PLANT SPECIES BIOLOGY

Plant Species Biology (2013) 28, 21–30 doi: 10.1111/j.1442-1984.2011.00352.x

Interaction between floral color change and gender transition in the protandrous weed *Saponaria officinalis*

SHABNAM G. JABBARI, SANDRA L. DAVIS and EMILY J. CARTER
Department of Biology, University of Indianapolis, 1400 E. Hanna Ave., Indianapolis, Indiana 46227, USA

Abstract

Natural selection has directed the evolution of floral traits so that pollinator visits are manipulated to maximize the fitness of individual plants by directing which other individual sires its seeds. In some plants, flowers change color over time and may have the ability to direct pollinators to rewarding flowers. In addition, by varying when pollen is available and when stigmas are receptive, protandrous plants can show variation in selfing rates. In this study, the association between color change and gender transition in flowers of *Saponaria officinalis* was examined. Anthocyanins were extracted from flowers of each gender stage to measure color using spectrophotometry. Female-phase flowers were found to have significantly higher anthocyanin concentration than male-phase flowers in both natural populations and experimental plots. This color change corresponded to a decrease in male sexual function, which was measured by the percentage of pollen grains stained as viable by lactophenol aniline blue and germinated on Brewbaker–Kwack media. Color change was phenotypically plastic. Plants grown in full sun had a more extensive color change than those grown in shaded experimental plots, and this effect was reversed the following year when the shading was removed. Pollinator observations documented both diurnal and nocturnal insect visitation. Fruit and seed set were equivalent on inflorescences bagged during daylight versus night, indicating that both diurnal and nocturnal insects are effective pollinators. If pollinators discriminate based on color, this could potentially reduce within-plant floral visits and also geitonogamy. This study is the first to document flower color change and moth pollination in *Saponaria officinalis*.

Keywords: anthocyanins, flower color change, geitonogamy, pollinator discrimination, protandry, *Saponaria officinalis*.

Received 4 March 2011; revision received 4 May 2011; accepted 23 June 2011

Introduction

Although pollen transfer occurs through biotic and abiotic pollination, plants do not play a passive role in their reproduction. Natural selection has directed the evolution of many floral traits, such as the timing of the presentation of sexual organs, petal color and display size, so that plants are able to influence what pollinators visit their flowers and increase the possibility that they may receive pollen from those individuals that will maximize the fitness of the offspring produced (reviewed in Willson & Burley 1983; Delph & Ashman 2006). In addition, these traits may interact with one another and with the environment in complex ways to further influence the pollination biology of plants. This paper examines how two of these traits, floral color change and protandry, may be interacting to influence the pollination biology of the common weed *Saponaria officinalis*, known as bouncing bet.

Floral color change is a common trait in angiosperms that can affect pollination. In these plants, the flowers, in addition to changing color as they age, often show a reduction in pollinator rewards such as pollen or nectar, and a loss of reproductive potential (Casper & La Pine 1984; Oberrath & Böhning-Gaese 1999; Ida & Kudo 2003; Nuttman *et al*. 2006). One hypothesis to explain why plants retain color-changed flowers is that flower color

Correspondence: Sandra L. Davis
Email: sdavis@uindy.edu

can act as a cue for insect pollinators. Several studies have indicated that plants that retain their color-changed flowers enhance their attractiveness to pollinators by increasing inflorescence size (reviewed in Weiss 1991; Oberrath & Böhning-Gaese 1999; Ida & Kudo 2003; Nuttman *et al*. 2006). However, a trade-off for plants with large displays is that subsequent visits to additional flowers on that plant by the pollinator could lead to an increase in intra-plant pollen transfer, or geitonogamous pollination. If the plant is self-incompatible or if inbreeding depression occurs, such self-pollination would not be beneficial (De Jong *et al*. 1993). Floral color change may reduce the level of geitonogamous pollination if, once a pollinator approaches an inflorescence, the pollinator preferentially visits flowers of a certain color, thereby reducing the number of flowers visited per plant (De Jong *et al*. 1992; Harder & Barrett 1996; Harder & Wilson 1998; Ida & Kudo 2003; Sun *et al*. 2005; Kudo *et al*. 2007). Alternatively, color change may not be adaptive as a mechanism involved in pollination, but may occur post-pollination, as the flower ages (Casper & La Pine 1984; Delph & Lively 1989).

To study the adaptive significance of floral color change, the impact on the pollination biology of the plant must be considered in the context of other potentially interacting floral traits and environmental factors. For example, Kudo *et al*. (2007) found that retaining color-changed flowers increased pollinator visitation to artificial single-inflorescence plants, but it did not have an effect in multiple-inflorescence plants. In addition, color change is known to respond to both biotic and abiotic factors and, therefore, may show phenotypic plasticity. Color change may be triggered by pollinator visitation so that unvisited flowers stay in their original color until they receive a visit. Therefore, the composition of colors within an inflorescence is dependent on the level of pollinator activity in the population (Nuttman *et al*. 2006). Furthermore, anthocyanin production in flowers and vegetative tissue has been shown to respond to both temperature and light exposure (Farzad *et al*. 2003; Hughes *et al*. 2005; Stiles *et al*. 2007; Gould *et al*. 2010). If the timing and extent of color change are plastic, plants would be able to respond to environmental cues to control pollinator activity differentially depending on such conditions.

A second floral trait that is dependent on flower age is dichogamy; when the development of the male and female sex organs within hermaphroditic flowers occurs sequentially. The most common form of dichogamy is protandry, in which the stamens mature and release pollen before the stigma becomes receptive (Lande & Schemske 1985). By varying when pollen is available and when stigmas are receptive, the degree of overlap between male and female phases may differ, which would alter the potential for autogamous pollination. Therefore, plants that are protandrous often show mixed-mating or intermediate levels of self-fertilization and cross-fertilization (Willson & Burley 1983; Elle & Hare 2002; Galloway *et al*. 2002; Davis & Jones 2008). In many dichogamous species other floral traits that may reduce geitonogamy by influencing pollinator behavior have been observed. For example, display size may influence the number of flowers visited (Galloway *et al*. 2002) or the architecture of the inflorescence may encourage incoming pollinators to visit female-phase flowers before male-phase flowers (Harder *et al*. 2000).

