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COMPARATIVE FLORAL ECOLOGY OF BICOLOUR AND CONCOLOUR MORPHS OF *VIOLA PEDATA* L. (VIOLACEAE) FOLLOWING CONTROLLED BURNS

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Abstract—We compared pollinators, pollination rates and seed set of bicolour and concolour morphs in self-incompatible, *Viola pedata* over two seasons. The two populations grew on a wooded slope (CR) vs. an exposed glade (SNR) and were of unequal sizes. Both were burned in 2014. The number of flowers produced by concolour plants at SNR was higher in 2014 while the number of flowering bicolour plants increased significantly at CR in 2015. Petal temperatures, regardless of site, showed that the dark purple, posterior petals of bicolours were consistently warmer than their own mauve-lilac, anterior (lip) petals and the all mauve petals of concolours. Major pollen vectors were polylectic/polyphagic bees (Andrenidae, Apidae and Halictidae) but females of *Andrena carlinii* dominated at both sites. Bees foraged on flowers upside down or right side up but neither mode correlated with either morph. Bees foraged preferentially on concolour at both sites. Pollen tube counts were higher in concolours at both sites with a marginally greater number of pollen tubes penetrating concolour ovules regardless of site or year. While both populations produced more seeds in 2014 SNR plants always produced more seeds than CR plants. The increasing numbers of bicolour plants at CR in 2015 suggested that bicolours may equal or outnumber concolours as dark petals offer additional warmth to ecto-thermic pollinators foraging in a cooler, shady forest vs. an open, sunny glade. Subtle environmental factors may give a floral trait a selective advantage influencing fitness when an unbalanced polymorphism persists in discrete and localized populations.

Keywords: Bees, bicolour, concolour, morphs, ovules, pistils, pollen tubes, posterior petals

INTRODUCTION

Colour polymorphisms have been well documented in flowers of unrelated species. Unlike plants with heterostylous flowers (e.g. *Linum*, see Armbruster et al. 2006) most colour morphs are interpreted as unbalanced polymorphisms (sensu Futuyma 2013) as the frequencies of 2–4 colour morphs vary broadly with natural distribution over time (Irwin & Strauss 2005; Pelligrino et al. 2008). A population's shift in colour morph frequencies may have more than one explanation (Rausher 2008). While the population's response to the selective foraging of its dominant pollinators is anticipated (Epperson & Clegg 1987; Irwin & Strauss 2005; Malberla & Nattero 2011; Russell et al. 2016) there are other factors. These may include florivory/herbivory (Carlson & Holsinger 2013; de Jager & Ellis 2014; Sobral et al. 2016), differential rates of self-pollination in discrete morphs (Fehr & Rausher 2004), variation in inflorescence display (Gomez 2000), genetic trends that cause unidirectional changes in

pigments (Rausher 2008) and/or an indirect response to selection related to pleiotropic, non-floral traits (Armbruster 2002).

Curiously, *Viola* species have not been used to study variation in frequencies of floral colours although they are recorded throughout the genus in North America and Europe (McKinney 1992; Hildebrandt et al 2006; Mereda et al. 2008; Pellegrino et al 2008; Marcussen & Borgen 2011). Instead flowers of *Viola* species were more likely to be used as model systems in genetic variation (Clausen 1926; Culley 2002), systematic variation (Nieuwland & Kaczmarek 1914; McKinney 1992), developmental morphology (Johri et al 1992; Weberling, 1989), molecular development (Wang 2008) and reproductive ecology (Gurevitch et al. 2006; Winn & Moriuchi 2009). In some *Viola* species endemic to Europe petal colour frequency is predictable according to whether populations grow on old soils polluted by zinc or lead (Hildebrandt et al. 2006). Gradations in petal colour in the “zinc” violets are also results of a past history of interspecific introgression (Migdalek et al. 2013).

There should have been a continuous interest in uniting demographic studies of colour morphs in *Viola* species with

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their pollination ecology as interpretations of floral adaptations in their chasmogamous flowers started in the 19th century (Darwin 1876; Müller 1883). However the much later work of Beattie (1969; 1971a; 1972; 1974; 1976) remains the definitive introduction to pollination mechanisms and breeding systems in this genus. Specifically, Beattie's observations showed that *Viola* petals and pedicels changed positions and angles over their respective lifespans encouraging visits by many pollinators representing at least three insect Orders (Diptera, Hymenoptera, Lepidoptera). Some insects were more likely to forage for nectar in an inverted position acquiring ventral depositions of pollen (sternotribic) while others foraged after landing prone on the liplike anterior petal receiving dorsal depositions (nototribic). Beattie (1974) attributed these foraging behaviours to the evolution of two overlapping syndromes based on floral architecture, petal ornamentation and modifications of the terminal surfaces of anthers and pistils.

With more than 400 species in the genus *Viola* (Mabberley, 1997) it is not surprising that a few recent studies challenge the earlier descriptions of generalist entomophily reported by Davidse (1968) and Beattie (1969; 1971; 1974). Herrera (1990; 1993) concluded that floral traits of *V. cazorensis* most probably evolved under disruptive selection as only one hawkmoth, *Macroglossum stellatarum* was the primary pollinator. Freitas & Sazima (2003) found that flowers of two, Neotropical, high elevation species produced little or no nectar and depended primarily on pollen harvesting bees (*Anthenoides*; Andrenidae). This conflicts with the generalization that insects forage on *Viola* flowers for nectar exclusively, and this ends in the passive deposition of pollen onto the vector's body (sensu Bernhardt 1996).

In fact, *Viola pedata* shows floral characteristics atypical for the genus that may make it ideal for studies on colour morph frequencies. As it produces no cleistogamous flowers and appears to be the only *Viola* species studied so far with a self-incompatible (late-acting) breeding system (Becker & Ewart 1990). Unlike the much used *Ipomoea purpurea* (Fehr & Rausher 2004) it is an obligate out-crosser. It is not known to hybridize with allied, acaulescent species (McKinney 1992). Floristic taxonomists recorded a white-flowered form (alba) in *V. pedata*, a second form in which all petals are lavender-mauve (concolour) and a third in which three petals are lavender-mauve while the two posterior petals are dark purple (bicolour). These last two forms are so distinct that children living in the Missouri Ozarks called the concolour forms hens and bicolour forms roosters (Steyermark 1963) but are these morph frequencies pollinator driven? Carroll & Goldmann (1994) found that dusky winged skippers (*Erynnis* species; Lepidoptera) spent the same amount of time foraging on bicolour and concolour morphs of *V. pedata*. In fact, these insects foraged on each morph in proportion to morph frequencies found in the same population.

