, eds. *Genetic diversity and erosion in plants*. Springer International, New York: pp. 55–88.
- Takebayashi N, Morrell PL. 2011. Is self-fertilization an evolutionary dead end? Revisiting an old hypothesis with genetic theories and a macroevolutionary approach. *American Journal of Botany*. 88:1143-1150.
- Tang R, Li Y, Xu Y, Schinnerl J, Sun W, Chen G. 2020. In-situ and ex situ pollination biology of the four threatened plant species and the significance for conservation. *Biodiversity and Conservation*. 29:381-391.
- U.S. Fish and Wildlife Service /Negron-Ortiz V. 2018. Technical/Agency Draft Recovery Plan for *Spigelia gentianoides* (Gentian pinkroot). 5-Year Review: Summary and Evaluation. https://ecos.fws.gov/docs/recovery_plan/Spigelia%20gentianoides%20Recovery%20Plan%20Amendment.pdf >

- Wagenius S, Lonsdorf E, Neuhauser C. 2007. Patch aging and the S-Allee effect: Breeding system effects on the demographic response of plants to habitat fragmentation. *The American Naturalist*. 169:383-397.
- Walker J. 1993. Rare vascular plant taxa associated with the longleaf pine ecosystems: patterns in taxonomy and ecology. *Proceedings of the Annual Tall Timbers Fire Ecology Conference*. 18:105-126.
- Wall MA, Teem AP, Boyd RS. 2002. Floral manipulation by *Lasioglossum zephyrum* (Hymenoptera: Halictidae) ensures first access to floral rewards by initiating premature anthesis of *Xyris tennesseensis* (Xyridaceae) flowers. *Florida Entomologist*. 85:290-291.
- Wang Q, Shao S, Su Y, Hu X, Shen Y, Zhao D. 2019. A novel case of autogamy and cleistogamy in *Dendrobium wangliangii*: A rare orchid distributed in the dry-hot valley. *Ecology and Evolution*. 9:12906-12914.
- Weakley AS, LeBlond RJ, Sorrie BA, Witsell CT, Estes LD, Gandhi K, Mathews KG, Ebihara A. 2011. New combinations, rank changes, and nomenclatural and taxonomic comments in the vascular flora of the southeastern United States. *Journal of the Botanical Research Institute of Texas*. 5:437-455.
- Weeks AR, Sgro CM, Young AG, Frankham R, Mitchell NJ, Miller KA, Byrne M, Coates DJ, Eldridge MD, Sunnucks P, Breed MF. 2011. Assessing the benefits and risks of translocations in changing environments: a genetic perspective. *Evolutionary Applications*. 4:709-725.
- Wilcock C, Neiland R. 2002. Pollination failure in plants: why it happens and when it matters. *Trends in Plant Science*. 7:270-277.
- Willmer P. 2011. *Pollination and floral ecology*. Princeton University Press, Princeton.
- Wright SI, Lauga B, Charlesworth D. 2002. Rates and patterns of molecular evolution in inbred and outbred *Arabidopsis*. *Molecular Biology and Evolution*. 19:1407-1420.
- Wright SI, Ness RW, Foxe JP, Barrett SC. 2008. Genomic consequences of outcrossing and selfing in plants. *International Journal of Plant Sciences*. 169:105-118.

- Wright SI, Kalisz S, Slotte T. 2013. Evolutionary consequences of self-fertilization in plants. *Proceedings of the Royal Society B: Biological Sciences*. 280:20130133.
- Wyatt R. 1988. Phylogenetic aspects of the evolution of self-pollination. In: Gottlieb L, Jain S, eds. *Plant evolutionary biology*. Chapman & Hall, London: pp. 109-131.
- Zhang T, Qiao Q, Novikova PY, Wang Q, Yue J, Guan Y, Ming S, Liu T, De J, Liu Y, Al-Shehbaz IA. 2019. Genome of *Crucihimalaya himalaica*, a close relative of *Arabidopsis*, shows ecological adaptation to high altitude. *Proceedings of the National Academy of Sciences*. 116:7137-7146.