In the protandrous herb *S. officinalis*, in addition to the gender transition, flowers also appear to undergo a correlated change in color from white to pink. Like most protandrous plants, *S. officinalis* is pollinated by animals (Faegri & Van Der Pijl 1976; Bertin & Newman 1993). Bouncing bet appears to be mainly pollinated by moths (Jürgens *et al*. 1996; Witt *et al*. 1999); however, personal observations have also suggested that it may be pollinated by bees (S. Jabbari, unpubl. data, 2008). Bees have been shown to have different color preferences than moths (Faegri & Van Der Pijl 1976). A correlation between gender and flower color may direct or attract pollinators to specific gender stages. This may influence selfing rates by reducing the number of flowers visited within the same plant and, therefore, geitonogamous pollination. However, these two traits have not previously been studied in concert. In addition, the production of floral pigments is influenced by environmental factors. In the present study, five populations across central Indiana were studied to further understand if: (i) there is indeed a correlation between gender and flower color; (ii) diurnal and nocturnal pollinators are visiting and effectively pollinating the flowers; and (iii) color change is influenced by genetic variation or environmental effects.

Materials and methods

Study populations

Saponaria officinalis (Caryophyllaceae) L., commonly known as bouncing bet, is an invasive perennial herb that was introduced to the USA from Europe, and is now distributed across continental USA and northern Canada (US Department of Agriculture 2010). It is found along roadsides, disturbed areas and railroad tracks. In addition to reproduction through seed, *S. officinalis* is able to spread clonally through the growth of underground rhizomes and can form dense clusters of inflorescences making it highly susceptible to geitonogamous pollination (Jürgens *et al*. 1996; Witt *et al*. 1999; Davis & Jones 2008).

The flowering season of bouncing bet in Indiana is from June to August. Plants produce inflorescences of flowers

in terminal clusters. Individual flowers are protandrous, opening in the evening and remaining open for approximately 72 h. Flowers develop through four stages, with stage one consisting of an early male phase when the flower opens and the first of two whorls of stamens extends from the corolla tube and dehisces. Stage two, the late male phase, is marked by the emergence of the second whorl of stamens. The third stage, the early female phase, corresponds to the initial protrusion of the three stigmas from the corolla tube. The fourth and final stage, the late female phase is associated with the stigmas curling back towards the petals. At this stage, the anthers are typically no longer present on the stamens. As the flower transitions from male to female, there appears to be a correlated transition in petal color from white to pink. Plants in all of the populations used in the present study have the potential for geitonogamous pollination, as flower counts indicated that both male-phase and female-phase flowers are open simultaneously on all individuals (S. Jabbari, unpubl. data, 2008). Davis and Jones (2008) confirmed through pollination studies that autogamy and geitonogamy can occur in bouncing bet, but outcrossing through the delivery of pollen by insect pollinators contributes significantly to seed production.

In the present study, five natural populations of *S. officinalis* distributed across central Indiana were examined. The populations were located in Marion and Morgan counties, and included roadsides, walking trails and undisturbed grassy areas. The populations also differed in environmental characteristics, such as sun exposure, soil type and distance to water resources. Owing to the possibility of clonal reproduction in *S. officinalis*, the plants selected for experimental study were a minimum of 1 m apart.

Flower color change in natural populations

To document the change in flower color as the flower transitions from its male to female phase and to determine if there are population-level differences in color, between 12 and 20 flowers from each of the four gender stages were collected from plants within each of four populations, for a total of 268 flowers. The four populations were chosen based on the number of flowering plants and to represent a variety of environmental conditions, such as sun exposure. To quantify color, anthocyanins, the pigments responsible for the pink color of the flowers, were extracted from the petals. The flower petals were diced using a clean scalpel, the mass was determined to the nearest 0.001 mg, and the petals were placed in Eppendorf tubes with 1.5 mL of acidified methanol (16 MeOH : 3H_2O : 1 3 mol/L HCl) in a refrigerator overnight. On the following day, the Eppendorf tubes were centrifuged to settle the diced petals. The supernatant was collected with a micropipette and placed into a cuvette, which was then used to measure the absorbance of the anthocyanin solution at a primary wavelength of 530 nm and a secondary correction wavelength of 625 nm using a Spectronic Genesys 5 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). The absorbance was then converted to the units ABS/g/mL, which correlates to anthocyanin concentration, using the protocol in Weiss and Halevy (1989). The data obtained were then analyzed using a mixed-model ANOVA with anthocyanin concentration as the dependent variable and flower stage as a fixed independent variable and population as a random independent variable. All analyses were conducted using SPSS 16.0 software for Macintosh (IBM, Armonk, NY, USA).

Environmental and population effects on flower color in a common garden

To separate the effects of the environment and the genetic population source on flower color, 20 plants from each of five populations, for a total of 100 plants, were transplanted in July 2009 into a common garden on campus at the University of Indianapolis. The common garden was divided into four plots, each 9 m^2. Within each plot, plants were spaced with 0.3 m between each plant. Two of the plots were manipulated for sunlight exposure by using frames constructed from polyvinyl chloride (PVC) pipes that were then covered with 50% shade cloth. The plants were allowed sufficient sun exposure to ensure that new buds were formed and flowers began to open (approximately 3 weeks). Flower petals were then collected from each of the four gender stages from each plant. Anthocyanins were extracted and analyzed as described above. A mixed-model ANOVA was conducted on the anthocyanin concentrations with anthocyanin concentration as the dependent variable, treatment (shade *vs* sun) as the fixed-effect independent variable and population as a random independent variable.

Timing of pollen viability and stigma receptivity

To document the length of the male and female phases and their potential overlap, which would allow for autogamous pollination, pollen viability and stigma receptivity were tracked in plants growing in the common garden experimental plots.