We require more information on the pollination ecology of *V. pedata* as insects observed foraging on its flowers in past studies were not always identified to species and collectors failed to note whether euthanized foragers carried

the pollen of the host flower. Gibson & Davie (1901) proposed that the species was pollinated by a combination of Lepidoptera and long tongue bees. Beattie (1974) was far more specific recording 63% of all visits to the flower by bees in the family, Andrenidae (species unidentified) and 21% to Lepidoptera including day-flying hawkmoths (*Hemaris*; Sphingidae). In contrast, Carroll & Goldman (1994) described a more specialized system in Missouri based on small Lepidoptera. For Beattie (1974), floral presentation in *V. pedata* expressed intermediate floral characters based on its floral architecture and observations of how insects foraged on the flower. About 36% of all flowers of *V. pedata* received insects landing on the anterior (lip-like) petal leading to dorsal (nototribic) depositions of pollen while 64% foraged in an inverted manner leading to ventral (sternotribic) depositions.

This paper attempts to address and clarify eight interrelated questions regarding the pollination dynamics and relative fitness of two, colour morphs of *Viola pedata* in two populations in Missouri following exposure to controlled burns. First, do colour morphs offer the same numbers of flowers/plant (floral presentation) over time? Second, as dark colours absorb heat more efficiently, are the exposed and reflexed, deep purple petals of bicolour flowers warmer than the nodding, mauve-lilac posterior (lip) petal on the same flower and in the posterior petals of concolour flowers? Third, does pollinator diversity vary according to colour morph and site over time? Fourth, do pollinators approach and then forage on bicolour and concolour flowers in the same way? Fifth, are pollinators more likely to be generalist or specialist foragers as this species produces both nectar and pollen as edible rewards? Sixth, do natural rates of pollination (pollen tubes penetrating pistils) vary in two colour morphs according to site and season? Seventh, do bicolour and concolour morphs produce the same number of ovules/pistil over time? Finally, do bicolour and concolour morphs produce the same numbers of seeds in different sites over time? Ultimately, these combined results will allow us to ask which morph is fittest according to site, season, pollinator activity and history of fire-regime.

MATERIALS AND METHODS

Study sites and field states

The first site used from 5/2/13 – 5/7/13, from 4/14/14 – 5/27/14 and from 4/2/15 – 6/3/15 was on Oak Ridge, adjacent to the 11 km Sugar Creek trail within Cuivre River State Park (CR), Lincoln County, Missouri. Less than 200 flowering plants of *V. pedata* were found on the slope under mixed hardwood (*Quercus* dominated) forest annually for all three seasons (Fig. 1). The site is usually burned in late winter in alternate years by park staff and was burned in 2014 but not in 2013 or 2015. Voucher specimens were deposited in the herbarium of the Missouri Botanical Garden (MO).

The second site used from 4/16/14 – 5/28/14 and from 4/8/15 – 6/4/15 was at Shaw Nature Reserve (SNR) at Gray Summit, Franklin County (Fig. 2). The population under study was confined to the relatively sterile,



FIGURE 1. Broad habitat view of field site at Oak Ridge, Cuivre River State Park, April 2015 (R. Edens-Meier, photographer).



FIGURE 2. Broad habitat view of field site at Shaw Nature Reserve, April 2015. Note the dead tillers of grass remaining from the previous autumn when the glade is not burned (Justin Zweck, photographer).

highly drained, sandy soil lower edges of the dolomite glade (Crescent Glade) dominated by grasses and forbs with some shrubs (*Rhus* species). In 2014 and 2015 this site produced > 1,000 flowering, basal rosettes of *Viola pedata*. The site was burned in 2014 by Shaw employees but not in 2015. Burns are conducted in the winter at 3-year intervals, coinciding with the burning of surrounding woodlands. However, some years do not burn well due to the sparseness of fuel in a true glade flora. Prior to 2014 this site was burned last in 2010 (James Trager, personal communication). Voucher specimens were deposited as above. Pooled field observations, bagging and collection

hours (see below) at both sites by co-authors from 2013 – 2015 totalled approximately 112 hours.

Morphs frequency and flowers per morph

We documented when the first flowers opened and the last flowers wilted at both sites in 2014 and 2015. Morphs of *V. pedata* at CR were restricted to the bicolour and concolour forms (McKinney 1992). Only two plants with white morphs were observed at the SNR in 2014 and 2015. Neither was measured and we did not take any time to observe insect visitation of these flowers. In 2014 and 2015 we kept counts of the number of rosettes of bicolour and concolour morphs (Figs. 3 & 4) at each site. We also

counted the number of flowers produced within each rosette. However, as the SNR population was so large we also established a 13.1 × 8.0 m quadrat where the majority of bicolour morphs were found and counted the number of bicolours in 2014 and 2015.

Attractants, rewards and petal temperatures

Viola pedata is among the few species native to North America lacking the characteristic trichome tufts (beards) towards the bases of both lateral petals (McKinney 1992). Flowers of both morphs were dissected to see if they had nectar glands attached to the connective filaments of two stamens as in most *Viola* spp. (Beattie 1974). To determine if the flowers produced a discernible scent we smelled flowers of each morph on plants between 10 AM – Noon. We also placed 1 or 2 flowers of the same morph in clean glass vials, capped the vial and then smelled the contents 20 and 30 minutes later. In 2015 we measured the spur lengths of living flowers of concolour and bicolour morphs, remaining attached to their pedicels, at the Shaw Nature Reserve using electronic digital calipers (Fisher Scientific).

As *V. pedata* is a vernal flowering species the heat generated by solar energy once absorbed and retained by flower petals, may reward ectothermic, insect pollinators (see review in Willmer 2011). One presumes that dark colours (deep purple) absorb more solar energy as heat than light (mauve – lavender). Petal temperatures of both morphs were recorded using an Omega Type T Thermocouple Cu-CuNi HH-25TC Thermometer, Range -80°C to 400°C and an Omega Hypodermic Tissue Probe MPI-30 ½-T-G-60 SMPW-M were used to determine petal temperatures. In each flower of each morph the tissue probe was inserted into one of the posterior petals by carefully weaving the probe through tissue three times (Fig. 3). The temperature was recorded after one minute. The same procedure was also used to determine the petal temperature for the lower, nodding, mauve-lilac, anterior (lip) petal in the same flower. Before taking each petal temperature we recorded the time of day, the ambient temperature and whether the sky was sunny or cloudy.

Floral foragers

Insect visitors were observed *in situ* at both sites. Videos of floral foragers were made by R. Edens-Meier using a Sony Full HD 1080 Handycam, HDR-CX760V, 24.1 Megapixels at the CR site. Three of these videos are shown on Youtube; https://www.youtube.com/results?search_query=viola+pedata. Insect foragers were netted only when they were observed visiting one or more flowers of *V. pedata* and could be observed extending proboscides down the floral tube or manipulating anthers. From 4/6 – 4/7/2013 at CR and from 2014–2015 all insects collected were euthanized using fumes of ethyl acetate and they were pinned, labelled and identified. From 2014–2015 insects were always euthanized in separate jars according to the morph on which they were captured.

Pollen load analyses

To identify and record pollen carried by foragers each specimen euthanized within 24 hours was first placed on a



FIGURE 3. Bicolour flower of *Viola pedata*, with thermocoupler probe inserted through the posterior petals (R. Edens-Meier, photographer).



