Abstract

Reproductive Plasticity in Two Subspecies of a Cleistogamous Plant, *Triodanis perfoliata*

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Dimorphic cleistogamy is an intriguing reproductive strategy in which a plant produces both closed (cleistogamous), obligately selfing flowers and open (chasmogamous) flowers. Phenotypic plasticity in the production of chasmogamous and cleistogamous flowers has been demonstrated in many cleistogamous species and has been argued to be adaptive. Two subspecies of *Triodanis perfoliata* (Campanulaceae) exhibit dimorphic cleistogamy but differ in allocation to cleistogamous and chasmogamous flowers. I hypothesized that the divergence in flower ratio evolved from a plastic response that allowed the species to persist in a new habitat. Supporting evidence of this hypothesis would be finding that when placed in an environment similar to *T. perfoliata* ssp. *biflora*, *T. perfoliata* ssp. *perfoliata* demonstrates traits typical of *T. perfoliata* ssp. *biflora,* the derived subspecies.

I demonstrated that the habitats of the two subspecies differ in soil texture and light intensity. Many of these habitat differences are factors that have been shown to induce plastic responses in other dimorphic cleistogamous species. Using a series of growth room experiments I tested the hypothesis that chasmogamous and cleistogamous flower production is plastic in the two subspecies. Plasticity in response to light environment was tested by exposing plants of each subspecies to high and low light

treatments while plasticity to soil type was tested using a reciprocal soil transplant. Flower production in both subspecies of *Triodanis perfoliata* was found to be phenotypically plastic in response to light. The proportion of chasmogamous flowers produced was three times higher in light than in shade for both subspecies. This response is the opposite of the expected response of *T. p.* ssp. *perfoliata* under the hypothesis that reproductive plasticity promoted divergence in *T. p.* ssp. *biflora*. In response to soil type no reproductive plasticity was observed in either subspecies of *Triodanis perfoliata*. Although the results did not support the hypothesis that plasticity allowed the divergence of these two subspecies, the finding of plasticity in chasmogamous flower production could provide insights into the maintenance of cleistogamy in *Triodanis perfoliata*.

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INTRODUCTION

Plant Mating Systems

The diversity of mating systems in plants has been a topic of longstanding interest for evolutionary biologists and botanists. There are numerous ways in which plants can reproduce both sexually and asexually. Sexual reproduction can occur either by cross-fertilization (outcrossing) or self-fertilization (selfing). Outcrossing is the mating of two plants that are not closely related to produce offspring. The major selective advantage of outcrossing is the avoidance of inbreeding depression, the reduction in fitness of a population due to inbreeding. Inbreeding depression is caused primarily by the expression of deleterious recessive alleles in inbred offspring (Charlesworth and Charlesworth 1987).

Many species have evolved mechanisms that prevent self-fertilization and therefore increase outcrossing. These include herkogamy (separation of male and female floral parts in space), dichogamy (separation of male and female floral parts in time) and self-incompatbility (mechanism that allows a plant to recognize its own pollen and stop self-fertilization). In contrast, some species reproduce by self-fertilization or the fertilization of a flower by its own pollen. Two hypotheses that have been put forward to explain the evolution of selfing are the reproductive assurance hypothesis and the transmission advantage hypothesis. The reproductive assurance hypothesis simply states that self-fertilization will be favored when mates or pollinators are limited (Baker 1955). If there are no pollinators or few mates present in an area it would be advantageous for a species to reproduce by self-fertilization. The transmission advantage hypothesis states that a self-fertilization allele will increase its own transmission and therefore spread throughout an outcrossing population. If an individual partakes in selfing and outcrossing it will gain the advantage of fertilizing its own ovules, fertilizing others ovules, and

having its ovules fertilized by another individual. This creates a three to two advantage over an individual that only outcrosses (Fisher 1941). This leads to the prediction that complete outcrossing will evolve when inbreeding depression is larger than 50% and complete selfing will evolve when inbreeding depression is smaller than 50% (Lande and Schemske 1985). This is clearly not always the case, however, as some plants use both selfing and outcrossing for reproduction in what is termed to be a mixed mating system.

Mixed Mating Systems

Mixed mating systems, though thought to be evolutionarily unstable, are not uncommon in the botanical world. The major argument that has been put forward for the instability of mixed mating is the purging hypothesis (Lande and Schemske 1985). When an individual self-fertilizes, the chance that a homozygous recessive offspring will be produced increases. Deleterious recessive alleles that are expressed will be removed by selection from the population. Because of this a population that exhibits outbreeding will often contain more deleterious alleles than a population that continually uses self-fertilization. Because deleterious recessive alleles are purged with constant selfing, the threat of inbreeding depression is removed from the equation. Based on this hypothesis it would seem very unlikely that a species that has adopted self-fertilization would continue to also use outcrossing. The reality however, is quite to the contrary. There are many successful plant species that have adopted a mixed mating method that encompasses both selfing and outcrossing as a means of reproduction. Goodwillie et al. (2005) report that 42% of 345 species for which data currently exist demonstrate a mixed mating system, defined as mating in which the proportion of selfed offspring is between 0.2 and 0.8. Though mixed mating systems are found in many species, their maintenance is still yet to be fully explained (Goodwillie et al. 2005).

Cleistogamy

The central focus of this study is a type of mixed mating system that involves cleistogamy. Cleistogamy is an intriguing reproductive strategy in which a plant produces closed, obligately selfing, sometimes apetalous flowers (reviewed in Culley and Klooster 2007). Fully cleistogamous species are uncommon; most cleistogamous species produce both cleistogamous and chasmogamous flowers, which are typical open flowers that are capable of outcrossing. The production of both flower types is referred to as "dimorphic cleistogamy" by Culley and Klooster (2007). Some cleistogamous species have also been documented to create a third type of flower known as an induced cleistogamous flower. Though these flowers are morphologically similar to chasmogamous flowers they never fully open or partake in anthesis (Culley and Klooster 2007). Schoen and Lloyd (1981) attribute the production of this flower type to a halt in the development of a chasmogamous flower due to environmental stress.

Cleistogamy has been found to be present in 50 families and a total of 693 species (reviewed in Culley and Klooster 2007). It has evolved independently a number of times which is demonstrated by its appearance in many taxa that are not related (reviewed in Lord 1981). This information coupled with the knowledge that there are limited cases of the evolutionary loss of cleistogamy supports the statement that cleistogamy has adaptive value under certain circumstances (Oakley et al. 2007). This unique type of mating system is expected to be beneficial because a plant gains the advantages of reproductive assurance through the cleistogamous flower but maintains the ability to outcross through chasmogamous flowers (Oakley et al. 2007).

Despite its apparent advantages, the maintenance of this mixed strategy has posed a challenge for evolutionary biologists. Cleistogamous corollas are usually smaller, sometimes

apetalous and closed which means the plant can devote less of its resources to producing and maintaining them (Schemske 1978). In addition, cleistogamy confers reproductive assurance, since pollinators and mates are not needed for reproduction. The presumed advantage to producing larger, outcrossing flowers is avoidance of inbreeding depression; however, when a plant is regularly self-fertilizing the frequency of inbreeding depression is expected to be low (Lande and Schemske 1985). With the advantages of continuous self-fertilization clearly demonstrated, the maintenance of chasmogamous flowers in this system is puzzling.

It has been proposed that plasticity of chasmogamous and cleistogamous flower production could be an important benefit that encourages the maintenance of this mixed mating system. The argument for plasticity in flower type (cleistogamous and chasmogamous) was first presented by Schoen and Lloyd (1984). They created a basic model that lays out all the conditions proposed to favor the evolution of cleistogamy. Out of the basic model comes the "complex habitat" model, which states that the flower type produced will vary according to which type is more successful in a heterogeneous environment.

Phenotypic Plasticity

Phenotypic plasticity refers to the phenomenon when organisms with the same genotype are capable of producing different phenotypes in response to different environmental factors (Bradshaw 1965; Thibert-Plante and Hendry 2011). This ability to change phenotype in unpredictable environments allows an organism to cope with changes in a habitat within its lifetime, unlike adaptation which is a gradual genetically-based change. Phenotypic plasticity is particularly beneficial for species that have longer generation times, as the evolutionary reactions due to natural selection will not allow the organism to change quickly enough to moderate the effects of being exposed to a less than favorable or variable environment (Williams et al. 2008).

Plants need this ability because of their inability to move from an unfavorable environment. Phenotypic plasticity can be either 1) adaptive, a genotype that expresses a phenotype that will increase the organisms likelihood of survival in each environment or 2) nonadaptive, expression of a phenotype that does not improve fitness (Padilla and Savedo 2013). Some of the most notable adaptive plastic traits found in plants are height and leaf size (Zervoudakis et al. 2012). When grown in shady environments plants will increase in height or leaf surface area. These are recognized as adaptive traits as they increase the survival ability of the plant. Though phenotypic plasticity does not involve any genetic change it is possible that a trait acquired through this method could eventually become genetically encoded into a population. Therefore, it has been argued that phenotypic plasticity can play an essential role in evolutionary change (Thompson 1991).

The American psychologist, James Mark Baldwin, first put forth the idea that phenotypic plasticity might promote speciation in an 1896 paper "A New Factor in Evolution." Baldwin stated that a novel trait acquired through plasticity in response to a variable environment can become slowly assimilated into the population. Though he focused more on learning ability and behavior of the species, Baldwin's overall idea is essentially the same in regards to plasticity promoting speciation. The hypothesis was dubbed the "Baldwin effect" by George Simpson who agreed that this notion was conceivable but had doubts about its rate of occurrence.

Waddington (1953) suggested that phenotypic plasticity influences evolution and speciation through genetic assimilation. Genetic assimilation is a method by which a phenotype first produced by an organism in response to environmental variation becomes genetically fixed through natural selection. There are two means by which genetic assimilation has been proposed to occur. The first is when selection eliminates plasticity causing one phenotype in the

population to become fixed. The second is the loss of plasticity through mutational degradation or genetic drift (selection of alleles through chance, Pfennig et al. 2010). Diggle and Miller (2013) demonstrated the phenomenon of genetic assimilation using members of the plant family, Solanaceae. They started with a phylogenetic analysis and ancestral reconstruction of 19 species of sections *Lasiocarpa* and *Acanthophora*. They found a high probability of plasticity in the ancestral state using parsimony and Bayesian reconstructions. In an experiment the authors examined the proportion of staminate flowers produced in each of the fourteen species when subjected to variable environments and compared them to the ancestral reconstructions. They found that for nine of the fourteen species staminate flower reproduction was phenotypically plastic while the five other species showed no sign of plasticity when subjected to the treatment. Three of the five nonplastic species consistently produced greater amounts of staminate flowers while the other two produced very few. They concluded that the evolution from a plastic ancestor to the now nonplastic staminate flower production in certain species suggests genetic assimilation.

Phenotypic plasticity might allow populations to persist in new habitats, allowing time for genetic adaptation to the new environment to evolve (Figure 1). In her "developmental plasticity hypothesis of speciation", West-Eberhard (2003) argued that a strong relationship between the environment and a phenotype due to adaptive phenotypic plasticity can result in speciation in a few steps. In the first step fixation of the original phenotype and an alternative phenotype (acquired through plasticity) occur in different populations with little to no genetic change. Second, each phenotype will then undergo the process of genetic assimilation due to divergent selection (these two phenotypes are superior in their respective environments). Last, the two populations will become reproductively isolated because of adaptive divergence.

In the developmental plasticity hypothesis of speciation, adaptive phenotypic plasticity and ecological speciation (populations become reproductively isolated through evolution as a result of ecological selection) (West-Eberhard 2003). Without plasticity, it would be more difficult for the species to colonize a new environment or survive in current changing environment. Because of its plastic ability the organism is able to persist in the new habitat, allowing time for selection to act on the newly produced phenotype. Once selection has acted on the population the frequency is moved closer to the new fitness optimum which may eventually result in fixation of the new phenotype (Fitzpatrick 2011).

Phenotypic Plasticity and Cleistogamy

Plasticity in the production of cleistogamous vs. chasmogamous flowers has been demonstrated in various studies and plant species (Table 1). In general, chasmogamous flowers are more expensive than cleistogamous flowers and have been found to be favored in environments with ample resources (Schemske 1978). Many factors such as light intensity, soil composition, water availability, pollen limitation, and competition have been shown to induce a plastic response in allocation to chasmogamous vs. cleistogamous flowers (Table 1). These factors can affect reproductive mode either directly or indirectly as most of these factors (light, water availability, and nutrients) can also affect plant size. Plant size has also been shown to influence the production of flower types. For example, an increase in nutrient availability may lead to an increase in plant size, and increased plant size might cue greater production of chasmogamous flowers, making nutrient availability an indirect cause of plasticity.

In many cases an increase in light intensity has led to an increase in production of chasmogamous flowers (Schemske 1978, Waller 1980). Trapp and Hendrix (1988) found that light had indirect impacts on flower type in *Amphicarpaea bracteata* (L.) Fernald, through its effect

on plant size. In their study, plants grown in higher light intensity resulted in larger plants that produced more chasmogamous flowers. Some soil properties such as composition and moisture have also been shown to produce a plastic response in cleistogamy (Bell and Quinn 1987; Waller 1980). Wilken (1982) found that with low sand content of the soil, the production of chasmogamous flowers in *Collomia grandiflora* Douglas ex Lindl. increased along with an increase in plant size. This increase in chasmogamy was expected because soil with a higher sand content will typically be drier and have lower nutrient availability. In another example, Bell and Quinn (1987) studied the effects of soil moisture in *Dichanthelium clandestinum*, a species with dimorphic cleistogamy. *Dichanthelium clandestinum* was found to produce fewer chasmogamous flowers in soils with less moisture.

Another factor shown to affect reproductive mode is pollen limitation. Using *Collomia grandiflora,* Albert et al. (2011) found that a limitation in pollen resulted in an increase in cleistogamous flower production. They argued that pollen limitation and phenotypic plasticity could be a key factor in the maintenance of cleistogamy.

Evolution of Cleistogamy in Triodanis perfoliata

An annual cleistogamous species, *Triodanis perfoliata* (L.) Nieuwl. (Campanulaceae) also known as clasping Venus' looking-glass is a unique taxon for study of the role of cleistogamy and phenotypic plasticity in evolution. Two subspecies of *Triodanis perfoliata* are present in North Carolina and throughout North America: subspecies *perfoliata* and subspecies *biflora (*hereafter referred to as *T. p.* ssp. *perfoliata* and *T. p.* ssp. *biflora).* Although the subspecies often co-occur, genetic and morphological evidence indicates that they have a high degree of reproductive isolation (Stewart 2013). Reproductive isolation or, the inability of a species to interbreed with another species and produce viable offspring, can result from a number

of factors (Mayr 1963). An isolating mechanism that may be responsible for divergence of *Triodanis perfoliata* is difference in habitat. Anecdotally, *T. p.* ssp. *perfoliata* has typically been observed in shady areas in sandy drained soils at the edge of woodlands while *T.p.* ssp. *biflora* is often found in open areas with high light in clay-dominant soils.