The length of the male phase was assessed by using two methods to determine pollen grain viability: (i) collection and staining of viable pollen grains with lactophenol aniline blue; and (ii) collection and growth of pollen tubes on Brewbaker–Kwack media. Five flower buds on 20 plants were selected at random for each procedure and marked with jewelry tags for a total of 100 flowers. Pollen

from each flower was collected at 0, 12, 24, 36 and 48 h after anthesis. The lactophenol aniline blue stain was prepared according to the procedure outlined by Kearns and Inouye (1993). Pollen grains were collected by dusting the stamens of the flower against a microscope slide along with a drop of stain. The lactophenol aniline blue stains the viable pollen grains dark blue, whereas abortive grains remain unstained (Kearns & Inouye 1993). The numbers of non-abortive and abortive pollen grains were counted and the percentages of viable pollen grains were calculated.

A solution of Brewbaker–Kwack media and 2% agarose was prepared and poured into individual Petri dishes (Kearns & Inouye 1993). Pollen grains were collected by dusting the stamens of the flowers against the surface of media within separate Petri dishes. The plates were incubated at room temperature for 2 h and pollen tube growth was observed under a dissecting microscope. The percentage of pollen grains that germinated was calculated.

Data for the percentages of viable pollen grains and pollen germinated were arcsine transformed to meet the assumptions of normality. To determine if there was an effect of flower age on male sexual function, two separate one-way ANOVAs were carried out with either arcsine percentage viability or percentage germinated as the dependent variable and flower age (0, 12, 24, 36 or 48 h) as the independent variable. A post-hoc Tukey's test was carried out to determine if there were significant differences between specific floral stages.

Finally, female sexual function was examined by evaluating stigma receptivity with fluorescence microscopy as outlined in Kearns and Inouye (1993). Five individual flower buds from 20 plants, for a total of 100 flowers, were emasculated using forceps to remove the anthers from all 10 stamens through the closed petals. The buds were then marked with jewelry tags, covered with bridal veil and pollinated by hand at 0, 12, 24 and 36 h after anthesis. These flowers were then collected 12 h after pollination and were fixed and stored in 70% ethanol. The styles of each flower were separated from the rest of the floral structures and soaked in 8 mol/L sodium hydroxide (NaOH) for 24 h. The stigmas were then rinsed with distilled water and stored in a 0.5% aniline blue solution, which was dissolved in 0.1 mol/L tripotassium phosphate (K_3PO_4). After storage, the stigmas were placed on a slide with distilled water and observed under an epifluorescence microscope equipped with a 520 nm filter cube. The numbers of pollen grains adhering to the stigma and those that formed pollen tubes were counted. To determine if there was an effect of flower age on female sexual function, two separate one-way ANOVAs were carried out with either the number of pollen grains adhering or the number of pollen tubes formed as the dependent variable and flower age as the independent variable. A post-hoc Tukey's test was carried out to determine if there were significant differences between specific floral stages.

Relative pollinator contribution and behavior

Pollinator observations were conducted with an observer watching two or more flowers of various stages on each plant, growing in the common garden in 2009, for 30-min intervals during different times of the day, for a total of 14 h. To monitor different pollinators during their active pollinating times, flowers were watched in the common garden. Flowers were monitored during the mornings and afternoons to observe bees and other diurnal insects, and at dusk to observe moths and nocturnal insects. Plants were randomly chosen for observation based on the number and type of flowers within the inflorescences to ensure equal numbers of male-phase and female-phase flowers in the inflorescence. Each time an insect landed on a flower and probed its interior, it was documented as a visit and the sexual stage of the flower was recorded.

To determine the relative contribution of diurnal (those active mostly in daylight) versus nocturnal (those active at dusk and night) pollinators, 25 plants were selected at random from the common garden and two inflorescences with no open flowers were chosen on each plant. One inflorescence was covered with a polyurethane pollination bag for 12 h, from approximately 9.00 PM until 9.00 AM, exposing it to diurnal pollinators. The second inflorescence was covered in the same manner from 9.00 AM to 9.00 PM, exposing it to nocturnal pollinators for 12 h. These times were chosen to allow equal times for diurnal and nocturnal pollinators and, because of daylight savings time, represent times shortly after sunrise and before sunset. The process was continued, on the chosen inflorescences, daily for 2 weeks until the flowers in bud began to show fruit formation. Each inflorescence was collected after an additional 2 weeks and the fruit set (number of fruits per flower) and seed set (number of seeds per fruit) were counted and recorded. Data were arcsine transformed to meet the assumptions of normality, and two separate one-way ANOVAs were carried out with either arcsine (fruit set or seed set) as the dependent variable and treatment (diurnal or nocturnal pollinated) as the independent variable.

Phenotypic plasticity in flower color

As *S. officinalis* is perennial, plants grown in the common garden in the summer of 2009 were studied once again in the summer of 2010 to determine if the species had the ability to respond to environmental changes from year to year. All plants were given equal treatments in 2010 by removing the shade cloth that covered two of the plots in 2009. To study the ability of the plants to respond to

Table 1 Results of a mixed-model ANOVA analyzing the effects of flower stage (fixed effect) and population (random effect) on the dependent variable of anthocyanin concentration in the protandrous species *Saponaria officinalis*

Source	Type III sum of squares	d.f.	*F*	*P*
Stage	644.066924	3	25.833885	0.000083
	76.291160	9		
Population	44.440601	3	1.764385	0.223450
	75.844133	9		
Stage × population	75.743373	9	2.287382	0.017541
	927.180013	252		

environmental changes, the stage with the most significant color difference, stage four late-female flowers, was collected from all 38 plants that were flowering in August 2010. Anthocyanins were once again extracted and a mixed-model ANOVA was conducted with color at stage four as the dependent variable, and year and treatment as fixed independent variables. These 38 plants represented a subset of the plants that were studied in 2009, as not all plants that flowered in 2009 were flowering at the time of collection in 2010 and vice versa. For this subset of plants an analysis was conducted on the difference in anthocyanin concentrations between 2009 and 2010 to document the difference in color from year one to year two. The data were then log transformed to correct for the inequality of variances between groups. Following this correction, a paired *t*-test was conducted to determine if there was a significant difference between the color changes seen in the 'treatment (shade cloth in year one) to no treatment' (no shade cloth in year two) plots versus 'no treatment to no treatment' (control) plots.