FIGURE 4. Concolour flower of *Viola pedata* (R. Edens-Meier, photographer).

glass slide and bathed in 1–2 drops of ethyl acetate and/or the scopal load was removed with a probe and added to the slide surface. Grains left on the slide following ethyl acetate evaporation were stained in Calberla's fluid and mounted with glass cover slips for light microscopy after the stain dried. All techniques for washing, staining, mounting, observing grains and co-referencing the label on the slide to the label under the pinned insect followed Bernhardt et al. (2014). As more than one insect was euthanized in the same morph jar pollen of a known species was considered present on a slide when > 25 grains of that morphotype were counted. Therefore, pollen loads from each foraging insect were classified as one of the following; No pollen (< 25 grains of *V. pedata*), pure loads (> 25 grains of *V. pedata*), mixed load (> 25 grains of *V. pedata* + > 25 grains of at least one other co-blooming species) and alien load (> 25 grains of other species but no *V. pedata*).

Landing orientation and foraging bouts on morphs by bees

Bees were the most frequent visitors to these flowers (see below). At the CR site in 2014 and 2015 we observed how bees oriented themselves upon approach and landed on the flowers of each morph prior to feeding on nectar or collecting pollen. Two modes were observed. The bee could land directly on the liplike anterior petal or on a lateral petal of the same flower. This was recorded as right side up (Beattie, 1976 used the term, *nototribe*). Otherwise, the bee landed on one or both of the posterior petals continued to cling to the posterior petals with its third pair of legs and so reached the anther cone or spur by foraging upside down (Beattie 1976 used the term *sternotribe*). Insects that landed on the flowers but failed to forage for nectar or pollen were not recorded. While we observed bees foraging on flowers at the SNR site in 2014 and 2015 the population was so large in both years it was not possible to discern when a bee actually entered the site to start foraging and when it exited (see above).

In 2015 a new protocol was added. We followed bees of several species (most were females of *Andrena carlinii*) at CR to determine how many flowers of each morph they visited during a foraging bout. Bouts were recorded when the bee entered the field site and visited its first flower within the population. Counts stopped when the bee either left the site or was observed foraging on the flowers of another species (see Bernhardt & Montalvo 1979). This protocol could not be used at SNR due to its size as described above.

Natural rates of ovule number, pollination (tubes penetrating pistil tissue) and seed set

Pedicels of both morphs in both populations were selected at random and tagged with jeweller's tags while flowers were in bud each week. As the populations at each site remained in flower less than three weeks each year tagging occurred over a two-week period at both sites in 2014 and 2015. Only one pedicel/basal rosette was tagged and the perianth of the tagged bud was allowed to complete its floral lifespan. Each bicour or concolour flower was collected one to three days after we observed the wilting of petals. The wilted flower was fixed in 3:1 95% ethanol:glacial acetic acid for 2-6 hours then preserved in 70% ethanol. Pistils were excised, softened in sodium sulfite solution under incubation and squashed in decolourized aniline blue prior to viewing under epifluorescence using the Zeiss Axioskop 40 as described in Edens-Meier et al. (2010) to view pollen tubes germinating on the stigmas and penetrating styles, ovaries and ovules. Due to the rigidity of the style each pistil had to be softened for 60 minutes at 42°C but, as the stigma and style are so small, it was not necessary to split them lengthwise to view pollen tube progress. The ovary was butterflied with a scalpel, prior to squashing to permit a count of the number of ovules and observe pollen tube penetrations of micropyles.

To record seed set basal rosettes were selected at random. An open flower in each rosette was then selected at random and tagged, as above. After the petals wilted we bagged the pedicel in a marked organza bag. After four

weeks we recovered and collected as many bags as possible recording the number of mature, filled seeds in each bag. Seeds produced at each site were donated to the seed bank maintained by the Missouri Botanical Garden at the Shaw Nature Reserve.

Statistical analyses

The overall design of this study is a three-way factorial design with colour morph (concolour vs bicour) of the flower as a fixed effect, whereas location (SNR vs CR) and year (2014 vs 2015) were considered random effects. We used the package lme4 (v. 1.1-7) in the R computational environment (v. 3.1.0, R Core Team 2014) in order to perform the mixed effects ANOVA's. Given that all the data collected from the flower squashes were counted data, e.g., number of pollen tubes, we used a squared root transformation to meet the assumptions of the test.

RESULTS

Morphs' frequency and the number of flowers per morph

At the CR (Cuivre River) site in 2014 (burn year) we counted a total of 28 bicour morph plants and 32 plants with concolour flowers (approximately 1:1.02 morph ratio). The difference in morph ratios was not significant (binomial test, $P = 0.5654$). In 2015 we counted 161 plants producing bicour flowers and 88 producing concolours, resulting in a ratio of 1.8: 1.0 respectively. This ratio was a significant deviation from one to one ($P < 0.0001$). At the SNR (Shaw Nature Reserve) in 2014 and 2015, we stopped counting flowering basal rosettes after 1000 and morph ratios remained self-consistent in both seasons (bicour 40.0: concolour 1.0). In 2015, the main area where bicour was most common, produced 25 bicour plants and 71 concolour plants.

While the number of flowers per plant at the CR site was significantly lower in 2014 than in 2015 there was no significant difference between the number of flowers/plant in bicour vs. concolour morphs (Fig. 5). In contrast, the Mean number of flowers/concolour plant at SNR was far higher in the burn year of 2014 (6.9 flowers/plant) compared to the bicour plants (2.7). In 2015, when the site was not burned, there were no significant differences between the morphs (bicour 3.7: concolour 4.0; Fig. 5).

Floral phenology and presentation (attractants, rewards and petal warmth)

Populations at both sites remained in bloom for 14 to 20 days each year, flowering from mid-April to early May. Once the corolla of either morph opened at each site it wilted within seven days or less. Melanism in the bicour flower varied at the CR site where they were most common in 2015. In most plants only the two, top posterior flowers were dark purple but we found some other specimens in which melanism extended to the tips of the lateral petals as described and illustrated in the two species of Eurasian, melanism pansies by Clausen (1926). We also found one bicour plant in which all five petals showed some degree of melanism. In the absence of lateral beards the anther cone