Plasticity in chasmogamous and cleistogamous flower production has been argued to be an adaptive advantage of dimorphic cleistogamous species. However, the plastic ability of *Triodanis perfoliata* has not yet been evaluated. Intriguingly, the two major environmental factors that appear to differ in the habitats of the subspecies -- light level and soil type -- have been shown to induce plastic responses in other dimorphic cleistogamous species. The subspecies differ in their allocation to the two flower types: *T.p.* ssp. *perfoliata* produces a large inflorescence of chasmogamous flowers while *T.p.* ssp. *biflora* produces mostly cleistogamous flowers and only one or two chasmogamous flowers. *Triodanis p.* ssp. *perfoliata* is assumed to be the ancestral species in this case because all other members of the *Triodanis* genus also produce a large inflorescence of chasmogamous flowers. Therefore, *T. p.* ssp. *biflora*, the diverged species provides the most parsimonious explanation of evolution. The difference in flower allocation provides an opportunity to explore the evolutionary processes and selective factors that have played a role in this recent divergence in reproductive strategy.

In this study I test the hypothesis that chasmogamous and cleistogamous flower production is plastic in the two subspecies of *Triodanis perfoliata.* If present, I ask whether plasticity in flower type production could have played a role in subspecies divergence in reproductive strategy. This response is hypothesized to be the key factor that has allowed the species to persist initially in a habitat typical of *T.p.* ssp. *biflora*. Supporting evidence of this hypothesis would be the finding that plasticity of *T.p.* ssp. *perfoliata* when placed in an

environment similar to *T.p.* ssp. *biflora* will parallel changes in *T.p.* ssp. *biflora* and show reduced allocation to chasmogamous flower production. Further, *T. p.* ssp. *biflora*, the derived species is predicted to show less plasticity than *T. p.* ssp. *perfoliata* under this hypothesis.

This study aims to address the following questions: 1. When and where are chasmogamous and cleistogamous flowers produced in each subspecies?, 2. Do *T.p.* ssp. *perfoliata* and *T.p.* ssp. *biflora* exhibit plasticity in allocation to chasmogamous and cleistogamous flowers?, 3. Which environmental factors, if any affect the floral reproductive plasticity of *T.p.* ssp. *perfoliata* and *T.p.* ssp. *biflora*., 4. Do the habitats of *T.p.* ssp. *perfoliata* and *T.p.* ssp. *biflora* differ in light and soil properties?, 5. How do these environmental factors affect the overall success of each subspecies?, 6. What role, if any, did phenotypic plasticity play in the divergence of *T.p.* ssp. *perfoliata* and *T.p.* ssp. *biflora*? That is does plasticity in flower type production, if present, parallel differences observed in habitat features and reproduction in the two subspecies?

METHODS

Study Species

Triodanis perfoliata (L.) Nieuwland, a weedy annual member of the Campanulaceae family. Two subspecies of *T. perfoliata* can be found throughout much of North and Central America occurring in disturbed areas (Goodwillie and Stewart, 2013). Both *Triodanis perfoliata* subspecies *perfoliata* (L.) Nieuwl. and *Triodanis perfoliata* subspecies *biflora* (Ruiz and Pavon) Lammers produce chasmogamous and cleistogamous flowers. Chasmogamous flowers of both subspecies have a five petalled corolla (1-1.5 cm) that is violet blue in color, five anthers and a three-lobed stigma. Cleistogamous flowers of both subspecies are bud-like in appearance and apetalous. Flowers are produced during the early summer, continuing for about a month. After pollination the plant produces a capsule containing small reddish- brown lens shaped seeds.

Though they are not a prominent topic in the literature, there is some documentation of pollinators of *Triodanis perfoliata*. Gara and Muenchow (1990) noted that the megachilid bee (Hymenoptera), sphecid wasp (Hymenoptera), and leatherwing beetle (Coleoptera) were the three most common visitors of the chasmogamous flowers in *Triodanis perfoliata*. In populations in Pitt County, NC, chasmogamous flowers of both subspecies are visited by a large variety of insects; the most commonly observed were from the orders Diptera, Hymenoptera, and Coleoptera (C. Goodwillie, unpublished data).

The chasmogamous flowers of each subspecies also exhibit protandry and delayed selfing (Figure 2). When the flower first opens it is in the male phase; by then the anthers have dehisced depositing their pollen on the outside of the style. One or two days later the flower enters its female phase where the stigma matures and opens up into three lobes, making it possible for

fertilization to take place. After a few more days the three lobes begin to curl under (reflex), exposing the lobes to the self-pollen that was previously deposited on the outside of the stigma (Faegri and Van der Pijl 1979, C. Goodwillie, unpublished data).

Flower allocation in the two *Triodanis perfoliata* subspecies differs; T. p. ssp. perfoliata produces a large number of chasmogamous flowers while *T. p.* ssp. biflora produces mostly cleistogamous flowers. Certain morphological features can also be used to distinguish the two subspecies of *Triodanis perfoliata*. The floral bracts of *T.p.* ssp. *perfoliata* are broader and have cordate (heart-shaped) bases whereas the bracts of *T.p.* ssp. *biflora* are longer with cuneate (wedge-shaped) based. The location of the capsule pore also differs. In subspecies *T.p.* ssp. *perfoliata* the pore is located at the center or base of the capsule while in *T.p.* ssp. *biflora* the pore is located at the top of the capsule (Bradley, 1975). A difference in the color of the pollen between the two subspecies has also been observed; *T.p.* ssp. *biflora* has white pollen and *T.p.* ssp. *perfoliata* has light to dark purple pollen.

Developmental Study

As a starting point for the study of plasticity in chasmogamous and cleistogamous flower production, I conducted an observational study of the developmental pattern of flower production in field-grown plants. The two populations of each subspecies used were located in Pitt County (P0102, P0304, B1314, and B2728, Table 2). In each population, six marked individuals were followed through the 2014 field season from initiation of flowering to senescence, May 2 through June 29 after the last plant senesced. I recorded data on the location on the plant and the timing of the initiation and fruit maturation of chasmogamous and cleistogamous flowers. The timing of chasmogamous flower opening was also recorded. Plants were observed every other day, and the number, type (chasmogamous or cleistogamous) and

status of flowers in each node of each plant was recorded. The data were analyzed through graphical representation of the average number and type of flower per node and average date of dehiscence.

Habitat Study

The objective of this study was to quantify differences in light and soil composition of habitats typical of each of the subspecies that had been noted anecdotally. Sampling sites were located throughout Pitt County. Thirteen sites were sampled for each of the *Triodanis perfoliata* subspecies (Table 2 and Table 3). In some sites, a single subspecies was present. In others, one subspecies was predominant, but the other was present at low frequencies. All habitat sampling took place June 2-3, 2014.

***Light environment*--**A densiometer was used to assess the light environment of plants at each study site. A densiometer is a small hand held device used to quantify canopy cover. It contains a concave reflective sphere divided into 24-1/4'' squares. To use this device the operator holds it above the area of interest and counts the number of squares covered by the canopy above, giving an estimate of percent of canopy coverage. Each of the dots represents roughly one percent of coverage. Densiometer readings were taken above two haphazardly chosen plants at each of the sites. After averaging the two replicates for each site, a two-sample *t*-test was performed to test whether mean canopy coverage significantly differed between the habitats of the two subspecies.

***Soil environment*--**Two soil samples were collected at the thirteen sites using a 10 cm carbon steel, sand auger. Samples were taken from haphazardly chosen areas with at least one or more plants close by. All soil samples were initially air-dried and passed through a 2-mm sieve to remove any rocks or other debris.

Soil chemical analysis--Portions of the two samples taken at each site (excluding sites P0506, P1516, P2526, B0708, B1112, and B2324) were pooled into one individual sample and sent to NCDA&CS Agronomic Division for analysis. Approximately 100 mL of each soil sample was mixed and placed into a sample box provided by the Pitt County Agricultural Center. Soil sample boxes were prepared using the general guidelines set forth by North Carolina State University in the following document: http://content.ces.ncsu.edu/a-gardeners-guide-to-soil-testing.pdf

A two-sample *t*-test was used to analyze the difference between each measured soil variable between the two subspecies. A modified Bonferroni method (Rice 1989) was used to adjust significance level and reduce the possibility of having a significant *P*-value based solely on chance.

Soil texture analysis--Hydrometer analysis was used to quantify variation in soil texture (Bouyoucos 1962). A hydrometer is a glass tool consisting of a stem and a mercury weighted bulb used to measure the relative density of liquids. The depth to which the hydrometer sinks depends on density of particles suspended in the solution. Particle size distribution can be determined by differences in settling rates; large sand particles will sink faster than smaller silt or clay particles resulting in a lower hydrometer reading.

A sub-sample of 100 g was taken from each soil sample and oven dried at 105 °C for 24 h. Each sub-sample was then ground lightly with a mortar and pestle and placed in a glass Mason® jar with 100 mL of 5% sodium hexametaphosphate solution and 250 mL of distilled water. Sodium hexametaphosphate is used as a dispersing agent, keeping the suspended soil particles from aggregating. The soil and solution was thoroughly mixed and allowed to sit overnight. On the next day the sample was remixed for five minutes by shaking vigorously. A

1000 mL glass cylinder "blank" with 100 mL of hexametaphosphate plus 900 mL of distilled water was used to calibrate the hydrometer.

The soil mixture was then placed in a glass cylinder, and distilled water was added to obtain a final volume of 1000 mL. The temperature of the sample was measured before each series of hydrometer readings. After mixing the column thoroughly by inversion, the hydrometer was gently placed into the column containing the sample. Readings were taken at 15, 30, and 60 sec. These three readings were then repeated two more times, remixing the sample before each replicate. Two final readings were taken after the column was left undisturbed, one at 1 h, followed by another at 24 h.

Before analysis each hydrometer reading was corrected using the temperature and blank readings. The temperature was used to correct the readings by adding 0.2 units for every degree above 20°C and subtracting 0.2 units for every degree below. The value of the blank reading was subtracted from the sample reading. Corrected values from the two replicates were averaged for each set of readings, and subspecies were compared using a repeated measures analysis of variance with sampling sites as subjects, subspecies as the between-subjects variable and the five time frames as the within-subject variables. All analyses were performed using SPSS, version 22 (SPSS, Armonk, NY).

Soil water retention--A cone-tainer® (Stuewe and Sons, Corvallis, OR) with a 14 cm depth and 3.8 cm diameter was filled with a sample of soil from each of the thirteen sites per subspecies. The cone-tainers were subirrigated for two days to become completely saturated with water. The saturated samples were then removed from the tub and allowed to sit for an hour to drain any excess water. At this point, I used a soil moisture probe (Decagon EC5, Pullman, WA) to obtain a soil moisture reading of capacitance (the dielectric resistance of the soil) to estimate

the proportional content of water in soil volume. Soil moisture levels were taken again the following day and at two-day intervals for up to eight days.

The soil moisture measurements taken on each day were averaged for each site and then compared between subspecies using a repeated measures analysis of variance with sampling sites as subjects, subspecies as between-subjects variable and days as the within-subjects variable.

Plasticity Study

Growth room experiments tested for plasticity in the production of cleistogamous and chasmogamous flowers in response to two environmental factors light levels and soil type. All experiments were carried out between September 2014 and June 2015. Seeds were collected from three sites for each subspecies (P0102, P0304, P1314, B0506, B2324 and B2526) at the end of May 2014. Maternal seed families were collected from at least 30 individuals in each population and taken only from the cleistogamous capsules of each plant. For each experiment (light and soil), seeds from each maternal family were sown in separate cone-tainers and raised in a growth room at East Carolina University (Greenville, NC).

***Light*--**To test for plasticity in response to light environment, plants of each subspecies were exposed to high and low light treatments. The low light treatments were created using PVC pipe and unbleached muslin to make shade canopies. Using a MQ-200 Quantum Separate Sensor, the light treatment was measured to have 265 µmol m-2 s-1 on average, while the shade treatment was at around 25 µmol m-2 s-1. Approximately 2 weeks after seedlings germinated, each cone-tainer was thinned until only one plant remained. Plants were then randomly assigned to treatments such that each plant in a treatment group was from a different maternal family. Twenty plants from each population were assigned to each treatment for a total sample size of 60 plants per subspecies per treatment. The cone-tainers were placed in a tray with a large tub

underneath it for subirrigation. Trays in high light treatments were placed directly under greenhouse sodium or metal halide light fixtures. Plants were randomly assigned to twenty four trays (blocks, twelve per treatment). Trays in each treatment were moved around the growth room twice weekly to minimize positional effects. Plants were watered using subirrigation and fertilized once (12-55-6 nitrogen - phosphate – potassium, Super Bloom ®, Green Light Company, San Antonio, Texas).

Variables measured for each plant include: number of each flower type (CH, CL, and ICL) on the main stem, height, bract size, number of lateral stems, and number of each flower type on one representative lateral stem. Total flower number was calculated as the total number of each flower type on the main stem plus the total number of flowers on the lateral stems (product of flower number on one lateral stem times the number of lateral stems). For bract size measurement, the tenth floral bract of each plant was collected from the main stem, counting from the base up. After collection, the width of each bract was measured using a digital caliper. Data on height and flower number were collected at plant senescence. Once all data were obtained the plants were dried and biomass was measured to test if overall performance in two environments differed.

ANOVA was used to analyze each dependent variable, with light treatment as a fixed factor, population as a random factor, and block set as a random factor nested within treatment. The dependent variables used to test for plasticity in each experiment were the proportion of chasmogamous flowers, total number of flowers, height, node number, bract size, and biomass. All proportion data were arcsine transformed and the square root of all count data taken before analysis. Generalized Estimating Equation (GEE) analysis was used to test for differences in the proportion of induced cleistogamous flowers with subject variable set as individual plant,

treatment and population set as predictors, using a linear model. GEE analysis was used because it is more robust and less sensitive to violations of assumption that resulted from the abundance of zeros found in this data set (Ballinger 2004).

Soil--To test for reproductive plasticity in response to soil type, a reciprocal soil transplant was performed. Soil samples were collected from a depth no lower than 30 cm at the three sites from which seeds were collected for each subspecies (P0102, P0304, P1314, B0506, B2324, and B2526). Ten samples were randomly collected from each site, and soils from all three sites were pooled for each subspecies. Hereafter, these pooled samples will be referred to as "biflora soil" and "perfoliata soil". Before use all soil samples were frozen overnight at 20°C temperature to kill any insects that may have been present.