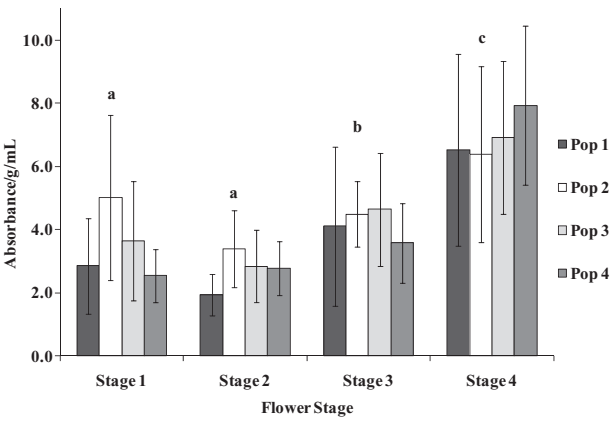

Fig. 1 Anthocyanin concentrations (mean ± standard deviation) in petals across flower gender (stage 1, early male; stage 2, late male; stage 3, early female; stage 4, late female) in four populations of *Saponaria officinalis* growing in Central Indiana. Different letters indicate stages that are statistically different (post-hoc Tukey's test).

Results

Flower color change in natural populations

The flower color data from natural populations indicated that there was no significant effect of the main factor of population on flower color; however, there was a significant effect of flower stage (Table 1). A post-hoc Tukey's test showed that both stages one and two, the male-phase flowers, had significantly lower anthocyanin concentrations than the female stages three and four and that female stage four flowers had significantly higher anthocyanin concentrations than all other stages (Fig. 1). In addition, there was a significant flower stage × population interaction (Table 1), indicating that the color of the flower depends on the combination of what stage it is in as well as what population the plant is growing.

Environmental and population effects on flower color in a common garden

The common garden experiment also illustrated that there was a significant effect of floral stage on petal color, with late-stage female flowers being significantly darker pink than early-stage male flowers. The environment also affected flower color; flowers in the sun treatment had a higher anthocyanin concentration than those in the shade treatment (Table 2). An effect of stage × treatment interaction and a post-hoc Tukey's test indicate that stage four was significantly different from all other stages in both the sun and shade treatments, and that stage four flowers in the sun treatment had a significantly higher anthocyanin concentration than stage four flowers in the shade treatment (Fig. 2). Unlike the results from plants growing in natural populations, in the common garden, there was no significant effect of population or a significant stage × population interaction (Table 2).

Timing of pollen viability and stigma receptivity

The percentage of pollen grains staining positive for viability is high in recently opened flowers (88%), but begins to drop off quickly after 24 h and falls to nearly 50% by 48 h after opening (ANOVA, $P = 0.005$) (Fig. 3a). The percentage of pollen grains capable of germination showed the same trend (ANOVA, $P < 0.001$). The percent-

Source	Type III sum of squares	d.f.	*F*	*P*
Treatment	1324.606	1	10.609	0.028
	540.024	4		
Stage	6586.313	3	56.042	< 0.001
	609.365	16		
Stage × treatment	1702.163	3	15.394	< 0.001
	583.395	16		
Population	453.363	4	0.857	0.558
	522.885	4		
Stage × population	445.918	12	1.072	0.453
	415.879	12		

Table 2 Results of a mixed-model ANOVA analyzing the effects of treatment, stage and population on the concentration of anthocyanin in flowers of *Saponaria officinalis*

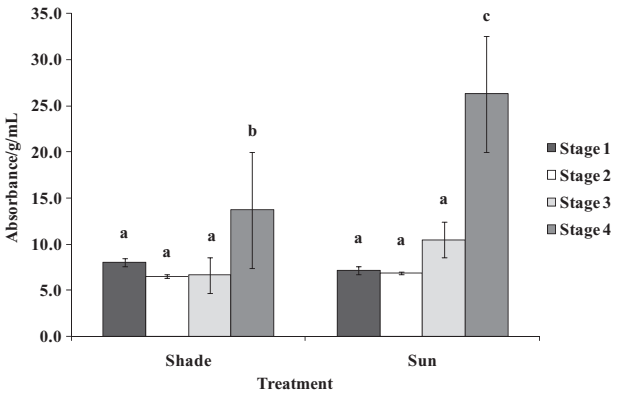

Fig. 2 Anthocyanin concentrations (mean ± standard deviation) in petals from four stages of flowers of *Saponaria officinalis* growing in a common garden at the University of Indianapolis. Plants were subjected to two treatments, shade and sun.