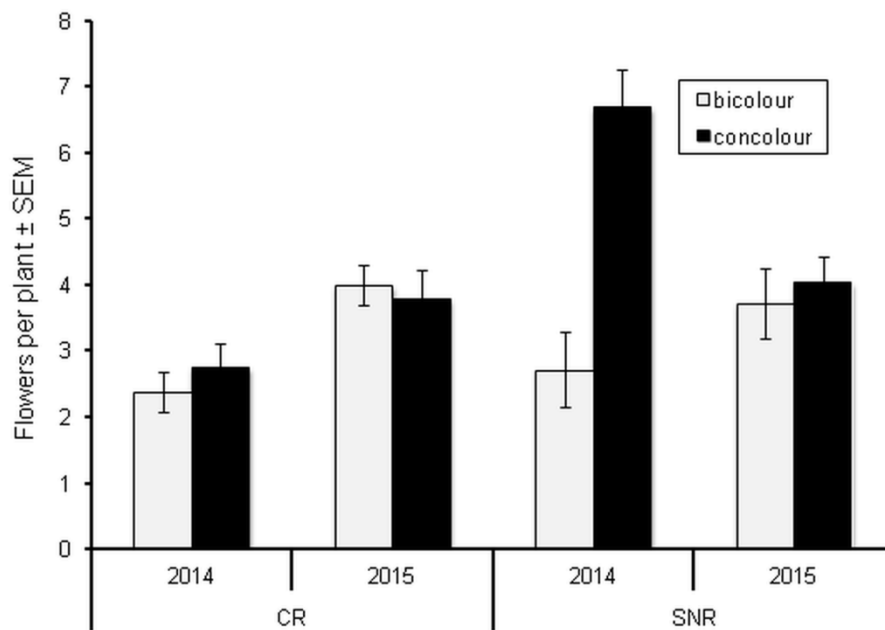


FIGURE 5. Mean number of flowers per plant of both morphs of *Viola pedata* at two sites and two years (2014 and 2015), Missouri, USA.

was fully visible at anthesis in both morphs. While the style is straight and lacks a rostellum (as noted by Beattie 1974) we also report that it is also very stiff and persists for several days following the withering of the corolla.

No discernible scent was detected in the flowers of either morph at either site and that includes sampling flowers in capped vials. Dissections of both morphs showed that each of the two, lower, anther connectives in each flower wore a large, elongated, green gland that protruded up to 66% the length of each narrowly, keeled spur. Spur lengths of bicolour and concolour morphs at SNR were greater than 5.0mm, but differences between morphs were not statistically significant ($t = 0.81$, $P = 0.427$); (concolour, 5.2 ± 0.70 ; $N = 18$) and (bicolour 5.4 ± 0.71 ; $N = 0.71$).

Petal temperature data at both sites was pooled. Regardless of colour morph the two, top (posterior) petals were anywhere between 1 – 3 degrees warmer than the lower, nodding, and often shaded, anterior (lip) petal ($F = 6.36$, $P = 0.0179$). For the concolour morph, though, there was no statistically significant difference between either of the two, petal temperatures. In contrast, the deep purple, posterior petals were always ~3 degrees warmer than the flower's anterior petal and 2 degrees warmer than the posterior petals of any concolour flower at either site ($t = 2.26$, $P = 0.0317$). Thus, it seems likely that the overall temperature differences we recorded were driven by the bicolour morph. Under shady conditions (e.g., under CR tree canopy or cloudy periods at SNR) the differential between the posterior and anterior petals decreased in magnitude but remained consistent.

Forager and foraging diversity, and pollen load analyses

Collections of insects at CR began on 5/2/13 when we observed bees on both morphs. We netted and euthanized 12 specimens that day and all were identified as females of *Andrena carlinii* but we did not record the morphs on which

each bee was caught. Two *A. carlinii* collected on 5/2/13 failed to carry the pollen of *V. pedata* but did carry the pollen of *Oxalis violacea*. The ten remaining *A. carlinii* collected the same day carried mixed loads of *V. pedata* pollen with the pollen of *O. violacea* and/or *Hypoxis hirsuta*. On 5/6/13 and 5/7/13 we caught one female *A. carlinii* and one female *Andrena nasonii* on concolour morphs respectively. The single *A. nasonii* carried the pollen of *Viola pedata* mixed with the pollen of *O. violacea*. In contrast, the female *A. carlinii* carried only grains of *H. hirsuta*.

An additional seven bee species were collected and identified on *V. pedata* when the contents of both sites were pooled from 2014-2015 but females of *Andrena carlinii* remained the most commonly recorded foragers on both morphs at both sites in both seasons (Tab. 1; Fig. 6). This brought the total of bees captured from 2013-2015 to 56. While three more bee species were collected at SNR, compared to CR, two of those species represented single captures over two seasons. As concolour morphs represented the vast majority of the SNR population (see above) it is not surprising that we collected only four bees, including a male of *Anthophora ursina*, on the bicolour morph over two seasons (Tab. 2). Over a two-year period the number of bees collected at both sites had almost identical pollen loads. At CR, 68% of bees collected carried the host flower's pollen while 70% carried the host flower's pollen at SNR (Tab. 1).

Females of *A. carlinii* rarely visited more than one or two open flowers produced in the same rosette at CR but they commonly visited more than one rosette in the same patch or clump. We observed that, if an *A. carlinii* landed on the same flower for a second time during the same foraging bout it did not stay long enough to drink nectar or collect pollen a second time. In 2015, at both sites, we noted that, sometimes, *A. carlinii* often appeared to lose its sense of direction if it landed on the two posterior petals, regardless of morph. When these bees were unable to find the common

TABLE 1. Pollen loads of bees collected on *Viola pedata* at Cuivre River and Shaw Nature Reserve, Missouri, USA, 2013-2015.

Location Species (sex)	Morphs visited Bicolor/Concolor	Pollen Loads			
		<i>Viola</i>	<i>Viola</i> + other spp.	Other species only	No Pollen
CR 2013					
<i>Andrena carlinii</i> (f)	NA*	0	11	3	0
CR 2014 – 2015					
<i>Andrena carlinii</i> (f)	0/6	1	10	5	0
<i>A. nasonii</i> (f)	1/0	0	0	0	1
<i>A. nasonii</i> (m)	0/1	0	1	0	0
<i>A. perplexa</i> (f)	0/1	0	1	0	0
<i>A. pruni</i> (f)	0/1	0	1	0	0
<i>Anthophora ursina</i> (f)	0/1	0	0	1	0
<i>Anthophora ursina</i> (m)	1/1	0	2	0	0
Sub Totals	2/11	1	15	6	1
SNR 2014 – 2015					
<i>Andrena carlinii</i> (f)	2/8	2	6	2	0
<i>A. cressoni</i> (f)	0/2	0	2	0	0
<i>A. perplexa</i> (f)	0/1	0	1	0	0
<i>Anthophora ursina</i> (f)	1/2	0	1	2	0
<i>Augochlorella aurata</i> (f)	0/1	0	0	0	1
<i>Lasioglossum bruneri</i> (f)	0/1	0	1	0	0
<i>L. cressoni</i> (f)	1/0	0	0	0	1
Sub Totals	4/15	2	11	4	2
Grand Totals	6/24	3	37	13	3

*NA = Not assessed

entrance to the anther cone or spur they flew to another flower or left the site.

We observed a total of 39 foraging bouts at the CR site by females of *A. carlinii* from 4/10 – 5/2/2015. On 4/14/15 we observed that one, female, *A. carlinii* visited 31 flowers on 27 basal rosettes over a 20 minute period. It visited 4 bicolor flowers and 10 concolor flowers before resting on an inflorescence of an *Antennaria* species and probing the florets ending the first bout. It then began a second bout returning to *V. pedata*, less than 30 seconds later, to forage on 10 bicolor flowers and seven concolor flowers before leaving the site.