Individuals of *T.p*. ssp. *perfoliata* were planted in biflora soil while individuals of *T.p.* ssp. *biflora* were planted in perfoliata soil. Plants of each subspecies were also placed in their "home" soil type. Seed families of the three populations of each subspecies were initially sown in cone-tainers in a greenhouse mix. Twenty plants from each population were assigned to each treatment for a total sample size of 60 plants per subspecies per treatment. Plants were assigned randomly to treatments such that each plant in a treatment was from a different family. Seedlings were transferred to their respective treatments three weeks after germination. Trays were randomly arranged throughout the growth room, and moved at regular intervals to minimize the effects of variation in light distribution. Every other day plants were rotated between three watering applications: subirrigation, top watering and no watering. Devised to mimic both the drawing up of ground water and water acquired through rain, this watering scheme is designed to capture the full effect of the difference in drainage between the two soil types (i.e., more rapid drainage of water from sand compared to clay) .

Variables measured for each plant include; number of each flower type (CH, CL, and ICL) on the main stem, height, bract size, number of lateral stems, and number of each flower type on one representative lateral stem. Total flower number was calculated as the total number of each flower type on the main stem plus the total number of flowers on the lateral stems (product of flower number on one lateral stem times the number of lateral stems). Mortality of plants before seed maturation was also recorded as a component of fitness. In this experiment, data were not collected after plant senescence but rather when the majority of plants in the sample had reached maturity.

ANOVA was used to analyze each dependent variable, with soil treatment as a fixed factor, and population as a random factor. The dependent variables used to test for plasticity in each experiment were the proportion of chasmogamous flowers and total number of flowers. All proportion data were arcsine transformed and the square root of all count data taken before analysis. Again, Generalized Estimating Equation (GEE) analysis was used to test for differences in the proportion of induced cleistogamous flowers with subject variable set as individual plant, treatment and population set as predictors, using a linear model. A contingency table using Fisher's Exact Test was used to determine if there was an effect of treatment on the frequency of deaths occurring before reproductive maturity in each subspecies.

RESULTS

Developmental Study

Initial dehiscence date of cleistogamous and chasmogamous flowers in the *T. p.* ssp. *perfoliata* populations differed. Across 10 plants in two populations, capsules of cleistogamous flowers began to dehisce on May 12 (Julian date of 132) and chasmogamous flowers starting at May 20 (140). Production of chasmogamous flowers began later and ended earlier than cleistogamous flower production for both subspecies. For *T. p.* ssp. *perfoliata* 95% of the capsules that dehisced in the last 10 days of the study were cleistogamous. Induced cleistogamous flowers, which are found only in *T. p.* ssp. *perfoliata* populations, were seen only at the end of the season, with a dehiscence range from June 9 (160) to June 27 (178). The nodal position at which the two flower types first appeared also differed in *T.p*. ssp. *perfoliata*. On average, cleistogamous flowers were first presented in the 10th node of the plant while chasmogamous flowers appeared in the 21st node (Figure 3, Table 4). The cleistogamous flowers typically appeared in the lower nodes of the plant compared to chasmogamous flowers (Figure 4). The distribution of dehiscence dates for cleistogamous, chasmogamous, and induced cleistogamous flowers have a nonsymmetrical bimodality, a left skewed modal distribution, and a unimodal distribution, respectively (Figure 5).

The dehiscence range of cleistogamous flowers in *T. p*. ssp. *biflora* following 11 plants across two populations ranged from May 10 (130) to June 13 (164) while the chasmogamous dehiscence range is May 24 (144) to June 1 (152). As with *T. p.* ssp. *perfoliata* chasmogamous flowers appeared later and dehisced earlier than cleistogamous flower capsules. In the last 10 days of the study 100% of the capsules in *T. p.* ssp *biflora* that dehisced were cleistogamous. On average, cleistogamous flowers were first presented in the 5th node of the plant while

chasmogamous flowers first appeared in the 26th node; in most cases, a single chasmogamous flower was produced (Figure 6, Table 4). Cleistogamous flowers also begin to appear in the lower nodes of *T.p.* ssp. *biflora*, while there are typically only one or two chasmogamous flowers found only in the terminal node of the plant (Figure 7). The timing of dehiscence of cleistogamous and chasmogamous flowers graphed as nonsymmetrical bimodal distribution and a unimodal distributions, respectively (Figure 8).

Habitat Study

Light-- Nine of 13 *T. p*. ssp. *perfoliata* sites were found to have 50% or more canopy coverage compared to two of 13 *T.p* ssp. *biflora* sites. On average *T. p.* ssp. *perfoliata* sites had 53% canopy coverage compared to 15% at *T. p.* ssp. *biflora* sites. Grubb's test for outliers revealed that *T. p.* ssp. *biflora* had one outlier at site B1112 and subspecies *perfoliata* had one outlier at site P1516. All outliers were excluded from the *t*-test analysis. Mean percent canopy coverage was significantly greater at *T. p.* ssp. *perfoliata* sites than *T. p.* ssp. *biflora* sites (t = 2.18 df = 12, $P < 0.001$).

***Soil chemical analysis*--**Of the 15 soil parameters, percent humic matter, weight per volume and potassium index were significantly different between subspecies ($\underline{P} < 0.05$) level. Only the $\underline{P}$-value for weight per volume was still significant when adjustments were made for table-wide significance (Table 5).

***Soil texture analysis*--**A higher hydrometer reading indicates a greater amount of particles suspended in a solution. Grubb's test revealed that biflora soil had one outlier at the 1 and 24 hr time interval; perfoliata soil had no outliers. These outliers were excluded from the analysis. The repeated measures ANOVA found that there was a highly significant effect of time, subspecies, and a significant interaction between time × subspecies in soil texture (Table 6). The

interaction of time × subspecies indicates that there is a difference between subspecies in the rate at which readings change through time. On average biflora soil (15s = 35, 30s = 29, 60s = 26, 1hr = 15, 24hrs = 10) had significantly higher readings across all time frames compared to that of perfoliata soil (15s = 22, 30s = 17, 60s = 15, 1hr = 10, 24hrs = 7) indicating that perfoliata soil settles faster than biflora soil (Figure 9, Table 6). The faster settling rate found in perfoliata soil is likely because of its higher sand content when compared to that of biflora soil.

Soil water retention-- The repeated measures ANOVA showed a highly significant interaction between day, day × subspecies, and between subspecies in soil water retention (Table 6). The significant test value of day indicates that soils became drier with time, while day × subspecies indicates that there was a difference between subspecies in the rate at which the soils dry. The between subspecies significant *P* value indicates an overall difference in soil moisture between the subspecies. On average, soil moisture readings for biflora soil (day 1 = 0.37, day 2 = 0.31, day 3 =0.24, day 4 = 0.17, day 5 = 0.09) were significantly higher than perfoliata soil (day 1 = 0.37, day 2 = 0.27, day 3 =0.19, day 4 = 0.15, day 5 = 0.07), indicating that perfoliata soil drained more rapidly (Figure 10, Table 6). This difference is likely to be due to the higher sand content of perfoliata soil.

Plasticity Study

Light-- Unfortunately, fungus gnats caused a high degree of early mortality, especially in the shade treatment. The final sample size for *T. p.* ssp *perfoliata* was 60 individuals in the light treatment and 42 in the shade. For *T. p.* ssp. *biflora* there were 52 individuals in the light treatment and 37 in the shade treatment surviving the fungus gnat outbreak. Reduction in the sample sizes resulted in an unbalanced design that did not have a representative from each population in every block. As a result, the data could not be analyzed using the full model, and

ANOVAs for population and block were run separately, each with treatment. In analyses of all variables in both subspecies either population and block or both were found to be non-significant. When population was found to be significant but block was not the results for the population ANOVA were used and vice versa. For *T. p.* ssp. *perfoliata*, population and block were found to be non-significant in most cases. For the proportion of chasmogamous flowers population was significant while block was not, and for biomass block was significant and population was not (Table 7). In *T .p.* ssp. *biflora* population was not significant in all cases. However, the interaction between treatment and population was found to be significant (or marginally significant* defined as slightly above 0.05 but below 0.06) for height, node number*, bract size, and biomass. For proportion of chasmogamous flowers population was not significant but block was marginally significant (Table 8). Proportion of induced cleistogamous flowers in *T. p.* ssp. *perfoliata* was found to be significant for treatment, block, and population (Table 9).

I found evidence of plasticity in plant height in *T. p.* ssp. *perfoliata*. Plants grown in the shade were taller on average than plants grown in high light. No significant difference in height was seen for *T. p.* ssp. *biflora* (Table 10 and 11). Another vegetative trait that showed variation between the two treatments was bract size. In *T. p.* ssp. *perfoliata* bract size was significantly wider in the shade when compared to bracts from the light treatment (Table 7). Again, the difference was not significant in *T. p.* ssp. *biflora* (Table 8). As for reproductive plasticity, the proportion of chasmogamous flowers produced increased when subjected to high light conditions for both subspecies (Figure 11 and 12). In contrast, the proportion of induced cleistogamous flowers increased in the light in plants of *T. p.* ssp. *perfoliata*. Plants of both subspecies generally performed better in light than in shade. *Triodanis p.* ssp. *perfoliata*, biomass was two times higher in the light treatment than in the shade; *T. p.* ssp. *biflora* biomass was 3.6 times higher.

On average, total flower number was 1.5 times higher in *T. p.* ssp. *perfoliata* in the light treatment and 2.5 times higher in *T. p.* ssp. *biflora*.

***Soil*--** Analyses did not find any plasticity in the production of flower types; soil treatment did not have a significant effect on proportion of chasmogamous flowers in either subspecies (Figure 16 Table 12 and 13). In *T. p.* ssp. *biflora*, however, populations differed significantly in the proportion of flowers that were chasmogamous. Neither subspecies differed in the production of induced cleistogamous flowers in the soil experiment (Table 14). Soil treatment did have some effects on plant performance. In *T. p.* ssp. *perfoliata* both treatment and population were found to have a significant effect on total flower number. Both subspecies produced a greater total number of flowers when grown in biflora soil. On average, *T. p.* ssp. *perfoliata* produced 45 flowers in biflora soil and 32 in its own soil while *T. p.* ssp. *biflora* produced 103 flowers in its own soil and 85 in perfoliata soil (Figure 17 and 18, Table 12 and 13). The soil transplant experiment had little mortality in all of the treatments except for *T. p.* ssp. *perfoliata* in biflora soil. Final sample size for *T. p.* ssp *perfoliata* was 59 individuals in the home soil treatment and 40 in the biflora soil treatment. In *T. p.* ssp. *biflora* there were 57 individuals in the home soil treatment and 59 in the perfoliata soil treatment. The contingency table results showed that the number of deaths occurring before reproduction in *T. p.* ssp. *perfoliata* was significantly higher in biflora soil than in perfoliata soil ($P = 0.0022$). In *T. p.* ssp. *biflora* no difference in mortality was found between soil types ($P = 0.6186$). In *T. p.* ssp. *biflora*, the growing tips of plants were observed to die in nine plants, all of which were on perfoliata soils. The frequency of this differed significantly between soil types ($P = 0.0038$).

DISCUSSION

Plasticity

The light and soil plasticity studies offer a mix of results. Little to no phenotypic reproductive plasticity was seen in response to soil type in the two subspecies of *Triodanis perfoliata*. This contrasts with other studies that have looked at the influence of soil type on flower production in cleistogamous species (Bell and Quinn 1987, Wilken 1982). Wilken (1982) found that a decrease in sand content led to an increase in chasmogamous flower production in *Collomia grandiflora*, which is consistent with the idea that an increase in resources will lead to an increase in chasmogamous flower production. A decrease in sand content will increase resources because water does not drain as quickly from less sandy soil. It is perhaps not surprising that my results were incongruent with previous studies, as the two soils used were quite similar on many levels. Both are mineral soils with only subtle differences in texture and no significant differences in most aspects of chemical composition. In contrast, Wilken (1982) compared flower production in soils that differed dramatically with one being a 2:1 soil to sand ratio and the other being 1:2. Interestingly, when compared to plants from the light studies which were grown in a greenhouse soil mix, *T. p.* ssp. *perfoliata* in biflora soil had 45% chasmogamous flower production whereas the light study plants displayed only 19%. Therefore, it appears that plasticity in response to soil type might have been observed with more distinct soil types.

Chasmogamous flowers production increased in higher light environments. Both the number and proportion of chasmogamous flowers increased in the light treatment of the two subspecies. The number of chasmogamous flowers was 4.4 times higher in light than in the shade in *T. p.* ssp. *perfoliata* and 7.4 times higher in *T. p.* ssp. *biflora*, on average. Proportion of

chasmogamous flowers produced was three times higher in light than in shade for both subspecies. This finding is expected as many other cleistogamous species have also been shown to be plastic in this direction (Schemske 1978, Trapp and Hendrix 1988, and Waller 1980). Masuda and Yahara (1994) observed the same pattern in *Impatiens noli-tangere* L., with higher production of chasmogamous flowers in high light conditions than in shaded conditions. In many cases an abundance of resources leads to an increase in chasmogamy (Waller 1980, Le Corff 1993, Imiazumi et al. 2008, Munguía-Rosas et al. 2012). In her study of the effects of light and nutrient availability on *Calathea micans,* Le Corff (1993) observed that an increase in light and nutrient availability led to an increase in chasmogamy. Waller (1980) found that when exposed to low light and drought like conditions *Impatiens capensis* produced only cleistogamous flowers whereas chasmogamous flowers were produced when plants were given adequate water and more light. Because chasmogamous flowers are more expensive to produce it makes sense that their production would increase as resource availability increases (Schemske 1978).

Light also significantly increased biomass and total flower number in both subspecies. On average, biomass of plants in the light treatment doubled in *T. p.* ssp. *perfoliata* and by 3.6 times in *T. p.* ssp. *biflora* when compared to shade plants. Total flower number of *T. p.* ssp *perfoliata* increased 1.5 times in the light treatment while *T. p.* ssp. *biflora* increased 2.5 times. This increase in plant size and total flower number in the light treatment raises the possibility that the increase in chasmogamous flowers with light could be caused by effect of plant size rather than a direct cue from light. Plant size has been shown to affect chasmogamous flower production (Diaz and Macnair 1998). Though resources may directly increase plant size the actual cue to make more chasmogamous flowers is not the increase in resources but the increase in size caused by the abundance of resources. Light has been found to have indirect effects on

chasmogamous flower production through plant size in other dimorphic cleistogamous species (Trapp and Hendrix 1988, Waller 1980). To evaluate whether light has a direct or indirect effect on proportion of chasmogamous flowers in this study, ANOVA was run with light treatment and biomass as fixed factors, population as a random factor, and block set as a random factor nested within treatment. The model was first run with an interaction term between light treatment and biomass. After determining there was no significant interaction between light treatment and biomass for *T. p.* ssp. *perfoliata* ($P = 0.5332$) or *T. p.* ssp. *biflroa* ($P = 0.5289$) the interaction term was dropped from the model. The model was then run again with biomass as the main effect. It was determined that biomass does not have a significant effect on proportion of chasmogamous flowers for *T. p.* ssp. *perfoliata* ($df = 75$, $F = 2.39$, $P = 0.1260$) or *T. p.* ssp. *biflora* ($df = 62$, $F = 1.29$, $P = 0.2596$) therefore, I concluded that the increase in proportion of chasmogamous flowers is primarily due to a direct effect of light.