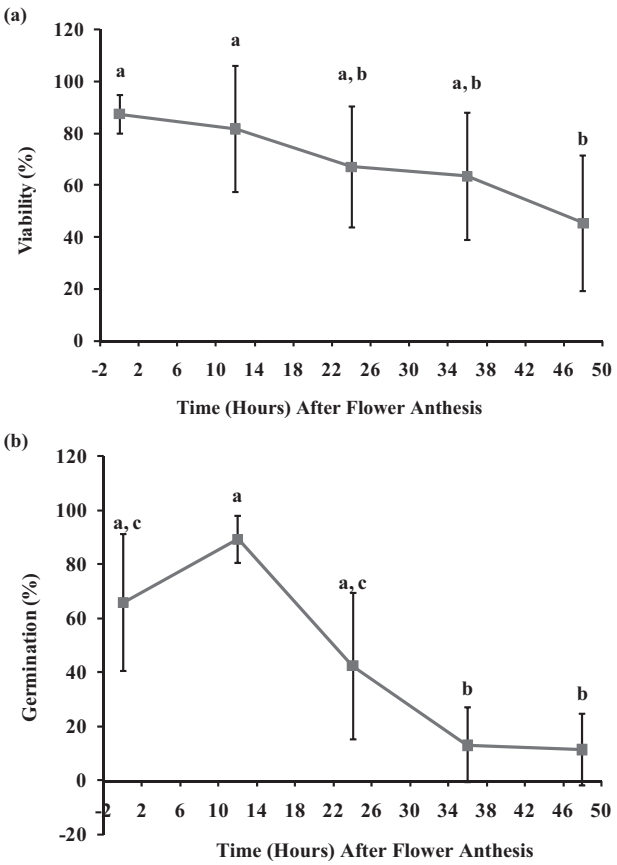

Fig. 3 Timing of male sexual function in protrandrous flowers of *Saponaria officinalis*. (a) Percentage of pollen grains that stained as viable using lactophenol aniline blue (mean ± standard deviation) and (b) the percentage of pollen grains that grew pollen tubes after incubation on Brewbaker–Kwack media (mean ± standard deviation). Different letters indicate stages that are statistically different (post-hocTukey's test) from one another.

age of pollen grains that germinated was close to 70% for grains collected 12 h after anthesis, but fell to 13% and 11% by 36 h and 48 h after anthesis (Fig. 3b). This indicates that there is likely little viable pollen remaining for autogamy 36 h after flower opening.

There was no significant increase in the number of pollen grains adhering to the stigma or the number of germinated pollen grains for flowers pollinated in the first 36 h after opening ($P = 0.559$ and $P = 0.517$, respectively). This represents a flower age of 48 h, as the stigmas were not collected until 12 h after hand pollination. The low numbers of pollen grains adhering and germinating indicate that the stigmas (Fig. 4a,b) were most likely not fully receptive by 36 h.

Relative pollinator contribution and behavior

Both diurnal and nocturnal visits by a variety of insect types were recorded (Table 3). There was no significant difference between the number of visits between male-phase and female-phase flowers. However, there may be a trend for a preference toward female-stage flowers for both diurnal and nocturnal pollinators ($P = 0.18$).

There was no significant difference in fruit set (ANOVA, $P = 0.398$) or seed set (ANOVA, $P = 0.221$) between inflorescences bagged during the day versus those bagged at

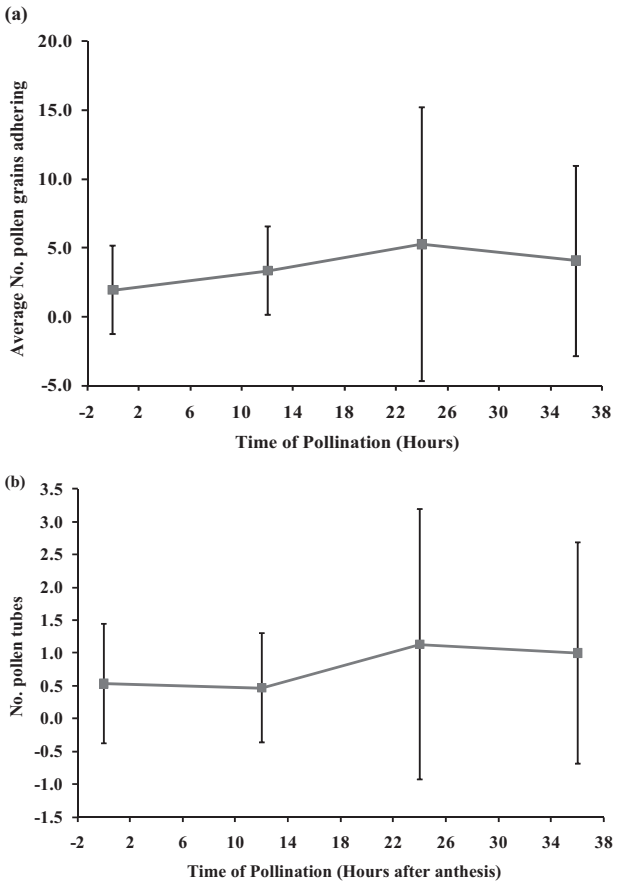

Fig. 4 Timing of stigma receptivity of *Saponaria officinalis* as measured by (a) the number of pollen grains adhered and (b) the number of pollen tubes germinating on stigmas that were hand pollinated 0–36 h after flower anthesis (mean ± standard deviation).

Table 3 Types of pollinators observed and their number of visits to male-phase and female-phase flowers of *Saponaria officinalis* during day and night time monitoring

Time of day	Insects observed	Mean no. visits per hour per flower	
		Male	Female
Diurnal	Flies (Diptera) Bumble bees (*Bombus*) Honey bees (*Apis*)	1.5	2
Nocturnal	Crane flies (Tipulidae)	1.5	2.2

night (Fig. 5). This indicates that both diurnal and nocturnal insects are contributing to pollination in this species and are equally efficient.

Phenotypic plasticity in flower color

The comparison of all plants growing in the common garden in year one and year two indicates that floral color

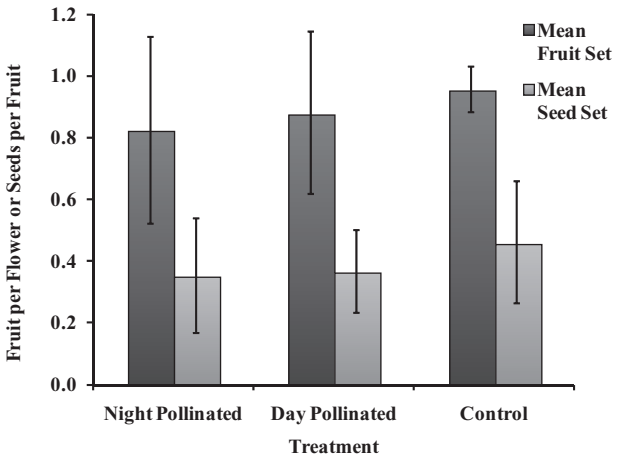

Fig. 5 Reproductive success of inflorescences in which either diurnal or nocturnal pollinators were excluded by pollination bags (mean ± standard deviation).