In 2014 the ratio of concolor to bicolor flowers was almost equal at the CR site (see above). In that season, we observed that bees of four species and varying sizes visited 17 flowers of bicolor and 25 flowers of concolor. At the same site in 2015, when bicolor morphs greatly outnumbered concolours (see above), we observed bees of different species and sizes visiting 51 flowers of bicolor and 59 concolor flowers. Over a two-year period of observation at SNR, where the ratio of bicolor to concolor was always 40:1, only six observations of bee foraging observed were to bicolor morphs.

Male bees of all species collected were observed probing the spur regardless of site or morph. They were not observed to forage on the anther cone, regardless of colour morph. In contrast, after females of each *Andrena* species foraged for

nectar, they were observed to clasp the cone of anthers around the style repeatedly while they scraped out pollen with their forelegs. These grains were transferred to their scopae (Figs.6, 7; and see <https://www.youtube.com/watch?v=I90rBkdvVXE>).

While gynes of *Bombus* species nested at both sites they only hovered near the flowers, or touched the petals momentarily without foraging, then flew away. Pollen loads



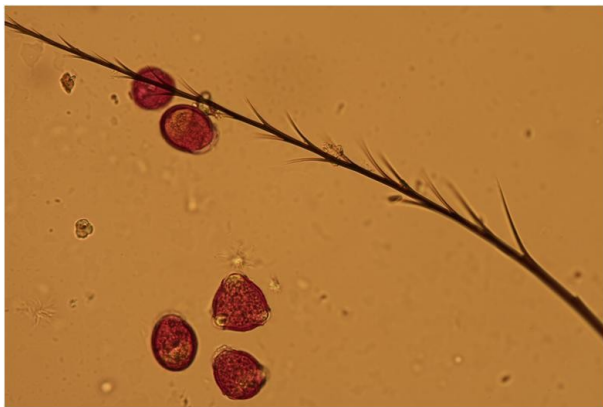
FIGURE 6. Female of *Andrena carlinii* foraging upside down on a bicolor flower. (Zong-Xin Ren, photographer).

TABLE 2. Insect-mediated rates of pollination in *V. pedata* at Cuivre River and Shaw Nature Reserve, Missouri, USA, in 2014 and 2015.

Location	Year	Morph	Number of flowers	Grains /tubes in Pistils Absent
CR	2014	Bicolour	18	10 (55.6%)
		Concolour	31	8 (25.8%)
	2015	Bicolour	15	9 (60.0%)
		Concolour	15	5 (33.3%)
Grand total			79	32 (40.5%)
SNR	2014	Bicolour	32	4 (12.5%)
		Concolour	34	1 (3%)
	2015	Bicolour	15	5 (33.3%)
		Concolour	15	0 (0%)
Grand total			96	10 (10.4%)

TABLE 3. Comparative averages of ovule, pollen tube penetration, and seed production of *V. pedata* at two sites over two years. Values in parentheses represent the standard error of the mean, except for seed production, which represents the range.

Location	Year	Morph	Ovules	Pollen tubes	Pollen tubes	Seed production
CR	2014	Bicolour	32.9 (2.3)	24.1 (4.2)	11.4 (2.5)	4.5 (0–27)
		Concolour	37.0 (2.2)	23.5 (3.8)	12.4 (2.5)	3.7 (0–38)
	2015	Bicolour	34.1 (3.8)	18.8 (7.3)	3.7 (2.1)	6.5 (0–37)
		Concolor	29.0 (2.4)	17.7 (3.1)	7.1 (1.8)	6.0 (0–38)
SNR	2014	Bicolour	31.1 (2.4)	27.3 (3.6)	14.3 (2.1)	15.3 (0–38)
		Concolour	38.2 (2.7)	28.2 (2.6)	16.2 (1.9)	21.1 (0–40)
	2015	Bicolour	21.1 (2.7)	19.8 (3.2)	6.7 (1.4)	13.5 (0–35)
		Concolour	24.7 (3.5)	44.8 (9.1)	16.4 (4.3)	12.5 (0–40)

FIGURE 7. Pollen of *Viola pedata* and scopal hair of *Andrena carlini*. (D. Jocson, photographer)

of male bees, regardless of species, indicated they were all polyphagic while pollen loads of females indicated that most were polylectic (see above). While a male of the oligolectic species *Andrena violae* was collected on coblooming *Viola palmata* in 2013 at CR we did not catch this species on *V. pedata* at either site over the next two seasons.

Small bombylid flies were observed at both sites in both years but they did not contact the anther cone or stigma while they foraged. They did not carry significant loads of

the host flower's pollen (see above) in 2014 and we stopped collecting them.

As visits by Lepidoptera at either site were so infrequent; we observed but did not collect them. We did not observe *Erynnis* species at either site. In 2014 we observed one visit by *Papilio glaucus* in which the butterfly visited two flowers (one flower on each morph). An unidentified *Papilio* species with black forewings and greenish-black hind wings visited two flowers on two rosettes (morphs not recorded). *Papilio* species were observed most commonly foraging for nectar on co-blooming *Phlox divaricata* at CR. An unidentified, yellow-winged member of the Pieridae (resembling *Phoebis sennae*) visited three flowers of concolour. In all three cases butterflies did not land on the posterior petals and did not forage in an inverted position. They landed on the lateral and/or anterior petals extending their proboscides under the anther cone to reach the spur.

Orientation of bees on morphs

In 2014 at the CR site we recorded 59 visits of bees to flowers of *V. pedata*. That was the year we collected bees within less than 60 seconds of their entry into the site while they foraged on their first flowers. We did not wait for them to finish their bouts (see above). A total of 36 (0.64) visits were made using the right side up orientation. In contrast, when we allowed CR bees to finish their foraging bouts in

2015 we counted 104 visits to these flowers in which (0.27) of the landing orientations were right side up.

Therefore, when observations of the first visit of a bee to a *V. pedata* at the CR site are combined for 2014 and 2015 the bee was more likely to land on the anterior (lip) petal right side up, and insert its proboscis under the anther cone, than it was to land on the posterior petals first and then forage upside down when that foraging bout began. However, as the same bee visited additional flowers during the same foraging bout in 2015 at CR the more likely it would change its foraging pattern from right side up to upside down. As these bouts progressed and ended in 2015 a total of 76 (0.74) of these orientations were made upside down. When a bee foraged upside down we did observe and record infrequent cases in which its third pair of legs clung to one or both of the two lateral petals. This usually occurred when posterior petals were askew (see, <https://www.youtube.com/watch?v=qNAcOkcl9Ak>). In the majority of observations, a female of *Andrena carlinii* clutched the posterior petals with its third pair of legs and the bee's abdomen also appeared to contact these petals (Fig. 6; <https://www.youtube.com/watch?v=190rBkdvVXE>) regardless of colour morph.