Vegetative traits of the two subspecies were also found to be phenotypically plastic. Plants in the shade treatment were 1.4 times taller on average than plants in the light treatment and no taller in *T. p.* ssp *perfoliata* and *T. p.* ssp. *biflora*, respectively. Also, floral bracts were found to be significantly larger in the shade treatments than in the high light treatment for *T. p.* ssp. *perfoliata*. On average, bracts were 1.2 times wider in the shade treatment than in the light treatment. These are typical responses observed in most plants (Rice and Bazzaz 1989). The increase in height and bract size under the shade treatment can be interpreted as an attempt to intercept as much sunlight as possible in order to maximize photosynthesis (Zervoudakis et al. 2012). In terms of resources, it is not surprising that plants devoting more energy to growing taller and making larger bracts would produce fewer chasmogamous flowers. Cheplick (2005) tested the effect of light on reproductive allocation in a cleistogamous grass (*Microstegium*

vimineum). He found that plants grown in the shade showed a greater allocation of resources to leaf size and reduced allocation to both flower types. Because chasmogamous flowers are more expensive and not essential for reproduction, it is possible they would be the obvious source from which the plant could borrow. In general plants have a predetermined pattern of resource allocation (Brock et al. 2005). When resources are limited this allocation can be altered to ensure the survival of the plant (Yang and Midmore 2005). If resources are limited most of these resources will be allocated to growth of the plant rather than reproduction, as reproduction will only be possible if the plant survives through its juvenile stage. It is interesting that *T. p.* ssp *biflora* exhibited no significant plasticity in either plant height or bract size (Table 8 and 11). I originally hypothesized that plasticity allowed the divergence of the subspecies. Thus, I expected that *T. p.* ssp. *biflora* would be less plastic than *T. p.* ssp. *perfoliata*. This expectation was formed on the basis that *T. p.* ssp. *biflora* is the newly emerging species and would likely have more genetically assimilated traits.

The light plasticity study showed that the typical pattern of chasmogamous flower production can be altered due to light limitation. Similarly, the developmental study also provided support for the argument that chasmogamous flowers are produced when resources are more abundant. Though cleistogamous flowers appear throughout the season, chasmogamous flowers have only an optimal range in the middle of the growing season at which they appear (Figure 3 and Figure 6). The distribution of cleistogamous flowers over time has a bimodal appearance over all. This bimodality is more than likely due to the appearance of secondary cleistogamous flowers after the primary cleistogamous flowers and chasmogamous flowers have begun to dehisce. Cleistogamous species such as *Impatiens capensis* Meerb. show a similar pattern of cleistogamous flower production first and chasmogamous second (Lu 2002). The

production of induced cleistogamous flowers typically occurred at the end of the growing season and was only observed in *T.p.* ssp. *perfoliata* (Figure 5). This evidence coincides with the results from the plasticity study. Chasmogamous flowers are only found during the middle of the season when presumably resources are more abundant. Meanwhile, the induced cleistogamous flowers only occur at the end of the season, when conditions become hotter and drier.

Though they explain very little of the plasticity seen in the light experiment, induced cleistogamous flowers are still an interesting piece to this puzzle. It appears there are two "layers" of plasticity present, meaning there are multiple times at which a developmental "decision" is made pertaining to flower type. The first decision appears to be made early in development based on the fundamental differences in morphology. Chasmogamous flowers typically have 5 sepals and petals; cleistogamous flowers have three sepals and produce no petals. However, induced cleistogamous flowers typically have sepal numbers similar to that of a chasmogamous flower and contain a few petal-like structures at the tip of the flower. Thus it appears that the switch from chasmogamous to induced cleistogamous flower is made later in development. Because induced cleistogamous flowers are uncommon, most of the plasticity seen in this study can be attributed to the first stage of decision-making. On average there were more induced cleistogamous flowers found in the light treatment than in the shade treatment, which is the opposite to pattern of increased allocation to chasmogamy in light. This may just be a reflection of the increase in chasmogamous flowers providing more possibilities for a switch from chasmogamy to induced cleistogamy to occur. In the field, induced cleistogamous flowers are typically observed at the end of the growing season just before plant senescence (Figure 3, 5). The switch to induced cleistogamous flowers may be advantageous because there will not be enough time for fruit of a chasmogamous flower to mature. In other cleistogamous species these

intermediate flower morphs typically appear in between the production of cleistogamous and chasmogamous flowers (Ruiz de Clavijo and Jimenez 1993). However, few of these studies have explored the possible link between these intermediate flowers and phenotypic plasticity.

Evolution

The two subspecies of *Triodanis perfoliata* differ in their allocation of chasmogamous and cleistogamous flowers. If the *T. p.* ssp. *perfoliata* is plastic in its flower production it could be the key factor that has allowed it to persist in a habitat typical of *T.p.* ssp. *biflora* and could have played a role in divergence. The first step in testing the hypothesis was confirming that the habitats of the subspecies are different. After sampling both light and soil in the two habitats I found significant evidence that is consistent with this hypothesis. Densiometer data confirmed canopy coverage differed significantly between the sites of the two *Triodanis perfoliata* subspecies. On average, *T. p.* ssp. *perfoliata* sites had three and a half times more canopy coverage than *T. p.* ssp. *biflora* sites. Hydrometer readings revealed a difference in settling rate between the soils of the two subspecies. Soil of *T. p.* ssp. *biflora* had consistently higher readings across all time frames compared to that of *T. p.* ssp. *perfoliata,* indicating there is a significant difference in texture between the two soils. The lower readings of the *T. p.* ssp. *perfoliata* soil are a result of its higher sand content.

If phenotypic plasticity played a role in speciation, we might expect to see that the direction of plasticity in the ancestral species in response to environmental variation mirrors the divergence of daughter species in those environments. For example, Shaw et al. (2007) observed this pattern in the courtship behavior of three spine stickleback, *Gasterosteus aculeatus*. Under laboratory conditions male sticklebacks in two benthic populations with high cannibalism participated in “zigzagging” (courtship dance) less than males belonging to a limnetic population

with low cannibalism. Differences in behavior between the ecotypes parallel plasticity seen in the ancestral oceanic fish in response to attacks. Similarly, in *T. p.* ssp. *perfoliata* we expected to find that *T. p*. ssp. *perfoliata* created fewer chasmogamous flowers in the high light, lower sand treatments that are typical of *T. p.* ssp. *biflora*. Interestingly, it was found that the plasticity in the light treatment showed the opposite trend. In fact proportion of chasmogamous flowers increased in the high light treatment for *T. p.* ssp. *perfoliata*, the opposite of what was expected, while no plasticity was observed in the soil plasticity study.

Although we found no support for the hypothesis that plasticity was involved in the divergence of these two subspecies in chasmogamous flower production, we did see the expected pattern in bract size. Bract width was significantly different between the two light treatments in *T. p.* ssp. *perfoliata*. When placed in an environment similar to that of *T. p.* ssp. *biflora* the smaller bract size produced parallels that of *T. p.* ssp. *biflora*, which occurs in a high light environment. Another expectation of this hypothesis was that *T. p.* ssp. *perfoliata*, the presumed ancestral subspecies, would be more plastic than *T. p.* ssp. *biflora*, the derived subspecies. Though this is not true for the reproductive traits, vegetative traits were found to be more plastic in *T. p.* ssp. *perfoliata* than in *T. p.* ssp. *biflora*. It is possible that *T. p.* ssp. *perfoliata* colonized a high light environment and its plasticity in bract size allowed it to adjust to this new habitat. After some time of small bract size being selected for the trait became fixed through genetic assimilation.

Reproductive Isolation

The final and arguably the defining step of speciation is reproductive isolation. Prezygotic isolation (behavioral, mechanical, and spatial) occurs before the formation of a zygote, while postzygotic isolation (zygote mortality and hybrid sterility) occurs after mating has

already taken place and a hybrid offspring has been produced (Ramsey et al. 2003). One of the known mechanisms of prezygotic isolation is a difference in habitat. When two diverging populations use different habitats, it will limit the amount of gene flow between the populations. *Triodanis p.* ssp. *perfoliata* and *T. p.* ssp. *biflora* significantly differ in two habitat factors, soil and light. The two subspecies soil types differ, as shown in the soil texture analysis. Hydrometer readings for biflora soil had significantly higher readings across all time frames compared to that of perfoliata soil. A high hydrometer reading means there are more particles suspended in the solution. The lower readings for perfoliata soil indicate a higher percentage of sand because large particles settle faster than do small soil particles such as clay and silt. Difference in light between the two habitats was shown through an estimation of canopy coverage using a densiometer. On average *T. p.* ssp. *perfoliata* sites had 53% canopy coverage compared to 15% canopy coverage at *T. p.* ssp. *biflora* sites.

Other studies have shown that extreme differences in soil can allow colonization of different species and contribute to ecological speciation (Kruckeberg 1986). Different levels of edaphic tolerance are often used to distinguish closely related species (Rajakaruna and Whitton 2004). Many of the examples in the literature concern adaptation to serpentine soil. Perhaps the most extensively studied system is that of *Mimulus guttatus* and *Mimulus nudatus*. Flowering time of the predecessor (*M. guttatus*) is later than that of the serpentine endemic *M. nudatus*. These two species also differ in pollinator due to differing floral morphologies (reviewed in Rajakaruna 2004). Macnair and others (Macnair and Christie 1983, Christie and Macnair 1987, Macnair et al. 1989, Macnair and Gardner 1998, Gardner and Macnair 2000) believe that these differences in flower timing and morphology have resulted from this species adaptation to stressful/limiting soil conditions. These adaptations then led to reproductive barriers between

populations, those adapted to serpentine soil and those only capable of growing in normal soil conditions.

Despite significant differences in habitat parameters, the growth room experiments found little evidence that each subspecies did best in its home soil and light environment. It was expected that *T. p.* ssp. *perfoliata* would perform better in the shade than in the light treatment, but.in fact both subspecies performed better in the high light treatment. *Triodanis p.* ssp *perfoliata* did not perform as poorly as *T. p.* ssp. *biflora* did in the shade, which provided some evidence for adaptation to the light environment. On average, total flower production in *T. p.* ssp. *perfoliata* was 1.5 times higher in the light than in the shade while *T. p.* ssp. *biflora* was 2.5 times higher. Biomass also reflected this trend with *T. p.* ssp. *perfoliata* plants being two times larger in the light than in the shade and *T. p.* ssp *biflora* being 3.6 times larger. Though *T. p.* ssp *biflora* did perform more poorly in the shade than *T. p.* ssp. *perfoliata*, overall the results did not show that each subspecies did better in a setting similar to its own habitat. It is possible that the fabric used to create the shade treatment was too opaque. The shade and light treatments differed in the level of light intensity by an order of magnitude that may have exceeded the differences seen in the field. Ideally I would have replicated the actual light levels found in the field. Also, a high degree of early mortality caused by fungus gnats especially in the shade treatment resulted in an unbalanced design and limited sample sizes.

In the soil study, the performance of each subspecies in its own soil versus in the other subspecies' soil was quite similar. There were significantly more deaths of *T. p.* ssp *perfoliata* in biflora soil than in perfoliata soil at both the juvenile stage and the reproductive stage. Oddly, however, the *T. p.* ssp. *perfoliata* plants in biflora soil that did survive tended to do better in terms of total flower production. Overall, *T. p.* ssp. *biflora* had low mortality in both soil types,

one death in perfoliata soil and three in its own soil. *Triodanis p.* ssp. *biflora* produced 103 flowers in its own soil and 85 in perfoliata soil, on average. I multiplied the proportion surviving to reproduction by total flower number to achieve a lifetime fitness parameter. The cumulative fitness values revealed that each subspecies performed 1.1 times better in biflora soil than in perfoliata soil. Therefore, *T. p.* ssp. *perfoliata* performed slightly better in biflora soil and *T. p.* ssp. *biflora* had slightly higher fitness in its own soil. One interesting observation provided further evidence for soil adaptation in *T. p.* ssp. *biflora*. Fifteen percent of the *T. p.* ssp. *biflora* plants in perfoliata soil exhibited an odd phenomenon of necrosis at the top of the plant that was not observed in biflora soil. In total, however, there is relatively little evidence that these plants are adapted to their own soils though habitat differences have been demonstrated. It is possible some soil factor that affects plant performance, such as nutrients or microbes was lost during collection and freezing of the soil.

Conclusions

Through varying light levels it was revealed that *T. p.* ssp. *perfoliata* and *T. p.* ssp. *biflora* produce more chasmogamous flowers in higher light environments. Both the number and proportion of chasmogamous flowers increased in the light treatment of the two subspecies. The plasticity seen in the light study and also the observation of induced cleistogamous flowers in the developmental study may provide a starting point for future studies looking at the maintenance of cleistogamy.

Although the plasticity in response to light was observed, it does not appear to explain the divergence in flower allocation between the subspecies. The habitat study results confirmed the differences between the subspecies, but those differences did not cause the expected effect on flower allocation in the growth room studies. Though the plasticity seen in flower allocation in

the light study did not support the hypothesis that plasticity was involved in the divergence of these two subspecies, it could still provide insights to the maintenance of cleistogamy. When resource allocation is taken into consideration, this result is not surprising. The developmental study results also fall in line with the argument that allocation to chasmogamy occurs when resource availability is high. The only support found for the evolutionary argument was *T. p.* ssp. *perfoliata* bract size mirroring *T. p.* ssp. *biflora* when placed in a high light environment. This observation coupled with *T. p.* ssp. *biflora's* less plastic nature suggests that some of the subspecies vegetative traits may be due to genetic assimilation. This avenue may be an interesting topic for future research.

Overall, evidence to support the hypothesis that difference in habitat is keeping these subspecies separate is mixed. Fitness in the greenhouse was little affected by habitat differences. Other studies performed in our lab have attributed most of the reproductive isolation found between the subspecies to cleistogamy (E. Arthur, unpublished data). Because *T. p.* ssp. *biflora* creates cleistogamous flowers almost exclusively there is less of a chance for hybridization between the two subspecies. However, it is unlikely that a single mechanism is causing the divergence of these subspecies (Widmer 2009).