Table 4 Results of an ANOVA illustrating the effect of year and treatment on anthocyanin concentration in stage four late female flowers of *Saponaria officinalis* growing in a common garden at the University of Indianapolis

Source	Type III sum of squares	d.f.	*F*	*P*
Year	3619.419	1	51.677	< 0.001
Treatment	438.211	1	6.257	0.014
Year × treatment	924.770	1	13.204	< 0.001

in *S. officinalis* depends on current environmental conditions (Table 4). There was a significant effect of year, with flowers overall having a lower anthocyanin concentration in year two than in year one (Fig. 6). In year two, all of the plants were uncovered and therefore received no treatment and, as expected, there was no difference between the plots as there was in year one (Fig. 7). For the subset of individual plants in which data were available for both years there was also a change in color from year one to year two. The paired *t*-test indicated that the difference in flower color between year one and year two was significantly smaller between plants that were in the shade treatment in year one versus those plants that were in the sun treatment in year one (Fig. 7). In other words, individual plants that were growing in the shade treatment in year one had less of a change in flower color in year two, whereas plants that were growing in full sun in year one had a much larger change in flower color in year two.

Discussion

The present study illustrates that protandry and color change are correlated processes that are potentially influencing the pollination biology of *S. officinalis*. Extraction of

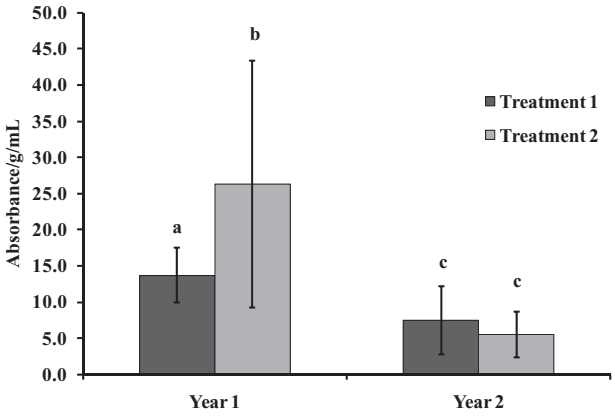

Fig. 6 Comparison of flower color (mean ± standard deviation) of late-stage female flowers in *Saponaria officinalis* plants grown in a shade treatment in year one and no treatment in year two (Treatment 1) and grown in full sun in year one and no treatment in year two (Treatment 2).

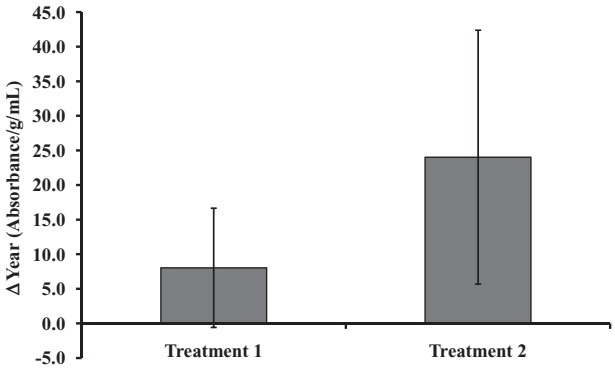

Fig. 7 Average change in anthocyanin concentrations (mean ± standard deviation) of late-stage female flowers of individual plants of *Saponaria officinalis* grown in a shade treatment in year one and no treatment in year two (Treatment 1) and grown in full sun in year one and no treatment in year two (Treatment 2).

anthocyanins showed that there was a significant effect of flower stage on color, with male-phase flowers having a lower concentration of anthocyanins than female-phase flowers (Fig. 1). This color change was seen across all naturally growing populations, but a population × stage interaction indicates that the degree of color change is dependent on the population in which the plants are growing (Table 1).

To further investigate the source of the population effect, in the second year of the study, plants were transplanted to a common garden. Data from the common garden indicated that flowers exhibited the same effect of stage as they did in their natural populations, with female-phase flowers having a significantly higher concentration of anthocyanins than male-phase flowers (Fig. 2). There was no longer a stage × population interaction, indicating that this effect from the previous study resulted from environmental differences in the natural populations rather than genetic differences among the populations (Table 2). As anthocyanin concentration always increased in female-phase flowers in both natural populations and in the common garden, it is possible that color change is a physiological change with age (Delph & Lively 1989). However, the degree of color change was significantly greater in those plants that were in the full sun treatment compared with the shade treatment (Fig. 6). Studies indicate that anthocyanin production can be an indirect photoprotection mechanism of photosystem II. Anthocyanin induction usually requires light exposure, and an increase in exposure leads to upregulation of anthocyanin biosynthesis (Gould *et al*. 2010). In other words, with increased light exposure, the anthocyanin concentration rises, resulting in a darker pink color in the flowers, leaves and stems of plants. Therefore, although the development of a color change is fixed across all plant populations, the degree of the color change is influenced by the environment and is phenotypically plastic.

In addition to being phenotypically plastic within a season, the color comparison data of two successive years for flowers growing in the common garden indicated that individual plants of *S. officinalis* are capable of phenotypic plasticity in flower color in response to changing environmental conditions between seasons. All plants in both treatments showed reduced anthocyanin concentration in year two compared with year one (Fig. 7). This difference may be a result of the fact that flowers were collected in August of year two and July of year one. Allocation to reproduction has been shown to vary temporally within a season (Young & Stanton 1990; Brunet & Charlesworth 1995; Mazer *et al*. 2003; Huang *et al*. 2004). In general, later in the season plants may be devoting more of their resources to fruit production in older flowers rather than pollinator attraction in new flowers (Huang *et al*. 2004). Therefore, the reason for the lighter or diminished flower color in year two may have been a result of this change in allocation. Another possible cause of diminished flower color in year two may result from reduced sun exposure; that is, the days were shorter during the time in which these flowers were collected in year two, resulting in diminished anthocyanin biosynthesis (Gould *et al*. 2010). Finally, the diminished color in year two may be correlated with the general condition of the second year plants. Plants growing in the second year of the common garden were larger in size. Gould *et al*. (2010) showed that the degree of red color in leaves and stems was dependent on their position on the plant. In the present study, flowers collected in the second year may have been on lower positions of the plant and may have been shaded by leaves from above.