In 2014 and 2015 there were no statistically significant differences in bee orientation to bicolour vs. concolour morphs at the Cuivre River site. All but four bee orientations observed at SNR from 2014-2015 were always to concolour morphs and were always made upside down with one exception. The collection of the male *Anthophora ursina* in 2014 was made after the bee landed right side up on the bicolour flowers. As related above, we were not able to determine when bees began and ended foraging bouts on *V. pedata* at SNR. Regardless of morph, bees foraging upside down at CR and SNR continued to cling to the two posterior petals via their third pair of legs.

Ovule number and rates of pollination

The number of ovules in ovaries (Tab. 3) was consistent between sites ($F = 0.0158$, $P = 0.9972$), but not years ($F = 35.3$, $P < 0.0001$). In 2015 ovule production was consistently lower at SNR compared to SNR in 2014 and at CR in 2014 and 2015 (Fig. 8). There was no difference between the numbers of ovules inside an ovary between morphs at either site in either year ($F = 0.7863$, $P = 0.3831$).

Results of squashes and fluorescence analyses (Tab. 3, Figs. 10-13) at both sites indicated that rates of insect-mediated pollination were far higher at the SNR vs. CR, regardless of year ($F = 5.7$, $P < 0.0001$). However, at both sites, analyses of pollen tubes in pistils showed that bicolour morphs were visited less frequently compared to concolour morphs regardless of year (Tab. 2). Rates of pollination did not vary much, at either site, according to whether the area was burned or not (Tab. 3). At CR the number of pistils lacking pollen grains or pollen tubes increased slightly in both morphs the year after the burn (2015). At SNR, though, the number of bicolour pistils that were not pollinated in 2015 increased over 20% the year after the burn (2015). In contrast, the number of concolour pistils at

SNR lacking grains and tubes in 2015 actually declined slightly with 100% of pistils analyzed containing pollen tubes (Tab. 2, 3).

However, the mean number of pollen tubes actually germinating and penetrating a pistil did not vary between sites ($F = 0.04$, $P = 0.8345$) or years ($F = 1.75$, $P = 0.1957$). We did detect weak interaction effects between morphs and years ($F = 3.87$, $P = 0.0519$). This effect seems to be driven by the increased number of tubes in the style in the concolour morph at SNR in 2015 (Fig. 9).

As this is a species with late-acting self-incompatibility the number of pollen tubes that actually entered the ovary and penetrated ovule micropyles within 7 days after the flowers opened, required comparison. At both sites, ovule penetration was higher in 2014 (burn year) compared to 2015 ($F = 35.3$, $P < 0.0001$; see also Tab. 3). The average number of tubes penetrating ovules in 2014 and 2015 was marginally higher in concolour pistils regardless of site ($F = 3.9143$, $P = 0.0581$; Fig. 10).

Seed set

Seed production varied greatly between sites, morphs and years (Tab. 3). There were no main effects in seed set among years ($F = 0.6592$, $P = 0.6877$), location ($F = 0.3571$, $P = 0.6910$), or flower morph ($F = 0.2404$, $P = 0.4818$). There was a significant interaction between year and location ($F = 5.9292$, $P = 0.0161$), with seed set at SNR in 2014 being significantly higher than in 2015 and it was significantly higher compared to either year at CR.

DISCUSSION

Variation in colour morph frequencies

Unlike studies on zinc violets (Hildebrandt et al 2006) we can't attribute variation in colour morph frequencies in our populations to either a history of interspecific hybridization or soil pollution. However morph ratios in *V. pedata* may vary, at least in part, on other environmental factors according to habitat. We note that irregular burning regimes had little negative effect on morph frequencies in a rocky glade (SNR). A glade burn may be very hot but it must also be brief as only dead, thin stems of forbs and grass culms provide fuel. In contrast a positive but short-term effect, caused by the brief release of micronutrients in ash after rain probably stimulated reproductive effort (flower production) in concolour morphs at SNR. This has been well studied in some geophytes native to Mediterranean biomes in Australia and South Africa. Cyclical burns stimulate flowering in many herbaceous species but they belong to floras that evolved with cyclical fires (Le Maitre & Brown 1992; Lamont & Downes 2011). In fact cyclical fire regimes also occur in xeric, North American glades. In the absence of fires the herbaceous vegetation is succeeded by woody species (Martin & Houf 1993).

Compare this to our mixed hardwood forest (CR) where burns were performed in alternate years due to a greater accumulation of biomass represented by fallen branches and leaf detritus as fuels. This residue may smolder for hours following late-winter - early spring burns (unpublished

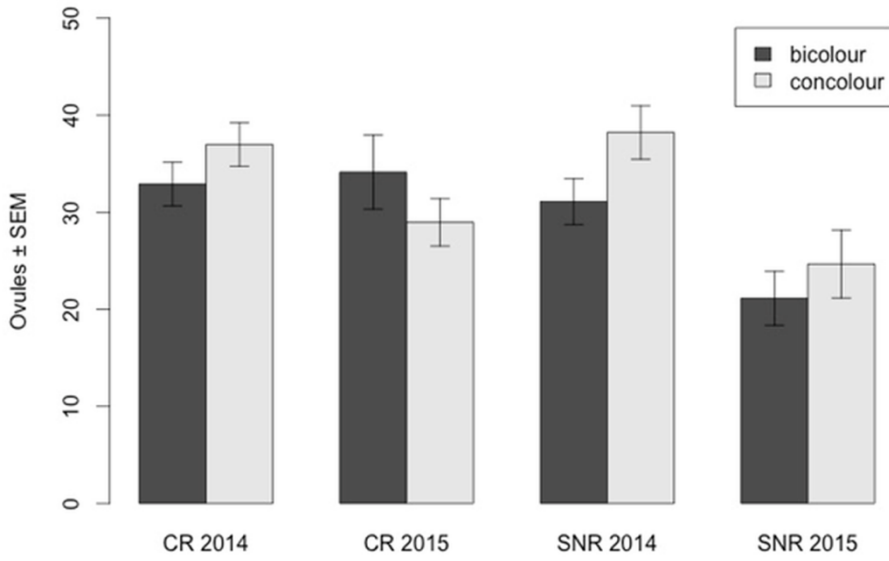


FIGURE 8. Mean number of ovules per flower in two morphs of *Viola pedata* at two sites and two years in Missouri, USA.