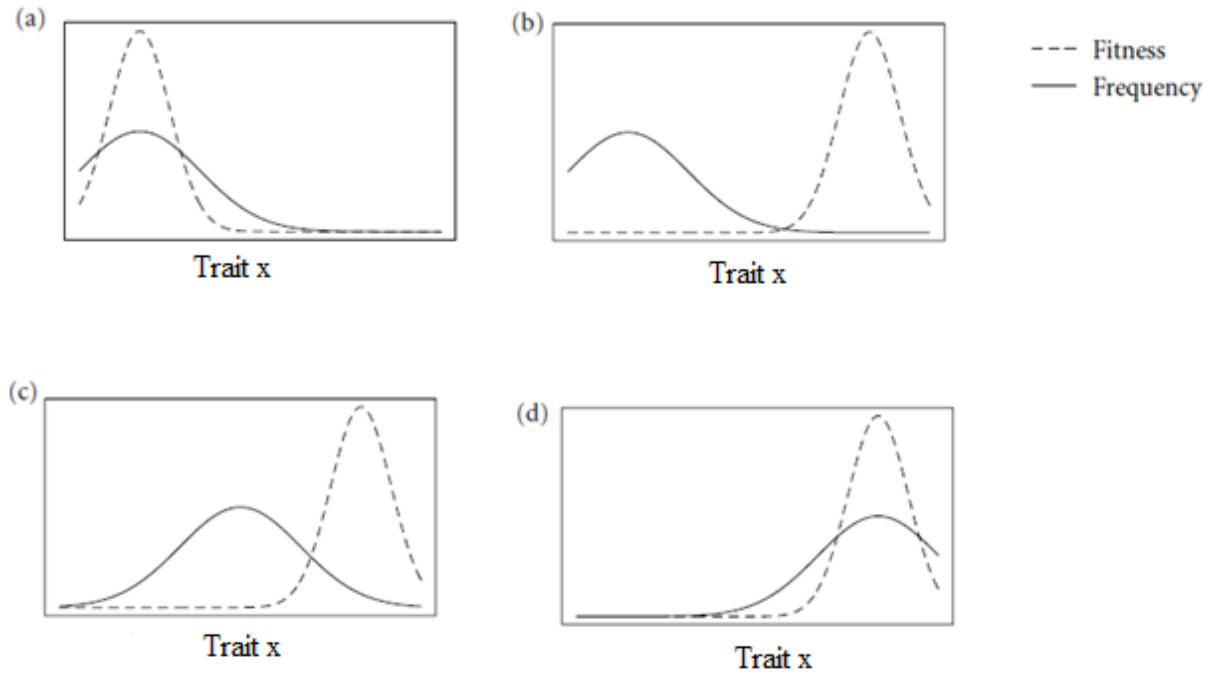


Figure 1: Modification of ancestral phenotype through phenotypic plasticity. Adapted from Fitzpatrick (2012). (a) The ancestral phenotype of a plastic organism (b) change in the environment changes the fitness optimum (c) phenotypic plasticity allows ancestral phenotype to be altered (d) selection acts on modified phenotype eventually fixing the new phenotype resulting in a newly diverged species.

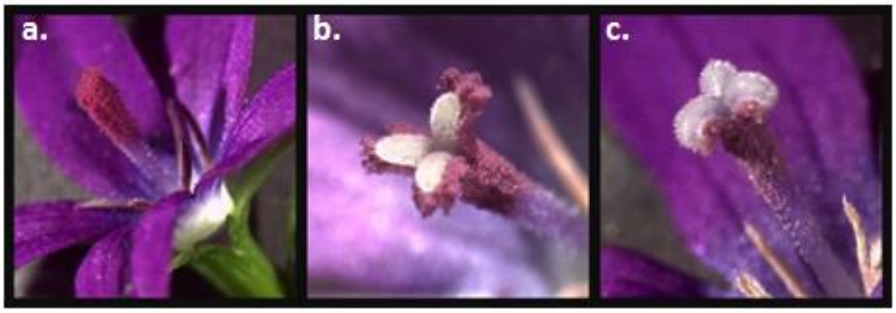


Figure 2: Delayed selfing in a chasmogamous *T. p.* ssp. *perfoliata* flower. a.) In the male phase the anthers have deposited pollen onto the outside of the stigma. b.) In the female phase the stigma has opened and is available to receive outcross pollen. c.) The lobes of the stigma are curling under, which may allow the previously deposited pollen to come in contact with the stigma lobes. Credit for photos goes to J. Thigpen.

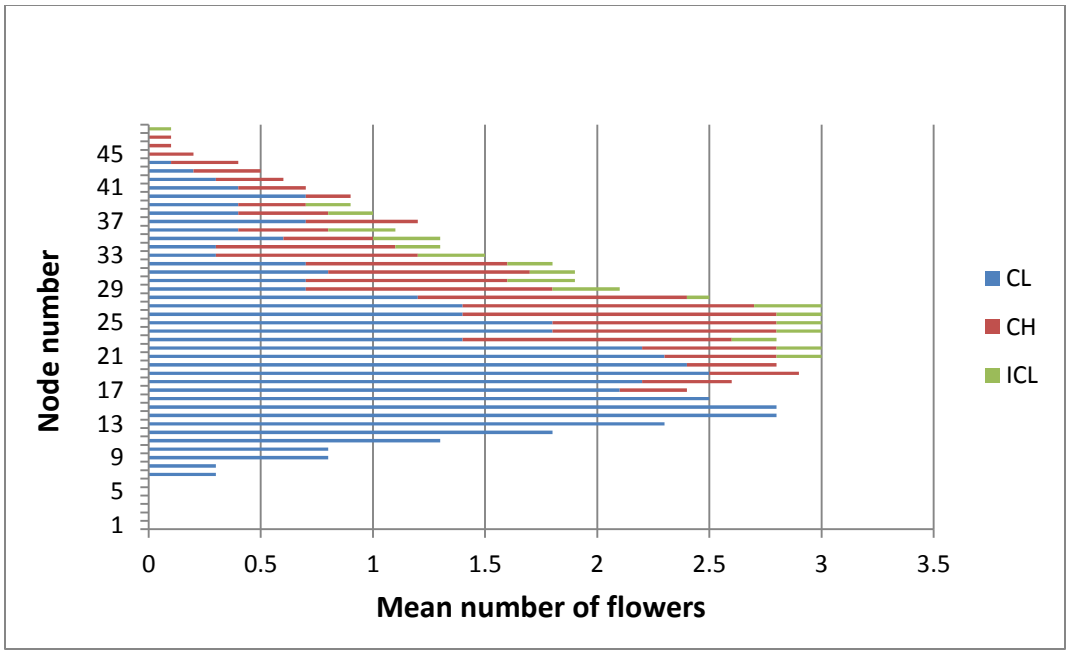


Figure 3: Node position of flowers in *T. p.* ssp. *perfoliata*. Mean number and type of flower found in each node of a plant across 10 plants in two populations of *T. p.* ssp. *perfoliata*. CL = cleistogamous, CH = chasmogamous, and ICL = induced cleistogamous. Nodes were numbered sequentially from bottom to top of the stem.

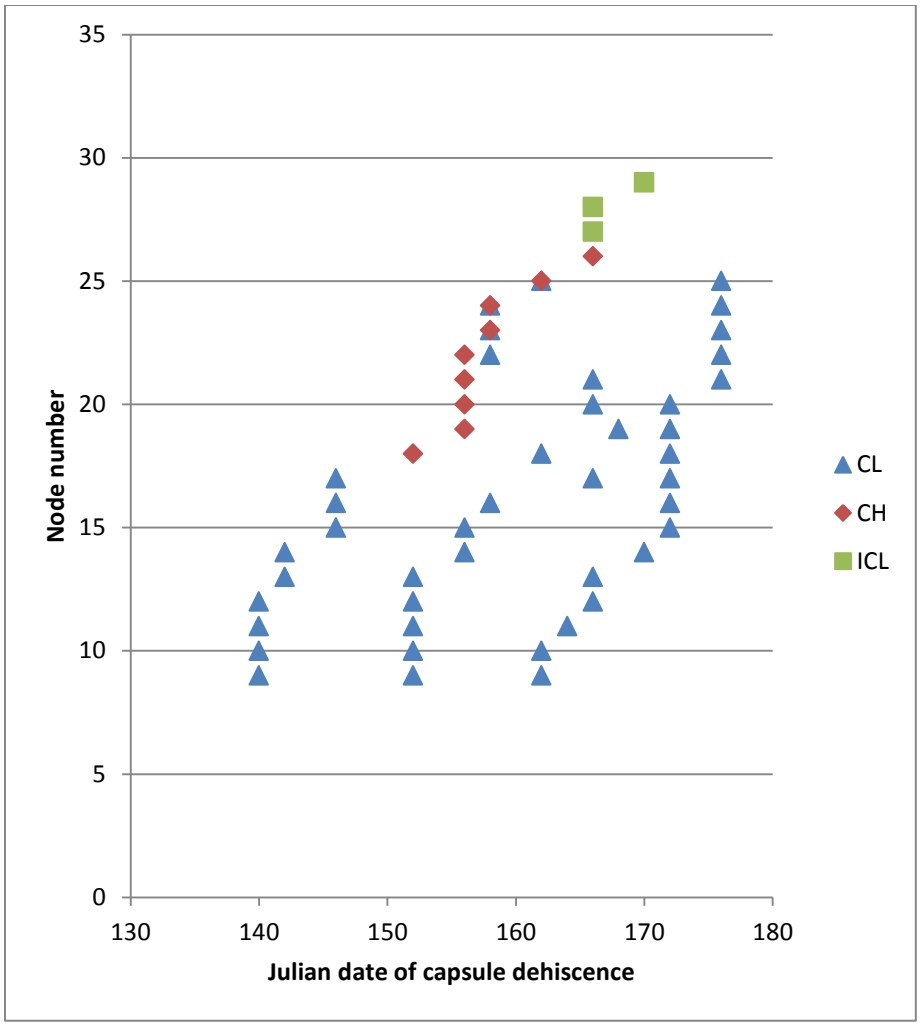
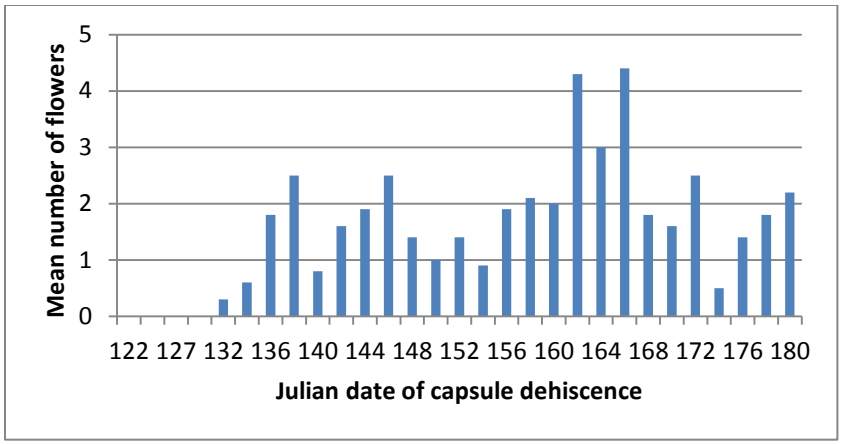
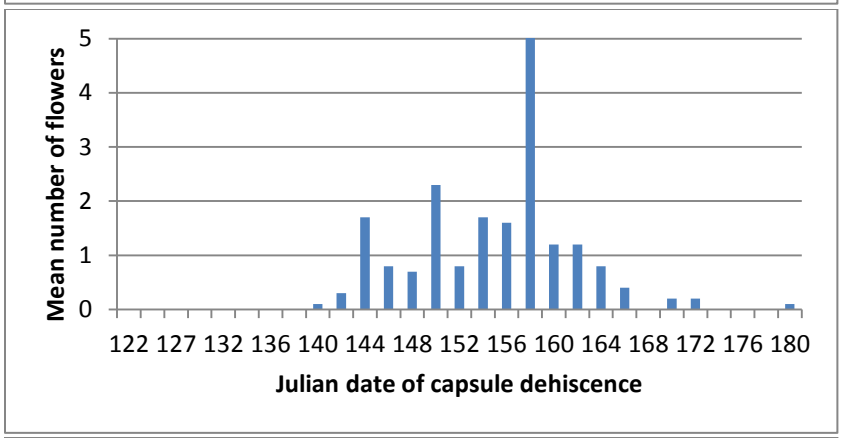


Figure 4: Capsule dehiscence in one representative plant of *T. p.* ssp. *perfoliata*. Date of capsule dehiscence and location of all types of flowers in a single plant of *T.p.* ssp. *perfoliata*. CL = cleistogamous, CH = chasmogamous, and ICL = induced cleistogamous. Nodes were numbered sequentially from bottom to top of the stem.

a)



b)



c)

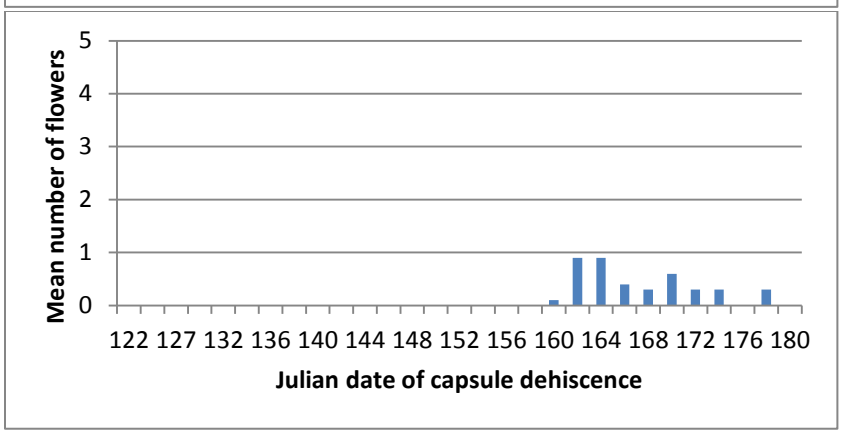


Figure 5: Mean capsule dehiscence date in *T. p.* ssp. *perfoliata*. Mean number of capsules of each flower type that dehisced on a specific Julian date across 10 plants in two populations of *T. p.* ssp. *perfoliata*, a) cleistogamous, b) chasmogamous, c) induced cleistogamous.