For the correlation of color and gender transition to be adaptive, it must have an impact on the fitness of the plant.

Dichogamy is often thought to decrease autogamous selfing rates; however, in large inflorescences it may not reduce geitonogamy (Galloway *et al*. 2002). Protandry appears to be limiting the potential for autogamous pollination in this species. The data indicate that pollen germination ability sharply declines by 36 h after opening (Fig. 3), and hand pollinations showed that stigma receptivity is not complete in most individuals by this time. This corresponds to data presented by Davis and Jones (2008) that showed that autonomous-only self-fertilization in bagged flowers produced significantly lower seed set than in control flowers that had insect visitors. However, in the data presented here the standard deviation for both the number of pollen grains adhering and the number of pollen tubes increased at 24 h, indicating that some individuals may be becoming receptive and there is variation in the timing of receptivity (Fig. 4a,b). This in turn would lead to variation among individuals in the level of autogamous pollination possible. The pollen viability and germination data also indicate that the color change in the flowers is corresponding to the change in gender. Stage one and two flowers that have significantly lower anthocyanin concentrations correspond to the first 24–36 h after flower opening, or the male stage in which pollen is still viable.

The pollinator observations in the common garden indicated that there was not a significant effect of time of day on the number of visits received, indicating that diurnal pollinators were visiting as frequently in the day as moth pollinators were at dusk (Table 3). Bagging experiments showed that diurnal and nocturnal pollinators are equally efficient, which indicates that bees are important pollinators for *S. officinalis* (Fig. 5). Bouncing bet is an expected candidate for bee pollination owing to its sucrose-rich nectar, and this is significant because bees are known to have innate preferences for flower colors (Baker & Baker 1983; Jürgens *et al*. 1996; Witt *et al*. 1999). Preliminary data demonstrated a trend toward a preference for female-phase flowers (S. Jabbari, unpubl. data, 2009) and insect visitors may be using color to make this distinction. However, further studies need to be conducted to account for the number of flowers open and the percentage of flowers that are in the male versus female stage. Witt *et al*. (1999) demonstrated that nectar production in *S. officinalis* is at its peak on the evening of the second day following anthesis, which correlates with the time indicated as the female phase in the present study. If insects are preferentially visiting female-phase flowers when they visit a plant, this will bring outcross pollen to these flowers before visiting male-phase flowers, reducing intra-plant pollen transfer, and increasing the amount of pollen being exported and, hence, siring success. The placement and timing of male-phase and female-phase flowers can reduce geitonogamous pollination in some species (Harder *et al*. 2000), but less is known about loosely structured inflorescences such as *S. officinalis* (Galloway *et al*. 2002).

The ability to respond to the environment and show plasticity in floral color change could also increase the plant's ability to manipulate its pollinator interactions. Bees have been shown to be more active visitors in sunnier, warmer temperatures (McCall & Primack 1992; Totland 1993; Lázaro *et al*. 2008), when *S. officinalis* displays a more pronounced flower color change. Therefore, in conditions of increased sun exposure, pollinators may be visiting more frequently and staying on individual plants longer, which increases the risk of geitonogamy. A more pronounced color change in this type of environment might increase the effectiveness of directing pollinators to specific flowers. In contrast, when sun exposure is low and pollinators may be scarce, it may be advantageous to reduce the amount of color change so that pollinators visit as many flowers on the plant as possible and geitonogamy may serve as a potential means of pollination, thus allowing for reproductive assurance. In our case, all plants showed color change as the flowers transitioned from male to female and this response increased with sun exposure; however, there may be genotypic differences in the degree of color change. This remains to be investigated.

This study is the first to document that color change is correlated with gender transition in *S. officinalis* and the first to document that *S. officinalis* is bee pollinated. To determine if the covariation between color change and gender transition, and the phenotypic plasticity it shows is adaptive, further studies will need to be conducted. More detailed pollinator observations need to confirm if pollinators indeed show a preference for color and if this preference can be distinguished from a gender preference. Future studies will also determine if there is genetic variation among individuals in color change and if this is correlated to fitness. If color change does affect fitness, whether or not it is a result of fewer geitonogamous pollinations must be determined.

Acknowledgments

The authors wish to give special thanks to Corey A. Woods for constructing the frames for the common garden experiment, Dr Dana A. Dudle for her advice on a previous version of this manuscript, the University of Indianapolis for the Summer Research Grant to Dr Sandra L. Davis, and also to the University of Indianapolis physical plant for clearing the plots for the common garden.

References

Baker H. G. & Baker I. (1983) Floral nectar sugar constituents in relation to pollinator type. In: Jones C. E. & Little R. J. (eds). *Handbook of Experimental Pollination Biology*. Van Nostrand Reinhold Company Inc, New York, pp. 117–141.

Bertin R. I. & Newman C. M. (1993) Dichogamy in angiosperms. *The Botanical Review* **59**: 113–146.

Brunet J. & Charlesworth D. (1995) Floral sex allocation in sequentially blooming plants. *Evolution* **49**: 70–79.

Casper B. B. & La Pine T. R. (1984) Changes in corolla color and other floral characteristics in *Cryptantha humilis* (Boraginaceae): cues to discourage pollinators? *Evolution* **38**: 128–141.

Davis S. L. & Jones L. (2008) The potential for mixed mating in the protandrous perennial *Saponaria officinalis* (Caryophyllaceae). *Plant Species Biology* **23**: 183–191.

De Jong T. J., Klinkhamer P. G. L. & Staalduinen M. J. (1992) The consequences of pollination biology for selection of mass or extended blooming. *Functional Ecology* **6**: 606–615.

De Jong T. J., Waser N. M. & Klinkhamer P. G. L. (1993) Geitonogamy: the neglected side of selfing. *Tree* **8**: 321–325.

Delph L. F. & Ashman T. L. (2006) Trait selection in flowering plants: how does sexual selection contribute? *Integrative and Comparative Biology* **46**: 465–472.