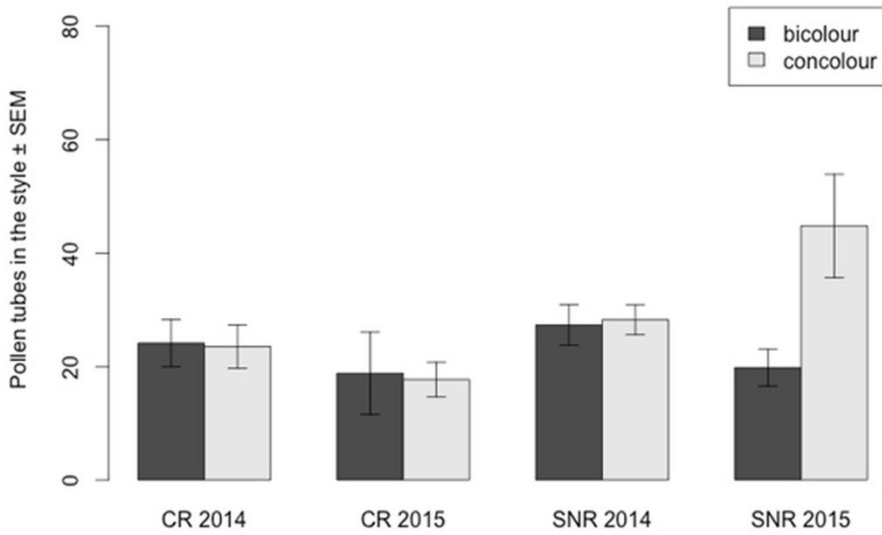


FIGURE 9. Mean number of pollen tubes germinating and penetrating styles in two morphs of *Viola pedata* at two sites and two years in Missouri, USA.

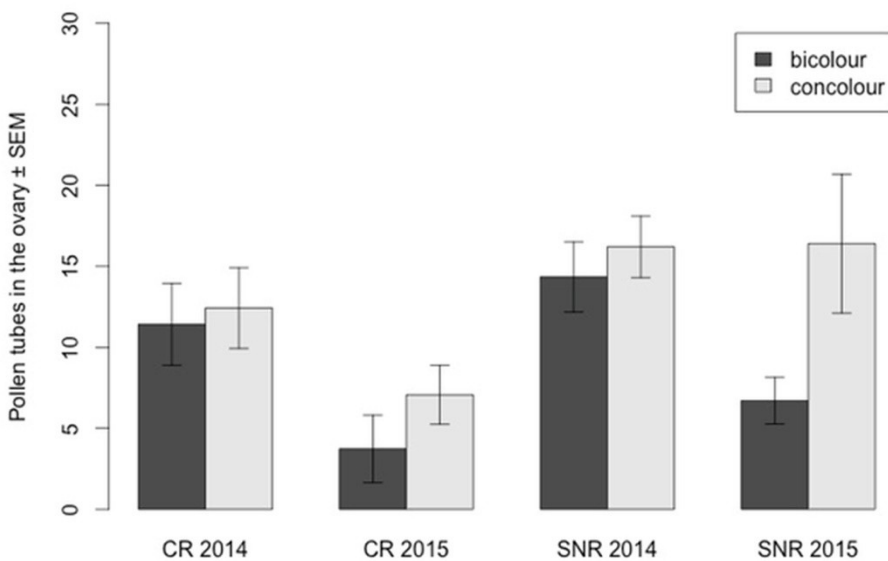


FIGURE 10. Mean number of pollen tubes penetrating ovules in two morphs of *Viola pedata* at two sites and two years in Missouri, USA.

observations). Perhaps it damaged more budding rosettes poised to flower in 2014 as bicolour rosettes appeared in twice the numbers (compared to concolours) at CR in 2015.

Comparative lack of pollinator diversity and foraging preferences between sites

We now have a third record of active (vector mediated) pollen collection (sensu Bernhardt, 1996) in a *Viola* species. The difference between this study and Freitas & Sazima (2003) was that their Neotropical species offered pollen as their primary (only?) reward. This did not appear to be the case in *V. pedata*. It maintained prominent nectar glands and spurs in both morphs. Our populations reflected the potential plasticity of generalist pollination systems found in *Viola* species in general. In other parts of its range Lepidoptera and male bees visit *V. pedata* exclusively for nectar (Beattie 1974; Carroll & Goldman 1994). While the apices of anthers of *V. pedata* wear the modified “snow shovels” (sensu Beattie 1974), associated with maximization of passive release of pollen onto nectar foraging insects, our females of *A. carlinii* remained active pollen collectors regardless of site. Bisexual flowers pollinated by a combination of nectar-drinkers and active pollen-collectors are not unique. Some Neotropical *Fuchsia* species are pollinated by a combination of hummingbirds and *Bombus* species. The birds consume only nectar (passive anther contact) while *Bombus* species drink nectar but also make active pollen collections (Bernhardt & Montalvo 1979; Breedlove 1969).

Within our two sites studied over three seasons the dominant pollinators were female, short-tongued bees in the family, Andrenidae as noted previously by Beattie (1974). We note that, at both sites over two years, females of *A. carlinii* dominated visits despite obvious differences in habitat landscapes and differences in prescribed burn cycles (see above). This is not surprising when the distribution of this bee is reviewed. Its North American range far exceeds the distribution of *V. pedata*. *Andrena carlinii* is polylectic also foraging on flowers of *Vaccinium* species and ephemeral woodland herbs. It nests in woodlands but is also native to open sites, not obscured by vegetation (Schrader & LaBerge 1978). This explains its presence in a mixed hardwood forest and in an exposed glade. In general, our insect collections failed to show that pollen dispersal of *V. pedata* at either site depended either on oligolectic bees (e.g. *Andrena violae*) or on insects foraging exclusively for nectar.

Beattie (1969; 1971b; 1972; 1974) provided ample evidence that many temperate zone, *Viola* species have generalist pollination systems. However, we should also consider the possibility that *V. pedata* may have a regionally narrow spectrum of pollinators when its populations are discontinuous, discrete and disjunctive. In our case we were disappointed repeatedly by the comparative lack of participation by native Lepidoptera at both of our sites compared to the observations of Carroll and Goldman (1994) in Missouri. Over three seasons at two sites we failed to observe the diurnal sphingid moths first described visiting *V. pedata* in West Virginia by Beattie (1974). Potential variation in guilds of anthophilous insects, based on the broad distribution of *V. pedata*, must be expected as this

species is recorded in almost half of the eastern, continental United States and south-eastern Canada. We wonder whether a combination of insecticide use and global warming over the past two decades accounted for the absence of *Erynnis* species at our sites?

Variation in bee orientation on morphs vs. morph preference

The orientation of bees on flowers of *V. pedata* appears to have little or nothing to do with foraging on either colour morph. Bee orientation mode appears to be based on when an insect begins and then completes its foraging bouts. We presume that when a bee first enters the site it first flies down to the flower and lands prone on the anterior petal. As the bout progresses the same bee appears to be more likely to fly above the flowers, lands directly on the posterior petals and then inverts while its hind legs continue to cling to the posterior petals. Consequently, our observations of orientation and landing differed from that of Beattie (1974). If one records only the visit of a bee to the first *V. pedata* flower it visits during a foraging bout then the right side up mode of visitation will dominate leading to some nototribic deposition of pollen as dorsal regions of its head and thorax contact the anther extensions. However, if one watches a full bout this right side up orientation declines as foraging and cross-pollination progresses. The majority of these later visits must be inverted leading to stenotribic depositions (sensu Beattie 1972).