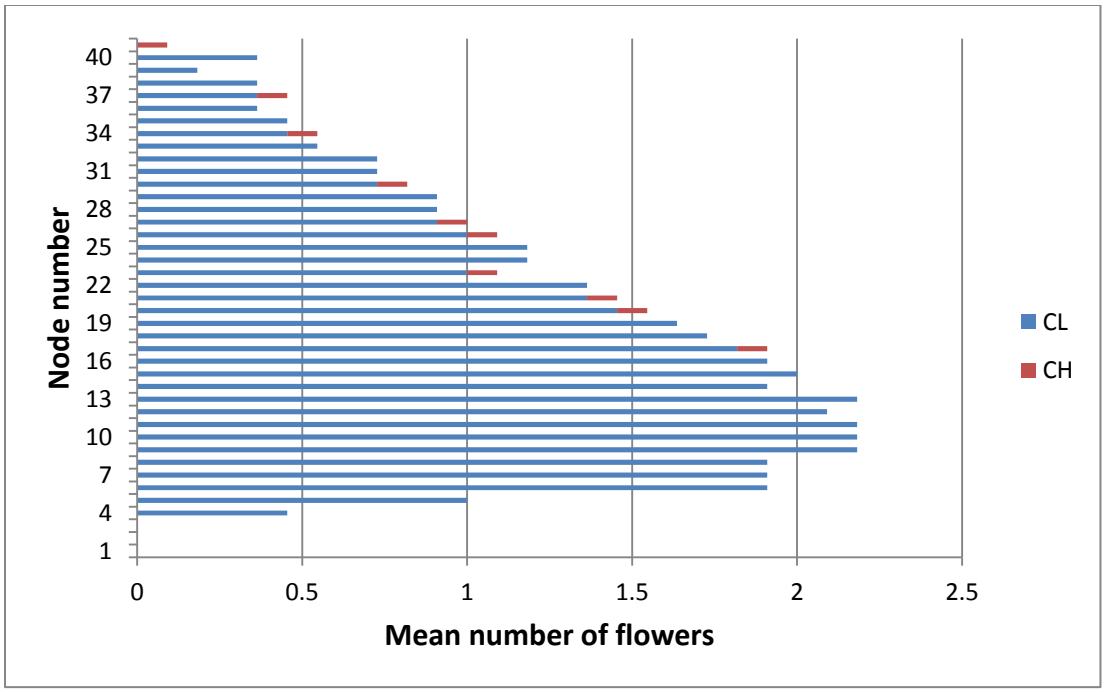


Figure 6: Node position of flowers in *T. p.* ssp. *biflora*. Mean number and type of flower found in each node of a plant across 11 plants in two populations of *T. p.* ssp. *biflora*. CL = cleistogamous and CH = chasmogamous. Nodes were numbered sequentially from bottom to top of the stem.

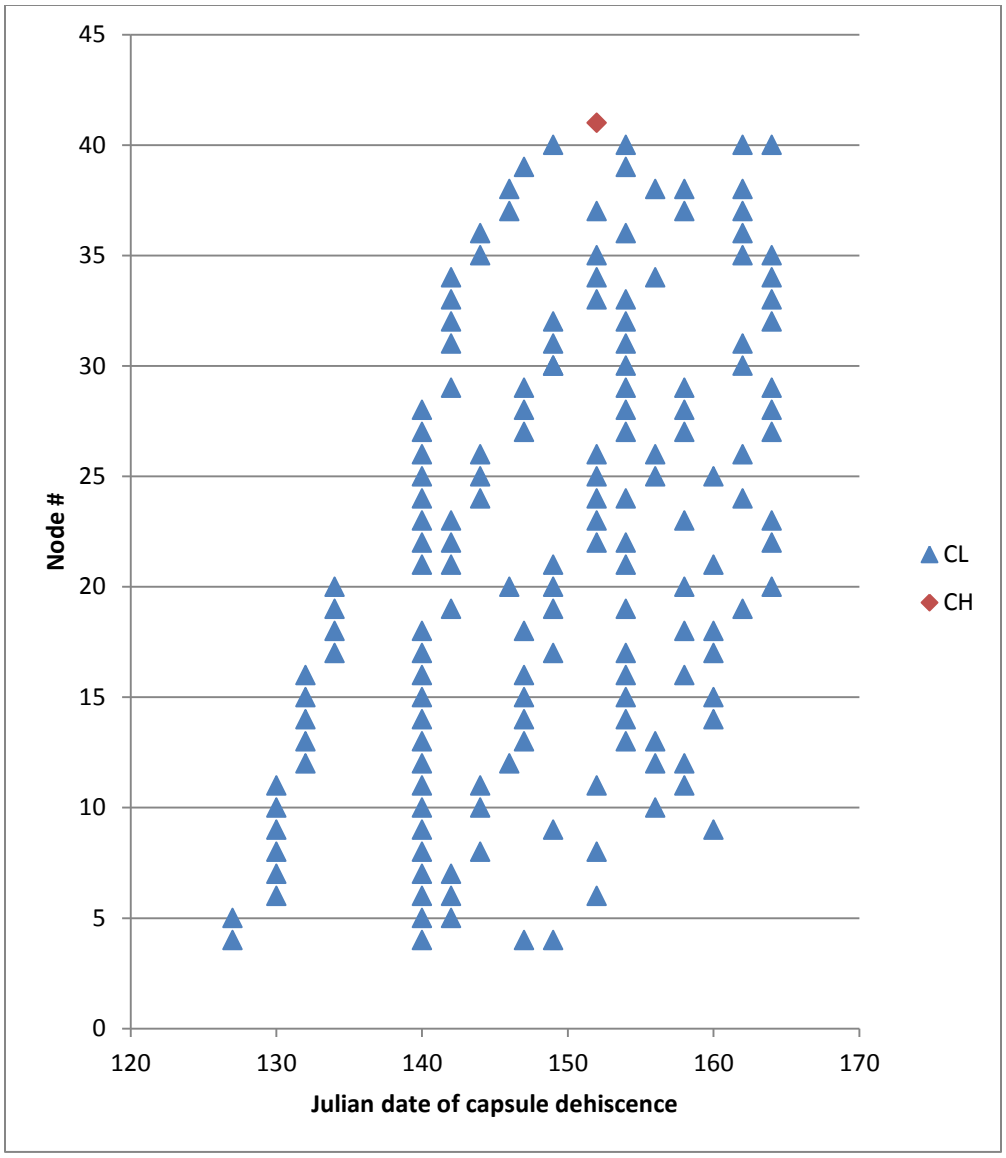
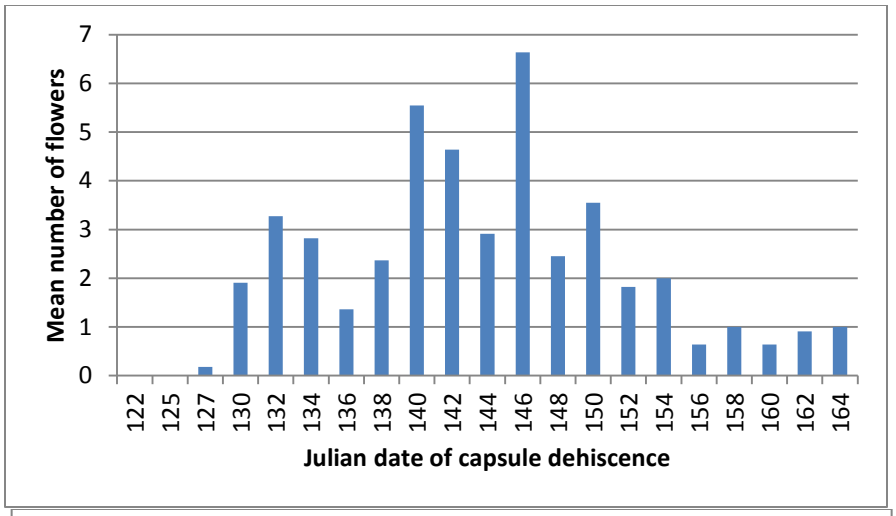


Figure 7: Capsule dehiscence in one representative plant of *T. p.* ssp. *biflora*. Date of capsule dehiscence and location of all types of flowers in a single plant of *T.p.* ssp. *biflora*. CL = cleistogamous and CH = chasmogamous. Nodes were numbered sequentially from bottom to top of the stem.

a)



b)

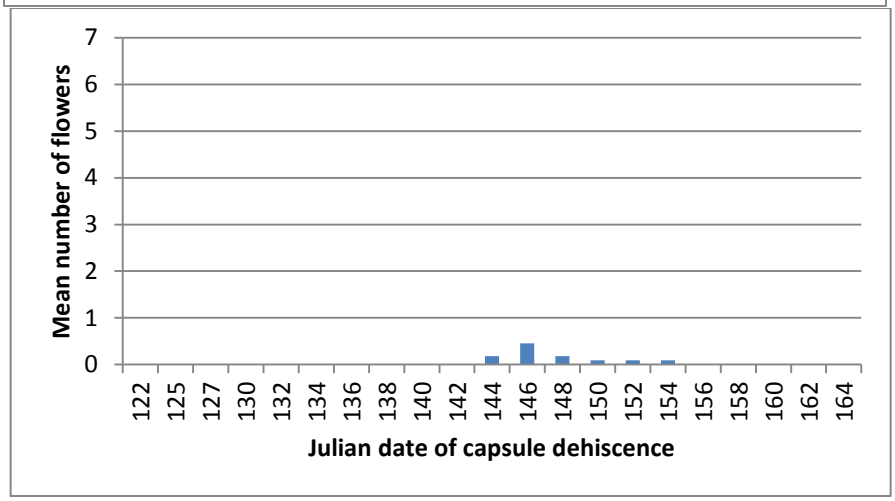


Figure 8: Mean capsule dehiscence date in *T. p.* ssp. *biflora*. Mean number of capsules of each flower type that dehisced on a specific Julian date across 11 plants in two populations of *T. p.* ssp. *biflora*, a) cleistogamous and b) chasmogamous.

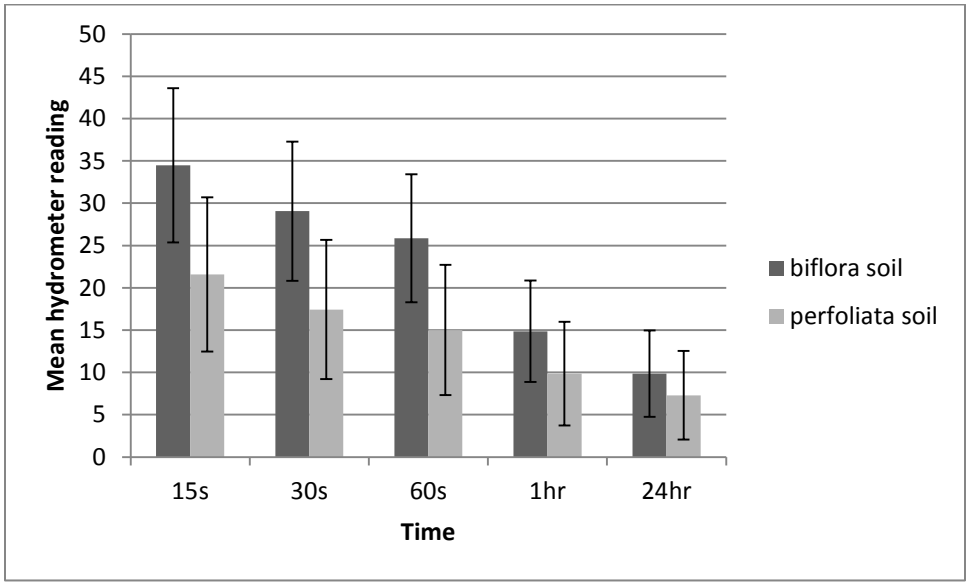


Figure 9: Mean hydrometer readings for soils collected in populations of two subspecies of *Triodanis perfoliata* at different time periods. The rate at which values decline is a function of soil particle size. Error bars depict standard deviation. N = 13 soil collection sites for each subspecies.

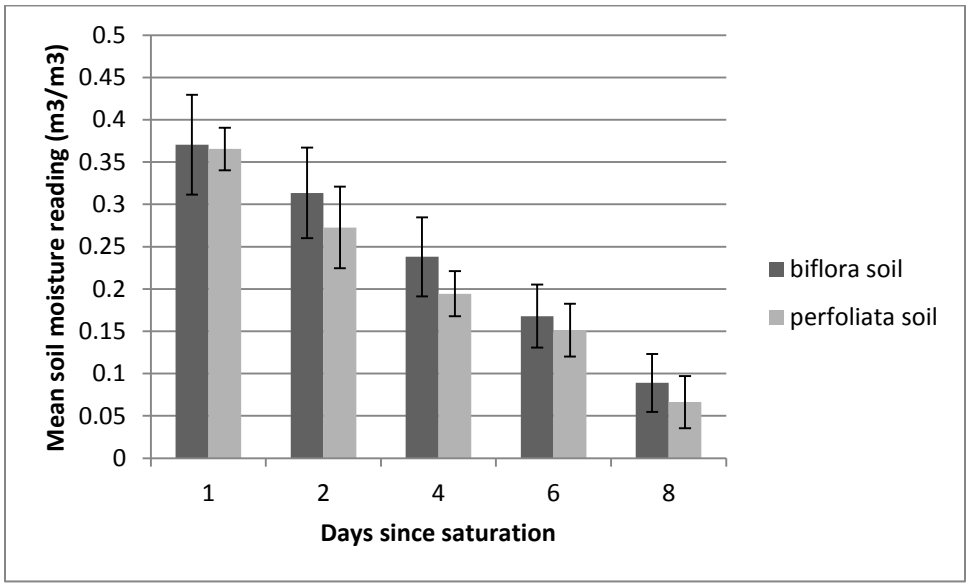


Figure 10: Mean soil moisture readings for soils collected in populations of two subspecies of *Triodanis perfoliata* taken across an eight-day time span. Soils were fully saturated on day zero and measured at a two day interval after the first two days. Error bars depict standard deviation. N = 13 soil collection sites for each subspecies.

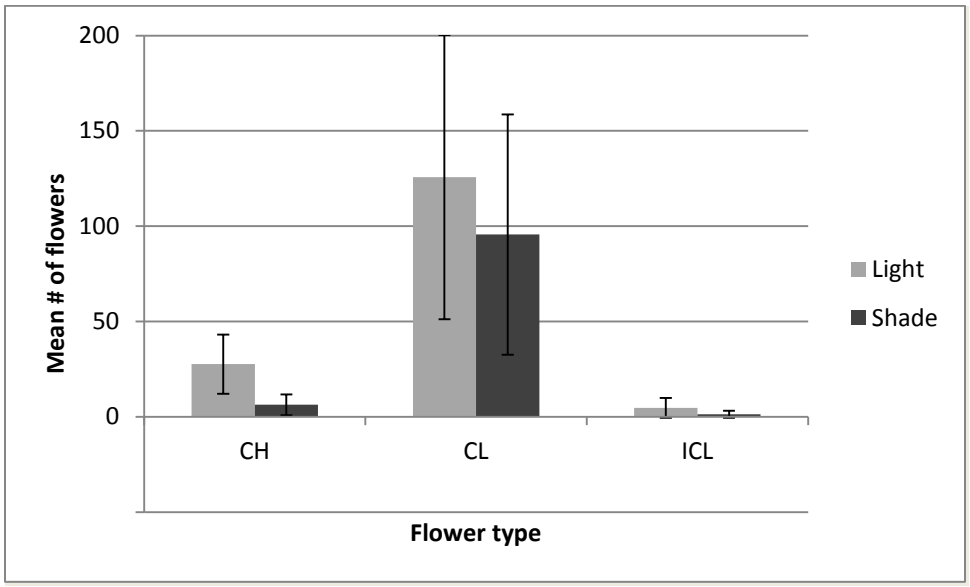


Figure 11: Mean number of the three flower types produced in each treatment for *T. p.* ssp. *perfoliata*. N= 60 plants for the light treatment and N=42 for the shade treatment.