Delph L. F. & Lively C. M. (1989) The evolution of floral color change: pollinator attraction versus physiological constraints in *Fuchsia excorticata*. *Evolution* **43**: 1252–1262.

Elle E. & Hare J. D. (2002) Environmentally induced variation in floral traits affects the mating system in *Datura wrightii*. *Functional Ecology* **16**: 79–88.

Faegri K. & Van Der Pijl L. (1976) *The Principle of Pollination Ecology*. Pergamon, Oxford.

Farzad M., Griesbach R., Hammond J., Weiss M. R. & Elmendorf H. G. (2003) Differential expression of three key anthocyanin biosynthetic genes in a color-changing flower, *Viola cornuta* cv. Yesterday, Today, and Tomorrow. *Plant Science* **165**: 1333–1342.

Galloway L. F., Cirigliano T. & Gremski K. (2002) The contribution of display size and dichogamy to potential geitonogamy in *Campanula americana*. *International Journal of Plant Sciences* **163**: 133–139.

Gould K. S., Dudle D. A. & Neufeld H. S. (2010) Why some stems are red: cauline anthocyanins shield photosystem II against high light stress. *Journal of Experimental Botany* **61**: 2707–2717.

Harder L. D. & Barrett S. C. H. (1996) Pollen dispersal and matting patterns in animal-pollinated plants. In: Lloyd D. G. & Barrett S. C. H. (eds). *Floral Biology, Studies on Floral Evolution in Animal-Pollinated Plants*. Springer, New York, pp. 140–190.

Harder L. D., Barrett S. C. H. & Cole W. W. (2000) The mating consequences of sexual segregation within inflorescences of flowering plants. *Proceedings of the Royal Society, Series B* **267**: 315–320.

Harder L. D. & Wilson W. G. (1998) Theoretical consequences of heterogeneous transport conditions for pollen dispersal by animals. *Ecology* **79**: 2789–2807.

Huang S., Tang L., Yu Q. & Guo Y. (2004) Temporal floral sex allocation in protogynous *Aquilegia yabeana* contrasts with protandrous species: support for the mating environment hypothesis. *Evolution* **58**: 1131–1134.

Hughes N. M., Neufeld H. S. & Burkey K. O. (2005) Functional role of anthocyanins in high-light winter leaves of the evergreen herb *Galax urceolata*. *New Phytologist* **168**: 575–587.

Ida T. Y. & Kudo G. (2003) Floral color change in *Weigela middendorffiana* (Caprifoliaceae): reduction of geitonogamous pollination by bumble bees. *American Journal of Botany* **90**: 1751–1757.

Jürgens A., Witt T. & Gottsberger G. (1996) Reproduction and pollination in central European populations of *Silene* and *Saponaria* species. *Botanica Acta* **109**: 316–324.

Kearns C. K. & Inouye D. W. (1993) *Techniques for Pollination Biologists*. University Press of Colorado, Niwot.

Kudo G., Ishii H. S., Hirabayashi Y. & Ida T. Y. (2007) A test of the effect of floral color change on pollination effectiveness using artificial inflorescences visited by bumblebees. *Oecologia* **154**: 119–128.

Lande R. & Schemske L. (1985) The evolution of self-fertilization and inbreeding depression in plants. I. Genetic Models. *Evolution* **39**: 24–40.

Lázaro A., Hegland S. & Totland Ø. (2008) The relationships between floral traits and specificity of pollination systems in three Scandinavian plant communities. *Oecologia* **157**: 249–257.

McCall C. & Primack R. B. (1992) Influence of flower characteristics, weather, time of day, and season on insect visitation rates in three plant communities. *American Journal of Botany* **79**: 434–442.

Mazer S. J., Lowry D. E. & Hansen T. (2003) Effects of nutrient availability on primary sexual traits and their response to selection in *Spergularia marina* (Caryophyllaceae). *Journal of Evolutionary Biology* **16**: 767–778.

Nuttman C. V., Semida F. M., Zalat S. & Willmer P. G. (2006) Visual cues and foraging choices: bee visits to floral colour phases in *Alkanna orientalis* (Boraginaceae). *Biological Journal of the Linnean Society* **87**: 427–435.

Oberrath R. & Böhning-Gaese K. (1999) Floral color change and the attraction of insect pollinators in lungwort (*Pulmonaria collina*). *Oecologia* **121**: 383–391.

Stiles E. A., Cech N. B., Dee S. M. & Lacey E. P. (2007) Temperature-sensitive anthocyanin production in flowers of *Plantago lanceolata*. *Physiologia Plantarum* **129**: 756–765.

Sun S. G., Liao K., Xia J. & Guo Y. H. (2005) Floral colour change in *Pedicularis monbeigiana* (Orobancheacea). *Plant Systematics and Evolution* **255**: 77–85.

Totland Ø. (1993) Pollination in alpine Norway: flowering phenology, insect visitors, and visitation rates in two plant communities. *Canadian Journal of Botany* **71**: 1072–1079.

US Department of Agriculture (2010) PLANTS profile for *Saponaria officinalis* (bouncing bet). [Cited 6 Nov 2010.] Available from URL: http://plants.usda.gov/java/profile?symbol=SAOF4

Weiss M. R. (1991) Floral colour changes as cues for pollinators. *Letters to Nature* **354**: 227–229.

Weiss D. & Halevy A. H. (1989) Stamens and gibberellins in the regulation of corolla pigmentation and growth in *Petunia hybrida*. *Planta* **179**: 89–96.

Willson M. F. & Burley N. (1983) *Mate Choice in Plants*. Princeton University Press, Princeton.

Witt T., Jürgens A., Geyer R. & Gottsberger G. (1999) Nectar dynamics and sugar composition in flowers of *Silene* and *Saponaria* species (Caryophyllaceae). *Plant Biology* **1**: 334–345.

Young H. J. & Stanton M. L. (1990) Temporal patterns of gamete production within individuals of *Raphanus sativus* (Brassicaceae). *Canadian Journal of Botany* **68**: 480–486.