As we did not measure the nutrients in the nectar and pollen grains of bicolour vs. concolour morphs we can't account for the bees' preferences for concolours. If both sites remain under their current maintenance programs it appears likely that frequencies in the bicolour morph should continue to decline at SNR as concolour is preferred by bees at both sites. Why then, should bicolour morphs persist at CR and increase and surpass numbers of concolour rosettes in 2015? One reason we suggest is that, while bicolour is less preferred by pollinators, field observations and pollen tube analyses showed that native bees continued to visit this morph at CR in the non-burn year (2015) with 40% of bicolour pistils containing pollen tubes. Residual warmth in the purple, posterior petals may mean that pollen-collecting bees will continue to forage on the anthers of some bicolour flowers as microhabitats become shadier and cooler at different times of the day due to changes in the angle of sunlight coupled with the irregular density of the forest canopy. Video and photos indicate that these dark purple petals may warm parts of the bee's third pair of legs and its abdomen. In contrast, at SNR the herbs on the floor of the glade do not stand under trees and are probably more exposed to more direct sunlight for far longer periods especially after a controlled burn (see below).

Floral warmth vs. foraging preferences

There is further precedence for this interpretation in Beattie (1971a) who studied *Viola glabella* in a much shadier, conifer forest. He noted that the pollinators were itinerant foragers on these yellow flowers visiting only during those brief periods when plants stood in direct sun. Beattie recorded ambient temperature, not floral temperature, but

also used a light meter. In addition, Bernhardt et al (2014) did not take ambient temperatures but noted that small to medium size-bees didn't visit generalist, food mimic, *Cyrtopodium montanum*, when these flowers were in deep shade in the course of the day.

We suggest that the warmer, deep purple petals of bicolor may be selectively advantageous but only under very specific environmental conditions. They may encourage some pollinators to forage in situ for longer periods after ambient temperatures start to drop as light gaps shift over the day. During some extended foraging bouts (see above) bees appeared more likely to forage first on the dark, bicolor petals at the CR site. These flowers are not heliotropic or paraboloid in shape (see Kevan 1972; 1975). Therefore, a bee that visits one flower cannot engage in long-term flower basking (sensu Heinrich 1993) and visits several genotypes over relatively short periods effecting cross-pollination. As *V. pedata* has such a broad distribution a useful, future exercise may be to record morph frequencies according to habitat, light intensity and whether dominant woody species are deciduous or evergreen.

Otherwise, there remains only one more untested possibility. Some pollinators (sphingid moths?) may prefer bicolor across parts of the range of *V. pedata* but those pollinators were not observed or collected in our populations for two years. If this is the case than skewed frequencies of colour morphs of *V. pedata* may be driven, in part, by resident pollinator preferences and may be more common than anticipated. Currently, publications that follow the density and diversity of specific pollinators throughout the broad, natural distributions of one animal-pollinated species remain uncommon (but see, Espindola et al. 2011). It is also intriguing to note that Steyermark (1963) reported a pure white population in Barton Co., Missouri and "mostly white" specimens from Polk County but never reported when or which insects visited the flowers.

Variation in ovule production pollination rates and seed set

Ovule production does not vary much between morphs in this species but burning in 2014 appeared to stimulate ovule production in concolours at SNR. Once again we credit the potential release of micronutrients in a habitat in which there is little soil and these plants grow between cracks in rocks. By killing or depressing the growth of some taller plant species that shade *V. pedata* our plants at SNR may have had greater access to water and sunlight channelling vernally produced sugars into greater ovule production. Once again, these are also the standard explanations for increased flower production in fire-cycle habitats in temperate Australia (see above). In contrast, the CR population grows under trees, is subjected to daily shading throughout vernal growth periods and was unlikely to manufacture enough carbohydrate to compete with ovule production at SNR over a two-year period converting fewer ovules into seeds.

As we noted previously, bees are more likely to visit concolour flowers. It comes as no surprise, then, that more concolour pistils contained pollen tubes in 2014 at both

sites. In 2015 the sheer number of pollinated pistils at SNR was > 0.50 higher in concolours than bicolors.

As the sheer number of flowering rosettes increases one presumes that pollinators will visit fewer plants as they become satiated more rapidly. This may also result in fewer compatible exchanges of pollen. When the number of flowering rosettes increased at CR in 2015 the number of ovules containing pollen tubes dropped dramatically. One wonders whether the 2014 burn also stimulated visitation by itinerant pollinators as fire should have removed debris or glade thatch at SNR making flowers blooming at about a centimetre above the ground more visible to foragers? Furthermore, concolour plants at SNR produced their largest visual display of flowers following the 2014 burns. Flowers of concolours were at a maximum visual presentation at SNR that year while, at CR, the ratio of the two colour morphs was almost identical.

However, once a flower was pollinated the sheer number of tubes per pistil was usually the same regardless of morph. We interpret this as evidence that a pollinator usually leaves the same number of viable grains on a receptive stigma, regardless of colour morph, restricting the number of pollen tubes that reach the ovary. Of course, when pistils express some form of late acting self-incompatibility the mere presence of tubes in entering ovules does not guarantee seed set. In some late acting SI expressed by some unrelated angiosperms (Kenrick & Williams 1986; Sage et al. 1999; Sage & Sampson 2003; Ramos et al 2005) recognition and rejection of shared alleles may continue to occur after tubes enter respective micropyles. Seed set at CR did not vary significantly over two years regardless of annual changes in morph frequencies. At SNR, while seed set in 2014 (burn year) was significantly higher than in 2015 we note that rates of pollen tube penetration were far higher in concolour flowers in 2015.

There are, of course, a number of alternative explanations for lower rates of seed set in any population from year to year. However, when we compare high pollen tube penetration of pistils at SNR in 2015 vs. lower seed production in the same season it should suggest an increase in insect mediated, self-incompatible pollinations based on bees visiting more than one flower on the same plant (geitonogamy) or crosses between genets sharing one or more of the same SI alleles (Kenrick et al. 1982). In 2014, at SNR, individual plants produced more flowers and this could have increased the frequency of geitonogamous crosses.

Once again, the 2014 burn at SNR appeared to benefit *V. pedata* producing more seed than in 2015. This also suggests that seed production is higher in an open glade compared to shady woodland. Fertility rates in *V. pedata* may be more dependent on the growth habits of surrounding vegetation (trees vs. grasses and forbs) according to burn cycle.

In conclusion, like most unbalanced polymorphisms the morphs expressed by *V. pedata* vary in frequency due to differences in regional modes of selection (see review in Futuyma 2013). As in other species with colour-based morphs (Rausher 2008) foraging preferences by the

dominant pollinators may drive differential rates of reproductive success because acts of cross-pollination in this species appear to be assortative according to morph preference (sensu Richards 1986; Rymer et al. 2010). While bees prefer concolour flowers the warmer posterior petals of bicouours may provide a novel adaptation increasing their fitness but only when they grow in specific habitats. We also note that burn regimes may also play a role in fitness benefitting flower and ovule production in the concolour morph but, once again, this selective advantage may occur only within a specific habitat. As the number of flowers produced by a plant and the number of ovules in an ovary must be interpreted as floral traits we suggest they may be linked directly to the colour polymorphism instead of as a nonadaptive pleiotropic effect (Armbruster 2002).

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