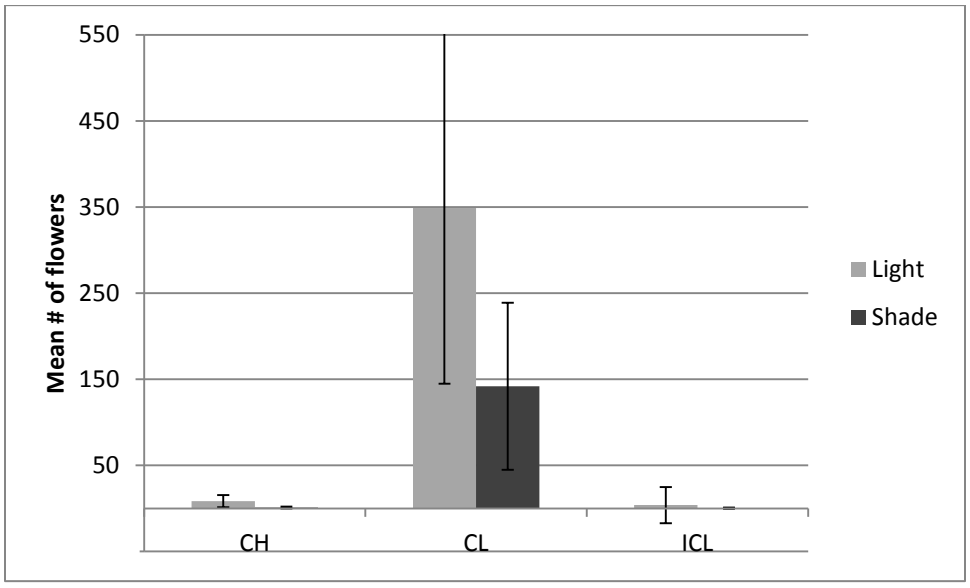


Figure 12: Mean number of the three flower types produced in each treatment for *T. p.* ssp. *biflora*. N= 52 plants for the light treatment and N=37 for the shade treatment.

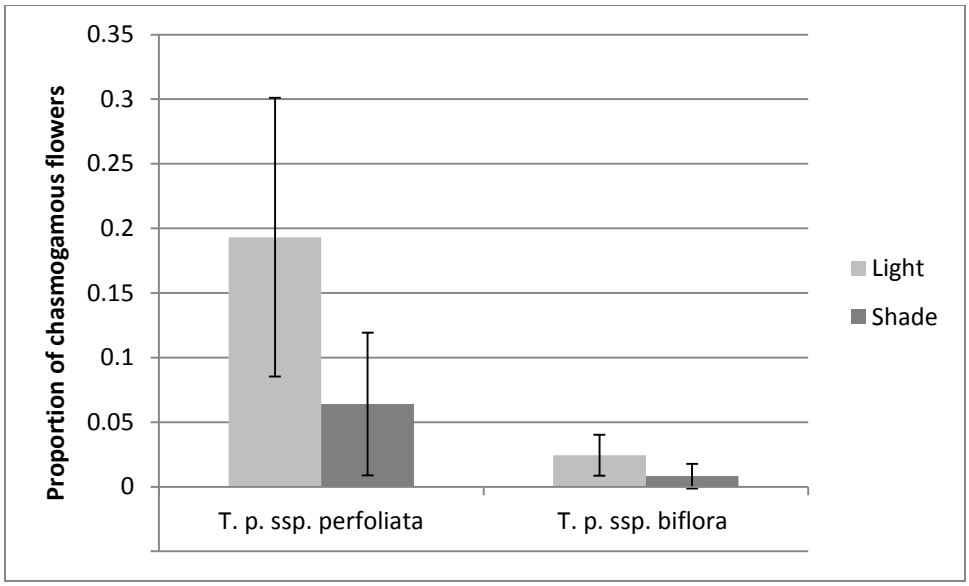


Figure 13: Mean proportion of chasmogamous flowers produced in the light study for *T. p.* ssp. *perfoliata* and *T. p.* ssp. *biflora*. N= 60 plants for the light treatment and N=42 for the shade treatment for *T. p.* ssp. *perfoliata*. N= 52 plants for the light treatment and N=37 for the shade treatment for *T. p.* ssp. *biflora*.

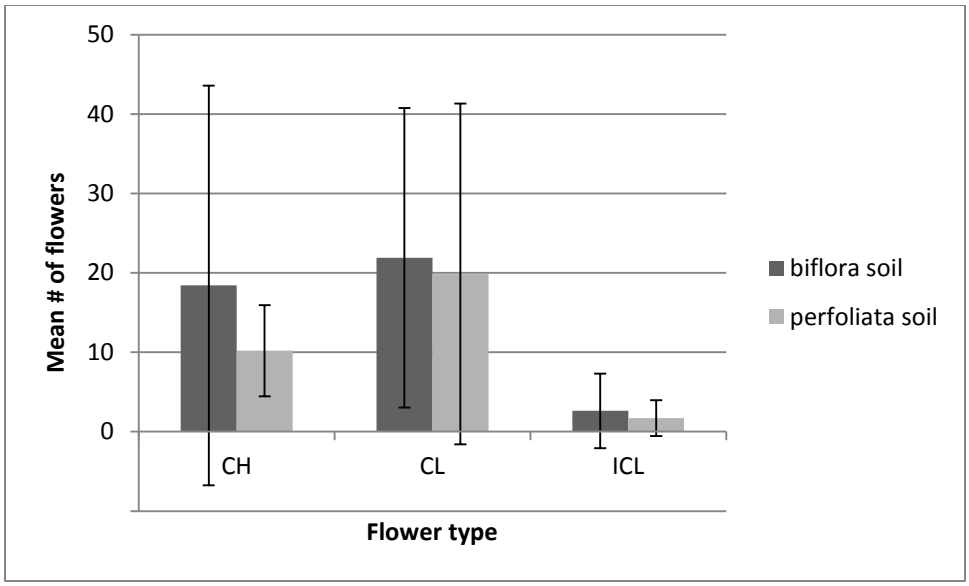


Figure 14: Mean number of the three flower types produced in biflora soil and perfoliata soil treatments for *T. p.* ssp. *perfoliata*. N = 49 plants for the biflora soil treatment and N = 59 for the perfoliata soil treatment.

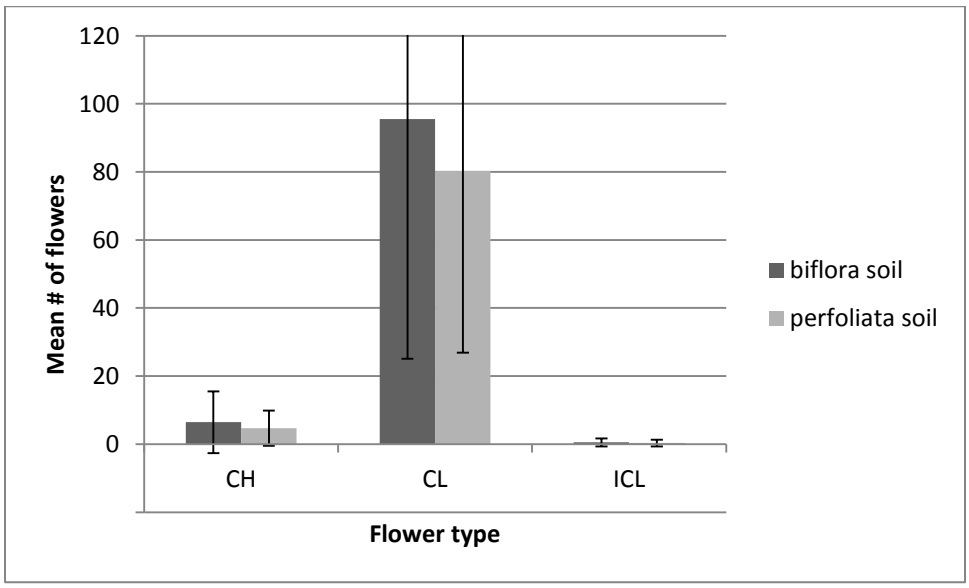


Figure 15: Mean number of the three flower types produced in biflora soil and perfoliata soil treatments for *T. p.* ssp. *biflora*. N = 57 plants for the biflora soil treatment and N = 59 for the perfoliata soil treatment.

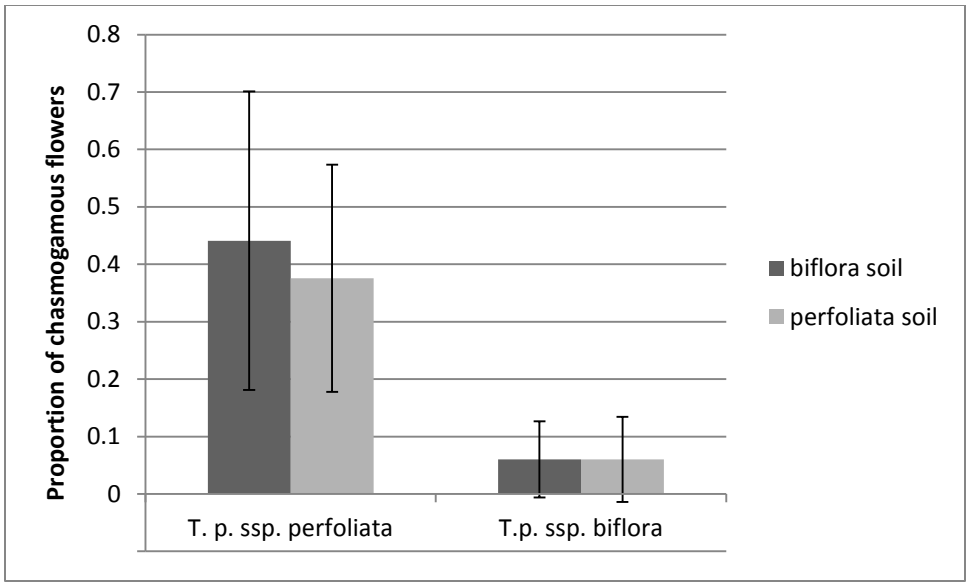


Figure 16: Mean proportion of chasmogamous flowers produced in the soil study for *T. p.* ssp. *perfoliata* and *T. p.* ssp. *biflora*. N = 49 plants for the biflora soil treatment and N = 59 for the perfoliata soil treatment for *T. p.* ssp. *perfoliata*. N = 57 plants for the biflora soil treatment and N = 59 for the perfoliata soil treatment for *T. p.* ssp. *biflora*.

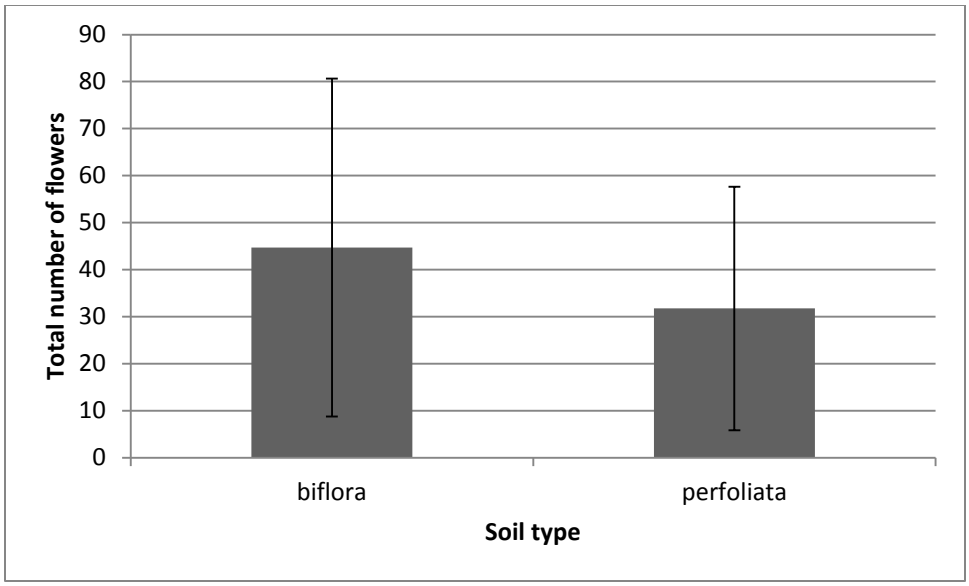


Figure 17: Mean total flower number produced by *T. p.* ssp. *perfoliata* in biflora soil and perfoliata soil. N = 49 plants for the biflora soil treatment and N = 59 for the perfoliata soil treatment.

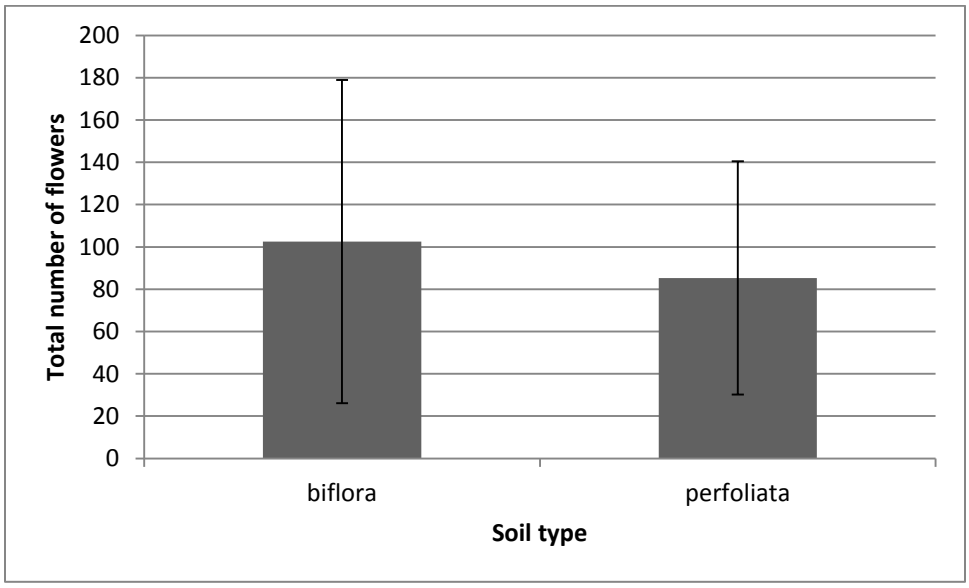


Figure 18: Mean total flower number produced by *T. p.* ssp. *biflora* in biflora soil and perfoliata soil. N = 57 plants for the biflora soil treatment and N = 59 for the perfoliata soil treatment.

Table 1: Sample of studies showing plasticity in cleistogamy. A sample of various studies documenting the plasticity of cleistogamy in different plant species.

Species	Condition altered	Change in condition thought to affect flower type	Effect on flower type production	Literature reference
Collomia grandiflora	Pollen amount	Less pollen	Increase in CL	Albert et al. 2007
Dichanthelium clandestinum	Light and soil moisture	Decrease in light and soil moisture	Light- No change Soil-Decrease in CL	Bell and Quinn 1987
Mimulus nasutus	Plant size	Increase	Increase in CH	Diaz and Macnair 1988
Calathea micans	Light and nutrient availability	Increase	Increase in CH	Le Corff 1993
Lamium amplexicaule	Temperature/Day length	Cooler/ Shorter day	Increase in CL	Lord 1982
Collomia grandiflora	Water availability	Less water	Decrease in CH	Minter and Lord 1983
Impatiens pallida **and** ***Impatiens biflora***	Light	More light	Increase in CH	Schemske 1978
Impatiens capensis	Vegetative herbivory	Leaf damage	Increase in CL	Steets and Ashman 2004
Amphicarpaea bracteata	Plant size and light intensity	Larger plants and increase light	Increase in CH	Trapp and Hendrix 1988
Impatiens capensis	Light intensity and soil moisture	Increase	Increase in CH	Waller 1980
Collomia grandiflora	Density, light, and sand content	Decrease	Increase in CH	Wilken 1982
Viola septemloba	Season	Beginning of season	Increase in CH	Winn and Moriuchi 2009

Table 2: Habitat study field sites for *Triodanis perfoliata* subspecies *perfoliata.* Description and location of field sites used in habitat study of *Triodanis perfoliata* subspecies *perfoliata*. Sites labeled as mixed were made up of at least 90% of the same subspecies. Population size was roughly estimated using the following scale: small (20 or less), medium (20-300), and large (300 or more).

Site	Pure or Mixed	Type	Population Size	GPS coordinates
P0102	Pure	Field/Woodside	Large	N 35° 43.249’ W 077° 30.936’
P0304	Pure	Roadside	Medium	N 35° 38.206’ W 077° 21.673’
P0506	Mixed	Creekside/Sidewalk	Small	N 35° 36.234’ W 077° 21.795’
P0708	Pure	Woodside/Fence	Small	N 35° 38.392’ W 077° 21.800’
P0910	Pure	Construction area/Roadside	Small	N 35° 37.484’ W 077° 24.629’
P1112	Pure	Roadside	Large	N 35° 37.134’ W 077° 24.838’
P1314	Pure	Roadside	Medium	N 35° 37.711’

				W 077° 21.540’
P1516	Pure	Ditch	Small	N 35° 36.701’ W 077° 24.600’
P1718	Pure	Roadside	Small	N 35° 37.080’ W 077° 19.521’
P1920	Mixed	Pathside	Small	N 35° 36.813’ W 077° 20.358’
P2122	Pure	Woodside/Clearing	Small	N 35° 35.328’ W 077° 18.563’
P2324	Mixed	Woodside/Park	Small	N 35° 33.373’ W 077° 22.64’
P2526	Mixed	Ditch/Park	Small	N 35° 35.495’ W 077° 23.168’

Table 3: Habitat study field sites for *Triodanis perfoliata* subspecies *biflora.* Description and location of field sites used in habitat study of *Triodanis perfoliata* subspecies *biflora*. Sites labeled as mixed were made up of at least 90% of the same subspecies. Population size was roughly estimated using the following scale: small (20 or less), medium (20-300), and large (300 or more).

Site	Pure or Mixed	Type	Size	GPS coordinates
B0102	Mixed	Pathside	Medium	N 35° 33.635’ W 077° 23.600’
B0304	Pure	Field/Clearing	Small	N 35° 36.873’ W 077° 24.054’
B0506	Mixed	Field/Clearing	Small	N 35° 33.417’ W 077° 25.600’
B0708	Pure	Clearing/Parking lot	Small	N 35° 34.140’ W 077° 23.928’
B0910	Pure	Clearing/Roadside	Medium	N 35° 35.347’ W 077° 23.774’
B1112	Pure	Woodside	Small	N 35° 36.071’ W 077° 21.459’
B1314	Mixed	Woodside	Large	N 35° 35.332’

				W 077° 18.484’
B1516	Mixed	Pathside	Medium	N 35° 36.794’ W 077° 20.147’
B1718	Pure	Field/Clearing	Small	N 35° 41.004’ W 077° 29.535’
B1920	Mixed	Field/Clearing	Medium	N 35° 53.172’ W 077° 32.793’
B2122	Pure	Roadside	Small	N 35° 36.434’ W 077° 23.725’
B2324	Pure	Roadside	Small	N 35° 35.435’ W 077° 639’21’
B2526	Pure	Clearing	Large	N 35° 35.600’ W 077° 22.642’
B2728	Pure	Field	Small	N 35° 43.249’ W 077° 30.936’

Table 4: Summary of plant reproductive traits for developmental study. Mean (and standard deviation) number of nodes, chasmogamous (CH) flowers per plant, cleistogamous (CL) flowers per plant, and induced cleistogamous (ICL) flowers per plant for each population studied.

	N	**# Nodes**	**#CH/plant**	**#CL/plant**	**#ICL/plant**
T. p.* ssp. *perfoliata					
Site 1	5	39 (9)	27 (13)	64 (59)	6(7)
Site 2	5	31 (5)	12 (4)	34 (10)	2(3)
T. p.* ssp. *biflora					
Site 1	5	30 (11)	1 (0)	64 (24)	-
Site 2	6	24 (4)	1 (0)	31 (9)	-

Table 5: Summary of chemical soil analysis. Mean and (standard deviation) of soil chemical analysis. *Significant at a table wide value using a sequential Bonferroni technique (Rice 1989).

Variable	*T. p.* ssp *perfoliata*	*T. p.* ssp *biflora*	*P*
Percent humic matter	0.441 (0.2)	0.73 (0.4)	0.049
Weight per volume	1.399 (0.06)	1.28 (0.08)	0.001*
Cation exchange capacity	4.18 (2)	5.96 (4)	0.189
Percent cation exchange capacity occupied by basic cations	55.3 (18)	66.6 (16)	0.155
Exchangeable acidity	1.61 (0.6)	1.59 (0.77)	0.949
Soil pH	5.27 (0.6)	5.29 (0.83)	0.952
Phosphorus index	113.4 (86)	75.6 (49)	0.245
Potassium index	17.8 (11)	31.7 (8)	0.005
Percent cation exchange capacity occupied by calcium	44.7 (17)	52.7 (17)	0.316
Percent cation exchange capacity occupied by magnesium	8.5 (3)	10.9 (3.2)	0.099
Sulfur index	25.4 (11)	29 (4)	0.359
Manganese index	71.8 (40)	54.1 (40)	0.332
Zinc index	233.7 (282)	102.2 (66)	0.169
Copper index	67.9 (57)	60 (42)	0.727
Sodium	0.05 (0.07)	0.02 (0.04)	0.264

Table 6: Repeated measures analysis of variance (ANOVA) for soil texture analysis (hydrometer reading) and soil moisture content.

Dependent Variable	**Source**	***df***	**Mean Square**	***F***	***P***
Hydrometer reading	Time	1	13117.831	394.184	0.000
	Time × Subspecies	1	1074.959	32.302	0.000
	Error(time)	50	33.278		
	Between Subspecies	1	0.043	7.509	0.008
	Error	50	0.006		
Moisture content	Day	2.524	1.053	1014.184	0.000
	Day × Subspecies	2.524	0.006	5.3	0.003
	Error(time)	126.223	0.001		
	Between Subspecies	1	2394.249	25.667	0.000
	Error	24	93.283		

Table 7: Analysis of variance (ANOVA) of light plasticity experiment for *T. p.* ssp. *perfoliata.* All proportion data were arcsine transformed and all count data were square root transformed before analysis. See text for description of model.

Dependent Variable	**Source**	***df***	**Mean Square**	***F***	***P***
Proportion CH flowers	Treatment	1	1.105	1969.951	0.000
	Population	2	0.064	259.585	0.004
	Treatment × population	2	0.000	0.014	0.986
	Error	96	0.017		
Total number of flowers	Treatment	1	132.272	119.294	0.002
	Population	2	14.856	15.613	0.060
	Treatment × population	2	0.952	0.100	0.905
	Error	96	9.530		
Height	Treatment	1	5421.962	65.308	0.012
	Population	2	354.623	4.340	0.187
	Treatment × population	2	81.712	0.534	0.588
	Error	96	153.139		
Number of nodes	Treatment	1	14.820	39.225	0.021
	Population	2	1.110	2.966	0.252
	Treatment × population	2	0.374	0.665	0.517
	Error	96	0.563		

Bract size	Treatment	1	97.1117	106.464	0.004
	Population	2	10.957	13.166	0.071
	Treatment × population	2	0.832	0.143	0.867
	Error	93	5.835		
Biomass	Treatment	1	3.519	49.510	0.000
	Block(treatment)	22	0.075	1.851	0.025
	Error	78	0.040		

Table 8: Analysis of variance (ANOVA) of ligh plasticity study for *T. p.* ssp. *biflora.* All proportion data were arcsine transformed and all count data were square root transformed before analysis. See text for description of model.

Dependent Variable	**Source**	***df***	**Mean Square**	***F***	***P***
Proportion CH flowers	Treatment	1	0.068	21.668	0.000
	Block(treatment)	22	0.003	1.709	0.050
	Error	65	0.002		
Total number of flowers	Treatment	1	985.225	30.752	0.030
	Population	2	75.395	2.347	0.299
	Treatment × population	2	32.127	1.736	0.183
	Error	83	18.507		
Height	Treatment	1	2071.215	2.775	0.237
	Population	2	1464.226	1.953	0.339
	Treatment × population	2	749.785	3.568	0.033
	Error	83	210.154		
Number of nodes	Treatment	1	2.666	1.508	0.344
	Population	2	0.947	0.533	0.652
	Treatment × population	2	1.776	3.093	0.051
	Error	83	0.574		
Bract size	Treatment	1	73.692	3.837	0.189
	Population	2	19.205	0.079	0.927
	Treatment × population	2	19.273	4.265	0.018

	Error	75	4.519		
Biomass	Treatment	1	8.750	50.864	0.019
	Population	2	0.150	0.870	0.535
	Treatment × population	2	0.173	4.177	0.019
	Error	83	0.041		

Table 9: Generalized estimating equations (GEE) analysis for proportion of induced cleistogamous (ICL) flowers in the light plasticity study. Generalized estimating equations analysis (GEE) for proportion of induced cleistogamous (ICL) flowers between high light and shade treatments for both subspecies. All proportion data were arcsine transformed before analysis. See text for description of model.

Dependent Variable	**Source**	**Wald Chi-Square**	***df***	***P***
T. p.* ssp. *perfoliata	Treatment	12.694	1	0.000
	Block	24.027	11	0.013
	Population	7.51	2	0.013
T. p.* ssp. *biflora	Treatment	1.752	1	0.186
	Block	4.317	11	0.960
	Population	0.950	2	0.622

Table 10: Summary of reproductive and vegetative traits in *T. p.* ssp. *perfoliata.* Mean and standard deviation of reproductive and vegetative traits in high light and shade treatments for *T. p.* ssp. *perfoliata*. CH = chasmogamous ICL = induced cleistogamous

Dependent Variable	Light	Shade
Proportion CH flowers	0.19 (0.11)	0.064 (0.06)
Proportion ICL flowers	0.033 (0.04)	0.014 (0.019)
Total number of flowers	157 (83)	103 (65)
Height (cm)	39 (9.5)	55 (15.9)
Number of nodes	36 (8.2)	46 (12.1)
Bract Width(mm)	8.8 (1.5)	10.6 (3)
Biomass(g)	0.74 (0.30)	0.36 (0.14)

Table 11: Summary of reproductive and vegetative traits in *T. p.* ssp. *biflora.* Mean and standard deviation of reproductive and vegetative traits in high light and shade treatments for *T. p.* ssp. *biflora*. CH = chasmogamous ICL = induced cleistogamous

Dependent Variable	Light	Shade
Proportion CH flowers	0.02 (0.015)	0.008 (0.009)
Proportion ICL flowers	0.023 (0.14)	0.0026 (0.005)
Total number of flowers	361 (205)	143 (97)
Height (cm)	42 (15.1)	52 (16.9)
Number of nodes	27 (7.3)	31 (8.8)
Bract Width (mm)	5.8 (1.8)	7.4 (3.3)
Biomass (g)	0.87 (0.27)	0.24 (0.13)

Table 12: Analysis of variance (ANOVA) of soil experiments in *T. p.* ssp. *perfoliata.* All proportion data were arcsine transformed and all count data were square root transformed before analysis. See text for description of model.

Dependent Variable	**Source**	***df***	**Mean Square**	***F***	***P***
Proportion CH flowers	Treatment	1	0.125	3.881	0.187
	Population	2	0.462	14.423	0.065
	Treatment × population	2	0.032	0.444	0.643
	Error	102	0.072		
Total number of flowers	Treatment	1	25.791	311.319	0.003
	Population	2	14.719	175.497	0.006
	Treatment × population	2	0.081	0.018	0.982
	Error	102	4.409		

Table 13: Analysis of variance (ANOVA) of soil experiments in *T. p.* ssp. *biflora.* All proportion data were arcsine transformed and all count data were square root transformed before analysis. See text for description of model.

Dependent Variable	**Source**	***df***	**Mean Square**	***F***	***P***
Proportion CH flowers	Treatment	1	2.603E-5	0.132	0.751
	Population	2	0.040	204.785	0.005
	Treatment × population	2	0.000	0.043	0.958
	Error	110	0.005		
Total number of flowers	Treatment	1	14.645	2.085	0.286
	Population	2	121.685	17.324	0.055
	Treatment × population	2	7.024	0.905	0.407
	Error	110	7.759		

Table 14: Generalized estimating equations (GEE) analysis for proportion of induced cleistogamous (ICL) flowers in the soil plasticity study. Generalized estimating equations analysis (GEE) for proportion of induced cleistogamous (ICL) flowers between each subspecies in its "home" soil and in the other subspecies "home" soil. All proportion data were arcsine transformed before analysis. See text for description of model.

Dependent Variable	**Source**	**Wald Chi-Square**	***df***	***P***
T. p.* ssp. *perfoliata	Treatment	0.806	1	0.369
	Population	0.749	2	0.688
T. p.* ssp. *biflora	Treatment	1.187	1	0.276
	Population	5.474	2	0.065

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